ANTIMALARIAL ACTIVITY OF METHYLENE BLUE AGAINST SELECTED DRUG RESISTANT *PLASMODIUM* PARASITES

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Antimalarial activity of methylene blue against selected drug resistant *Plasmodium* parasites

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DECLARATION

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

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DEDICATION

I dedicate this work to my loving and caring parents Mr. Joseph and Mrs. Anne Mwangi who have always supported and encouraged me pursue achievements in my studies. I also dedicate it to my brother Kigundu and my sisters Lynnette, Nancy, and Lucy. May the fruits of this achievement reflect perseverance, courage and joy in your endeavors.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS/ACRONYMS

ACT	- Artemisinin combination therapy
ACUC	- Animal Care and Use Committee
ANOVA	-Analysis of variance
AQ	-Amodiaquine
ATM	-Artemether
CQ	-Chloroquine
DHA	-Dihydroartemisinin
DHFR	-Dihydrofolate reductase
DMSO	-Dimethyl sulfoxide
dNTPS	-dinucleotide triphosphates
DNA	-Deoxyribonucleic acid
DPT	- Days post treatment
EDTA	- Ethylenediaminetetraacetic acid
G6PD	-Glucose-6-phosphate dehydrogenase
GFP	- Green Fluorescent Protein
GSH	-Glutathione

GR	-Glutathione reductase
HCL	-Hydrochloric acid
HEPES	- N-2 hydroxyethyl piperazine-N-2-ethane sulphonic acid
HMS	-Hexose-monophosphate shunt
IC_{50}	-50% Inhibitory concentration
IPR	-Institute of Primate Research
IRC	- Institutional Research Ethics Committee
KEMRI	-Kenya Medical Research Institute
LB	-Lysogeny broth
LM	-Lumefantrine
MB	-Methylene Blue
$NaHCO_3$	-Sodium hydrogen carbonate
PBS	-Phosphate buffered saline
PCR	- Polymerase chain reaction
PYR	-Pyrimethamine
SP	- Sulphadoxine-pyrimethamine
TE	-Tris EDTA

WHO	-World Health Organization
%	-Percentage
^{o}C	-Degrees Centigrade
µl	-microlitre
M	-Molar
mM	-millimolar
nM	-nanomolar
µM	-microMolar
ml	-Milliliter
g	-Grams
mg	-Milligrams
Wk	-Week

ABSTRACT

Malaria continues to be a major global health problem mainly due to emergence of parasites that are resistant to well-established antimalarials. This persistent threat of resistance has continually pushed for the search for new molecules with novel mechanisms of action and in some cases reversion to earlier phased out drugs. We aimed at developing Pyrimethamine (PYR) resistant *P. berghei* by gene manipulation using the transfection technique and determine *in vivo* activity of Methylene Blue (MB) on *P. berghei* parasites resistant to the standard antimalarials chloroquine, lumefantrine and pyrimethamine. In addition, the study set out to determine MB's activity against *P. falciparum in vitro.* PYR resistant *P. berghei* lines were developed through introducing plasmid DNA that conferred PYR resistance in wild parasites via transfection. The antimalarial activity of methylene blue was investigated *in vivo* on groups of Balb/C mice infected with either CQ, LM or PYR resistant rodent malaria parasite, *P. berghei*. Negative control groups were also included. Data collected was subjected to paired t-test for the comparison of means of the different experimental groups. Parasitaemia data generated from the *in vitro* experiments was subjected non linear regression analysis of log/dose response curves to determine the MB IC_{50}. Successful genetic transformation of wild parasites was confirmed by PCR detection of *TgDHFR* gene in the genomic DNA of transfected *P. berghei*. 45 mg/kg daily dose of MB was found to inhibit over 99% of the erythrocytic parasite growth of LM and PYR resistant *P. berghei* for up to 6 days post treatment (DPT) and 49.2% suppression in CQ resistant parasites to day 4 post treatment. MB treated mice survived longer than mice in the other treatment groups. At the same time, antiplasmodial activity against CQ resistant *P. falciparum* was observed and the IC_{50} determined at 27.33nM. Glutathione (GSH) plays a critical role in parasite detoxification by degrading heme and protection against oxidative stress. Methylene Blue, a glutathione reductase inhibitor prevents reduction of oxidized glutathione thus increasing levels of toxic cellular hydrogen peroxide. This mode of action provides a

new avenue in the production of antimalarials against resistant parasites. The minimal suppression against CQ resistant *P. berghei* could be linked to higher levels of GSH these parasites have in relation to their sensitive counterparts. Transfection provides a powerful tool in malaria research. The antimalarial activities displayed suggest that the phenothiazine structure could serve as a lead compound for further drug development and combination therapy. This may be the new approach to tackle increased prevalence of resistant malaria especially in Africa. In conclusion, the results obtained highlight that MB is effective in inhibiting *P. falciparum* growth and that it can be integrated in a new low cost antimalarial therapy against lumefantrine and pyrimethamine resistant parasites based on its activity on the drug resistant *P. berghei* and the *P. yoelii 17X* used. The suppressive activity demonstrated on the *P. yoelii 17X* shows it has potential activity across species. These findings suggest that MB provides a suitable candidate for first line drug in the management of resistant falciparum malaria as well as a potential candidate in the production of more effective and resilient combination-therapy drugs for malaria control.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

According to the latest WHO estimates, there were about 198 million cases of malaria in 2013 and an estimated 584,000 deaths worldwide, WHO African Region being the most affected with about 90% of all malaria deaths occurring there. Out of the estimated 528,000 deaths that occurred in Africa, 437,000 (75%) deaths were of children under five. Globally, 97 countries and territories had ongoing malaria transmission in 2014 (WHO Report 2014).

Following the development of chloroquine as an antimalarial, interest in the potential of methylene blue declined (Ehrlich, 1913). Recently, a number of authors have published papers on their studies on MB as an antimalarial. This recent interest in MB has developed only after reports of cases of resistance to chloroquine (Bountogo *et al.,* 2010; Zoungrana *et al.,* 2008).

Methylene blue (MB) is an old synthetic dye and it has been documented to have intrinsic antimalarial activity (Guttmann & Ehrlich, 1891). In addition, methylene blue is currently used in the treatment of various methemoglobulinemias, a serious complication of malarial anaemia as well as it is being investigated for its application in the management of Alzheimer's disease. Methylene blue is a specific inhibitor of *Plasmodium falciparum* glutathione reductase but has a number of desired and undesired side effects which are related to its redox properties (Mandi *et al.,* 2005). As an antiparasitic agent, methylene blue is pleiotropic meaning that it produces more than one effect: it interferes with hemoglobin and heme metabolism in digestive organelles (Schirmer *et al.,* 2003) and it is a selective inhibitor of *P. falciparum* glutathione

reductase by interfering with glutathione-dependant processes in *P. falciparum*-parasitized erythrocytes. The latter effect results in glutathione depletion.

In the red blood cells, carbohydrates are metabolized mainly by glycolysis, the pentose phosphate pathway (PPP), and 2, 3-bisphosphoglycerate (2, 3-BPG) metabolism. While the glycolysis path provides ATP for membrane ion pumps and NADH for reduction of methemoglobin, the PPP supplies the red blood cell with NADPH, which in turn maintains the reduced state of glutathione. The inability to maintain reduced glutathione in red blood cells leads to increased accumulation of toxic peroxides, especially hydrogen peroxide (H_2O_2), which in turn results in a weakening of the cell membrane and related hemolysis. Accumulation of H_2O_2 also leads to increased rates of oxidation of hemoglobin to methemoglobin that also weakens the cell membrane. Glutathione removes peroxides via the action of glutathione peroxidase. One of the uses of NADPH in the cell is to prevent oxidative stress. It reduces glutathione via glutathione reductase, which converts reactive H_2O_2 into H_2O by glutathione peroxidase. In the absence of glutathione reductase, the H_2O_2 would be converted to hydroxyl free radicals by fenton chemistry, which can attack the cell (Luond *et al.,* 1998).

Resistance to a large number of antimalarial drugs in the market like pyrimethamine, an antifolate, is due to mutations in the genes. These mutations like in the DHFR gene have been reported to decrease the binding affinity between pyrimethamine and dihydrofolate reductase via loss of hydrogen bonds and steric interactions (Sirichaiwat *et al*., 2004). The mutations are suggested to alter the shape of the active site cavity where the DHFR inhibitors bind the enzyme, reducing the binding affinities for drugs. A single point mutation causing a Ser-Asn change at codon 108 causes moderate pyrimethamine resistance, and the addition of Asn-Ile-51 mutations or Cys-Arg-59 mutations or both confers higher levels of resistance.

The existence of drug resistant *Plasmodium* lines as well as the increased prevalence of *Plasmodium* resistance to various first line drugs is not only very worrying but also poses a major hindrance to malaria control programmes. This has resulted in research and production of new antimalarials. A number of factors prove to be a hindrance in the search for new antimalarials and include: low economic returns on the side of pharmaceutical companies that produce them; high cost of research and production; poor success rates in comparison to quinine; inadequate control of the *Anopheles* mosquito vector and poor understanding of the basis of resistance. Hence the need for new antimalarials with a different mode of action and consequently provides the basis for searching new drugs and drug combinations that will significantly control cases of malaria morbidity and mortality (Kim & Schneider, 2013). Methylene blue presents a new avenue in the treatment of resistant malaria presumably by changing the redox potential of the infected cell. Although it was once noted for its antimalarial activity (Guttmann & Erhlich, 1891), it has been applied in other therapeutic uses and consequently minimal exposure to the strains of resistant malaria.

P. berghei is one of the many parasites that cause rodent malaria. It has extensively been used in studies and experiments that are designed to find drugs that can be used in the therapy and management of *P. falciparum* that causes human malaria. *P. knowlesi* on the other hand is associated with primate malaria, but has now been recognized as the fifth plasmodium parasite to infect humans as documented in South East Asia by Cox-Singh *et al.,* (2008). *P. knowlesi* offers an especially powerful experimental system, since the natural host, *M. fascicularis*, and an experimental host, *Macaca mulatta*, are phylogenetically close to humans. Genetic manipulation of this parasite species (van der Wel *et al.,* 1997) offers the unique possibility to study parasite-host interactions in a system that is highly predictive for the human situation (Kennedy *et al.,* 1997).

The findings of this study will assist in developing alternative drugs that can effectively combat the scourge that is caused by *P. falciparum* malaria. It will also provide information on the possibility of combining MB with other older drugs like pyrimethamine in the management of drug resistant malaria.

1.2 Statement of the Problem

Malaria is a disease of great concern to Africa and other tropical regions of the world. Despite the large arsenal of antimalarial drugs developed over the years, incidence and mortality of malaria is still high, mainly due to drug resistance. The existence of drug resistant *Plasmodium* lines as well as the increased prevalence of *Plasmodium* resistant to present treatment regime is not only worrying but also poses a major hindrance to malaria control programs

There is need therefore to come up with new molecules that have novel mechanisms of action against the malaria parasite. Methylene blue presents a potential antimalarial candidate and thus the need to assess its performance against various species and drug resistant clones of *Plasmodium* species.

1.3 Justification

Following the increase in reports on resistant malaria parasites to conventional drugs (Laxminarayan, 2004), there is the need to identify effective, affordable, alternative antimalarial compounds that are long lasting.

Although no longer the official first line anti-malarial in a number of countries (Talisuna *et al.,* 2004), SP is still in use. Current WHO policy dictates that SP should be provided to mothers at each scheduled focused antenatal care (ANC) visit in the second and third trimesters, with a minimum of three doses received during each pregnancy (WHO,

2013). This continued use of SP in the IPT provides drug pressure that selects for pyrimethamine resistant parasites. Thus pyrimethamine resistant parasites still remain in circulation. Therefore, the pyrimethamine resistant line will serve as a baseline in testing the efficacy of MB.

The rodent infecting *Plasmodium* species, *P. berghei* shows a similar basic biology to that of the human-infecting species and thus is commonly used in research (Tomas *et al.,* 1998). *P. berghei* is suitable for transfection thereby enabling the manipulation of their genome through the introduction of drug resistance transgenes into naïve parasites to facilitate the study on MB's antimalarial activity on resistant parasites. In addition, *P. falciparum* has been known to be virulent and is linked to cases of severe malaria in man. *P. berghei* which is non transmittable to humans, will be a safe choice for experimentation and observation in the animal model.

1.4 Hypothesis

Null Hypothesis: Methylene blue does not exhibit antimalarial activity against selected drug resistant *Plasmodium* parasites.

Alternate Hypothesis: Methylene blue exhibits antimalarial activity against selected drug resistant *Plasmodium* parasites.

1.5 Objectives

1.5.1 General Objective

To determine the antimalarial activity of methylene blue on selected drug resistant *Plasmodium* parasites.

1.5.2 Specific Objectives

- To genetically convert sensitive *P. berghei* parasites to pyrimethamine resistant parasites and confirm the conversion by PCR.
- To determine the antiplasmodial activity and IC_{50} of methylene blue against chloroquine resistant *P. falciparum* parasites *in vitro*.
- To determine the *in vivo* antimalarial activity of methylene blue against *P. berghei* resistant to chloroquine, lumefantrine and pyrimethamine respectively.

CHAPTER TWO

LITERATURE REVIEW

2.1 Classification of Plasmodium

Plasmodium, a protozoan*,* belongs to the family Plasmodiidae, order Haemosporidia and phylum Apicomplexa. There are currently 250 recognized species in this order. These organisms are obligate eukaryotic parasites, and are best associated with human malaria. Although they are best known for their infamous role as the etiological agent of human malaria, different species of *Plasmodium* have been found to also infect primates, rodents, ungulates, birds, and lizards. In humans, the most virulent parasites are *P. falciparum* and *P. vivax* (White, 2003).

2.1.1 The Life Cycle of *Plasmodium*

Plasmodium parasites have a life cycle with multiple stages in the definitive host and vector mosquitoes. Its infective stage is characterized by a sporozite that enters the vertebrate host after the mosquito vector bites. This sporozite is found in the salivary glands of the vector. Then there is the exoerythrocytic stage, in which the sporozoite travels to the liver and infects the hepatocytes (liver cells). It undergoes multiple rounds of asexual divisions (merogony or schizogony) and matures into merozoites which later burst out and infect red blood cells. The erythrocytic stage is mainly defined by the invasion of red blood cells by the merozoites. The organisms enter red blood cells (as merozoites), transform into the feeding stages (*trophozoites*), which then divide asexually into multiple new merozoites (*schizont* stage). During the schizont stage, some parasites differentiate into the reproductive forms (*gametocytes*) rather than the invasive merozoites. The gametocytes are classified as *microgametocytes* (that become male

gametes) and *macrogametocytes* (that become female gametes). The gametocytes must mature through five stages before they become infective to the mosquito.

Finally there is the reproductive stage; this begins when the vector takes a blood meal from the vertebrate host that contains mature gametocytes. In the vector the gametocytes transform into male and female gametes and merge to become a zygote (the only diploid stage in the organism's life-cycle). The zygote becomes an *ookinate* which invades the tissues of the vector midgut to become an oocyst. When the oocyst ruptures, thousands of *sporozoites* emerge and travel to the vector's salivary glands, as it is through the saliva that they will enter the next vertebrate host. This cycle is best summarized by Figure 2.1.

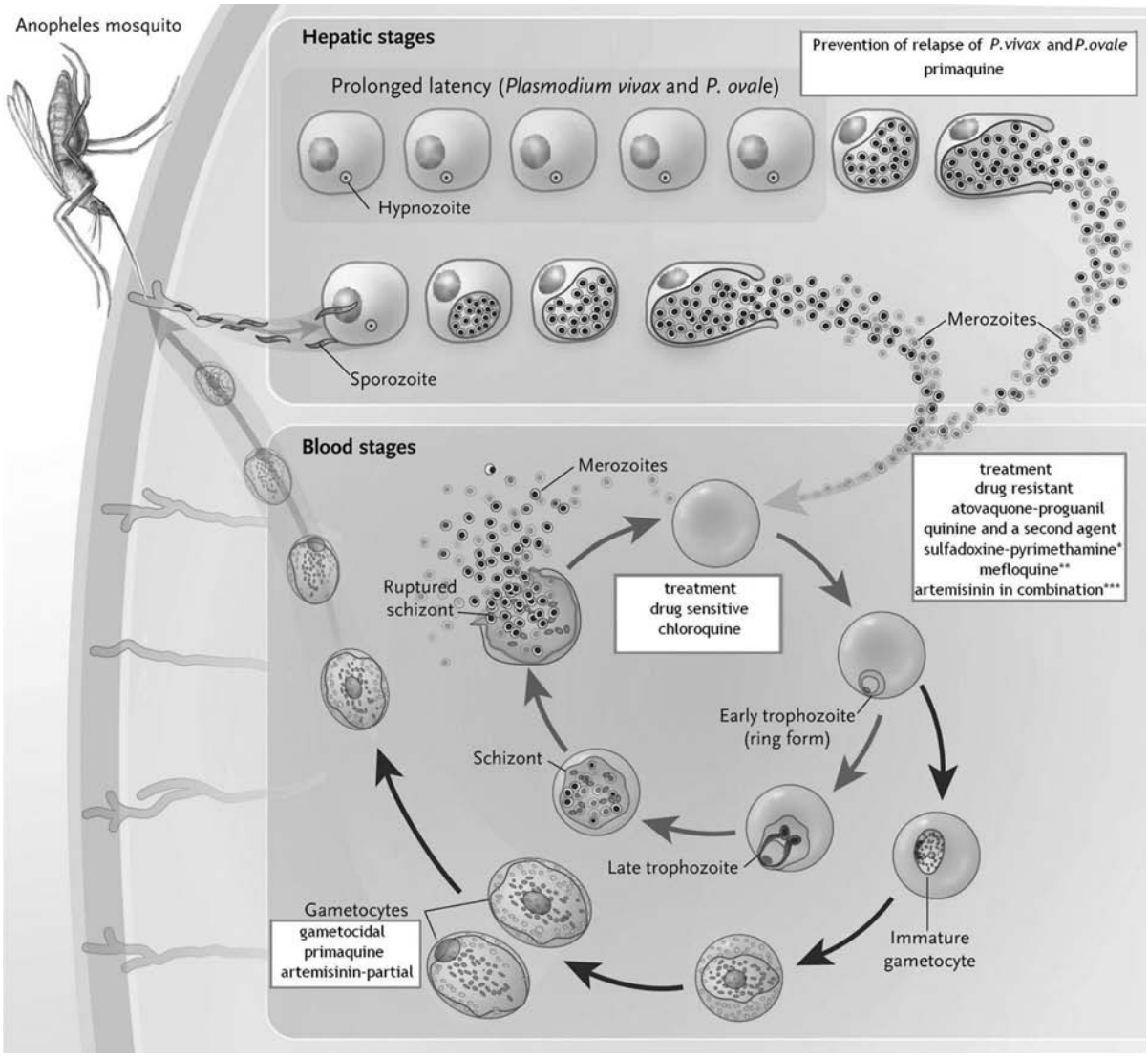


Figure 2.1: Life cycle of Plasmodium (Daily, 2006)

2.2 *P. berghei* as a model in malaria studies

P. berghei is one of the many species of malaria parasites that infect mammals other than humans. *P. berghei* is one of the four species that have been described in murine rodents of West Africa. The rodent parasites are not of direct practical concern to man or

domestic animals. The interest of rodent malaria parasites is that they are practical models for the experimental study of mammalian malaria. These parasites have proved to be analogous to the malarias of man and other primates in most essential aspects of structure, physiology and life cycle (Carter & Diggs,1977).

P. berghei infects African thicket rats and is transmitted by the mosquito *Anopheles dureni*. The parasite is used to model human hepatic malarias because it is transmissible to laboratory rats using *Anopheles*, which can be maintained in laboratory in full conditions.

Considering the fact that *Plasmodia* are highly similar in their patterns of infection and life cycle, this makes the *berghei* strain, which is not transmittable to humans, a safe choice for experimentation and observation.

2.3 Drugs used in the treatment of malaria

There are a variety of drugs used in the treatment of malaria. These drugs target different stages of the *Plasmodium* parasite in the human host as well as metabolic pathways and organelles that are essential for the survival of the parasite. Drug development is therefore based on pathways or components of a pathway that are either unique to the parasite or have sufficient differences from its host so that the drug will have little or no effect on the host. Most antimalarial drugs developed so far have been designed based on pathogen and host genomic and proteomic information (Rathore *et al.,* 2005).

Based on their role in parasite growth and survival, the parasite food vacuole, apicoplast and mitochondrion have been identified as major organelles for drug targeting. Several metabolic pathway components are also being developed as drug targets. For instance we have drugs that target the parasite food vacuole- the site of host haemoglobin

degradation; others are inhibitors of apicoplast activity like tetracycline, doxycycline and clindamycin, alone or in combination with other antimalarial agents to treat uncomplicated *P. falciparum* malaria (Fungladda *et al.,* 1998; Kremsner *et al.,* 1994). Some drugs like Atovaquone act by selectively inhibiting electron transport chain in the mitochondria of the parasite without affecting the host mitochondrial functions. Other antimalarial agents act by interfering with the parasite metabolic pathways like the folate pathway and glycolysis. Artemisinin has been found to act by inhibiting an endoplasmic reticulum-based Ca2+ -dependent ATPase of the parasite that is essential for its survival (Eckstein-Ludwig *et al*., 2003).

Inspite of all these variety of antimalarials, this study was centered on pyrimethamine and lumefantrine as the focus of resistance by the *Plasmodium* parasite.

2.3.1 Pyrimethamine (Inhibitor of the Folate Pathway)

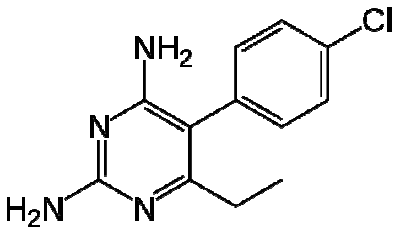


Figure 2.2: Pyrimethamine

Pyrimethamine, known chemically as 5-(4-chlorophenyl)-6-ethyl-2,4-pyrimidinediamine, is a dihydrofolate reductase (DHFR) inhibitor which exerts its antimalarial activity by interfering with folic acid synthesis by the enzyme, and such drugs are antifolates. Folates are essential metabolites, and the folate-dependent generation of DNA precursors in the form of deoxythymidine 5'-phosphate is essential

for the replication of malaria parasites. It is used widely in combination with longer-acting sulphonamides like sulfadoxine and sulfalene. This DHFR inhibitor blocks the development of the mature trophozites and also has sporontocidal activities. The inhibition of the folate pathway decreases pyrimidine synthesis with subsequent effects on DNA, serine, and methionine production thus terminating the parasite's life (Olliaro, 2001). Due to its structural likeness to that of pyrimidine as shown in the figure 2.2 it easily becomes pathway inhibitor.

Pyrimethamine is well absorbed into the body and is eliminated in a few days- it has a half-life of 3 days- thus allowing for single dose treatment. Oral administration of the drug provides a rapid absorption with high blood concentrations in 2-6 hours after an oral dose as compared with intramuscular injections (Winstanley *et al.*, 1992). It is mainly concentrated in the kidneys, lungs, liver and spleen, and about 80-90% is bound to plasma proteins. It is metabolized in the liver and slowly excreted via the kidneys. It can cross the blood-brain barrier and the placenta and is detectable in breast milk. The sulfadoxine-pyrimethamine (S-P) combination has a long half-life which provides protection from new infections for a prolonged period of time thus its use in prophylaxis (Daily, 2006).

2.3.1.1 Toxicity of Pyrimethamine

Although it is well tolerated, administration of the drug for prolonged periods may cause depression of haematopoiesis due to interference with folic acid metabolism. Skin rashes and hypersensitivity reactions also occur. Larger doses may cause gastrointestinal symptoms such as atrophic glossitis, abdominal pain and vomiting, haematological effects including megaloblastic anaemia, leukopenia, thrombocytopenia and pancytopenia, and central nervous system effects such as headache and dizziness.

Acute overdose of pyrimethamine can cause gastrointestinal effects and stimulation of the central nervous system with vomiting, excitability and convulsions. Tachycardia, respiratory depression, circulatory collapse and death may follow (WHO, 2006).

2.3.1.2 Resistance to Pyrimethamine

Following the failure of chloroquine as the first line of treatment in Kenya, S-P was introduced in 1998 as a replacement. Widespread treatment failures were observed within five years of implementing SP as the first-line antimalarial and therefore phased out in Kenyan hospitals soon afterwards. This was also supported by reports of widespread S-P resistance in Africa, S.E Asia and South America (White *et al*., 1999).

Although no longer the official first line anti-malarial, S-P remains available and is frequently used in the private sector (Watsierah *et al.,* 2010). It also plays an important role in the prevention of malaria in pregnancy when used for intermittent presumptive treatment of malaria in pregnant women (IPTp) (ter Kuile *et al.,* 2007) and has been included in several trials of intermittent presumptive treatment in infants (IPTi) (Kobbe *et al.,* 2007).

Investigations have revealed that in *P. falciparum*, pyrimethamine resistance is due to amino acid changes in DHFR, in particular a change at position 108 of serine (Ser) or threonine (Thr) to asparagine (Asn) (Peterson *et al.,* 1988; Wu *et al.,* 1996). Peterson *et al.* (1988) pointed out that DHFR resistance was associated with point mutations in the DHFR gene which led to reduced affinity (100-1000 times less) of the enzyme for the drug. A decrease in the affinity for an enzyme inhibitor or an increase in enzyme levels is an important factor in determining the degree of resistance. The resistance to S-P therefore is as a result of genetic changes that are accelerated by drug pressure thus facilitating drug associated selection of resistant strains.

In human infections, the *Plasmodium* is usually polyclonal. Resistance is thought to arise from natural point mutations in the chromosomes and independent of drug pressure but once formed they are more resistant mutants capable of surviving in the presence of antimalarial drugs (Wernsforfer,1991).

Resistant parasites are selected when the parasites are exposed to subtherapeutic drug concentrations. This happens when there is large scale use of the drug and if doses are in adequate. It may also arise if the drug is eliminated slowly from the body (White, 1992). Drugs like chloroqine and mefloquine are eliminated slowly from the body and thus persist in the blood and put selective pressure for a while after the drug was administered. The spread of resistance is also fueled by the reproductive advantage conferred by the resistance mechanism. This derives from the increased gametocyte carriage associated with treatment failure (both from the primary infection and the subsequent recrudescence).

Roper *et al*. (2004) observed that SP-resistant strains in South Africa had identical microsatellite markers flanking the associated mutated allele to the resistant strains from Southeast Asia. This result suggested that the drug-resistant strains are from a common ancestor and spread out to other regions. This distribution of pyrimethamine resistance is well demonstrated in the Figure 2.3.

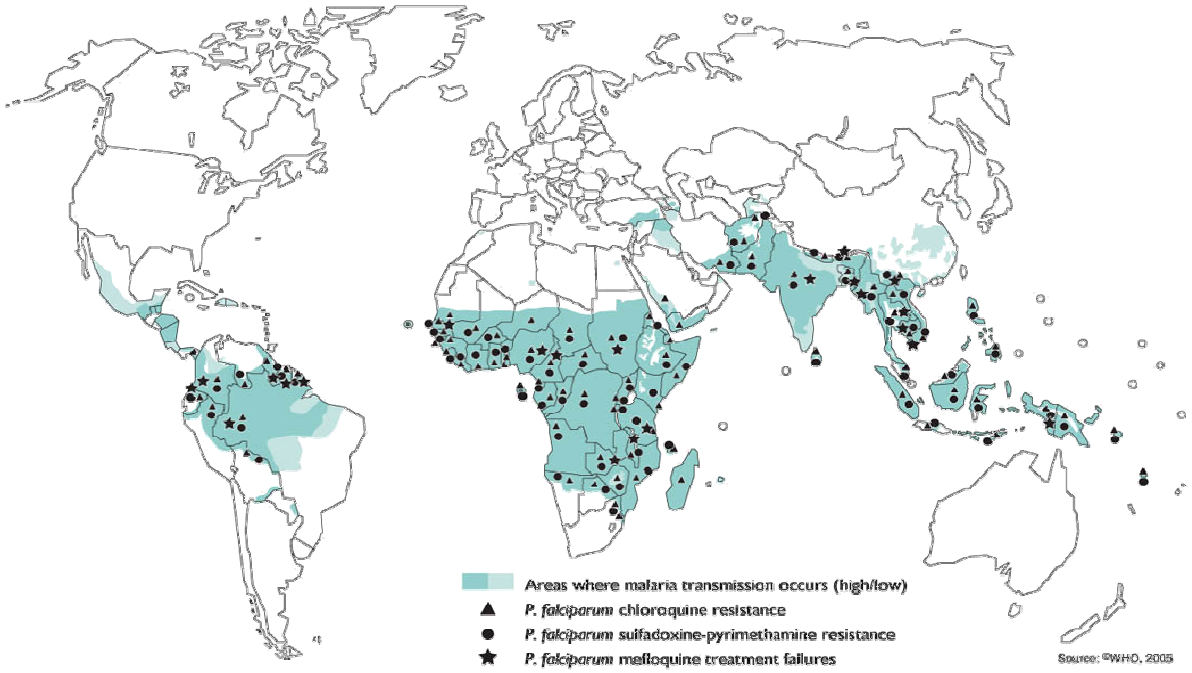


Figure 2.3: Drug resistance of *P. falciparum* in the world (WHO, 2005)

2.3.2 Lumefantrine

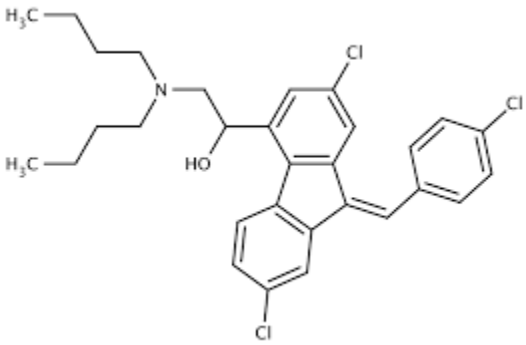


Figure 2.4: Lumefantrine

The above Figure 2.4 shows the structure of Lumefantrine, a racemic fluorene derivative with the chemical name 2-dibutylamino-1-[2, 7-dichloro-9-(4-chlorobenzylidene)-9H-

fluoren-4-yl]-ethanol. It conforms structurally, physicochemically and in mode of action to the aryl amino alcohol group of antimalarial agents including quinine, mefloquine, and halofantrine. Preliminary studies of the pharmacokinetic properties of lumefantrine are reminiscent of those of halofantrine.

It has a variable oral bioavailability that is augmented considerably by fats, a large apparent volume of distribution. The drug has a terminal elimination half-life for malaria estimated initially at approximately 4 to 5 days (Ezzet *et al.*, 2000). However it has the disadvantage of being slow acting when used alone. Lumefantrine is a highly lipophilic substance; oral bioavailability increases substantially if the drug is administered after a meal rich in fat.

2.3.2.1 Pharmacokinetics of Lumefantrine

Oral bioavailability is variable and is highly dependent on administration with fatty foods. Absorption increases by 108% after a meal and is lower in patients with acute malaria than in convalescing patients. Peak plasma levels occur approximately 10 h after administration. The terminal elimination half-life is around 3 days (WHO, 2006).

2.3.2.2 Toxicity of Lumefantrine

Despite similarities with the structure and pharmacokinetic properties of halofantrine, lumefantrine does not significantly prolong the electrocardiographic QT interval, and has no other significant toxicity. In fact the drug seems to be remarkably well tolerated. The reported side effects are generally mild and they include nausea, abdominal discomfort, headache and dizziness – and cannot be distinguished from symptoms of acute malaria (WHO, 2006).

2.3.2.3 Mode of Action of Lumefantrine

The exact mode of action of lumefantrine (LM) is unknown. However, it has been observed that *pfcrt* 76T carrying parasites are more susceptible to LM as compared to K76 carriers. This goes to suggest that in parasites carrying 76T, LM might be transported out of the digestive vacuole by PfCRT and into the cytoplasm thus making the parasites susceptible to the drug. The target of LM may be in the cytoplasm (Sisowath, 2009). Daily (2006) further points out that LM's targets include the parasite's metabolism and detoxification of hemoglobin.

2.3.2.4 Resistance to Lumefantrine.

Resistance to lumefantrine is multigenic, Other than *pfcrt* and *pfmdr1* genes, single nucleotide polymorphisms in the *pfmrp1* gene may also be involved in LM resistance especially PfMRP1 and 1390I as portrayed by Sisowath (2009).

2.3.3 Chloroquine

Chloroquine, Figure 2.5 below, is a 4-aminoquinoline that was first chemically synthesized in 1934 as a substitute for quinine. It is almost completely absorbed when taken orally, though peak plasma concentrations can vary. It is inexpensive, well tolerated, and available intramuscularly (IM) and intravenously (IV).

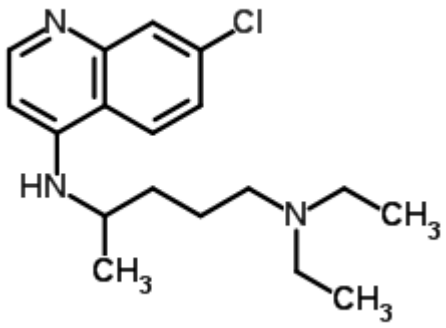


Figure 2.5: Chloroquine

This drug is an amphiphilic weak base and is selectively deposited in the food vacuole of the parasite because of a pH gradient. The food vacuole is the site of parasite metabolism of its main food source, hemoglobin and this is where chloroquine exerts its antimalarial effect by preventing the polymerization of the toxic heme to the non-toxic insoluble haemozoin (Sullivan *et al.,* 1996; Chong & Sullivan, 2003). The parasite detoxifies hemoglobin metabolites, and chloroquine interference with this process results in parasite death.

Chloroquine became prominent in the early 1950s when global eradication of malaria was declared by WHO and later on became a drug of choice in malaria treatment. However, chloroquine resistant parasites started appearing and now the drug is virtually ineffective in most parts of the world. The resistance to chloroquine observed in *P. falciparum* is not due to a change in haem processing but is attributed to mutations in a food vacuole transport protein (Fidock *et al.,* 2000). Interestingly, the same chloroquine resistance in *P. vivax* seems to be independent of mutations in this gene as noted by Nomura and colleagues (2001).

In Africa resistance had spread out in the entire continent in about 10 years since it was first reported in 1978 in East Africa. In 1993, Malawi discontinued the use of chloroquine because of a high prevalence of chloroquine resistance, and since then the prevalence of the *pfcrt* mutations dropped significantly over the following years, with parasites demonstrating an increase in *in vitro* chloroquine sensitivity, raising the possibility of recycling drugs (Kublin *et al*., 2003). At the moment, chloroquine remains effective for *P. falciparum* infections in very limited areas in the world.

2.3.4 Amodiaquine

Amodiaquine is a chloroquine derivative synthesized to replace chloroquine (see Figure 2.6 below). Therefore its antiparasitic effect is similar to that of chloroquine probably due to its structural similarity to chloroquine. It is available in the form of amodiaquine hydrochloride and is readily absorbed from the gastrointestinal tract. Its mode of administration is oral or intramuscular. In the liver, the amodiaquine is rapidly converted into the active metabolite desethylamodiaquine which is associated with the antimalarial effect (Winstanley *et al.,* 1990)

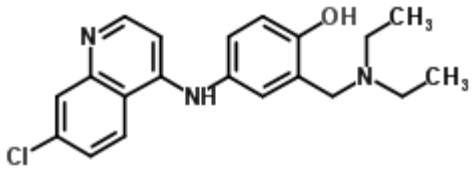


Figure 2.6: Amodiaquine

At the moment a challenge in the application of amodiaquine as an antimalarial is the cross-resistance with chloroquine, this could hamper effectiveness. Currently, amodiaquine is being used as part of combination therapy with SP or other agents like

artesunate and has been shown to improve treatment outcomes in highly drug-resistant areas (Gasasira *et al.,* 2003; Adjuik *et al.,* 2002)

2.3.5 Quinine

Quinine, Figure 2.7, is an aryl-amino alcohol derived from the bark of the *Cinchona* tree of the South American highlands. Its antimalarial activity was known by traditional healers and was regularly used on intermittent fever. It is one of two naturally derived antimalarial compounds. Quinine is rapidly and almost completely absorbed from the gastrointestinal tract, and peak plasma concentrations occur 1 to 3 hours after oral administration of the sulfate or bisulfate (Supanaranond, 1991). This drug has been used as the mainstay therapy for severe malaria and can also be given intravenous or intramuscular. However, hypotension secondary to rapid IV infusion and severe hypoglycemia are potential toxicities.

Quinine is usually reserved as a second-line therapy and for the treatment of severe malaria. Marked side-effects (tinnitus, dizziness, nausea) and a 7 day treatment course mean that it has a limited place as a first-line treatment for uncomplicated malaria.

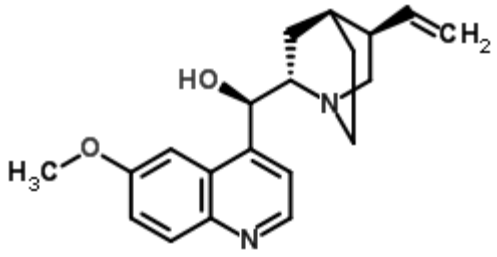


Figure 2.7: Quinine structure

Drug resistance has been very slow to develop. The parasite gene *pfmdr1* has been shown to modulate resistance to quinine and the drugs halofantrine and artemisinin *in*

vitro in a strain-specific manner. Allelic replacement with mutations in this gene has directly demonstrated their role in quinine resistance (Sidhu *et al.,* 2005). Quinine is typically used in combination with a second agent such as doxycycline, clindamycin, or SP.

2.3.6 Piperaquine

Piperaquine is a bisquinoline antimalarial drug introduced in the 1960s, to replace chloroquine as first-line treatment for *P. falciparum* and *P. vivax* malaria in many areas of China during the 1970s and 1980s (Chen *et al.,* 1982). Being a quinoline derivative, its structure in Figure 2.8 shows partial similarity to chloroquine.

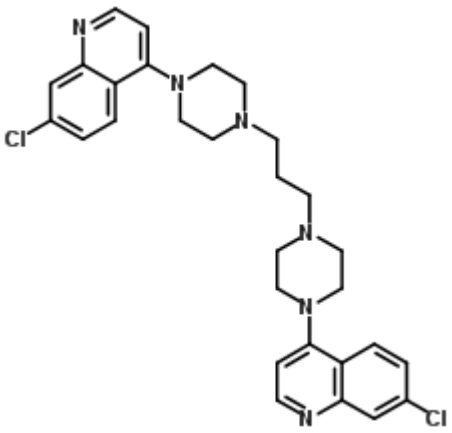


Figure 2.8: Piperaquine

Its extensive usage led to the development of resistance in China (Davis, 2005). On the bright side, due to its good tolerability, piperaquine is now being tried in combination with dihydroartemisinin (Denis, 2002). Safety and efficacy trials done on adults and children in Cambodia, Vietnam and Thailand showed that this combination had > 97% 28-day cure rates.

2.3.7 Mefloquine

Mefloquine, is a 4-quinoline methanol, was introduced in the mid-1980s to treat patients suffering from chloroquine-resistant malaria. It is structurally related to quinine and was designed as a synthetic replacement to quinine. It is well absorbed from the gastrointestinal tract; although the full treatment dose can result in gastrointestinal upset thus it is recommended to split the treatment dose to lessen the side effects. This drug's long half-life allows weekly dosing for prophylaxis, its dosage is limited to a single tablet/week for 4 weeks. Its downside includes the occurrence of neuropsychiatric symptoms that has reduced its acceptability by travelers (Schlagenhauf, 2003).

This drug acts as a blood schizonticide. Drug resistance has also been reported on the Thai-Myanmar border. The mechanism of mefloquine resistance has been associated with an increase in gene copy number and expression of *pfmdr1* in some studies (Price, 2004). Polymorphisms in *pfmdr1* have also been shown to correlate with varying susceptibility to mefloquine as noted by Duraisingh *et al.* (2000).

Although a long half-life of this drug provides a distinct advantage for treating malaria, its extended presence in the circulation has also been suggested to be the reason for the emergence of mefloquine-resistant parasites. Mefloquine has now been adopted as a component of artemisinin-based combination therapy.

2.3.8 Halofantrine

Halofantrine is a phenanthrene–methanol compound with activity against the erythrocytic stages of the malaria parasite. Its use has been especially recommended in

areas with multi-drug resistant *P. falciparum*. A second course of treatment is highly recommended after 7 days since recrudescence can occur with only one round of treatment. The use of halofantrine is restricted because it can induce heart arrythmia.

2.3.9 Primaquine

Primaquine is an 8-aminoquinoline antimalarial compound effective against *P. falciparum* gametocytes and eradicates the hypnozoite stages of *P. vivax* and *P. ovale* (Bunnag, 1980). To prevent a hypnozoite relapse of *P. ovale* or *P. vivax* infection, a 14-day treatment course is required to treat the liver latent stage after an active blood stage infection is treated with chloroquine. However, this drug is not suitable in patients who have a glucose-6-phosphate dehydrogenase deficiency (Baird, 2004) since it is associated with methaemoglobinaemia and haemolysis in the G6PD-deficient persons. Primaquine use is also associated with minor gastrointestinal disturbances, such as stomach pain and diarrhoea.

2.3.10 Atovaquone-Proguanil

The atovaquone-proguanil combination is used for the treatment and prevention of CQ resistant *P. falciparum.* Atovaquone is a hydroxynaphthoquinone that selectively inhibits the parasite mitochondria respiratory chain at the cytochrome bc1 complex by mimicking the natural substrate ubiquinone (Srivastava, 1999). Its oral bioavailability is improved when taken in fatty food. Atovaquone when used alone was prone to rapid development of resistance because of a very high rate of recrudescence. Drug resistance to atovaquone is mediated through mutations in its target cytochrome b.

Proguanil is a readily absorbed biguanide compound and is a DHFR inhibitor. It has a synergistic effect with atovaquone by reducing the concentration of atovaquone needed to interfere with the mitochondrial membrane potential (Srivastava, 1999). Atovaquone-

proguanil has a partial effect on the liver stage in *P falciparum* such that posttravel use is only 1 week as compared to 4 weeks for the other prophylactic drugs. However, resistance to this combination has been reported.

2.3.11 Artemisinin compounds

Artemisinin is a naturally produced antimalarial from *Artemisia annua*, sweet wormwood, and is a sesquiterpene lactone. Extracts of sweet wormwood were used to treat fevers in China for centuries before its antimalarial activity was identified in 1971.

Its mechanism of action is that it acts by inhibiting an endoplasmic reticulum-based Ca2+-dependent ATPase of the parasite that is essential for its survival (Eckstein-Ludwig, 2003). Once inside the cell, the peroxide bridge is cleaved by an iron-dependent mechanism. This results in the generation of carbon-centred short-lived radicals. These intermediate radicals react with the ATPase, resulting in its inactivation. They also are active against the development of early-stage gametocytes and lessen gametocyte carriage as compared to other antimalarials (Kumar, 1990).

Lipophilic and hydrophilic derivatives, artemether and artesunate respectively, have been developed that provide nonparenteral administration. The water-soluble artesunate is for IV injection while artemether is for IM injection. These derivatives have shown potent antimalarial activity. *In vivo*, they are metabolised to dihydroartemisinin and are 5 – 10-fold more effective than artemisinin.

Artemisinin and its derivatives shown in Figure 2.9 are absorbed and eliminated rapidly and have a half-life of < 60 min. In contrast, pyrimethamine, atovaquone, mefloquine and chloroquine remain at parasiticidal concentrations in the circulation for several days. To cure malaria, parasiticidal concentrations of the drug should be maintained in the circulation for a week, maintaining activity for at least four asexual cycles of the

parasite. Therefore, although long-lasting antimalarial agents are generally prescribed on a weekly dosage, artemisinin-based drugs need to be taken at least on a daily basis to be effective; their short half-life limits their monotherapeutic use, as it causes high recrudescence rates. Thus, these compounds are often used in combination with other antimalarials that have different modes of action for instance Artemether-lumefantrine (Coartem) and Artekin.

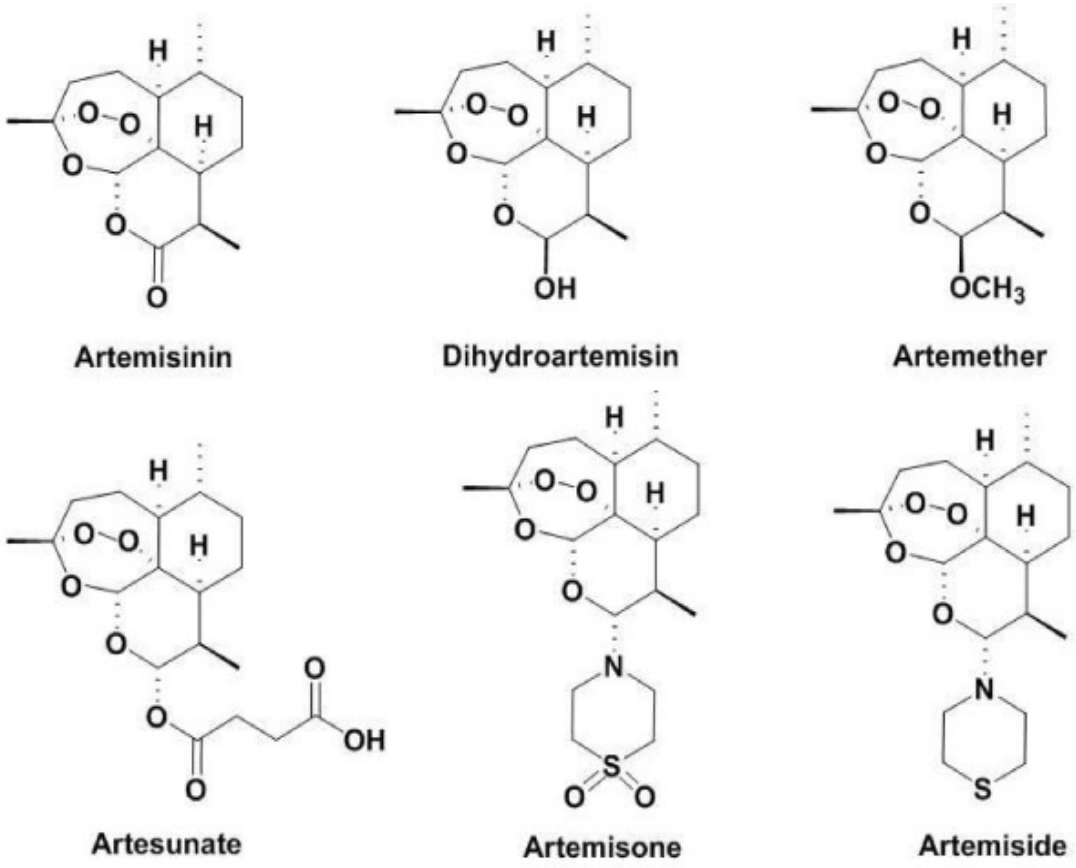


Figure 2.9: Artemisinin and derivatives

Combination therapy offers several advantages over the individual use of a drug. The combination of two antimalarials with different sites of action in the parasite leads to a simultaneous attack on two systems in the parasite. Additionally, the chances of

selection of a parasite resistant to both drugs will require an almost simultaneous change in at least two loci.

At the moment, the emergence of *P. falciparum* strains tolerant to artemisinin in the Thai-Cambodia border area is of great concern (WHO, 2010). This not only has direct implications for artemisinin therapy, but promotes the selection of strains resistant to partner drugs.

2.4 Chemotherapy in the control of malaria

Malaria control is too complex to be addressed by a single approach. It is important to tailor the strategy to the prevailing ecological and epidemiological conditions (Mouchet, 1998). Concerted malaria control efforts, using both vector control and treatment, have achieved great success, with massive declines in infection being seen throughout the Americas. This success was achieved by among others a strong infrastructure of personnel, regular house spraying, treatment of cases detected and active surveillance among others.

Various other methods have been formulated into controlling the advancement of malaria ranging from transmission control to chemotherapy and vaccines. Insecticide treated bednets, have been applied in controlling malaria transmission in endemic areas.

Chemotherapy implies the use of antimalarial drugs that have been classified into seven categories based on the various stages of the parasite: 1) tissue schizontocidal drugs that include causal Drugs that eliminate liver stage from initiating erythrocytic stage and hypnozoitocidal for radical cure of exo-erythrocytic hypnozoites of *P. vivax* and *P. ovale* after treatment of acute erythrocytic phase; 2) Blood schizontocidal drugs; 3) Clinical cure for fast action on erythrocytic stages, example of these drugs are artemisinin and quinolines; 4) Suppresive therapy drugs that have a slower suppressive

action on erythrocytic stages; 5) Gametocytocidal drugs designed to destroy sexual erythrocytes thus preventing transmission to the vector; 6) Sporontocidal drugs that prevent the formation in mosquito of oocyst and sporozoites; 7) Chemoprophylactic drugs that eliminate liver stage from initiating erythrocytic stage and those that arrest the development of merozoites in erythrocytes.

2.5. Malaria and drug resistance

Treatment using antimalarial chemotherapy has been the primary option in the fight against malaria in many countries (Oyakhirome *et al*., 2007). However, resistance to antimalarials compromise the efforts made in controlling the disease. Over time, virtually all antimalarials developed have been rendered ineffective against the disease causing parasite.

Most common antimalarial drugs used include chloroquine, amodiaquine (AQ), sulfadoxine-pyrimethamine (SP), quinine, mefloquine and artemisinin derivatives. Reduced susceptibility to chloroquine was reported in Kenya, as was the case with SP. Resistance to SP has been associated with point mutations in the genes for enzymes involved in obligatory parasite-folate biosynthesis pathway (Mbugi, *et al.*, 2006). AQ is more effective than chloroquine. However, there is cross-resistance to amodiaquine in the chloroquine resistant strains of *Plasmodium*. There have also been isolates with reduced susceptibility to mefloquine in west and central Africa with more pronounced resistance in the border areas between Cambodia, Myanmar and Thailand. The African strains still remain sensitive to quinine with decreasing sensitivity being reported in South-East Asia (RBM/WHO, 2001; Meng *et al.,* 2010).

Ever since the discovery of the first case of chloroquine resistance along the Thai-Cambodian border in the late 1950s, Southeast Asia has played an important role as a focus for development of drug resistance to *Plasmodium falciparum* infection

management. Although the first case of quinine resistance had been reported much earlier from South America, the onset of chloroquine resistance marked the beginning of a new chapter in the history of malaria in Southeast Asia and by 1973 chloroquine finally had to be replaced by the combination of sulphadoxine and pyrimethamine (SP) as first line drug for treatment of uncomplicated malaria in Thailand and more than 10 African countries also switched their first line drug to SP. In 1985, SP was eventually replaced by mefloquine (Farooq & Mahajan, 2004).

Rapid development of resistance to this new drug led to the introduction of artemisinin as a combination drug (ACT) in mid-1990s. However, resistance to ACT is already developing and although not yet fully characterized, there is evidence of decreased efficacy of the artesunate-mefloquine combination (Mugittu *et al.*, 2006, Laufer, 2009). Intermittent preventive treatment (IPT) is a control strategy in endemic areas that involves providing all pregnant women with at least two preventive treatment doses of an effective antimalarial drug during routine antenatal clinic visits. This approach has been shown to be safe, inexpensive and effective.

There are variations in the way this resistance manifests itself. In high transmission areas people have developed some immunity; there is the clinical manifestation due to prolonged infection and also risk of severe disease leading to high mortality. Low transmission areas on the other hand are characterized by epidemics as noted by Bjorkman and Bhattarai (2005) thus morbidity. Among other effects include the high costs involved in drug research and development, the decline in the region's economic status among others (WHO, 2006).

2.6 Drug repurposing in Malaria

Malaria elimination is becoming a reality for some countries and strategies for global malaria eradication are now being considered which will require new drug regimens

with improvements in cost, simplicity and efficacy against resistant strains (Alonso *et al.,* 2011). In particular, the emergence of *Plasmodium falciparum* strains that are tolerant to artemisinin in the Thai-Cambodia border area is of great concern (WHO, 2010). This not only has direct implications for artemisinin therapy, but promotes the selection of strains resistant to partner drugs. Thus new anti-malarial drugs are needed urgently (White, 2010). However, malaria projects take much longer than five years to go from discovery to having a clinical candidate. This is because of technical challenges and at times lack of funding.

It is clearly important to search for new approaches to make the process of getting new effective antimalarials more efficient. An alternative approach is that of drug repositioning or repurposing. This simply is taking a molecule that has been developed for one indication and showing its utility in another. Starting with a molecule that has already undergone clinical trials in another indication provides several potential advantages. Since MB has already been used in the treatment of other medical conditions and had earlier been noted for its antimalarial activity, we could argue this would be drug repurposing. Already the clinical safety profile is understood, and safe therapeutic doses have been established. Importantly, human pharmacokinetic data from the methemoglobinemia and Alzheimer trials exist and provide some indication of whether therapeutic concentrations in the new indication (malaria) can be achieved safely and maintained in patients. In malaria, there have also been other initiatives in drug repositioning (Chong *et al.,* 2006 and da Cruz *et al.,* 2012).

In malaria, drug repositioning has been from screening a library of compounds containing FDA-registered medicines and other molecules in clinical development. Other sources are existing drugs that are tested in an assay for activity against *Plasmodium* stages. A further potential source of drugs for repositioning is those molecules where clinical development has been discontinued before approval.

Compounds showing low micromolar activity and with a suitable pharmacokinetic and safety profile can further be evaluated *in vivo*.

Drug repurposing provides a prudent mitigating approach against resistant parasites as well as treatment failure of other antimalarials because it will reduce the time and cost of drug discovery. This will in turn lead to faster development of affordable drugs.

2.7 Current medical applications of Methylene Blue

Methylene blue was discovered in 1866, the first member of the phenothiazine family of dyes and redox indicators. It has a variety of uses including biomedical applications and biological activity (Moura & Cordeiro, 2003).

Methylene blue has been used in human and veterinary medicine for a number of therapeutic and diagnostic procedures including use as a stain in bacteriology, as a redox coloring agent, as a targeting agent for melanoma, as an antihemoglobinemic, and as a bacteriostatic genitourinary antiseptic and disinfectant (Merck, 2001). One of the most common clinical applications is for treating methemoglobinemia induced by overexposure to drugs, or to industrial chemicals such as nitrophenols at an intravenous dosage of 1-2 mg/kg (Harvey, 1980).

Another application is in the treatment of some psychiatric disorders because of the anxiolytic and antidepressant properties attributed to its ability to block activation of guanyl cyclase by nitric oxide (Naylor *et al*., 1986; Eroglu & Caglayan, 1997). In addition, MB has also been considered as an adjuvant therapy in treatment of schizophrenia (Deutsch *et al*., 1997) and as a chemotherapeutic agent for use by direct intratumoral injection in combination with photodynamic therapy (Orth *et al*., 1998). It is also being investigated in the management of Alzheimer's disease. Recently,

methylene blue was recommended for use in biopsies performed to identify lymphoma, indicating that new biomedical uses continue to be found for it.

In the event of ifosfamide neurotoxicity, methylene blue is used as treatment and prophylaxis. A toxic metabolite of ifosfamide, chloracetaldehyde (CAA), disrupts the mitochondrial respiratory chain, leading to an accumulation of NADH. Methylene blue acts as an alternative electron acceptor, and reverses the NADH inhibition of hepatic gluconeogenesis while also inhibiting the transformation of chloroethylamine into chloroacetaldehyde, and inhibits multiple amine oxidase activities, preventing the formation of CAA (Alici-Evcimen, 2007).

2.8 The potential of Methylene Blue as an antimalarial agent

Characterized by a short plasma half-life and a high bioavailability (18 h and 72%, respectively) (Walter-Sack *et al*., 2009), methylene blue (MB) was the first synthetic compound ever used in clinical therapy, dating back to the 1891 report of its antimalarial properties by the renowned chemist Paul Ehrlich.

As early as 1891, this thiazine dye methylene blue was reported to express antimalarial activity by Guttmann and Ehrlich (1891). However following the introduction of chloroquine and other drugs to the market, its potential as an antimalarial drug was not pursued (Ehrlich, 1913). It is best known as a synthetic dye.

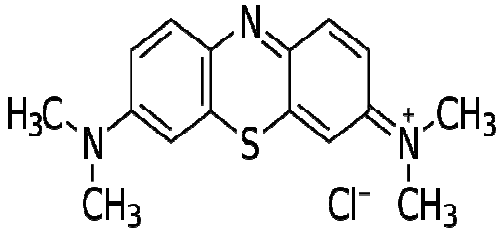


Figure 2.10: Methylene Blue

MB, shown in the Figure 2.10 above, is pleiotropic and exceptionally a weak base (pKa 0-l) accumulating inside malaria-infected red blood cell in which it is reduced and probably concentrated in the food vacuole where it inhibits the formation of hemozoin (Atamna *et al.*, 1996), just as 4-aminoquinolines do. Moreover, MB inhibits parasite glutathione reductase thus jeopardizes glutathione functionality, this effect results in glutathione depletion which sensitizes the parasite for chloroquine action.

Recently, both *in vitro* and *in vivo* studies as well as clinical trials have been done on MB in relation to its antimalarial activity with encouraging results. This goes to show that interest in MB has picked up again. It has been shown that the combination MB-CQ is safe in adults and children with and without G6PD deficiency (Rengelshausen *et al.*, 2004; Mandi *et al.,* 2005). Bountogo (2010) further recommended that MB needs to be combined with a rapidly acting partner drug like artemisinin. It was further pointed out that the main adverse effects whilst using the MB monotherapy were headache, dysuria and gastrointestinal symptoms. The headache and gastrointestinal symptoms could have been caused by the malaria itself.

Methylene blue has been categorized in a group of drugs considered to potentially cause haemolysis when given to persons with G6PD deficiency. However, in most of Sub-Sahara Africa, the class III G6PD deficiency dominates where there remains an enzyme activity of 15–25%, compared to only 0–5% in class II deficiency (Fleming, 2003). In addition, the methylene blue stain which has been given to malaria patients since the end of the 19th century has proven to be safe, well tolerated, and effective in combination with various malaria medications (Zoungrana *et al.,* 2008).

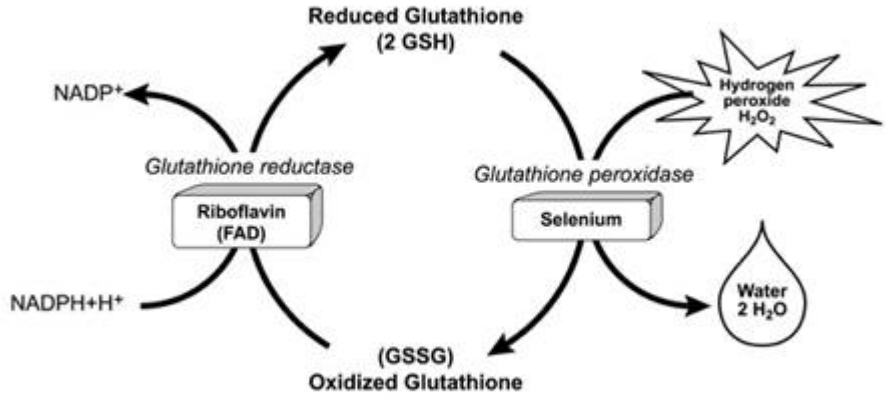


Figure 2.11: Glutathione redox cycle (Powers, 1999). The oxidized glutathione (GSSG) is redused by the flavin adenine dinucleotide (FAD) dependent enzyme, gluthathione reductase to glutathione (GSH). The GSH in a reaction catalysed by glutathione peroxidase, reduses the toxic hydrogen peroxide to two molecules of water.

It was noted that in the treatment of methemoglobinemia using MB, the MB also activated the hexose-monophosphate shunt of mammalian cells due to the oxidation of NADPH to NADP, and the consequent activation of glucose-6-phosphate dehydrogenase, which is the rate limiting enzyme of the shunt (Layne *et al.*, 1969). Atamna *et al.,* (1994) then demonstrated that MB also activated the shunt of *Plasmodium falciparum*. It is thus possible to infer from these studies that the antimalarial effect of MB could involve the oxidation of the parasite's NADPH, a co-factor needed for various essential metabolic activities which include the reduction of oxidized glutathione (through glutathione reductase) (Stryer, 1988). NADPH reduces glutathione via glutathione reductase, which converts reactive H_2O_2 into H_2O by glutathione peroxidase. In the absence of glutathione reductase, the H_2O_2 would be converted to hydroxyl free radicals by fenton chemistry, which can attack the cell (Luond *et al.,* 1998). Figure 2.11 above shows the overall glutathione mechanism.

Its other uses include treatment of methemoglobinemia, a condition that occurs when the blood cannot deliver oxygen where it is needed in the body. Methylene blue is also used as a dye to stain certain parts of the body before or during surgery.

2.9 Transfection technology in malaria research

The difficulty of working with human malaria parasites has resulted in a significant amount of research on different animal models of malaria like Baboon and mice. However, these models offer the only means to experimentally investigate natural host-parasite interactions *in vivo*.

An *in vivo* transfection technique has been developed and used on various species of the parasite. This has enabled investigation of the parasite at its genetic level and identification of new targets for chemotherapeutic or immunological intervention.

Transfection is a mechanism for mutating eukaryotic cells by introducing exogenous genetic material (Bothroyd, 1995). The introduced genetic material is expressed and retained either temporarily (transient transfection) or functionally maintained for longer periods (stable transfection) resulting in a genetically modified organism. In turn, transfection has been useful in determining the relationship between the structure of a gene and its expression and function following the genetic modification (Munyao *et al.,* 2008).

The development of transfection technologies in *Plasmodium* have led to substantial advances in malaria research (Carvalho *et al.,* 2005; van Dijk *et al*., 1995 and de Koning-Ward *et al.,* 2000). Van Dijk *et al*. (1995) showed that transfection by means of a pyrimethamine-resistance gene in *P. berghei* was stable. Initially, plasmid transfection vectors used exogenous dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) genes from either *Toxoplasma gondii* (*tgdhfr-ts*) or human sources (*hdhfr*) as selectable

markers (van Dijk *et al*., 1995; Wu *et al.*, 1996). The *tgdhfr-ts* gene is still widely used for transfection of the rodent malaria parasite *P. berghei* (van Dijk *et al.,* 1995). The DHFR-TS genes being selectable markers have point mutations that confer resistance to pyrimethamine thereby enabling the selection of recombinant *P. berghei* and *P. knowlesi* parasites (Tomas *et al.,* 1998). The *T. gondii* DHFR/TSR gene is generally preferred to reduce the possibility of unwanted recombination with the endogenous DHFR/TS gene, and because it confers resistance to higher concentrations of pyrimethamine.

The major advantage of using *P. berghei* over *P. falciparum* in transfection studies is that the transfection efficiency in *P. berghei* is much higher due to the ability to transfect blood stage merozoites directly, avoiding multiple membrane crossovers. One setback while transfecting the malaria parasites is their ability to maintain plasmids used for transfections as stable episomes (O'Donnell *et al.,* 2001). This problem can be circumvented in *P. berghei* by tranfecting blood-stage merozoites with linearized plasmids and only circular plasmids are used to transfect *P. falciparum* (Tomas *et al.,* 1998).

Transfection of *P. falciparum* is still limited by poor transfection efficiencies. The length of time required to obtain a stable transgenic culture can often be rate limiting and expensive. Electroporation of parasite-infected red blood cells is usually the technique of choice for introducing DNA in transfection experiments with *P. falciparum*, following the methods of Wu *et al*. (1996) (high voltage and low capacitance) and Fidock *et al*. (1997) (low voltage and high capacitance). Although there are other alternative approaches to transfection like the electroporation of noninfected red blood cells to allow spontaneous uptake of DNA and the use of polyamidoamine dendrimers to transfer DNA across membranes, they have been found to improve on transfection efficiency. However, it is not known if these techniques have an improved efficiency in establishing stable transfections. Transient transfection

efficiency is believed to be, 50 times higher than stable transfection. O'Donnell *et al.* (2002) had estimated that the transfection efficiency of plasmids into *P. falciparum* to be as low as $0.8x\ 10^{-6}$ parasites for stable transfections.

Transfection systems have been developed for both human and animal model species of *Plasmodium*, providing a broad range of genetic tools for the study of malaria parasite biology. Transient transfection has been used to provide insight into the regulation of gene expression by *Plasmodium* spp as well as promoter mapping. The development of stable transfection technologies has provided the opportunity to express transgenes in *Plasmodium* spp., as well as elucidate the function of proteins by disrupting, modifying, or replacing the genes encoding them. These genetic tools represent an important breakthrough for malaria research and will significantly contribute to our understanding of the biology of the parasite (de Koning, 2000)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

This was a laboratory based study, with both: *in vitro* and *in vivo* assessments. The study aimed at investigating the efficacy of methylene blue against drug resistant *Plasmodium* parasites both *in vitro* and *in vivo*. The *in vitro* study was done to test various concentrations of the MB on laboratory adapted parasite cultures of CQ resistant *P. falciparum* (W2 Strain). Owing to ethical reasons, this parasite was investigated *in vitro*.

Transfection technology was used to convert wild-type *P. berghei* ANKA into pyrimethamine resistant parasites. This was achieved through the introduction of plasmid DNA that had the *tgdhfr/ts* marker gene which conferred pyrimethamine resistance in the parasite. Upon completion of the transfection, PCR experiments were thereafter conducted to confirm transformation Plasmid vector used in the transfection was obtained from Promega Corporation; Wisconsin, USA through Hastings Ozwara.

In the *in vivo* approach, *P. berghei* resistant to chloroquine (CQ), pyrimethamine (PYR) and lumefantrine (LM) respectively were used. For comparison purposes, *P. yoelii yoelii 17X* (MR4, LOT: 58579702) was also tested. In summary different groups of Balb/C mice were infected with the said lines of the parasites and treated by intraperitoneal injections of MB regimen for four days at a predetermined dose following the establishment of a successful infection. Prior to using the MB dose for treatment, dose determining experiments were conducted to determine a suitable dose that would be

used and at the same time well tolerated by the animals as indicated by animal survivorship.

The efficacy of the treatment was determined by the *4*-Day Suppressive test using tail blood that was obtained by snipping 1-2mm off the tip of the mouse's tail using a pair of dissection scissors swabbed with 70% ethanol and on subsequent days, a cotton gauze swab was used to disturb the wound. Thin blood smears were prepared on frosted ended 1" x 3" microscope slides, fixed with absolute methanol, allowed to air dry then stained with 10% Giemsa stain solution for 10 minutes. Excess stain was washed off under running tap water and the stained smears air dried. Thin blood smears were examined using a binocular laboratory microscope (Zeiss Standard 20, Germany) at x100 objective lens under oil immersion and parasite load determined.

3.2 Experimental Animals

130 randomly bred BALB/c mice of either sex were used in the study. They were obtained from the Rodent Facility of the Institute of Primate Research (IPR) and the animal house of Kenya Medical Research Institute (KEMRI). This strain was preferred because it is inbred and is susceptible. Mice aged 8–10 weeks old, weighing of 20 ± 2 grams were used herein. The animals were housed in experimental rooms in a standard Macrolon type II cages clearly labeled with experimental details at 22^{o}C and 60–70% relative humidity and fed on commercial rodent feed and water *ad libitum*.

3.3 Parasites

Chloroquine resistant *P. falciparum* (Indo-China W2 strain) used were kindly donated by the Malaria Laboratory, KEMRI. In addition, the Lumefantrine resistant *P. berghei* ANKA strain parasites that were used in the experiments conducted were developed at and provided by KEMRI.

The blood-stage parasites of *Plasmodium berghei* ANKA strain that were used in this study to induce malaria infection in experimental mice for dose finding and transfection purposes were derived from the biobank at IPR. The Chloroquine resistant *P. berghei* (RC clone) used in the methylene blue studies were obtained from the MR4 repository (MR4, ATCC® Manassas, Virginia).

All mice infections were initiated by intraperitoneal (i.p) injection of 10^6 infected red blood cells (parasites) and examined by Giemsa stained thin smears.

3.4 Preparation of Plasmids conferring Pyrimethamine Resistance

3.4.1 Transformation of *E. coli* cells (DH5-α) using the transfection constructs.

To generate many copies of the transfection constructs (both experimental and control), competent DH5-α cells (*E. coli* cells) were transformed using heat shock method. Five microlitres of pUC-backboned plasmid sample (pD.D$_{TM}$.D./D.-.D.) that contained the *tgdhfr* gene were transferred into round bottomed tubes and 100µl of the electro competent DH5α cells then added to the plasmids. After 30 minutes of incubation, the tubes containing plasmid/bacterial cells mixture were put in a waterbath (Precision Scientific, USA, Cat No. 66798) set at 42^{o}C for 20 seconds. The tubes were thereafter returned on ice and allowed to incubate for 2 minutes. 1 ml of non selective liquid broth (LB broth) was then added to the mixture and incubated for 1 hour at 37^{o}C, 225 r.p.m in an incubator shaker. The cell suspensions were then plated on nutrient agar plates impregnated with ampicilin and incubated overnight at 37^{o}C. Growth was checked after the overnight incubation and the growth of white single colonies was an indication of transformation. Single colonies were then picked with each colony being put in a different tube containing 3ml selective LB broth with ampicilin (50µg/ml), and

incubated in an incubator shaker at 37^{o}C, 2.403 g for 16-18hrs to allow multiplication of the transformed cells. This was as described by Inoue *et al*., (1990).

3.4.2 Isolation of Plasmid DNA from DH 5-α Cells

Incubation of the transformed bacterial cells was aimed at increasing the quantity of plasmids of interest. The isolation of plasmid DNA was done according to protocols by Ehrt and Schnappinger (2003). The bacterial culture was divided into two 1.5 ml eppendorf tubes. The tubes were then centrifuged at 10,000 rpm in an eppendorf microfuge for 1 minute for separation of the supernatant that was later discarded. 100 µl of ice cold alkaline lysis solution I (P1) were then added into each pellet and vortexed then let to stand at room temperature for two minutes. 200µl of alkaline solution II (P2) were then added and severally inverted to mix. Lastly about 150µl of ice cold alkaline lysis solution III (P3) was added followed by mixing by inverting severally before incubating in ice for 5 minutes. After the incubation on ice, the solutions were centrifuged in a microfuge for 5 minutes to obtain a pellet and the supernatant was discarded. Isopropanol was then used to fill up the tubes and allowed to sit for 2 minutes to precipitate nucleic acids. This was later followed by centrifugation and supernatant poured off. During bacteria lysis using the solution P2 which contains sodium dodecyl sulfate and sodium hydroxide (Appendix I), chromosomal as well as plasmid DNA get denatured. Subsequent neutralization with potassium acetate (in solution P3) allows only the covalently closed plasmid DNA to reanneal and stay solubulized while most of the bacterial chromosomal DNA and proteins precipitate in a complex formed with potassium acetate and SDS. The complex is later removed by centrifugation (Ehrt & Schnappinger, 2003).

1 ml of ice-cold 70% ethanol was carefully added followed by several gentle inversions before spinning at 10,000 rpm for 3 minutes to precipitate the DNA plasmids. The

supernatant was poured off and the tube drained on paper towel. The pellets were later dissolved in 30µl of Tris EDTA containing RNAse (Anderson, 1997).

3.4.3 Restriction Digestion

Restriction digestion was done using the enzymes: *BamHI, EcoRI* and *EcoRV* (Invitrogen; Carlsbad; CA) to confirm that the correct plasmid was isolated. Three microlitres of plasmid samples were transferred into eppendorf tubes, 1 µl of 10x buffer and 1µl of restriction enzyme added respectively. The reaction solutions were topped up with 5µl of triple distilled water. The mixture was then incubated in a water bath at 37^{o}C for one hour for the restriction digestion to take place. An agarose gel electrophoresis was thereafter performed to view fragments and photos of the gel taken alongside a suitable marker ladder (Waters *et al.,* 1997).

3.4.4 Preparation of plasmid DNA for *P. berghei* transfection.

Ethanol precipitation was used to concentrate the DNA for transfection. Extracted plasmid DNA samples that were collected earlier were pooled into eppendorff tubes (approximately 300µl per tube). To each tube, 30µl of 5M potassium acetate were added. This was followed by the addition of 180µl of isopropanol. The contents in the tubes were thereafter well mixed and stored at -20^{o}C freezer overnight. The following morning, the tubes were thawed at 4^{o}C then centrifuged in a microfuge at 12000 rpm for 15 min at 4^{o}C. The supernatant was drained off on paper towels and 70% ethanol added at two volumes the original plasmid sample volumes. After adding the 70% ethanol, the mixture was incubated for 10 min and then centrifuged for 5 min at room temperature. The supernatant again was carefully drained off on paper towels and the pellet let to dry. Each plasmid DNA construct was resuspended in 300 µl of sterile cytomix ready for transformation of PYR sensitive *P. berghei* into PYR resistant lines via transfection (Waters *et al.,* 1997).

3.5 Transfection of wild-type *P. berghei*

Wild-type *P. berghei* ANKA strain stabilates were obtained from liquid nitrogen storage at IPR. Retrieval, infection and monitoring of parasitaemia of mice was done following IPR standard operating procedures (SOPs) (IPR, 2002). Cryopreserved *P. berghei* ANKA stabilates were retrieved from 9 x 9 cryoboxes from liquid nitrogen storage (Thermolyne Locator 4 Cryobiological Storage System, USA, Product No. CY50935) quickly thawed in a 37°C water bath (Precision Scientific, USA, Cat No. 66798) and diluted with an equal volume of sterile 1x phosphate buffered saline (PBS) pH 7.2. This preparation was then loaded into 1ml syringes with 29G x ½" needles (Ken Healthcare, China) and 100µl administered intraperitoneally into each of 5 BALB/c mice. Parasitaemia was assessed by microscopic examination of Giemsa-stained thin smears of tail blood starting day 3 post-infection.

At about 5% parasitaemia and with a high proportion of schizont stage, the mice were sacrificed and blood collected via cardiac puncture using a pre-heparinized 1ml syringes with 29G x ½" needles (Ken Healthcare, China) (IPR, 2002). The blood was immediately collected into a sterile 15ml centrifuge tube and put in an 18 hour culture. This culture contained 30 ml of complete culture medium (RPMI 1640 supplemented with 20% fetal calf serum with L-glutamine and 25mM HEPES, 2g of $NaHCO_3$ per litre of culture medium and 50000 I.U. of Neomycin). The standard culture conditions were as follows: RPMI 1640 medium, pH 7.3; 20% fetal calf serum; 4.9% O_2, 5.1% CO_2, 90% N_2 gas mixture (BOC gas); 1 - 4% cell suspension; 37^oC.

Once the presence of schizonts was confirmed in the culture, the culture was thereafter carefully layered on 10ml of 55% Nycodenz solution (27.5 ml Nycondenz stock and 22.5 ml PBS), and centrifuged at 1200 rpm in a swing out rotor at room temperature for half an hour. The brown layer that appeared contains schizonts; this layer was carefully collected out using Pasteur pipette. 20 ml of the culture media was added to the

collected schizonts (brown layer) and centrifuged at 12.478 g for 8 minutes to wash out excess nycodenz. The supernatant was discarded and the resulting pellet resuspended in 1 ml culture medium.

A 100µl schizont suspension was added to 300 µl of sterile cytomix (containing the plasmid DNA construct earlier developed) and subjected to standard electroporation conditions using the Gene Pulser II® (Bio-Rad) as described by de Koning *et al.* (2000) and Waters *et al.* (1997). A 0.4cm cuvette was used in this procedure. After the electroporation, the suspension was placed on ice for about 4 minutes.

The electroporation conditions result in rupture of fragile schizonts, thereby liberating the merozoites that in turn take up the transfection construct (plasmid DNA). The same effect can be achieved in intra erythrocytic stages of the parasites

3.5.1 Selection of transfected parasites in infected mice

The complete suspension (about 400 µl) of electroporated parasites was then topped to a volume of 500µl with complete culture medium. Using a 27-G needle, 100µl of the electroporated mixture were injected intravenously into 5 Balb/C mice via the tail vein.

After the inoculation, the mice were put on a pyrimethamine treatment at 0.07mg/ml in the drinking water. This was done to select out the successfully transformed PYR resistant *P. berghei.* The pyrimethamine solution in the drinking water was prepared by dissolving pyrimethamine in dimethyl sulfoxide (DMSO) to a final concentration of 7mg/ml (stock solution). Then diluted 100 times with distilled water and its pH adjusted to between 3.5 - 5.0 using 1M HCL. The treatment was done for 4 days; thin smears were made to monitor the progress of parasite growth. This dose effectively kills sensitive parasites, while the growth and multiplication of parasites containing the resistant DHFR/TS gene are normal.

3.5.2 Determining the efficacy of the parasite transformation by PCR.

To determine the whether the transfected DNA was present in the resistant parasites, PCR was carried out on blood samples obtained from the mice infected with the transformed parasites.

From group of mice earlier infected with transfected parasites, a mouse with parasitaemia of 5% the mouse was sacrificed and heart blood obtained using a pre-heparinized 23-G needle and 1ml syringe. The blood was mixed with 1ml of 1x PBS (pH 7.2), the blood/PBS mixture was carefully aliquoted into eppendorf tubes containing lymphocyte separation media at the ratio 2:1. The tubes were then centrifuged at 2000 x g for 25 min and the pellet suspended in 400µl of RBC lysis solution (0.15% saponin in RPMI). The tubes were incubated at room temperature for 10 min and then centrifuged at 1,062 g in a Beckman-GPR centrifuge. This step was repeated two more times until the pellet was lightly coloured. The pellet was vigorously vortexed to resuspend the cells, then 300µl of cell lysis solution (PureGene from Gentra Systems) added. The cell suspension was mixed gently with a pipette to ensure homogeneity. The mixture was centrifuged at 9,012 g for 3 min in an eppendorff 1540 microfuge. The supernatant was carefully transferred into a clean tube containing 300µl of isopropanol. This was mixed gently 50 times, and centrifuged at 13000 rpm in the microfuge to pellet the DNA. The supernatant was poured off and 1ml of 70% ethanol added and spun at 9,012g to wash the pellet. After the supernatant was poured off, the tubes were inverted on a paper towel to dry off for 15 min then the pellet resuspended in TE buffer for storage at 4°C.

3.5.3 Plasmid rescue of circular transfection vectors

To confirm that parasites were transfected with the correct plasmid, total parasite DNA was isolated as described in 3.5.2 above, and 1µg of parasite DNA was used to transform 50µl of competent *E. coli* by electroporation (Bio-Rad Gene Pulser I). This

was done by first mixing the bacterial cells and parasite DNA then incubating them on ice for 30 min, the DNA/cell mixture was then transferred into an electroporator set at 2.4Kv, 25μF and 200 Ω. Immediately after pulsing, the mixture was transferred to a culture tube and 950μl of LB broth added. This was incubated at 37°C for 1 hr with shaking at 225 rpm. The cell suspensions were streaked on selective LB agar plates containing 50μl/ml ampicilin incubated overnight at 37°C. The following day, singled bacterial colonies were picked and cultured in LB broth containing 50μg/ml ampicilin for 18hr after which plasmid isolation was carried out as described earlier in section 3.4.2. Plasmid DNA isolated was analyzed by digestion with restriction enzymes as earlier pointed in section 3.4.3 (Waters *et al.,* 1997).

3.5.4 PCR of plasmid DNA in transformed parasites

Total DNA isolated from the parasite was diluted to a concentration of 50ng/μl. PCR was performed to detect the *T. gondii* DHFR gene in the total parasite DNA collected. For each sample tube, a total or 25μl of reaction mixture was added containing the following: 2.5μl of 10x PCR buffer, 1.5μl of 25mM $MgCl_2$, 0.5μl of *Taq*, 2.5 μl of forward primer (5'-ATGCATAAACCGGTGTGTCTG-3'), 2.5μl of reverse primer (5'-CGTGATCAAAGCTTCTGTATTTCCGC-3'), 1μl of dNTPs, 1μl of sample DNA and 13.5μl of triple distilled water.

The PCR conditions were set as follows: Initial denaturation temperature of 94°C for 3 min, then 30 cycles of Denaturation (94°C, 1 min), Annealing (54°C, 30sec), Elongation (72°C, 1 min), and a final elongation at 72°C for 4 min as described by Menecuer *et al.* (2008). The amplicons were thereafter analyzed by electrophoresis in 2% agarose gel.

3.6 *In vivo* Assessment of Methylene Blue's Efficacy on malaria infected mice

3.6.1 Drug preparations

Methylene blue, pyrimethamine, artemether, lumefantrine and chloroquine used in the study for experiments or control purposes were weighed and dissolved as described. Methylene blue (Riedel-de Haen ®28514, Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) was weighed accordingly and dissolved in double distilled water to make a 45mg/kg dose solution for 4 days use. Before administration, the solution was vortexed to ensure thorough mixing. For the 4 different test groups, fresh MB solution was prepared. This preparation was administered by intraperitoneal injection.

Pyrimethamine (Sigma ®) (PYR) for a 1mg/kg dose for a 4 day treatment regimen was weighed and dissolved in 5% (v/v) DMSO. This was then vortexed gently to mix then topped up with 1X PBS and pH adjusted to 4.0. It was thereafter filter sterilized through 0.45µm pore-size membrane filter (Sartorius Stedim Biotech, USA, Ministart ® LOT: 1655501219103) and stored at 4^{o}C until use. Pyrimethamine was administered orally.

Artemether (ATM) and Lumefantrine (LM) were weighed and dissolved in a vehicle that consisted of 70% Tween 80 (d = 1.08 g/ml) and 30% ethanol (d = 0.81 g/ml) which was subsequently diluted 10-fold with double distilled water. On the day of administration the drug was freshly prepared. The drug was administered orally. The Artemether used was for a 10mg/kg dose while the lumefantrine was for a 50mg/kg dose.

Chloroquine diphosphate (CQ) was also weighed and dissolved in double distilled water to make a 10mg/kg dose. This solution was for oral administration. Drugs administered orally were given at 0.2ml per mouse

3.6.2 Parasite propagation and challenge infections

Retrieval, infection and monitoring of parasitaemia of mice was done in accordance with IPR standard operating procedures (SOPs) (IPR, 2002). Cryopreserved stabilates of sensitive *P. berghei* ANKA were retrieved from 9 x 9 cryoboxes from liquid nitrogen storage (Thermolyne Locator 4 Cryobiological Storage System, USA, Product No. CY50935) quickly thawed in a 37°C water bath (Precision Scientific, USA, Cat No. 66798) and diluted with an equal volume of sterile 1x phosphate buffered saline (PBS) pH 7.2. This preparation was then loaded into 1ml syringes with 29G x ½" needles (Ken Healthcare, China) and 100µl administered intraperitoneally into each of 5 BALB/c mice. Parasitaemia was assessed by microscopic examination of Giemsa-stained thin smears of tail blood starting day 3 post-infection (IPR, 2002).

On day 3 post-infection, tail blood was obtained by snipping 1-2mm off the tip of the mouse's tail using a pair of dissection scissors swabbed with 70% ethanol and on subsequent days, a cotton gauze swab was used to disturb the wound. Thin blood smears were prepared on frosted ended 1" x 3" microscope slides (IMED, China, Cat. No. 7105), fixed with absolute methanol and stained with 10% Giemsa stain for 10 minutes. Excess stain was washed off under running tap water and the stained smears air dried. Thin blood smears were examined using a binocular laboratory microscope (Zeiss Standard 20, Germany) at x100 objective lens under oil immersion (Cargille, USA, Cat No. 1648). Percent parasitaemia was calculated as follows:

$$\text{Percentage parasitaemia} = \frac{\text{(Number of infected erythrocytes)}}{\text{(Total number of erythrocytes counted)}} \times 100$$

Where the total number of erythrocytes counted was at least 2000.

At mean parasitaemia of 7-10%, mice were sacrificed and bled via cardiac puncture. Blood was then pooled to determine pooled parasitaemia. The approximate number of

RBCs in mouse heart blood is $9x10^6/\mu l$. The following calculation was used to determine the dilutions necessary to give the required number of parasitized RBCs for the challenge infection of 1 x $10^6/100\mu l$ in RPMI 1640 (Gibco, BRL).

Total number of RBCs = $9x10^6/\mu l$ x pooled blood volume
Number of parasitized RBCs = Pooled % Parasitaemia x Total number of RBCs
Number of parasitized RBCs required = 1 x 10^6 x Number of mice to be infected
Volume of parasitized RBCs required = (Number of parasitized RBCs required x Total volume of pooled blood) / number of parasitized RBCs
The volume of parasitized RBCs required was then diluted to a final concentration of 1 x $10^6/100\mu l$ using RPMI 1640 media (Gibco, BRL) per mice to be infected.

3.6.3 Dose Determination Experiments

Twenty five BALB/c mice were injected with the challenge infection of 1 x 10^6 parasites per mouse and randomly divided into 5 groups. Upon establishment of a successful infection, usually day 3-4 post infection, the groups received a 5mg/kg, 15mg/kg, 30mg/kg, 45mg/kg and 60 mg/kg dose of Methylene Blue dissolved in distilled water for 4 days by intraperitoneal injection. Parasitaemia was then determined 24 hrs immediately after the last treatment.

Parasitaemia was monitored daily until day 28 or when the mice died. Further to this, survivorship was also observed. The data collected on the five experimental doses of MB was analyzed and used to determine the most appropriate dose to use on the selected resistant *P. berghei* parasites.

3.6.4 MB experiments on resistant *P. berghei*

Three lines of *P. berghei* parasites were used in the *in vivo* assessment of methylene blue's antimalarial activity on resistant parasites. The analyzed parasites were the chloroquine (CQ) resistant (RC Clone), lumefantrine (LM) resistant and pyrimethamine (PYR) resistant *P. berghei.* The Lumefantrine resistant parasites were generously

donated by the Centre for Traditional Medicine and Drug Research, KEMRI thus experiments on this strain of parasites was done at KEMRI. Chloroquine resistant parasites were sourced from the MR4 repository in Virginia, USA. Pyrimethamine resistant parasites used were developed by transfection technology as earlier described.

Fifteen donor mice were obtained, labeled and divided into 3 groups. They were used for propagation of the resistant parasites as described in section 3.6.2. To further determine the activity of MB on other species of rodent malaria parasites, *P. yoelii* 17X was also propagated in BALB/c mice and subsequently used for challenge infections as in 3.6.2 above.

At mean parasitaemia of 7-10%, the donor mice were sacrificed and bled via cardiac puncture. Blood was then pooled to determine pooled parasitaemia. Using the calculations in 3.6.2 it was possible to determine what dilutions were necessary to give the required number of parasitized RBCs for the challenge infection of 1 x 10^6/100µl in RPMI 1640 (Gibco, BRL). For each parasite strain, groups of mice were infected with an innoculum of 1 x 10^6 parasites for the MB testing as demonstrated in Figure 3.1.

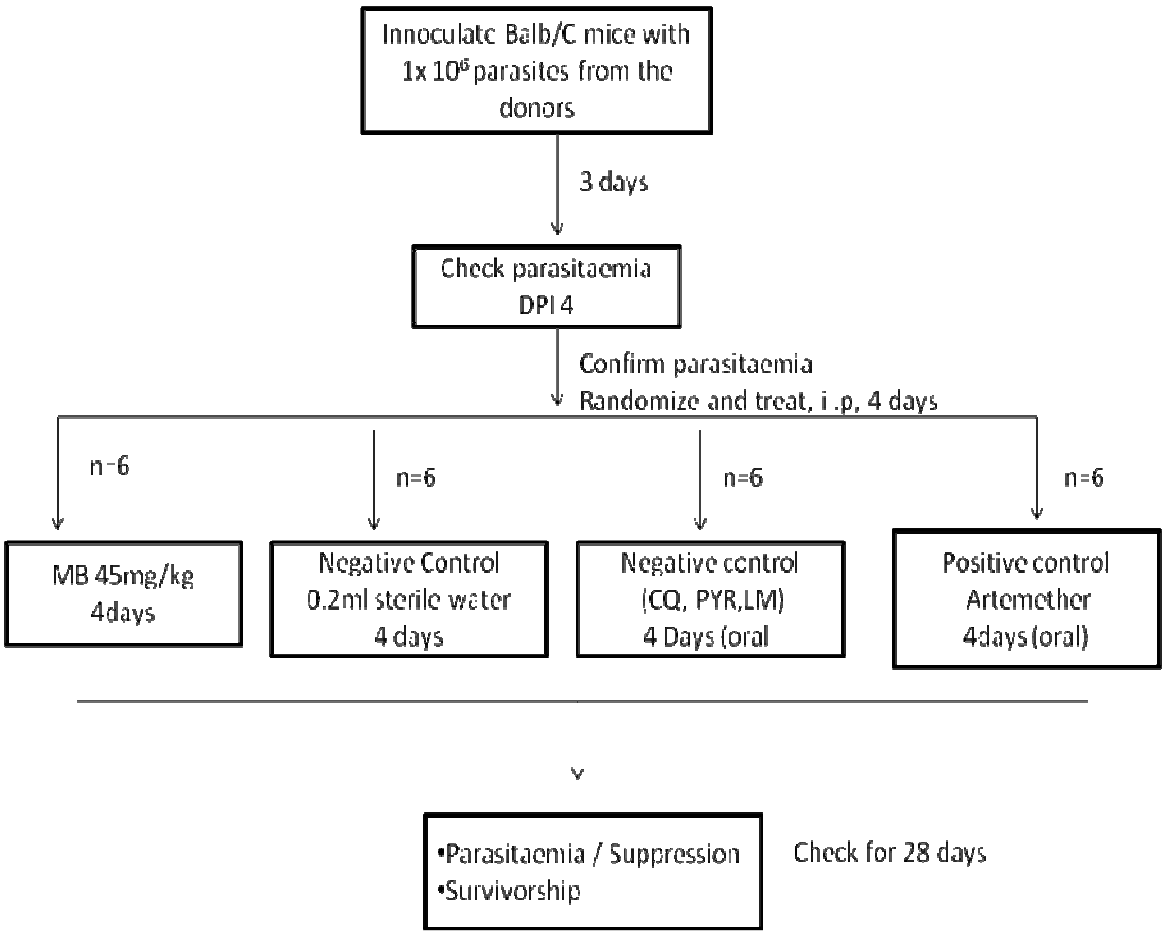


Figure 3.1: Intervention schedule for drug resistant *P. berghei* infected mice

For the *P. yoelii 17X* parasites, the donor mice were sacrificed and the blood pooled to determine the mean parasitaemia. The blood was used to determine what volume of the infected blood would be diluted with RPMI 1640 to get an infection dose of 1 x 10^6 parasites per mouse. Five experimental mice were thereafter infected with the *P. yoelii* parasites. After ascertaining a successful infection on day 3 post infection, just as with the experimental mice in the resistant *P. berghei* experiments, the mice got a 4 day dose of MB 45mg/kg.

3.6.5 Determining antimalarial activity of Methylene Blue in infected mice

Parasitaemia was used to determine how active MB was against the test parasites. Parasite count was estimated by microscopic examination of Giemsa-stained thin smears prepared from tail snips 24 hours after administration of the last dose for all the experimental and control groups. Thin smears were prepared and percentage parasitaemia was determined as earlier outlined in section 3.6.2.

Percentage chemosuppression of the test dose was then calculated as [(A -B)/A] x 100], where A is the mean parasitaemia in the negative control group and B is the parasitaemia in the test group. Parasitaemia was monitored for 28 days to determine how soon, if any, recrudescence would occur.

Survivorship was used to determine how long the mice would survive following administration of the MB preparations. Survivorship was calculated as a percentage of mice still alive per group following infection until all died or when experiment was terminated, and improved survivorship was attributed to the effect of the MB intervention used.

3.7 *In vitro* Assessment of Methylene Blue against Chloroquine resistant *P. falciparum*

Chloroquine resistant *P. falciparum* isolates (the W2 strain) were used in the *in vitro* assessment of MB. This parasite stabilate was obtained from Malaria Laboratory– KEMRI and maintained in continuous culture at IPR.

3.7.1 Preparation of culture media

The culture media was prepared by dissolving 10.4g of powdered RPMI 1640 (1X with GlutaMAX^{TM} and 25mM HEPES, GIBCO) in 1L of sterile double distilled water. The medium was then filtered through a 0.22µm filter unit (Nalgene®) and stored at 4^{o}C. 500ml of incomplete culture media was prepared by adding 19ml of sterile 5% $NaHCO_3$, 5ml of sterile 20% D-Glucose (Sigma, Cell culture tested), 5ml L-Glutamine (200mM), 19ml of HEPES (1M) (Sigma, HEPES Buffer solution 1M in H_2O) and 250µl Gentamicin solution (50mg/ml stock solution) to 452ml of the prepared sterile RPMI 1640 media. The incomplete media was kept sterile and stored at 4^{o}C until use. The complete culture media needed to grow the parasites was prepared by adding 1 volume of heat inactivated sterile human serum to 9 volumes of the earlier made incomplete media. This produced a 10% complete culture medium was developed. The complete media was also stored at 4^{o}C.

3.7.2 Thawing of cryopreserved *P. falciparum* stabilates and setting up continuous culture

Three sterile salt solutions were prepared for the thawing process (A -12% NaCl, B - 1.6% NaCl, and C - 0.9% NaCl). The solutions were filter sterilized using 0.22µm filter (Sartorius Stedim Biotech, USA, Ministart ®). A vial of cryopreserved *P. falciparum* (W2) parasites was removed from the liquid nitrogen and quickly thawed in water bath at 37ºC until fully thawed. The contents were then transferred into a sterile 50ml Corning centrifuge tube. For every 1ml of thawed blood 0.2ml of 12% NaCl was added dropwise while mixing constantly and the tube left to stand for 3 minutes. Then 10ml of the 1.6% NaCl solution for each 1ml of original thawed blood volume was added drop by drop again while mixing constantly. This was left to stand for 5 minutes. Finally, 10ml of 0.9% NaCl solution was added dropwise while mixing, per 1ml of original stabilate volume.

The suspension was then centrifuged at 1500 rpm for 5 minutes at room temperature (Beckman-GPR centrifuge). Supernatant was removed and the pellet resuspend in 5ml of pre-warmed complete medium. A drop of washed fresh O+ red blood cells was then added to the resuspended pellet and mixed well. The cells were there after transferred to a T25 culture flask (Corning ® Flask Plug seal cap 430168). The culture was later gassed for at least 1 minute with the special gas mixture (5%O_2, 5%CO_2 and 90%N_2). After gassing and the cap sealed airtight, the flask was incubated at 36.5°C (Trager and Jensen, 1976; Updated Methods in Malaria Research, 2008).

The media was changed after every 48hours and parasite growth monitored by microscopy. The culture was maintained at 2.5% hematocrit and 2% parasitaemia. This was achieved by dilutions of either adding more complete media or washed red blood cells.

3.7.3 Setting up a 96 well culture

Parasitized culture (from continuous culture in 3.6.2) was diluted with un-infected red blood cells (blood group O+) to obtain 0.4 - 0.5% parasitemia of >80% ring stage parasites and with complete culture media solution (supplemented with 10% heat inactivated serum of the same blood group or pooled A, B, and O) to 1.5% haematocrit.

A thin smear was then made to verify initial parasitaemia and give a start point for calculation of the required ‘parameters’ for diluting the culture to desired parasitaemia (Appendix II)

Two hundred microlitres of the diluted culture were added to each of the pre-dosed wells (starting with well A proceeding to well H, the lower drug concentrations). The lid was replaced and fastened in position with strip of autoclave tape. The plate was labeled

accordingly with the details on type of isolate and test drugs. The drugs tested were methylene blue, chloroquine, artemether and pyrimethamine at concentrations ranging from 7.813 to 1000 ng/ml.

3.7.4 Incubation of the plate culture

Before use, a glass desiccator jar was cleaned with 70% alcohol and moistened sheets of paper towel placed on the base of the chamber to provide a humid atmosphere for parasite growth. The plate was then gently placed on top of the moistened paper towel in the jar, a candle lit and the jar lid replaced. The jar was made airtight by sealing the lid with petroleum jelly at the mouth. Incubation was done at 36.5°C for 72 hours.

After the incubation period, the contents in the wells of the plate were transferred into pre-labeled eppendorf tubes and centrifuged at maximum speed for a minute using an Eppendorf Microfuge centrifuge 5414. The pellets obtained were used to make thin smears that were later fixed then stained with 10% Geimsa stain (as described in 3.6.2).

Thin blood smears were examined using a binocular laboratory microscope (Zeiss Standard 20, Germany) at x100 objective lens under oil immersion (Cargille, USA, Cat No. 1648). Percent parasitaemia was calculated as follows:

$$\text{Percentage parasitaemia} = \frac{\text{(Number of infected erythrocytes)}}{\text{(Total number of erythrocytes counted)}} \times 100$$

$$\text{Percentage inhibition} = \frac{\text{(parasitaemia in control)} - \text{(parasitaemia in drug-treated)}}{\text{(parasitaemia in control)}} \times 100$$

Where the total number of erythrocytes counted was at least 2000.

Data collected was used to determine the IC_{50} and percent inhibition of the test compounds used

3.7.5 Preservation of *P. falciparum* parasites

The pellet volume from the excess volume of *P. falciparum* infected culture from the parasite propagation experiment was determined by centrifuging at 1500rpm for 10 minutes at room temperature. The pellet was washed once with incomplete RPMI 1640. To the pellet 1.5 times equal volumes of heat inactivated human serum were slowly added dropwise with gentle mixing. This was later followed by the addition of 2.5 times equal volume of the sterile freezing solution (28% Glycerol, 3% Sorbitol and 0.65% NaCl in distilled water) also dropwise with gentle shaking to mix before aliquoting the suspension into cryovials (Greiner Bio One, CRYO.STM, Germany). The cryovials were placed into a freezing chamber (Nalgene Cryo 1^{o}C/min freezing container) and put in a freezer overnight at -80 ^{o}C .The following morning the *P. falciparum* parasite stabilates were transferred into a cryobox (Nalgene®, Thermo Fisher 31 Scientific, UK, Cat. No. CRY-180-020U) and placed in liquid nitrogen tank (Thermolyne Locator® 4 Cryobiological Storage System, USA, Product No. CY50935).

3.8 Ethical Considerations

Clearance to conduct this study was obtained from IPR's Institutional Research Ethics Committee (IRC) that comprises the Animal Care and Use Committee (IRC/12/11). The animals were maintained according to institutional animal care and use policy (ACUC), under the supervision of Animal Resources staff. Further to this, the mice received feed and water *ad libitum* throughout the experiment and were housed in standard Macrolon type II cages.

The biological waste generated during the experiments were first disinfected in 10% sodium hypochlorite solution before being disposed by incineration in accordance with IPR's waste management procedure. Precautions were taken when handling transgenic

resistant parasites. The parasites that remained after the experiment were cryopreserved in liquid nitrogen.

3.9 Data Management and Analysis

Data was stored on data sheets as both hard copy and as electronic copy in Microsoft Excel 2007 software. These were locked in safety cabinets accessible only by authorized persons. Soft copies were stored in computers and memory sticks. Backups made in both hard copy and electronic copy, were stored in a lockable cabinet. Graphs and charts were presented using MS Excel spreadsheet and GraphPad Prism 5.0. Statistical significance was calculated using the student t test for inter-groups comparisons. *P* values $\leq$ 0.05 were considered significant at 95% confidence intervals. Nonlinear regression analysis of log dose/response curves was used to determine the IC50 of the compounds used in the in vitro experiment.

CHAPTER FOUR

RESULTS

4.1 *TgDHFR* detection in transfected *P. berghei* parasites

Plate 4.1 exhibits PCR results from plasmid DNA isolated from transfected parasites compared to PCR products from positive control plasmid containing *TgDHFR* gene. This confirmed successful incorporation of the plasmid into the parasite. Subsequent data obtained from *in vivo* assays using the transformed parasites confirmed that the gene that conferred resistance to pyrimethamine was present in the transfected *P. berghei* (see Figure 4.7). From the bioassays, it was observed that there was steady increase in parasitaemia of the transfected parasites even when treated with 1 mg/kg pyrimethamine. This further demonstrated a successful transfection.

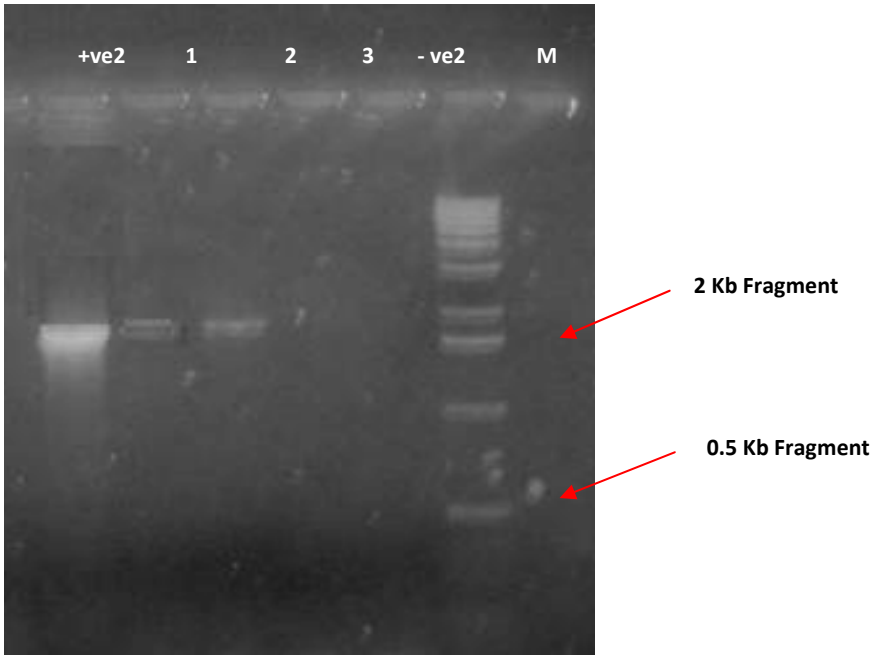


Plate 4.1 PCR detection of *TgDHFR* in transfected *P. berghei* DNA : +ve2- positive control plasmid containing *Tg*DHFR gene, 1 &2 DNA samples isolated from transfected

P. berghei, 3- DNA sample isolated from wild type *P. berghei*, -ve2 negative PCR control, M is the molecular ladder.

4.2 MB Dose Determining Experiments

Groups of experimental mice that had been infected with wild-type *P. berghei* ANKA parasites were treated with different doses of methylene blue in a experiments to determine the most effective and tolerable dose. Figure 4.1 shows parasitaemia curves of 3 doses of MB tested on wild-type parasites compared to that of parasites propagated in the absence of any intervention. The curve of wild-type parasites shows that the experimental conditions and setup were at optimum as shown by the steady rise in parasitaemia over time.

From Figure 4.1 there was significantly higher parasite clearing activity of MB 45 mg/kg compared to MB 15mg/kg (P=0.0329) whereas MB 5 mg/kg had no effective antiplasmodial activity. Methylene blue 45 mg/kg suppresses parasites in the first five days before the onset of recrudescence. This suggests that a higher dosage has better antimalarial activity on wild-type parasites. There was significant difference (P=0.0233) on parasitaemia when MB 45 mg/kg was used in comparison to parasitaemia in the absence of intervention. Methylene blue 45 mg/kg was thus chosen as the dose of choice in subsequent experiments to test this antimalarial activity on a variety of drug resistant parasites that were developed by passaging and drug pressure.

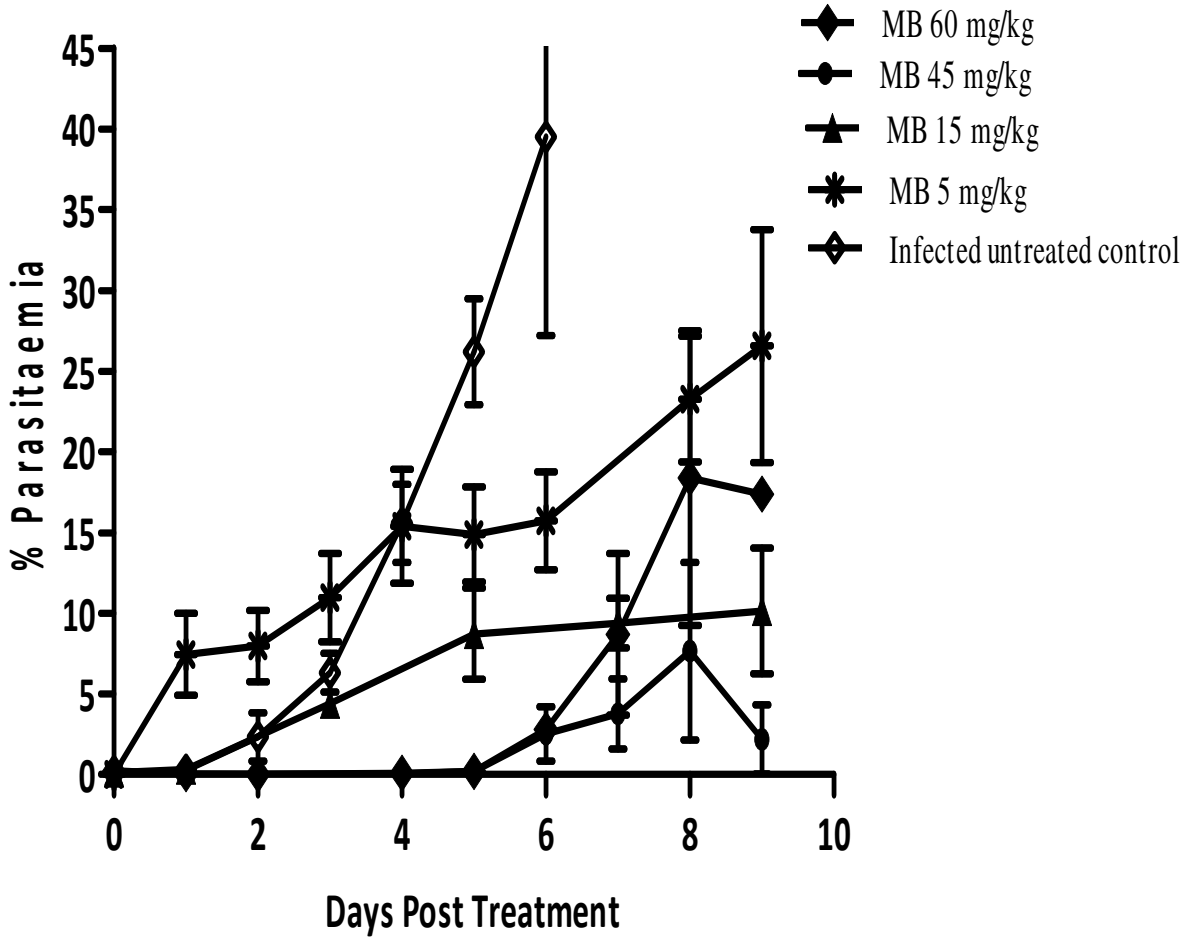


Figure 4.1: Parasitaemia of wild-type *P. berghei* responses to various doses of Methylene Blue (MB). Each treatment group had 5 infected mice

In addition, fur appearance and behavior of MB treated mice were normal compared to uninfected mice including mobility. The general animal health appeared good compared to mice that were infected but not treated. Mice in MB treated groups lived relatively longer than those in the infected-non-treated group.

The use of MB 45mg/kg resulted in up to 100% erythrocytic parasite clearance five days post treatment as shown in Figure 4.1. This provided the baseline evidence that MB at 45mg/kg was effective thus informing its choice of use in studies involving resistant parasites.

Plate 4.2 shows the effect of MB on parasitaemia. Yellow arrows in [A] show the *Plasmodium* parasites before the MB intervention. Following the treatment with MB as shown in [B], the parasites appeared to have disintegrated and declined in number - shown by red arrow.

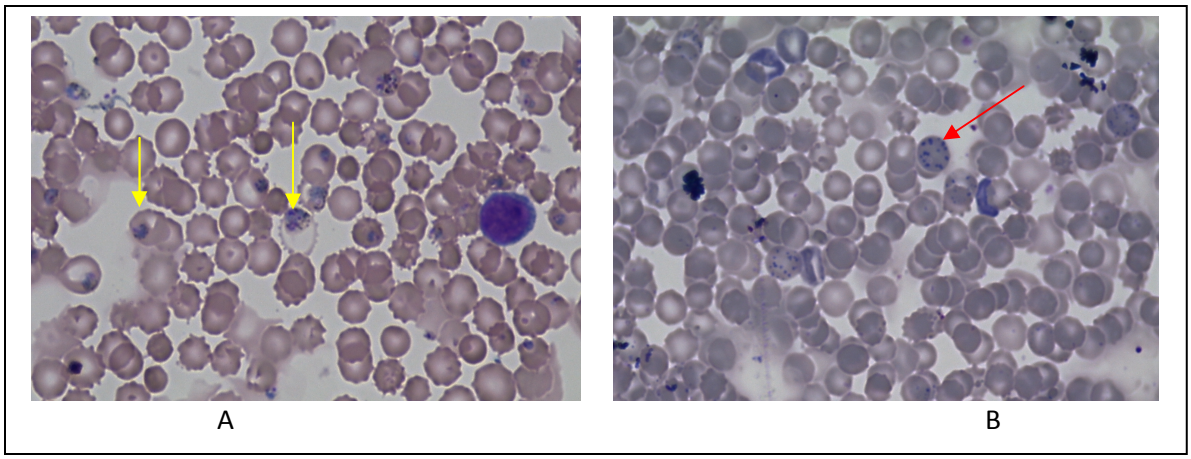


Plate 4.2: Parasitaemia before (A) and after (B) treatment with MB. There is parasite clearance after treating with MB

To further ascertain its activity against other rodent parasites, MB was tested on *P. yoelii 17X* as shown in Figure 4.2. When compared against MB 45mg/kg on wild-type *P. berghei*, there was no statistically significant difference in the means of the *P. berghei* and *P. yoelii 17X* parasitaemia (P=0.6067). The parasitaemia profile in Figure 4.2 show that MB is just as active on *P. yoelii* as it is on *P. berghei* demonstrating its antimalarial

potential despite the genetic difference in the species. However suppressive effect on the *P. yoelii* was for only 2 days.

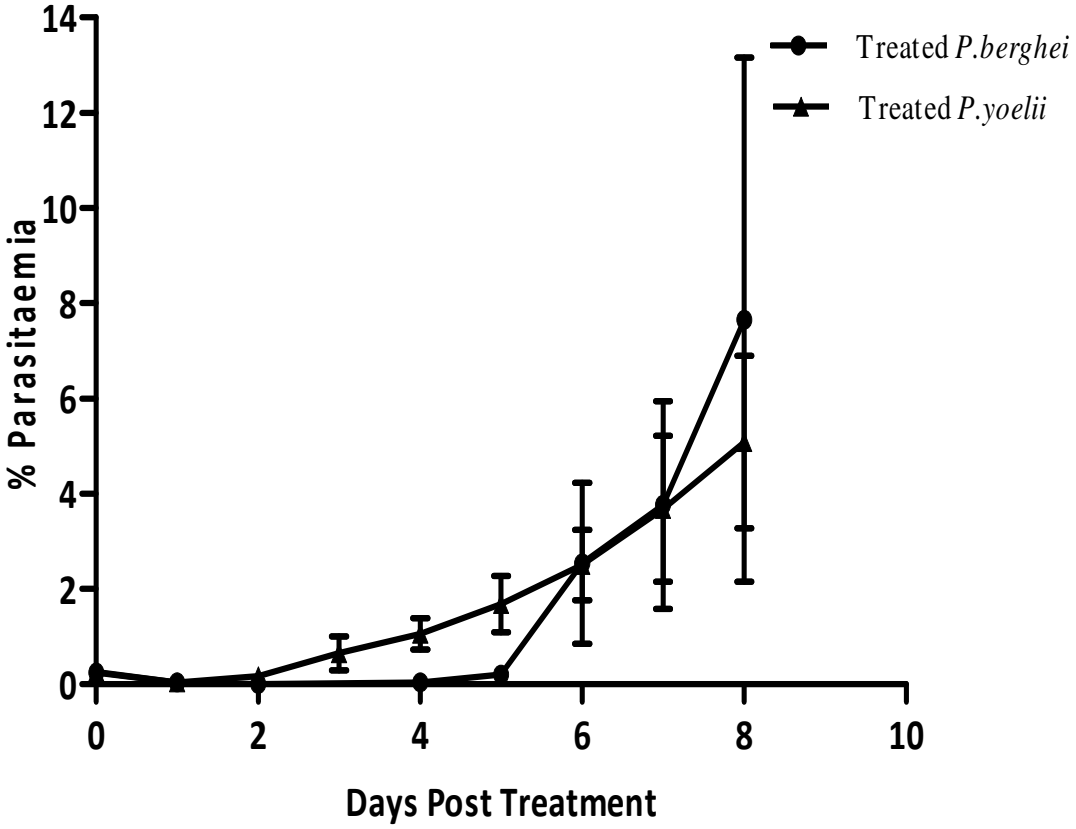


Figure 4.2: Parasitaemia curves of wild *P. berghei* and *P. yoelii* 17X after treatment with MB 45mg/kg. This suppressive activity in *P. yoelii* 17X shows methylene blue has antimalarial activity across *Plasmodium* species

4.3 Effect of Methylene blue against Lumefantrine resistant *P. berghei*

Lumefantrine resistant *P. berghei* used in this experiment were developed by passaging in increased drug pressure. The parasites were stable GFP luciferase expressing, from a 68th LM passage. MB at 45mg/kg had a suppressive effect for up to 7 days post

treatment before onset of recrudescence was observed. Activity of Methylene blue on the Lumefantrine resistant *P. berghei* is shown in Figure 4.3.

Figure 4.3 shows the suppressive effect MB has on parasites resistant to Lumefantrine (LM), whereas the parasite's resistance to lumefantrine is confirmed by the relative increase in parasitaemia in the LM50mg/kg treated group when compared to that of the MB 45 treated. At Day 6 post treatment, MB45 still exhibited a 100% chemosuppression; however this effect is not long lasting as recrudescence was observed from day 8 post treatment.

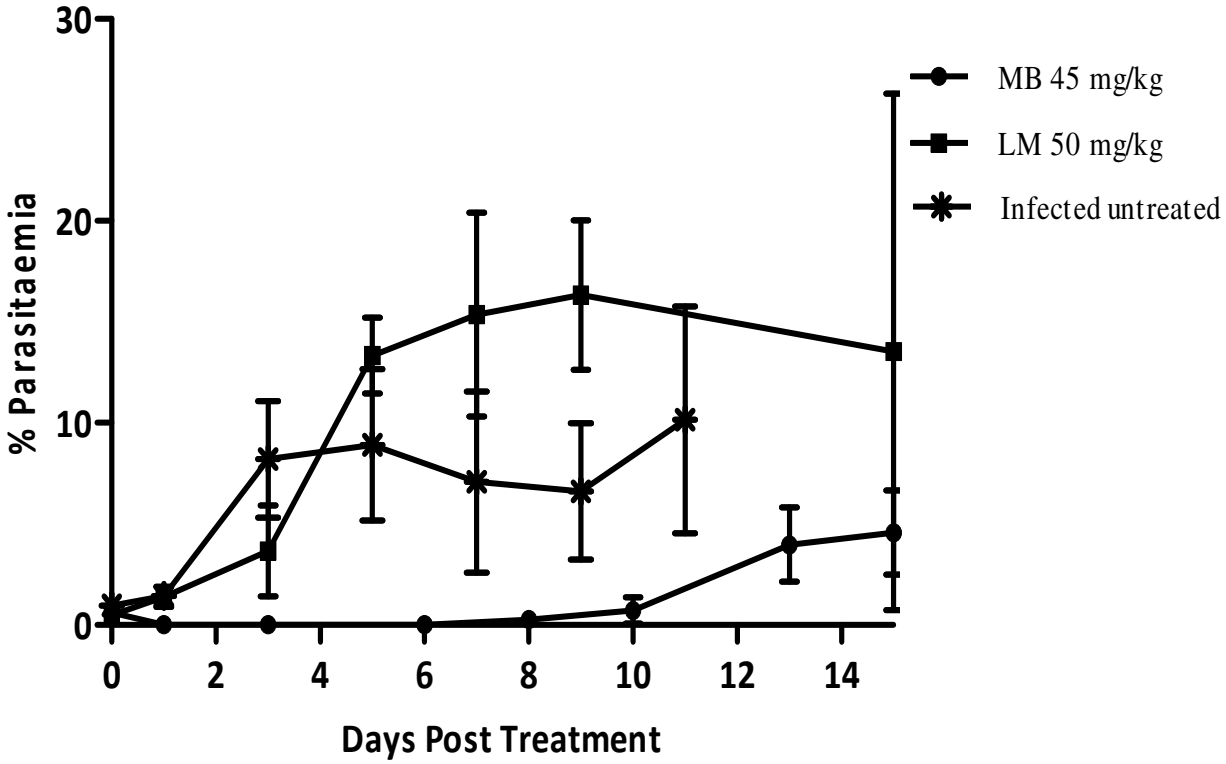


Figure 4.3: Antimalarial activity of MB 45mg/kg on LM resistant *P. berghei*. After 4 day treatment with MB, LM resistant parasites were suppressed to below detectable levels for 8 days

There was a significant difference in the antimalarial activity exhibited by MB against LM resistant *P. berghei* (P=0.0086) when compared to the LM 50 control.

4.4 Methylene blue against Chloroquine resistant *P. berghei* (RC Clone)

For parasites resistant to chloroquine, MB was tested on the RC clone of *P. berghei*. Figure 4.4 shows percentage parasitaemia response in RC clone after 4 day treatment with MB.

RC clone *P. berghei* showed relatively little response to the MB intervention as shown in Figure 4.4. There was no significant difference between the MB 45 mg/kg-CQ resistant and the CQ 10 mg/kg groups (P= 0.9826). The effect of MB 45 mg/kg treatment on resistant and wild type parasites was not significantly different (P = 0.6251), thus MB was more potent on wild type parasites compared to the CQ resistant (RC clone) parasites. Further analysis show that the parasitaemia curve in MB 45 mg/kg treated group was not statistical different from to the negative controls (infected non-treated) (P= 0.1486).

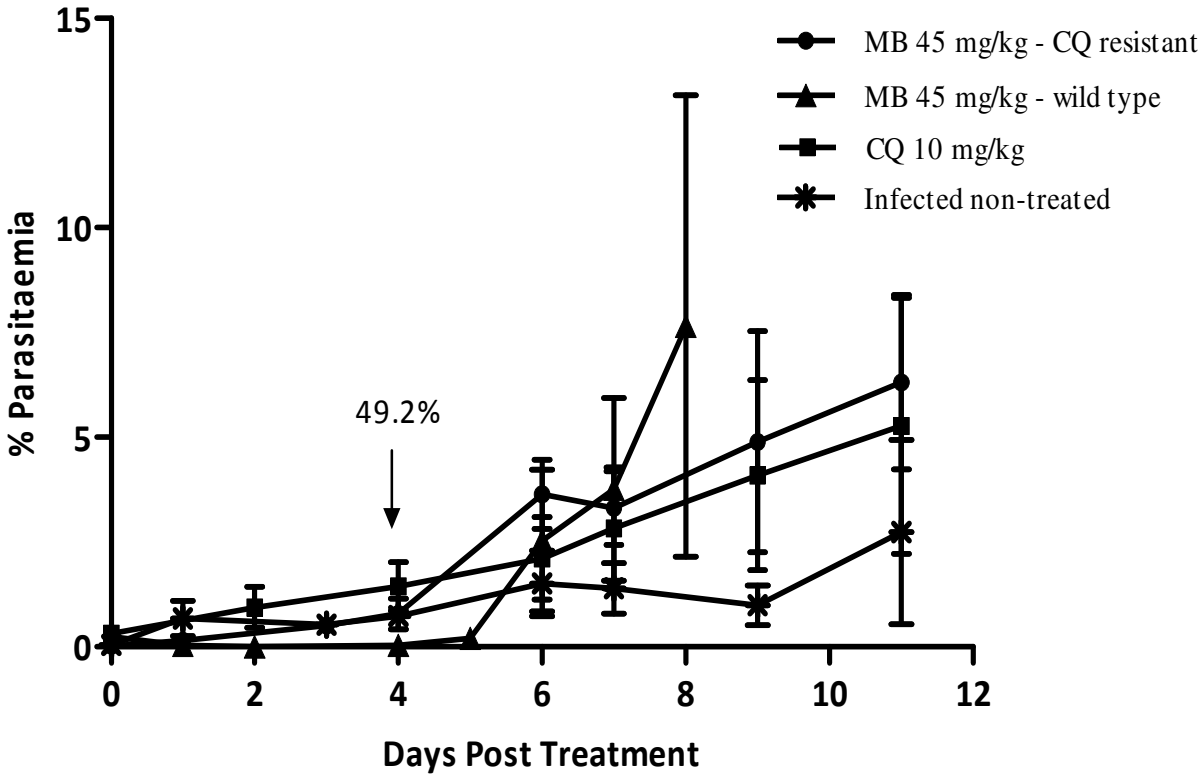


Figure 4.4: Effect of Methylene blue on CQ resistant *P. berghei*. At day 4 post treatment, MB had 49.2 % suppression on the CQ resistant parasite while parasitaemia in chloroquine treated mice steadily increased.

Upon treatment with CQ 10 mg/kg, animals that had been infected with the RC clone *P. berghei* displayed a gradual increase in parasitaemia (Figure 4.4). A drug resistance mechanism in the CQ resistant rodent malaria parasite confers a selective advantage under drug pressure that favored its proliferation.

4.5 Effect of Methylene blue against Pyrimethamine resistant *P. berghei*

Methylene blue was further tested on Pyrimethamine resistant parasites developed by incorporating drug resistant plasmid DNA in the parasite via transfection technology. Figure 4.5 shows the parasitaemia curves of groups treated with MB in comparison with a control group.

There was no significant difference in the MB antiplasmodial activity on PYR resistant and wild (sensitive) *P. berghei* (P=0.2912) as demonstrated by parasitaemia curves in Figure 4.5. A significant difference in parasitaemia between the MB 45 mg/kg-PYR resistant and PYR 1mg/kg groups (P=0.0191) was noted. At DPT 5, MB still exhibited a suppressive effect of over 99% on PYRr *P. berghei*; however some recrudescence was observed to take place from day 6 post treatment. Treatment with PYR 1mg/kg showed that the parasites were transformed to become PYR resistant and steadily increased even after exposure to pyrimethamine.

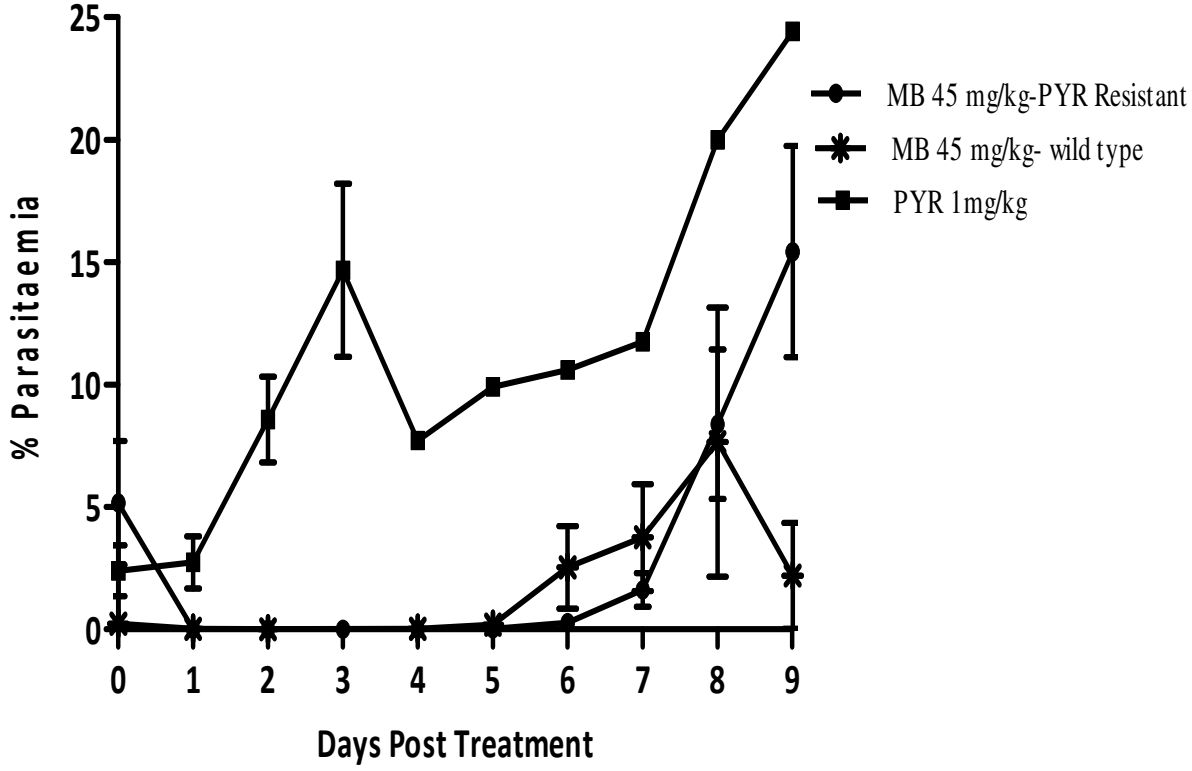
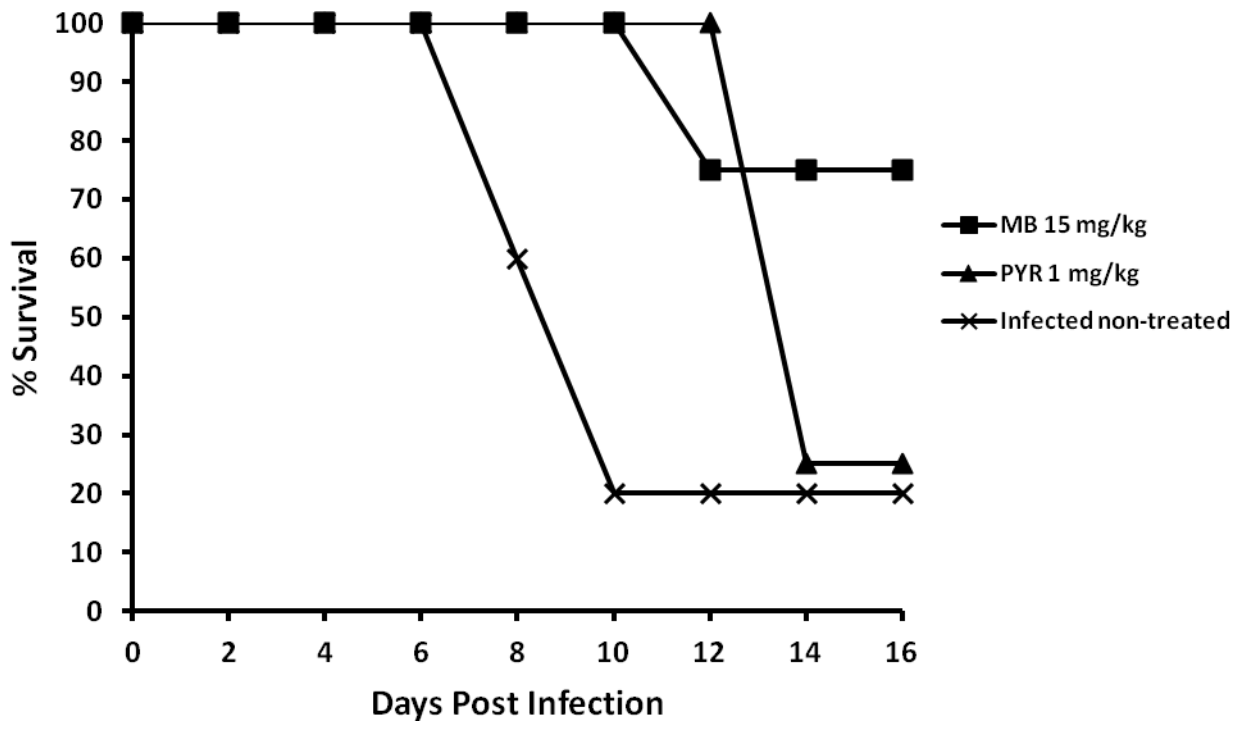


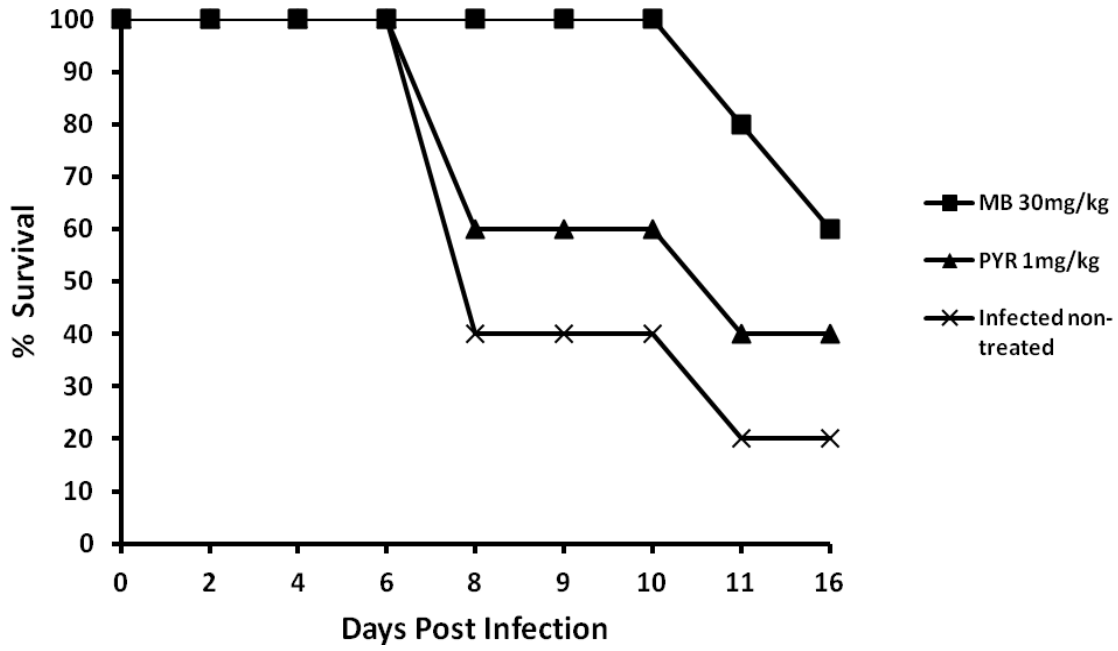
Figure 4.5: Methylene blue's antiplasmodial activity on Pyrimethamine resistant *P. berghei*. MB suppression of the resistant parasite line was comparable to that in wild type parasites. Parasite propagation despite treatment with PYR 1mg/kg showed that the transfection was effective.

4.6 Survivorship of infected mice

The overall effect on the survival of infected animals subsequently treated with MB was determined by number of days the mice stayed alive after the intervention in comparison to the controls. During the dosage experiments, MB treated groups generally had the best survivorship with a high portion of mice surviving longer than the controls as shown in Figure 4.6.


100
90
80
70
60
50
40
30
20
10
0
% Survival
0
2
4
6
8
10
12
14
16
Days Post Infection
MB 15 mg/kg
PYR 1 mg/kg
Infected non-treated

A


100
90
80
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40
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20
10
0
% Survival
0
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4
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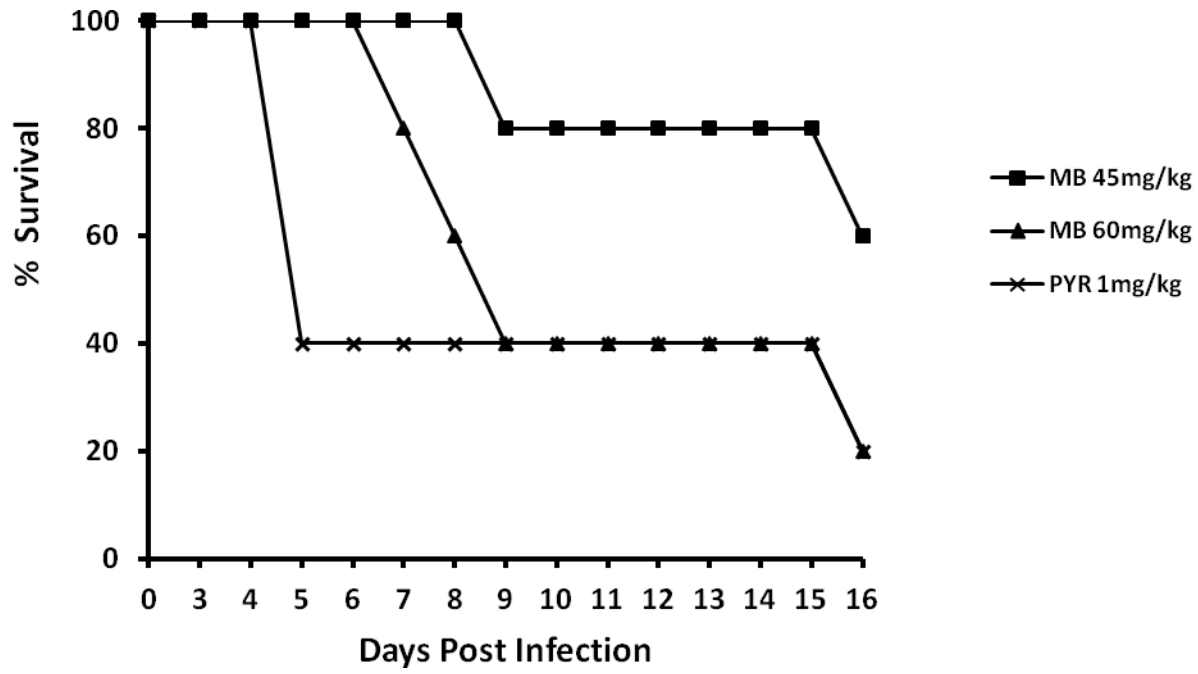
16
Days Post Infection
MB 30mg/kg
PYR 1mg/kg
Infected non-treated

B



C

Figure 4.6: Survival curves of malaria infected mice treated with Methylene Blue at A) 15 B) 30 C) 45 and 60 mg/kg/day. Mice treated with 60 mg/kg MB rapidly declined to 40% at DPI 9

When compared to the different doses tried, methylene blue at 45mg/kg (MB 45mg/kg) showed better chemosuppression of parasites as well as survival of mice with 80% surviving 15 days post infection followed by treatment.

In LM resistant *P. berghei* infected mice, mice treated with MB 45mg/kg showed better survival to the infection compared to both the negative and LM 50mg/kg controls as shown in Figure 4.7.

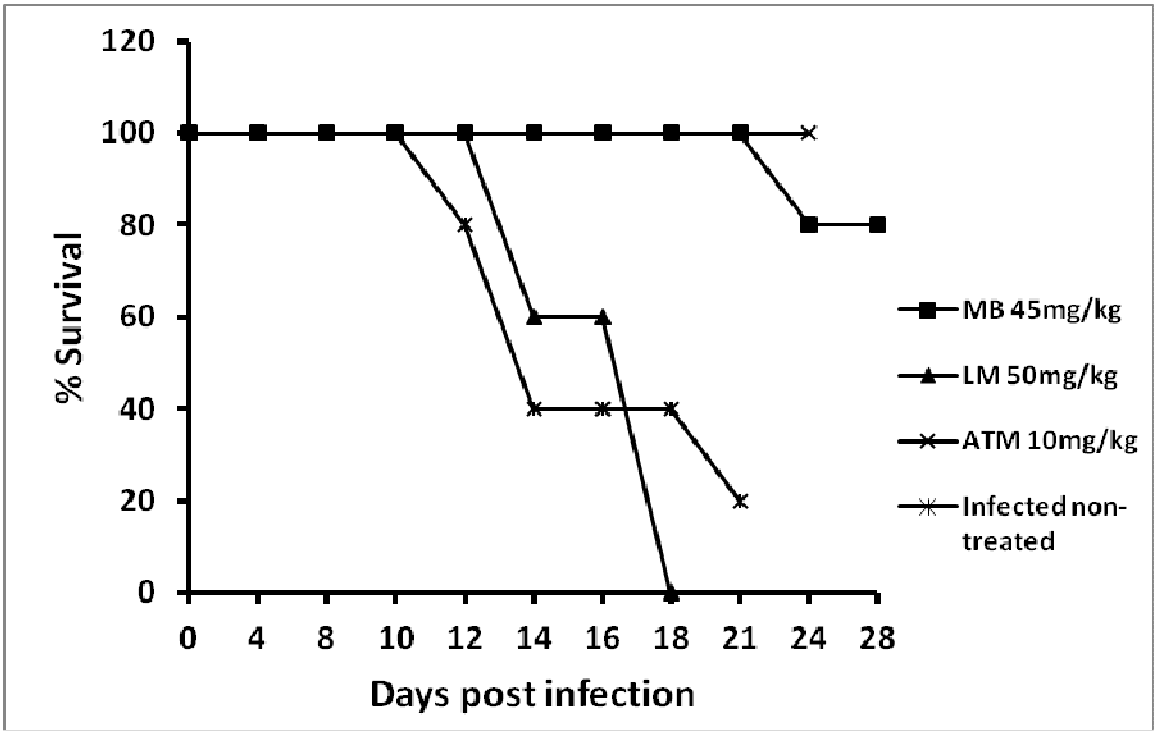


Figure 4.7: Survivorship curves of mice infected with LM resistant *P. berghei* and treated with MB. Methylene blue treated mice had over 80% survival 24 days post infection with the resistant parasite

A similar trend of a longer survival time in MB treated mice was observed in the CQ resistant infected mice that were treated with 45 mg/kg/day of methylene blue (Figure 4.8). However the positive control, ATM 10, showed relatively lower survivorship.

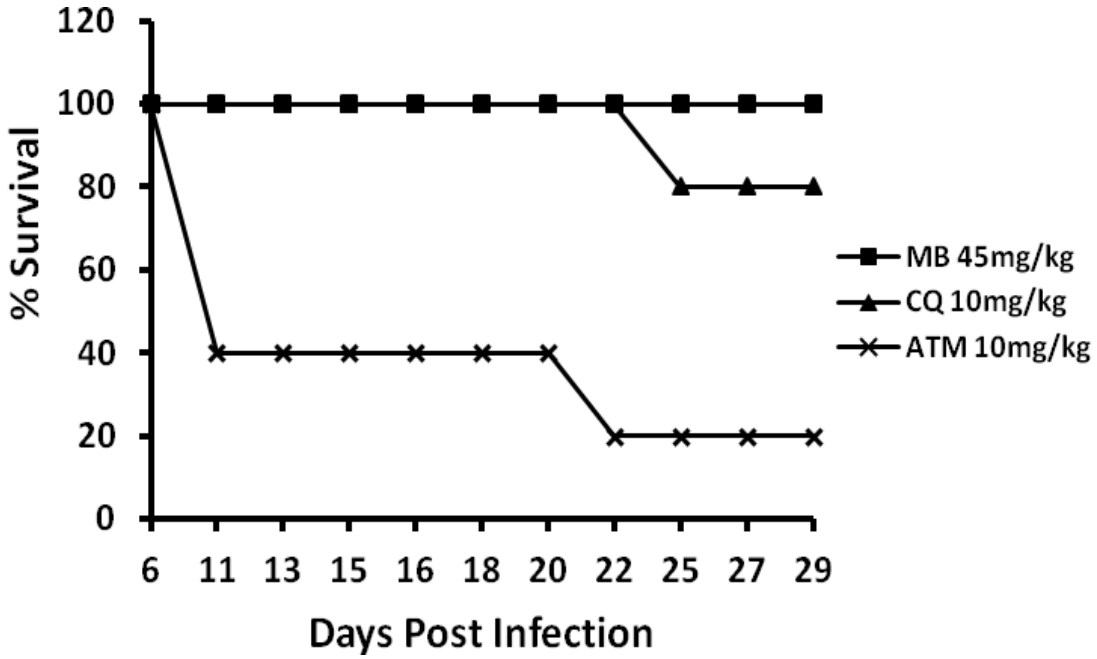


Figure 4.8: Survivorship of mice infected with CQ resistant *P. berghei* then treated with MB. Methylene blue treated mice sustained a 100% survival by day 29

When compared with the ATM and PYR treated groups, a major difference was observed in the group that had been infected with PYR resistant parasites then treated with MB 45mg/kg. There was a significant difference in the survivorship of the mice in the three treated groups, with those that received the MB treatment having an 80 % survival upto 10 days post treatment. Figure 4.9 shows a greater proportion of mice treated with MB lived relatively longer compared to the other groups. Immediately drug pressure was removed, the PYR treated mice started dying reducing survival to 20% at DPT 4. This can be argued to be as a result of the parasites losing the episomal DNA (plasmid DNA) that gave it resistance following the removal of drug pressure. The subsequent daughter parasites lacked the resistance and loss of fitness associated with resistance; thus making them more virulent on the host mice.

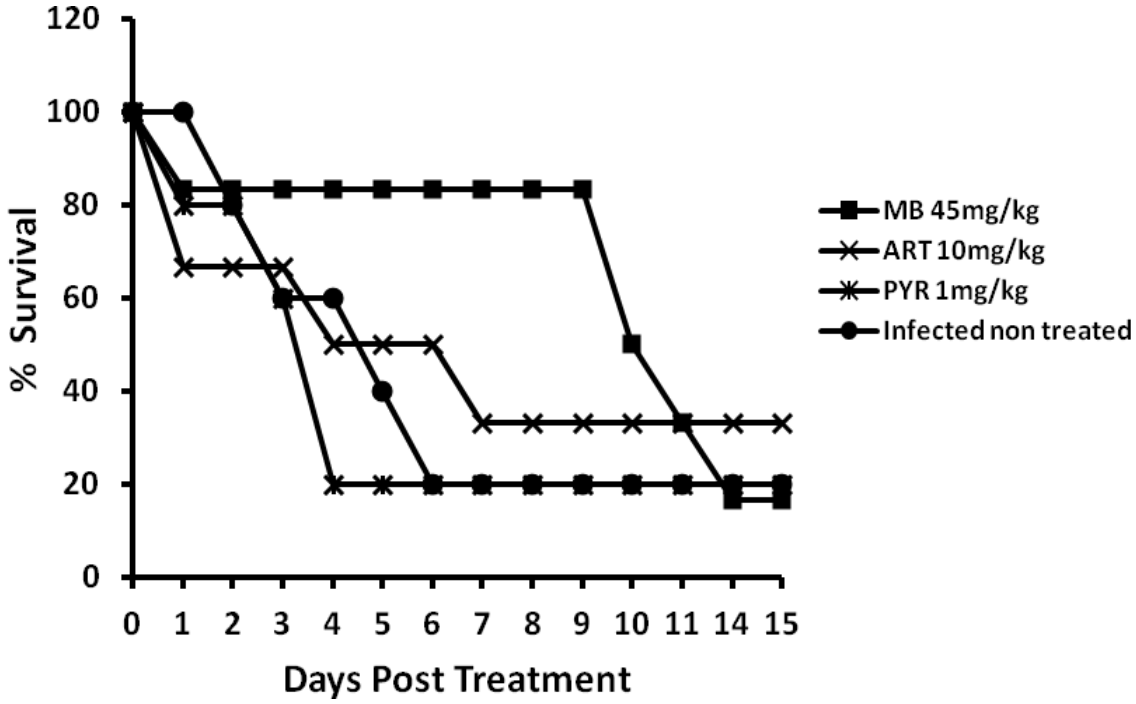


Figure 4.9: Survivorship curves of mice infected with PYR resistant *P. berghei* then treated with MB. MB treated animals had over 80% survival at DPT 9, survival in ART treated mice was at 50% at DPT 6.

In summary, MB was able to significantly lower parasitaemia levels in the PYR and LM resistant groups when compared to non-treated mice and consequently prolonged the survival time post-infection in all MB treated groups.

4.7 *In vitro* assessment of Methylene Blue on Chloroquine resistant *P. falciparum*

A general parasite inhibition profile of the compounds tested was generated. This profile curve was determined by having percentage inhibition graphed for various drug dilutions as shown in Figure 4.10. This gave a gross parasitaemia suppression activity of the test drugs.

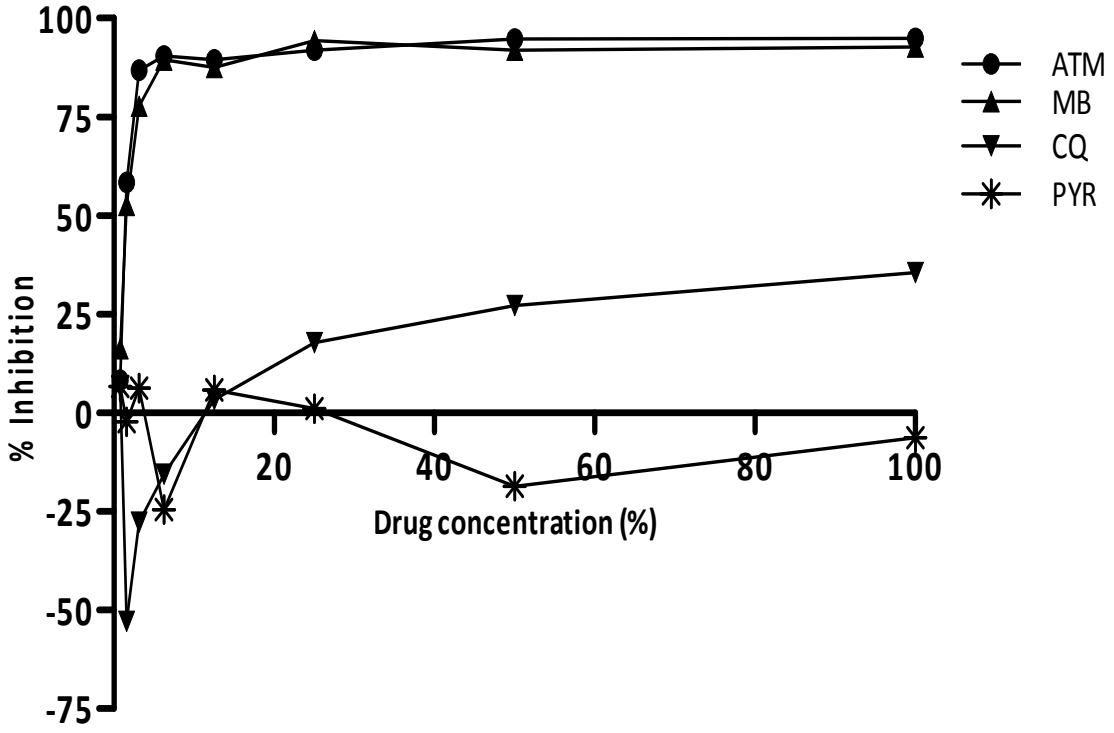


Figure 4.10: *In vitro* parasitemic drug-response as a function of the concentration following serial dilution. The *P. falciparum* W2 strain increased in parasitaemia in presence of PYR or CQ. ATM and MB had a comparable inhibitive effect

Parasite inhibition profile on *P. falciparum* W2 at different concentrations of the test compounds is shown in Figure 4.10. Methylene blue and artemether exhibited the greatest parasite inhibition across the various dilutions relative to the drug free control wells at 92.64 % and 94.85% respectively. Chloroquine and pyrimethamine on the other hand had minimal inhibitions at the same dilutions. The maximum inhibitions for CQ and PYR were 35.56% and 6.67% respectively. These low inhibitions by CQ and PYR confirmed the CQ resistance of the W2 strain used as well as the resistance nature of the isolate to PYR.

There was no significant difference in parasite inhibition between MB and ATM (P=0.9158), suggesting that MB had antimalarial activity on the chloroquine resistant W2 strain same as ATM. When compared against CQ and PYR, MB had a significant effect (P = 0.0001 and <0.0001) on parasite inhibition compared to the two controls. Similar results were established when ATM was compared to CQ and PYR.

Data collected in this study was fitted to a dose-response curve (using the Graphpad prism software 5.0) to determine IC_{50} by nonlinear regression analysis of log dose/response curves as shown in Figure 4.11.

The x values (drug concentrations) were transformed to Logs followed by normalizing of the Y values data (parasitaemia) so all curves begin at 0% and plateau at 100%. Zero was defined as the smallest value in the data set, and one hundred as the largest value on the data set.

Figure 4.11 below shows the dose-response curves (following a nonlinear regression analysis to determine the IC_{50}) of the drugs tested on the CQ resistant *P. falciparum* W2 strain. Results from the curves (Fig 4.11: A-D) show that MB was more active. This indicated a relatively high antimalarial activity of MB against *P. falciparum* W2 strain. This was further used to show that the experimental systems were functional in comparison to previously published studies.

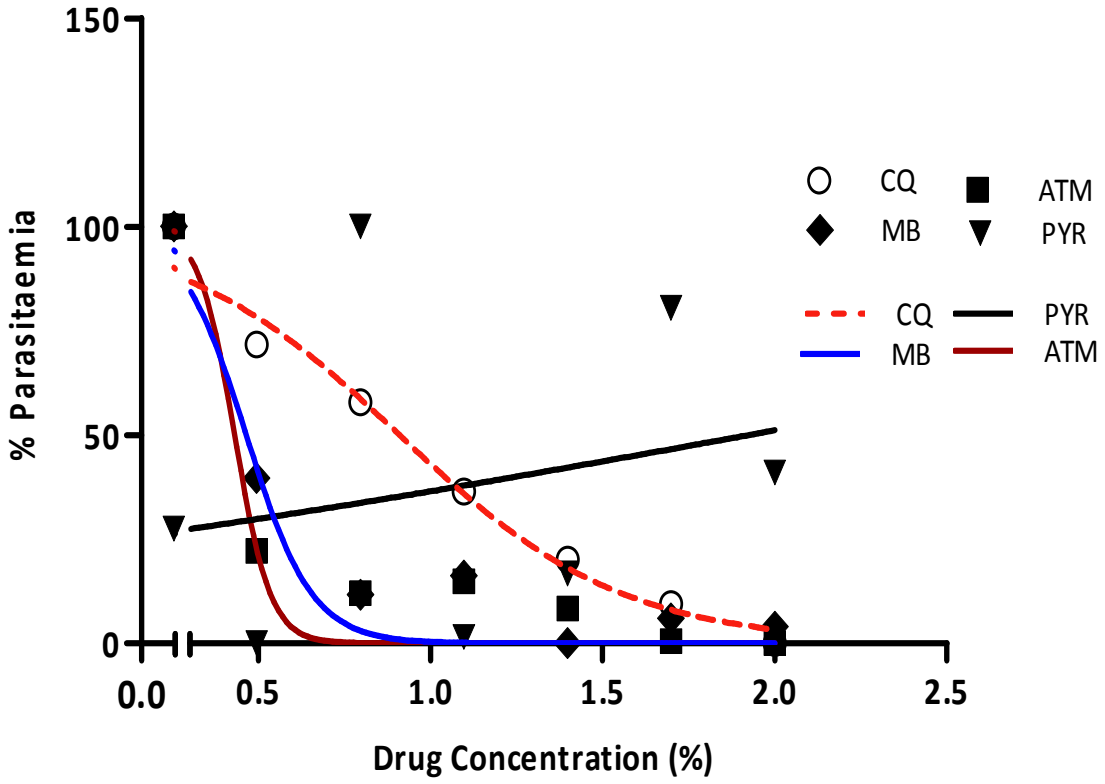


Figure 4.11: IC_{50} curves of test drugs on CQ resistant *P. falciparum*, W2 strain. Test drugs were methylene blue (MB), chloroquine (CQ), artemether (ATM) and pyrimethamine (PYR). A shift of the curve to the right is indicative of parasite resistance.

Nonlinear regression analysis show that the IC_{50} values of the compound tested were 27.33nM for MB (12.86 – 58.08nM; 95% CI); $1.267x10^5$nM for CQ ($4.139x10^4$-$3.88x10^5$nM, 95% CI); 23.31nM for ATM (9.46-57.43nM, 95% CI) and 516.2nM for PYR. The high IC_{50} values for CQ and PYR were as expected in that the W2 parasite is resistant to both CQ (IC_{50} > 100nM) and PYR (Delves *et al.,* 2012).

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Genetic conversion of wild *P. berghei* by transfection

Whereas animal models, rodent and primate *Plasmodium* parasites are essential in malaria research, transfection technology offers a promising platform for the investigation of many aspects of infection, parasite biology, host- parasite interaction as well as drug trials. Stable transfections have enabled the manipulation of the parasite genome thereby facilitating studies on functional analysis of genes, possible transgene expressions and gene knock-out.

This molecular transformation of the parasite genome has led to great advances in malaria research such as the successful expression of bioactive host cytokine (interferon gamma) by the malaria parasite's transcription and translation machinery (Ozwara *et al.,* 2003). Such applications of transfection may lead to generation of immunopotentiated parasites as well as enhanced protection.

Genetic modification of malaria parasites has therefore proven to be an invaluable tool and cannot be down played. With the work of Van Dijk *et al*. (1995) where it was shown that introduction of a pyrimethamine-resistance gene in *P. berghei* by means of transfection was stable, we adopted this to successfully generate pyrimethamine resistant *P. berghei* from wild type (sensitive) parasites by genetic modification.

In this study, transfection using electroporation conditions of 1000 V, 25 μF, single pulse for about 0.9 milliseconds succeeded using 50-75 μg of the transfection plasmid DNA and about $5x10^9$ merozoites. The conditions used were similar to those described by Waters (1997) and generated a suitable cohort of transfected parasites for use. This transfection technology facilitated in the generation of resistance at molecular level and

provided a population of pyrimethamine resistant parasites These parasites were thereafter exposed to MB and the results compared to those of resistant *P. berghei* parasites generated by continued drug pressure.

Transfection by electroporation succeeded as demonstrated by the *in vivo* selection of transfected parasites using oral administration of pyrimethamine in drinking water. The non-transfectants were selectively eliminated from the mice system by the pyrimethamine administered as observed in other studies where *P. knowlesi* was used on olive baboons (Munyao, 2008).

Successful transfection was further determined by PCR analysis of the DNA isolated from the pyrimethamine resistant transfected *P. berghei*. The DNA was positive for plasmid carrying the selectable marker *TgDHFR* gene, while DNA samples isolated from wild type *P. berghei* were negative.

An indicator of a successful transfection was the parasitemic growth profile of the transfected parasites in the presence of pyrimethamine pressure during selection. The longevity of the genetic manipulation induced resistance was dependent on the presence of continued drug pressure. A postulation is that after the 4 day treatment with PYR, the PYR treated mice died early because when the drug pressure was removed, the parasites lost the episomal DNA (plasmid DNA) that gave it its resistance. Subsequent daughter parasites lacked the plasmid DNA and were thus more virulent on the host mice. This observation was in line with the findings of van Dijk (1995). The daughter parasites were more virulent possibly because of the shift in cost of fitness after losing the plasmid DNA.

5.2 *In vitro* bioassay of methylene blue on chloroquine resistant *P. falciparum*

P. falciparum (W2 strain) was tested for its *in vitro* susceptibility to MB. Significant difference in antimalarial activity was demonstrated between the MB and the CQ control. Artemether also demonstrated significant antimalarial activity in comparison to CQ. The W2 strain was also resistant to pyrimethamine. The W2 strain was therefore CQ resistant (IC_{50}=126.7µM), PYR resistant (IC_{50}=516.1nM), but MB and ATM were active against them. No significant difference was noted in the inhibition curves of MB and ATM. These results have demonstrated that MB is active against CQ resistant *P. falciparum* and its antimalarial activity is associated with the phenothiazine group and not metal impurities in MB (Pascual *et al.,* 2011).

Ademowo *et al*. (2007) noted that MB was active against 75% of CQ-resistant field isolates from children in Southwest Nigeria. Pascual (2011) demonstrated that there was no significant correlation between the Proveblue (MB) responses and CQ, quinine, LM, or Dihydroartemisinin suggesting that no cross-resistance exists between Proveblue and the standard antimalarial drugs. This absence of cross-resistance suggests that MB and the antimalarials had either different modes of action or that different mechanisms of resistance were involved.

Earlier studies have shown that the thiazine dyes have antimalarial activity and methylene blue being notable for both its high antimalarial potency and selectivity (Vennerstrom, 1995). Other studies have been done to try and determine methylene blue's mode of action against malaria parasites. It has been reported that the leuko forms of MB exist largely as the neutral species at physiological pH and as a mixture of cationic and dicationic forms in acidic cellular compartments such as the parasite food vacuole (pH 5.0 to 5.4) (Krogstad *et al.,* 1985). Thus this pH differential would tend to have MB concentrate inside infected and less in non-infected erythrocytes on the

assumption that ionized forms of the leuko dyes are relatively membrane impairing. This could also help explain the activity against the resistant *P. falciparum* strain.

The results of this study have shown that MB has antimalarial activity against CQ resistant *P. falciparum* (W2) in addition to the K1 strain tested by Akoachere *et al.* (2005). It further seeks to propose that after further studies, it can be applied in CQ resistant malaria endemic regions.

5.3 *In vivo* bioassay of methylene blue activity against *P. berghei* resistant to chloroquine, lumefantrine and pyrimethamine respectively

Against the erythrocytic forms of rodent malaria parasites, MB was relatively more active on *P. berghei* than on *P. yoelii 17X*. This was noted in the number of days it took for recrudescence to appear in the two parasites. MB activity on wild-type *P. berghei* was 4 times more potent than on *P. yoelii 17X*. This was so in that recrudescence in the *P. yoelii* infection was noted on day 2 whereas in the *P. berghei* infection was on Day 5. MB thus demonstrated activity across species of rodent infecting *Plasmodium*. The MB 45 mg/kg dose was safer in terms of animal survivorship than the 60 mg/kg dose in a 4 day treatment regime.

The preliminary results on the activity of MB on resistant *P. berghei* were promising. Significant chemosuppression was noted on the LM and PYR resistant parasites for about 5 days post treatment. This indicated that MB was active on parasites resistant to common antimalarials and displayed a different mode of action. Some of the common mechanisms of resistance are the result of epigenetic changes such as gene amplification, protein over expression and protein modifications.

Lumefantrine is an arylaminoalcohol closely related to mefloquine (MQ), halofantrine and pyronaridine (Schlitzer, 2008). Although the exact mechanism of resistance to LM

is not completely understood, it is postulated that *pfmdr1* likely contributes to LM resistance. In *P. berghei* and *P. chabaudi*, amplification of the *pfmdr*1 orthologue is associated with mefloquine resistance as in *P. falciparum* (Sidhu *et al.,* 2005). Thus, *pfmdr*1 could also be involved in LM resistance in *P. berghei*. Therefore, the suggested acidity in cellular compartments such as the parasite food vacuole (pH 5.0 to 5.4) as a result of MB interaction could be the mode of antimalarial action against LM resistance. This acidity in the parasite food vacuole prevents the conversion of toxic heme to non-toxic hemozoin following hemoglobin digestion by the parasite. Thus the accumulation of toxic heme leads to parasite death. We can thus conclude that MB 45 mgg/kg was effective on the LM resistant parasites that were developed by passaging in increasing drug pressure. To validate the optimum experimental conditions, negative control (infected non-untreated) was included and showed continued parasite growth.

The antiparasitic activity of MB on the pyrimethamine resistant and wild-type *P. berghei* was comparable. In both populations of the parasite, MB had over 99% chemosuppression that lasted for 6 days before recrudescence. When the pyrimethamine resistant parasites were treated with MB at 45mg/kg and their parasitemic profile compared to those treated with pyrimethamine control (1 mg/kg), there was a disparity. Results showed parasite suppression by MB but proliferation in the presence of pyrimethamine drug pressure.

Pyrimethamine is a dihydrofolate reductase (DHFR) inhibitor which exerts its antimalarial activity by interfering with folic acid synthesis by the enzyme consequently interrupting the parasite's folate pathway. The results indicate that MB had a different mechanism of suppressing growth of the parasites and therefore a potential candidate in controlling PYR resistant malaria.

Pyrimethamine resistance in *P. falciparum* was demonstrated to be due to amino acid changes in DHFR, in particular a change at position 108 of serine (Ser) or threonine (Thr) to asparagine (Asn) (Wu *et al.,* 1996). Mechanisms of resistance to pyrimethamine have not been determined at molecular level. But due to closeness (genetic) of *P. berghei* to *P. falciparum,* treating the parasites with PYR 1mg/kg showed that indeed the parasites were transformed since parasitaemia steadily increased even after exposure to pyrimethamine. It also affirmed that the experimental conditions were optimum.

Treatment with MB had a minimal effect on the CQ resistant *P. berghei* parasites with 49.20% suppression on parasitaemia on day 4 post treatment. No significant difference between the MB 45 mg/kg-CQ resistant and the CQ 10 mg/kg groups (P= 0.9826) was noted. The overall effect of MB on the wild-type parasites was high levels of reduced parasitaemia up to day 4 post treatment. When compared with CQ resistant (RC Clone) parasites 0.79% parasitaemia was observed during the same time period. This low antimalarial activity against RC clones could be associated to the fact that MB (like 4-aminoquinolines) is concentrated in *Plasmodium* food vacuole where it inhibits the formation of hemozoin (Atamna *et al.,* 1996).

Other than accumulation in the *Plasmodium* food vacuole, MB is also known to inhibit the parasite's glutathione reductase (Färber *et al.,* 1998). The homodimeric flavoenzyme glutathione reductase (GR) which catalyzes the reduction of glutathione disulfide is a cornerstone of the malaria parasite antioxidant defense and repair mechanisms. In *Plasmodium falciparum*-infected red blood cells (RBCs), glutathione reductase regenerates reduced glutathione, which is essential for antioxidant defense. GR utilizes NADPH produced in the pentose phosphate shunt by glucose-6-phosphate dehydrogenase (G6PD). Thus, conditions affecting host G6PD or GR induce increased sensitivity to oxidants hence the use of MB as an antimalarial. Furthermore, GR deficiency resulting from insufficient saturation of the enzyme with its prosthetic group

FAD is common. Based on these naturally occurring phenomena, GR of malaria parasites and their host cells serve as attractive targets for the design of antimalarial drugs.

Other studies have shown that an elevation of glutathione (GSH) content in parasites leads to increased resistance to chloroquine (CQ), while GSH depletion in resistant *P. falciparum* strains is expected to restore the sensitivity to CQ. This implies that modulation of intracellular levels of GSH affects drug sensitivity (Meister, 1985), inferring that CQ resistant parasites have an elevated level of intracellular GSH.

It has also been demonstrated that mouse cells, infected with *P. berghei* strain that was selected for CQ resistance under drug pressure, contained higher levels of GSH than their sensitive counterparts, and that co-treatment with BSO (L-buthionine-[s,r]-sulfoximine) (which inhibits GSH synthesis) increased sensitivity to CQ (Dubois, 1995). This could explain the poor activity of MB observed on the CQ resistant *P. berghei.* The already high levels of GSH in the infected mouse cells bypassed the activity of the GR enzyme in converting the oxidized glutathione (GSSG) to the more desirable reduced glutathione (2 GSH) and consequently rendering the inhibitory effect of MB on GR insignificant. On this hypothesis we can propose that MB is not a good candidate drug for use to control CQ resistant malaria generated by drug pressure.

Although a dose of 30 mg/kg (low) of MB did not achieve 100% suppression, mice treated with this dose had a 60% survivorship at day 16 post infection. At high dose of 60 mg/kg, MB was found to be equally suppressive on the parasites as MB 45mg/kg but survivorship was greatly affected with a 40% survival at day 15 post infection compared to MB 45's 80%. Low survivorship was one indicator that methylene blue at high dose of 60 mg/kg wasn't safe. In the study on resistant *P. berghei*, there was over 80% survivorship in the MB 45mg/kg treated groups for upto, in some cases, 24 days post

infection. This high survivorship may be as a result of the relief from parasite burden that was caused by MB rapid parasite suppression and/or clearance. Another reason could be that mouse immunity which was initially primed and was given time to react to the malaria parasite by means of cytokines and antibodies.

MB reaches maximum plasma concentrations 1-2 h following oral administration at a dose of 100 mg in humans. Its plasmatic half-life is approximately 5-6.5 h and renal excretion is low, at approximately 20-30%. The rapid antimalarial effect of MB, combined with its pharmacokinetic properties, including a short half-life, might promote the slow development of resistance because exposure of parasites to sub-therapeutic levels of the drug would be very brief; this effect has been shown to occur with artemisinin.

With a 72% bioavailability of MB, this could be attributed to the MB's fast parasite clearance *in vivo* that in turn lessen the overall parasite burden on the mice. This antiplasmodial activity was indicated by the low mean cumulative parasitaemia in the MB treated groups. Despite the over 99% suppression of parasites in some cases, recrudescence was observed to occur on average at day 5 post treatment. This recrudescence affirms MB's short plasma half-life. The recrudescence can also be linked to not only *P. berghei* being a recrudescent parasite in nature but also possible parasite sequestration in organs such as the liver and spleen.

Conclusions

From the study, we can conclude that:

1. Transfection is a valuable technology in studies on parasite biology and generation of transgenic *P. berghei* systems. These systems can be used in the

design, screening and development of effective antimalarials as well as predicting drug-resistant mutations in an *in vivo* parasite/host setting.

2. Methylene blue at 45 mg/kg was more effective than at a low dose of 5 mg/kg and safer in terms of survivorship than at 60 mg/kg. High doses of MB above 45 mg/kg may not be safe for the mice.

3. Methylene blue has antimalarial activity against both wild-type *P. berghei* and its selected resistant clones; and *P. falciparum*. It was also equally active on resistant parasites generated through genetic manipulation by transfection and those generated through drug pressure methods Methylene blue is a promising antimalarial drug candidate against CQ, LM and PYR resistant strains of the malaria parasites.

4. From the study, methylene blue has cross species protection as displayed by its antiparasitic activity on the three species tested on namely *P. berghei, P. yoelii 17X* and *P. falciparum.*

5. The alternate hypothesis "methylene blue exhibits antimalarial activity against drug resistant *Plasmodium* parasites" is acceptable.

Recommendations

- There is need for studies to investigate the antimalarial effectiveness of MB combined with standard antimalarials and natural product derivatives. Since MB is fast-acting, its interactions with antimalarials with long half-life or bioactive molecules from medicinal plants against malaria parasites need to be elucidated.

- Further assays should be done targeting mixed infection scenarios and various strains of drug resistant field isolates such as artemisinin resistant isolates.
- There is need for studies on mechanisms of action of MB against CQ resistant *P. berghei* and *P. falciparum* parasites .
- Investigate effects of prolonged treatment course with MB on vital organs and parasite recrudescence. Studies on MB's safety, toxicity and carcinogenic effect on animal models also need to be done
- Further studies of MB's *in vivo* antimalarial potential using non-human primates like the baboon needs to be done.

Study Limitations

Financial constrains and procurement procedures.

In the transfection study, blood stage forms of *P. berghei* cannot be cultured through multiple cycles, necessitating *in vivo* selection protocols that are more demanding than those of cells in culture.

Occasional lack of adequate animals for use in the study coupled with delays in acquisition of reagents for the work.

Slow growth of parasites especially during the propagation phases in both the *in vivo* and *in vitro* set ups. At times there would be the untimely deaths of the donor mice.

Several challenges were encountered during the *in vitro* experiments which included culture contaminations; complications and delays in the procedures for acquisition of human serum and blood for *P. falciparum* cultures.

The microscopy method used for parasitaemia counts in the IC_{50} study of MB could be subjective and prone to human errors.

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APPENDICES

Appendix I: Preparation of Lysis Buffers

Preparing lysis solutions needed for isolating plasmid DNA from transformed *E. coli* cells

SOLUTION	**VOLUME (ml)**	**CONTENT**	**Amount**
P1	50 ml	50mM Glucose 1M TRIS (25mM) 0.5M EDTA	0.4504 g 0.1514g 9.305g
P2	250ml	0.2N Sodium hydroxide 1% sodium dodecyl sulfate (SDS) Distilled water	2g 2.5g 250ml
P3	50ml	5M Potassium acetate (KAC) Glacial acetic acid Double distilled water	14.7g in 30ml distilled water 5.75ml 14.25ml

Appendix II: Preparation of drug stock and working solutions

Stock and working solutions for the test drugs-Artemether (ATM), chloroquine (CQ) and Methylene blue (MB) - were prepared by dissolving them as follows to make 1mg/ml:

10.1 mg Methylene Blue (MB), dissolved in 10ml distilled water
10.1mg Chloroquine diphosphate (CQ), dissolved in10ml distilled water
10.5mg Artemether (ATM), dissolved in 10.5ml 70% Alcohol
10.1mg Pyrimethamine (PYR), dissolved in 10ml 70% Alcohol

Using an analytical balance (Mettler AE163), the drug powders were weighed into sterile 15ml centrifuge tubes and dissolved accordingly. Necessary safety precautions were undertaken. Occasionally they were vortexed to ensure a homogeneous solution. Thus 1mg/ml stock solutions were prepared.

The prepared drugs were then filter sterilized by first passing through a 0.45µm syringe adaptable filter then through a 0.22µm filter (Sartorius Stedim Biotech, USA, Ministart ®) and stored at 4^{o}C before its use. They were then diluted to the desirable working concentrations.

Pre-dosing of culture plates with test drug

To ensure drug stability, pre-dosing the micro-titer plate was done the same day as you set the assay. This is because some drugs (e.g. artemisinin derivatives) do not tolerate storage very well.

Pre-dosing of culture plates is necessary in order to facilitate easy and quick dilution of the test drugs. Using a multi-channel pipette, 25µl of complete RPMI 1640 medium

were added to wells B to H of a sterile 96-well flat bottomed micro culture plates (Linbro Tissue Culture Multi-well, Flow Laboratories CAT: 76-008-04).

Fifty microlitres of the prepared drug solutions from section 3.6.3 (the start concentration) were added to wells of row A at two wells per test sample for the duplicate set up as shown in Figure 3.2. The working solutions had a serial two fold dilution. The two fold serial dilution was achieved by transferring 25µl of the drug solution (start concentration) from wells of row A to row B, mixed well then 25µl of this solution transferred to row C. This was continued until H, 25µl from this well were discarded leaving 25µl of fluid in each well. Consequently well A contained the highest concentration (100%), well H contained only 0.7812% of the maximum concentration.

Calculation for diluting the parasite culture to desired parasitaemia

The following formula was used to calculate the setting parameters:

$$CiVi = CfVf$$

Where Ci= initial parasitaemia/ concentration of flask
Vi= initial volume of culture to be added
Cf=final parasitaemia/concentration in micro-culture plate
Vf=final volume of mixture per plate

Assuming that the percentage parasitaemia (%P) from the thin film is 4% in a culture maintained at 5ml and 6% haematocrit for use in setting (n=1) number of plates.
Ci=4%
Cf=0.4%
Volume of mixture for 1 plate
Vf= 1 plate x 100 wells (96 approximated to 100) x 200µl (volume of culture per well)

=20000µl

=20ml

Therefore, Initial volume of culture needed

4% x Vi = 4% x 20 ml

Vi = (0.4% x 20ml) /4%

=2mls

Since 5ml of culture has 6% hct, or 6/100 x 5ml= 0.3ml (100% RBC)

Thus, 2ml culture has 0.12ml (100% RBC)

To adjust haematocrit to 1.5% of Vf (20ml)

1.5/100 x 20ml=0.3 ml (100% RBC)

But the Vi (2ml) has 0.12 ml (100% RBC) and 0.3ml-0.12ml = 0.18ml (100% RBC) are required. This requires the addition of 50% RBC (RPMI/HEPES suspended). Since the remaining 0.18ml haematocrit is 100% RBC, 0.18 x 2=0.36ml of 50% RBC is needed.

The final volume of 20ml, needed is achieved by addition of CMS to 2ml test culture and 0.36ml of (50% RBC).

CMS needed= 20ml – (2ml+0.36ml)

= 20ml-2.36ml

=17.64ml

By this percentage parasitemia of 0.4%, Haematocrit of 1.5% is achieved.

For the negative control;

No. of wells = 4 per plate (approximate to 6 due to spillage)

Volume of mixture= 6 well x 200µl = 1200µl

=1.2ml

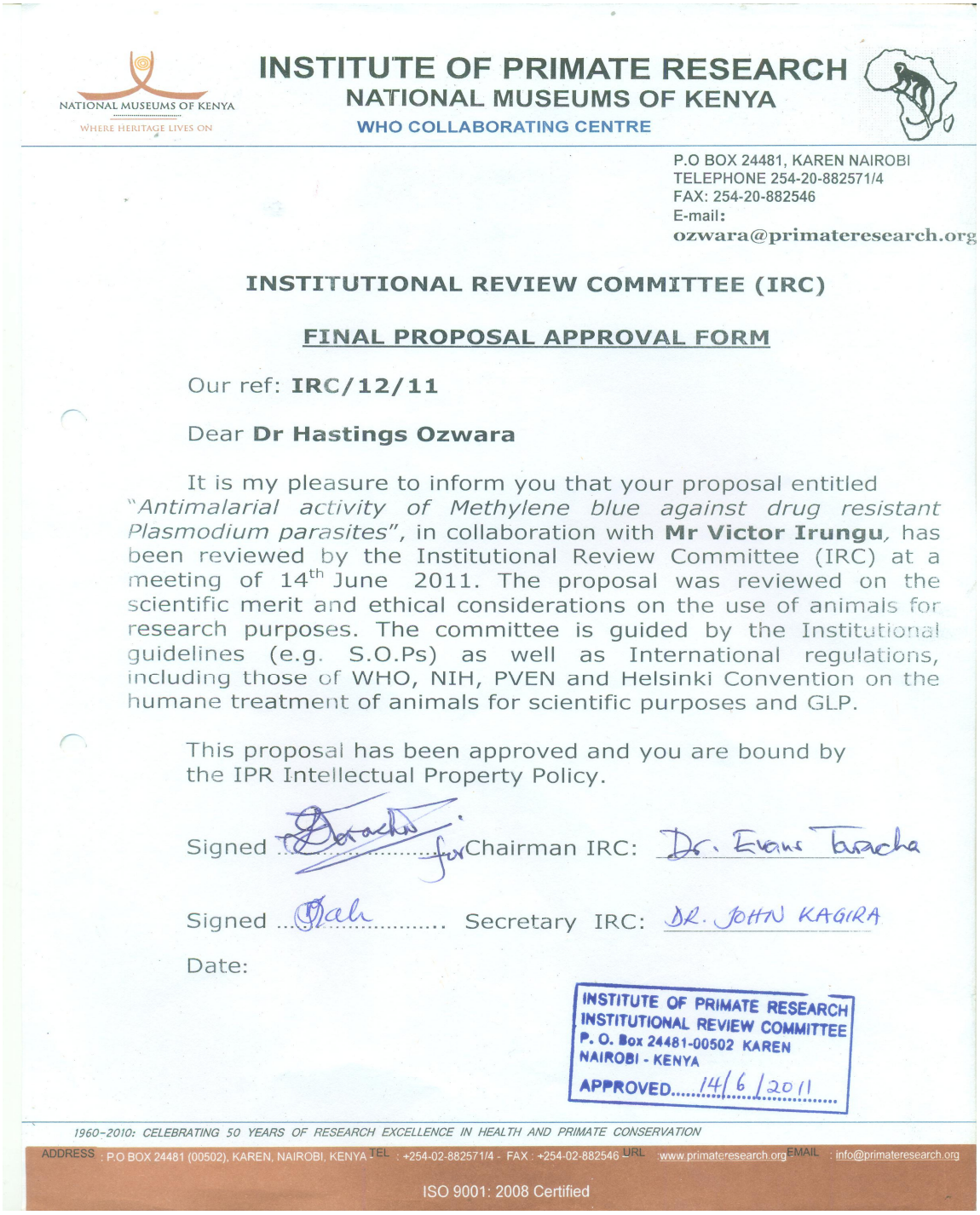
1.5% hct = 1.5/100 x 1.2 = 0.018ml (100% RBC)

=0.036ml (50% uninfected RBC)

CMS = 1.2-0.036

=1.164ml

Appendix III: Ethical approval form



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INSTITUTIONAL REVIEW COMMITTEE (IRC)

FINAL PROPOSAL APPROVAL FORM

Our ref: **IRC/12/11**

Dear **Dr Hastings Ozwara**

It is my pleasure to inform you that your proposal entitled "*Antimalarial activity of Methylene blue against drug resistant Plasmodium parasites*", in collaboration with **Mr Victor Irungu**, has been reviewed by the Institutional Review Committee (IRC) at a meeting of 14th June 2011. The proposal was reviewed on the scientific merit and ethical considerations on the use of animals for research purposes. The committee is guided by the Institutional guidelines (e.g. S.O.Ps) as well as International regulations, including those of WHO, NIH, PVEN and Helsinki Convention on the humane treatment of animals for scientific purposes and GLP.

This proposal has been approved and you are bound by the IPR Intellectual Property Policy.

Signed ..... for Chairman IRC: Dr. Evans Taracha

Signed ..... Secretary IRC: DR. JOHN KAGIRA

Date:

INSTITUTE OF PRIMATE RESEARCH
INSTITUTIONAL REVIEW COMMITTEE
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Appendix IV: Journal of Infections in Developing Countries (JIDC) published manuscript

JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Methylene blue inhibits lumefantrine-resistant *Plasmodium berghei*

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Abstract

Introduction: Chemotherapy still is the most effective way to control malaria, a major public health problem in sub-Saharan Africa. The large-scale use of the combination therapy artemether-lumefantrine for malaria treatment in Africa predisposes lumefantrine to emergence of resistance. There is need to identify drugs that can be used as substitutes to lumefantrine for use in combination therapy. Methylene blue, a synthetic anti-methemoglobinemia drug, has been shown to contain antimalarial properties, making it a candidate for drug repurposing. The present study sought to determine antiplasmodial effects of methylene blue against lumefantrine- and pyrimethamine-resistant strains of *P. berghei*.

Methodology: Activity of methylene blue was assessed using the classical four-day test on mice infected with lumefantrine-resistant and pyrimethamine-resistant *P. berghei*. A dose of 45 mg/kg/day was effective for testing ED90. Parasitemia and mice survival was determined.

Results: At 45 mg/kg/day, methylene blue sustained significant parasite inhibition, over 99%, for at least 6 days post-treatment against lumefantrine-resistant and pyrimethamine-resistant *P. berghei* ($p = 0.0086$ and $p = 0.0191$, respectively). No serious adverse effects were observed.

Conclusions: Our results indicate that methylene blue at a concentration of 45 mg/kg/day confers over 99% inhibition against lumefantrine- and pyrimethamine-resistant *P. berghei* for six days. This shows the potential use methylene blue in the development of antimalarials against lumefantrine- and pyrimethamine-resistant parasites.

Key words: methylene blue; lumefantrine; pyrimethamine; *Plasmodium berghei*; resistant; parasitaemia.

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Introduction

Malaria is a disease of great concern to Africa and other tropical regions of the world. About 90% of the estimated 584,000 worldwide malaria deaths in 2013 occurred in Africa [1], with children under five years of age accounting for 78% of all deaths. Chemotherapy is currently the most effective method to control malaria. Over time, monotherapy treatments have selected for resistant strains and consequently became ineffective. This has been witnessed with the use of chloroquine and sulfadoxine-pyrimethamine as first lines of treatments in Africa [2].

A major limitation of the existing antimalarial drugs is primarily resistance (and cross-resistance between closely related drugs) [3,4]. However, most of these drugs still have a place, and their lifespan could be prolonged if they were better deployed and used, and also rationally combined. Newer compounds are also being developed [5,6].

Current strategies for malaria treatment rely on the use of combinations of drugs. The combinational therapy rationale lowers the probability of the parasites having a resistant genetic profile, with mutations conferring resistance to both drugs surviving the treatment [7].

Lumefantrine (LM) is one of the current combinational therapy drugs with artemisinin, used as first-line treatment against malaria in Africa, including Kenya [8]. This artemether-lumefantrine therapy produces a > 95% cure rate [9-11]. However, the absorption and elimination half-life of LM is subject to very wide inter-individual variations, which produce chances of treatment failures [12]. There are reports that the use of LM in artemisinin combinational therapy

(ACT) selects for parasites that show increased tolerance to the combinational therapy. This selection, coupled with large-scale unsupervised usage and parasites having increased copy numbers of the *pfmdr1* gene associated with mefloquine (MFQ) resistance [13,14], is synonymous with emergence of LM resistance.

Pyrimethamine (PYR) is used widely in combination with longer-acting sulphonamides such as sulfadoxine and sulfalene. It is used in malaria-endemic regions of Africa and the Western Pacific region for its important role in the prevention of malaria in pregnancy when used for intermittent presumptive treatment of malaria in pregnant women (IPTp) [15], and it has been included in several trials of intermittent presumptive treatment in infants (IPTi) [16]. Although no longer the official first-line anti-malarial in a number of countries, sulfadoxine-pyrimethamine (SP) remains available and is frequently used as a prophylaxis by travelers. The continued use of SP in the IPT provides drug pressure that selects for PYR-resistant parasites. Thus, PYR-resistant parasites are likely to remain in circulation.

The progressive increase in drug-resistant malaria, including delayed clearance of *Plasmodium falciparum* after treatment with artemisinin derivatives [17], highlights the need for novel effective antimalarial drugs. In this context, efforts have been encouraged to focus on research into novel and safe alternative compounds that have a low propensity to generate resistance [18,19]. One possible source is repositioning compounds that have been approved for other treatments but which have been found to have parasiticidal or disease-modifying effects in malaria infection, such as methylene blue (MB). Methylene blue already has other medicinal applications such as the treatment of methemoglobinemias, management of Alzheimer's, and applications in ifosfamide-induced encephalopathy and neurotoxicity.

In vivo evaluation of antimalarial compounds begins with the use of rodent malaria parasite and murine models. Although studies have been carried out on the potential of MB in the management of malaria, none has been done to assess its effect on LM-resistant *Plasmodium berghei*. Other studies demonstrated that MB is effective, with 50% inhibitory concentration at 3.62–3.90 nM, on 23 *P. falciparum* strains resistant to several standard antimalarials, including chloroquine (CQ) [20]. Dormoi *et al.* [21] went on to further demonstrate significant efficacy of MB in the treatment and survival of cerebral malaria in mice infected with non-resistant *P. berghei* ($p = 0.0011$).

This study aimed to assess MB antiplasmodial activity on stable LM-resistant ANKA lines [22] and PYR-resistant *P. berghei*, with a view of predicting its applicability and efficacy in LM- and PYR-resistant malaria. This is important since resistance to ACTs, the first-line anti-malarial regimen, has been reported in western Cambodia [23,24].

Methodology

Drugs and chemicals

Anhydrous MB from Sigma-Aldrich Laborchemikalien GmbH, (Seelze, Germany) was used in this study. LM was a kind donation from Daniel Kiboi, Kenya Medical Research Institute. Pure PYR was from Sigma Chemical Company, St. Louis, USA.

MB was dissolved in distilled water, PYR was weighed and dissolved in 5% (v/v) dimethyl sulfoxide, then vortexed gently to mix before topping up with 1X phosphate-buffered saline (PBS). PH adjusted to 4.0. LM was freshly prepared by dissolving in a vehicle consisting of 70% Tween-80 ($d = 1.08$ g/mL) and 30% ethanol ($d = 0.81$ g/mL) and subsequently diluted tenfold with double-distilled water.

Parasites and animals

To study the activity of MB on resistant rodent malaria parasites, resistant lines of *P. berghei* ANKA (MR4 catalog number MRA-868, MR4, ATCC Manassas, Virginia, USA) were used. The LM-resistant line (developed by passaging [22]) employed in the study was obtained from Kenya Medical Research Institute. The parasite was stable green fluorescent protein luciferase expressing and was subjected to selective LM pressure to the 68th passage. PYR-resistant line (developed by transfection technology as described previously [25,26]) and wild-type strains were also used for comparison purposes. The PYR-resistant and wild-type parasites were obtained from the Institute of Primate Research biobank.

Adult BALB/c mice bred in the rodent facility of the Department of Animal Sciences, Institute of Primate Research, Kenya, were used. At the time of use, they were 7 to 10 weeks of age and weighed 20 ± 2 g. In the overall study, the female-to-male sex ratio of the animals used was 80:20. The animals were housed in an experimental room in standard Macrolon type II cages clearly labeled with experimental details at 22°C and 60%-70% relative humidity. They were fed a commercial rodent pellet diet obtained from Unga Farm Care (EA) Limited, Nairobi, and were given water *ad libitum*.

Parasite retrieval and infection

Cryopreserved stocks of *Plasmodium berghei* were revived by being thawed at 37°C in a waterbath before being intraperitoneally injected into donor mice. At average parasitemia of between 8% and 15%, the donor mice were sacrificed and cardiac puncture was performed on them to collect the infected blood. The heparinized blood collected from the donors was pooled and diluted in PBS. The parasite suspension was used to infect experimental mice intraperitoneally (10^6 infected erythrocytes per mouse). The inoculated experimental mice were randomly divided into groups of five. Control groups, each with five mice, were also included. Upon determination of successful infection, the mice were treated for four days with intraperitoneal injections of MB, while LM and PYR were administered orally also for four days. Parasitemia was assessed by microscopic examination of Giemsa-stained thin smears made from tail-vein blood.

Preliminary dose-determining experiments were earlier done on wild-type *P. berghei* at 5, 15, and 45 mg/kg/day of MB; 15 mg/kg had 67% activity, while 45 mg/kg had 100% activity. The effective dose (ED90) value was determined by its relative potency and so subsequent studies were done using 45 mg/kg/day of MB.

Drug efficacy was determined by overall percentage (%) parasitemia suppression after microscopic observation of Giemsa-stained thin blood smears. The mice survival rate (%) and drug curative effect to mice (%) relative to the untreated controls were also used as a measure of effect of these drugs on the infected mice.

Ethical approval

Approval for animal use was granted by the institutional research ethics committee of the Institute of Primate Research, Kenya that comprises the animal care and use committee. The animals were maintained according to institutional animal care and use policy.

Statistical analysis

Parasitemia and survivorship values are reported as mean ± standard error of mean. These were generated in Microsoft Excel. Statistical analysis of data to compare mean parasitemia of the appropriate parasite lines when treated with MB was carried out using student's two-tailed unpaired t-test while assuming 95% confidence limits. $P < 0.05$ was considered significant. The analysis of data obtained was done using the GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

This study aimed to determine how effective MB would be on resistant rodent parasites. Several doses of MB were tested to determine the effective dose value; 45 mg/kg/day was found to have the highest potency. This was similar to Garavito *et al.*'s [27] findings on MB efficacy on sensitive wild-type *P. berghei*. Based on this, subsequent tests were carried out using it. The dose had an inhibition activity that suppressed wild-type parasites to below detectable levels compared to 67% of 15 mg/kg and 43% of 5 mg/kg (Table 1).

Inhibition of parasite growth

Parasitemia patterns obtained after treatment with MB indicate MB inhibits parasite growth. It was observed to suppress parasites to below detectable levels for up to 11 days on the LM-resistant type of *P. berghei* (Figure 1). During MB administration, suppression was observed for the 4 days and for 7 days post-treatment (PT). Parasite recrudescence was detected on day eight PT.

Table 1. Effect of MB 45 mg/kg against CQr compared to LMr and PYRr parasites at day 5 post-treatment.

	Methylene blue chemosuppression		
	Parasitemia		
	Non treated	Treated	% suppression
LMr *P. berghei*	13.34	0	> 99.00
PYRr *P. berghei*	9.92	0	> 99.00
CQr (RC-clone) *P. berghei*	2.11	2.22 ± 0.59	0
P. yoelii 17X (CQ resistant)	0.22	1.68 ± 0.59	0
Wild-type *P. berghei*	26.21	0.21 ± 0.16	99.20
Wild-type *P. berghei* 15 mg/kg	26.21	8.73 ± 2.83	66.69
Wild-type *P. berghei* 5 mg/kg	26.21	14.88 ± 2.95	43.23

LMr: lumefantrine resistant; PYRr: pyrimethamine resistant; CQr: chloroquine resistant.

The stability of LM resistance in parasites used was proven by the steady parasitemia growth even after treatment with LM at 50 mg/kg. This shows that the resistance phenotype for the parasite had not been lost with dormancy.

The dose of 45 mg/kg of MB administered suppressed the bulk of parasitemia in the PYR-resistant *P. berghei* (Figure 2) for a total of 9 days. At day 5 post-treatment, suppression was maintained at 100% on the PYR-resistant parasites. MB-treated mice had undetectable parasitemia compared to 15% (1.5×10^6 parasites/μL of blood) of the PYR-treated group at day 3 post-treatment (Figure 2). However, recrudescence was observed to take place from day 6 PT. PYR at 1 mg/kg showed that the parasites (transfected with *tgdhfr*-bearing plasmids) were transformed since the parasitemia steadily increased even after exposure to PYR. Pyrimethamine exerts its antimalarial activity by interfering with folic acid synthesis by the enzyme, consequently interrupting the parasite's folate pathway. These observations suggest that MB has a different mechanism of action from PYR, thereby advocating its use in regions with reported PYR-resistant malaria.

The results of MB activity on other rodent malaria causing parasites are shown in Table 1. They reveal over 99% chemosuppression by MB at 45 mg/kg at day 5 PT on wild-type LM-resistant and PYR-resistant *P. berghei*. The same dose at the same time period did not achieve similar results on the chloroquine-resistant (RC) strain of *P. berghei* and non-lethal *P. yoelii* 17X (also CQ resistant), where suppression lasted 2 days. A low dose of MB (15 mg/kg) had a 67% suppressive effect on wild *P. berghei*.

Overall, the results show that MB inhibits LM-resistant and PYR-resistant *P. berghei* parasites. Against erythrocytic forms in rodent malaria parasites, MB at 45 mg/kg was more active on *P. berghei* than on *P. yoelii* after a 4-day treatment.

Mice survivorship

The results on mice survival in Figure 3A shows that the group treated with MB in the LM-resistant parasites category had 100% of mice surviving up to day 21 post-infection (PI) before one mouse died by day 24. The control group treated with 50 mg/kg LM had 60% of mice surviving at day 16 PI, and the last mouse died on day 18. The infected non-treated negative control group had only 40% of mice alive at day 18 PI, with the last one dying 3 days later (Figure 3A).

Mice infected with PYR-resistant parasites and treated with MB had a 100% survivorship at day 7 PI. By day 8 PI, one mouse died, reducing survivorship to 80%, which further dropped to 40% on day 16 PI (day 14 PT). In the artemether-treated control group, 60% of the mice survived from day 5 PI to day 9 PI, and 40% survived from day 7 to 12 PT (day 10–12 PI). The mice in the infected non-treated negative control had all died by day 10 PI. PYR (1 mg/kg)-treated groups had survivorship fall to 20% on day 10 PI from 80% on day 7 PI (Figure 3B).

Figure 1. Parasitemic profile of lumefantrine-resistant *P. berghei* 14 days post-treatment with methylene blue 45 mg/kg in 4-day curative test.

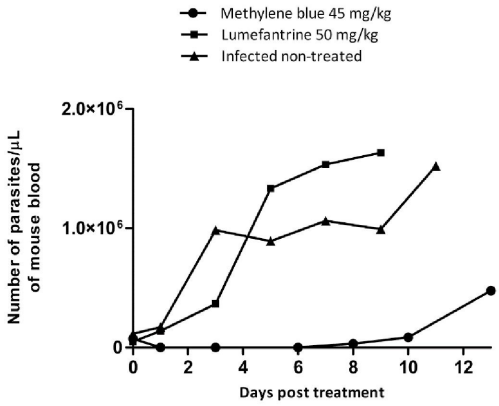


Figure 2. Parasitemic profile of pyrimethamine-resistant *P. berghei* 9 days post-treatment with methylene blue 45 mg/kg in 4-day curative test.

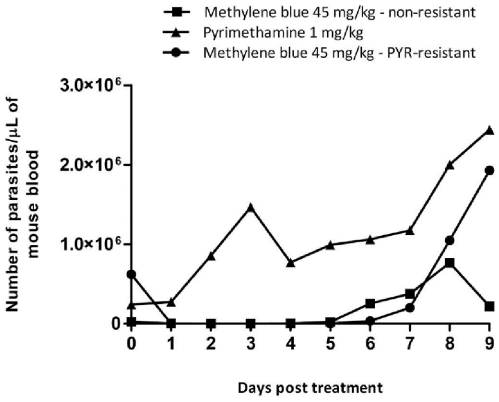
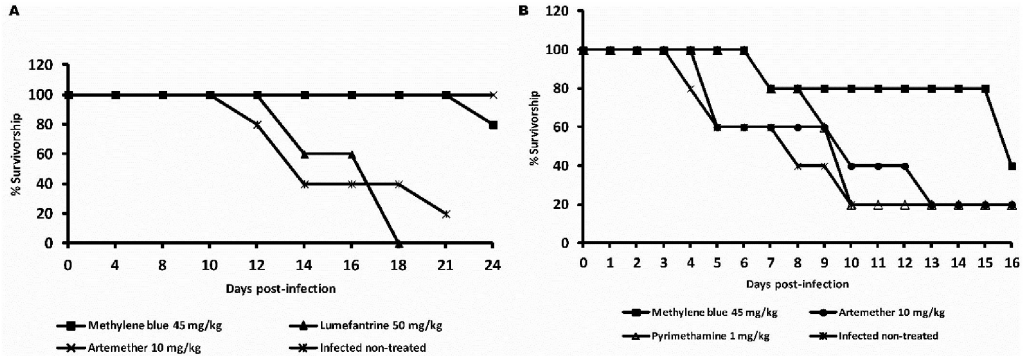


Figure 3. Survivorship curves of mice. **A)** infected with lumefantrine-resistant *P. berghei* and treated with methylene blue 45 mg/kg/day; **B)** treated with methylene blue 45 mg/kg/day after infection with pyrimethamine-resistant *P. berghei.*



Discussion

This study was designed to determine the effect of MB on *P. berghei* parasites resistant to LM and PYR. Results showed that MB is effective against the two resistant types of rodent parasites, suppressing parasitemia to below detectable levels for the duration under drug pressure. This potentiates MB into a candidate for drug repurposing against malaria.

MB demonstrated remarkable parasite suppression against the two resistant rodent malaria parasites at the onset of treatment. MB rapidly suppressed parasitemia to below detectable levels up to day 5 PT (Table 1) in relation to the negative controls. On the *P. berghei* parasites resistant to PYR and LM, MB demonstrated impressive chemosuppression for six and seven days post-treatment, respectively. This shows the potency of the 45 mg/kg dose of MB in treating LM-resistant malaria. The results obtained present the 45 mg/kg dose MB as a start in the development of alternative fast-acting treatment regimens, especially against non-CQ resistant parasites. The rapid antimalarial effect of MB, coupled with its short half-life, may promote slow development of resistance because exposure of parasites to sub-therapeutic levels of the drug would be very brief. It may next be possibly used as an alternative to artemisinin in combinational therapy. It is conceivable that MB can be considered for application in treatment of both uncomplicated and complicated malaria as well as for its potential for use in LM- and PYR-resistant malaria regions. This therapeutic potential is further highlighted in MB's capacity to efficiently manage cerebral malaria, a complication of untreated malaria [21].

Although the overall parasite burden was decreased from onset of treatment, response to treatment in this study can be categorized as clearance and recrudescence. Rapid parasite suppression was noted in both resistant and wild-type parasites before recrudescence was noted at least six days after the end of therapy. A factor that probably contributed to this observation is MB's short plasma half-life of 18 hours. The 72% bioavailability of the compound explains the fast parasite clearance *in vivo* [28]. It is also likely that inactive parasites are an omnipresent component of many malaria parasite infections, and act by introducing metabolic perturbations into active ones. Further studies need to be done to determine the reason for recrudescence and whether the parasite had possible resistance mechanisms to MB. More studies also need to determine a dose that would still be safe and completely cure the animals; this would substantially secure the need to have MB included in the armamentarium of malaria treatment. It would also be interesting to determine whether prolonged treatment at 45 mg/kg would result in increased recrudescence time or cure.

The findings indicate that MB was active on parasites resistant to the common antimalarials and displayed a different mode of action, which could be advantageous against the mechanisms of resistance displayed. Some of the common mechanisms of resistance are the result of epigenetic changes such as gene amplification, protein overexpression, and protein modifications.

The significant parasitemia suppression in mice treated with the MB dose was synonymous with longer survival time. Mice groups treated with MB had over

80% survivorship for at least 14 days in the two categories, which was indicative of better survival rates after treatment with MB. The improved survivorship could be attributed to the 6-day chemosuppression observed on parasites that, in turn, lessened the overall parasite burden on the mice. This possibly gave the immune systems of the mice time to recover and help control the parasite infection.

Recently, *PfMDR* N86, the chloroquine-susceptible allele, has been proposed as a molecular marker for LM resistance. Artemether-lumefantrine treatment selects for N86 in recurrent infections [29]. In Mozambique, there has been an increase in the prevalence of molecular markers associated with LM resistance since initial use of artemether-lumefantrine (AL), suggesting the need for continued surveillance for the emergence of resistance to the drug [30]. An increase in the gene copy number has been associated with increased risk of treatment failure with mefloquine, artesunate-mefloquine, or artemether-lumefantrine [31]. This further pushes the need to find antimalarials that would counter the foreseen problem of LM resistance.

Two main modes of action of MB have been suggested. One is its accumulation and probable concentration inside in the food vacuole where it inhibits the formation of hemozoin, just as the 4-aminoquinolines do. The other is inhibition of parasite glutathione reductase, which jeopardizes glutathione functionality, resulting in glutathione depletion, which sensitizes the parasite for chloroquine action [32,33].

Other than exhibiting inhibitor properties, MB has been reported to be a substrate that is reduced by flavoenzymes that produce reactive oxygen species [34] to generate oxidative stress. MB achieves generation of oxidative stress by inducing indirect structural changes in the interdimeric region of glutathione reductase. It targets the crucial redox machinery of *Plasmodium*, most effectively against the late ring/early trophozoite stages [28]. This further suggests the prospects of including MB in managing resistant malaria.

The over 99% suppression on the two resistant strains and remarkable survivorship observed in MB-treated mice demonstrates the high antimalarial potency of MB on LM- and PYR-resistant malaria. The results also present MB as safe to use since over 80% survivorship was observed at day 15 and 24 PI, respectively, in the mice groups infected with PYR- and LM-resistant parasites and treated with MB compared to the controls. It can thus be implied that the treatments with MB improve the survivorship of infected mice

Findings from this study have shown evidence that the thiazine dye is active against PYR- and LM-resistant parasites. This suggests that MB has the potential to be used in LM- and PYR-resistant malaria regions, having displayed suppression below detectable levels for 9 and 11 days, respectively, since the onset of treatment on the resistant *P. berghei*. Repositioning MB in the fight against malaria presents a different chemotherapeutic approach to resistant parasites that would, in turn, provide more options in terms of combinational therapy. Meissner *et al.* showed that MB is safe for use in the treatment of children presenting with uncomplicated *P. falciparum* malaria, with overall clinical and parasitological failure rates by day 14 of 10% and 24%, respectively [35].

Conclusions

This study has determined that MB could be a potential drug candidate in drug repurposing in the chemotherapeutic management of LM- and PYR-resistant malaria. It can, in the future, be considered as a replacement for LM in combination therapies. An in-depth study of its application against a wide range of resistant strains, including artemisinin-resistant *P. falciparum* and field isolates, and longer treatment periods would be warranted to determine its true utility and any possibilities of cross-resistance.

These results from resistant mutations obtained in a laboratory *in vivo* system may not be a clear representative of the natural populations of *P. falciparum*; therefore, further studies of MB on LM-resistant *P. falciparum* would be highly recommended. However, if these results obtained are general across parasite genotypes and species, they have significance especially for mutant parasites resulting from continued population-wide exposure to drugs.

Acknowledgements

We thank Kenya Medical Research Institute (Center for Traditional Medicine and Drug Research and Centre for Biotechnology, Research and Development) for assistance in acquiring test drugs and lumefantrine-resistant *P. berghei*. Special gratitude to the staff of Malaria Program – Department of Tropical and Infectious Diseases and the Animal Sciences Department (rodent facility) at the Kenya Institute of Primate Research for supplying us with mice used for this study and support in animal handling.

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Authors' contributions

VIM, HSO, and SAO were involved in the study conception and design. VIM developed the original concept of the study. DMK provided the lumefantrine drug and lumefantrine-resistant parasites for the study. VIM executed the laboratory work, produced the graphs, and wrote the drafts. RMM assisted in conducting the experiments. VIM and HSO analyzed and interpreted the data, reviewed the manuscript, and had primary responsibility for final content. ZWN reviewed the study design and reviewed the manuscript.

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Conflict of interests: No conflict of interests is declared.

Appendix V: Letter of invitation to present at the 2017 Keystone Symposia Conference (Malaria: From Innovation to Eradication)

KEYSTONE SYMPOSIA™
on Molecular and Cellular Biology
Accelerating Life Science Discovery

December 21, 2016

Victor Irungu Mwangi
Tropical and Infectious Diseases
Institute of Primate Research
P.O BOX 24481
Karen, NAIROBI, 00502
Kenya

Re: 2017 Keystone Symposia Conference
B5: Malaria: From Innovation to Eradication

Dear Victor Irungu Mwangi,

On behalf of meeting organizers Marcel Tanner, Sarah K. Volkman, Marcus V.G. Lacerda and Salim Abdulla, it is a pleasure to invite you to participate in the conference: *"Malaria: From Innovation to Eradication,"* to take place February 19, 2017-February 23, 2017, at the Speke Resort & Conference Centre, Kampala, Uganda.

Thank you for your completed registration. We have also received your poster abstract entitled, "Repositioning methylene blue for the management of resistant malaria." We look forward to your participation at the symposium.

Sincerely,

Jane L Peterson

Jane L. Peterson, Ph.D.
President and Chief Executive Officer

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Appendix VI: Letter of invitation to present at the 4TH KEMRI Annual Scientific and Health (KASH) Conference

KEMRI ANNUAL SCIENTIFIC AND HEALTH CONFERENCE

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

21st Jan 2014

Dear Dr. Mwangi V,

We are pleased to inform you that your paper titled "**Methylene Blue - Antimalarial Activity against Selected Drug Resistant Rodent Plasmodium Parasites"** has been accepted for **poster** presentation at the KEMRI Annual Scientific and Health (KASH) Conference in Nairobi, Kenya to be held February 5-7, 2014.

Please note the following:

Visit the KEMRI website www.kemri.org for regular KASH conference updates and to obtain complete author information. Your assigned abstract **#101 in the Traditional Medicine session** will be listed online on the preliminary program. Instructions for the presentation format will be available on the website and are also attached with this letter.

You are encouraged to submit a full paper after the conference to the KEMRI journal:

The African Journal of Health Sciences
Kenya Medical Research Institute
P.O Box 20752-00200,
Nairobi.
Email:info@ajhsjournal

We thank you for submitting this paper for consideration and look forward to your participation in the 4th KASH Conference.

Best regards,

R Nelia Karanja

Dr. Robert Karanja, Chair, KASH Conference Scientific-Sub Committee