CHEMICAL SYNTHESIS, EFFICACY AND SAFETY OF ANTIMALARIAL HYBRID DRUG COMPRISING OF SARCOSINE AND ANILINE PHARMACOPHORES AS SCAFFOLDS

Jean Baptiste Niyibizi

MASTER OF SCIENCE IN MOLECULAR BIOLOGY AND BIOTECHNOLOGY

PAN AFRICAN UNIVERSITY INSTITUTE FOR BASIC SCIENCES TECHNOLOGY AND INNOVATION

2017

Chemical synthesis, efficacy and safety of antimalarial hybrid drug comprising of sarcosine and aniline pharmacophores as scaffolds

Jean Baptiste Niyibizi

MB300-0011/15

A thesis submitted to Pan African University, Institute for Basic Sciences Technology and Innovation in partial fulfillment of the requirement for the degree of M.Sc. in Molecular Biology and Biotechnology

March 2017

DECLARATION

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

Signature: _____ Date: _____

Jean Baptiste Niyibizi

This thesis was submitted with our approval as University Supervisors.

Signature: _____ Date: _____

Dr Joseph K. Ng'ang'a

Department of Biochemistry

Jomo Kenyatta University of Agriculture and Technology, Kenya

Signature: _____ Date: _____

Dr Peter G. Kirira

Department of Physical Sciences

Mount Kenya University, Kenya

DEDICATION

I dedicate my project to my mother and my siblings.

ACKNOWLEDGEMENT

I am thankful to almighty God for his grace and mercy. My sincere thanks go to PAUSTI/JKUAT through African Union for this award. I highly appreciate my supervisors: Dr Joseph Ng'ang'aand Dr Peter G. Kirira for their invaluable support towards implementation of this project. I am also so grateful to Kenya Medical Research Institute (KEMRI) and Mount Kenya University (MKU) for providing some crucial facilities during my laboratory work. I also thank Francis KIMANI for his assistance at KEMRI/Malaria Laboratory. Last but not least, I thank all my fellow colleagues whom we shared ideas during this study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF CHEMICAL REACTIONS

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

ACT: Artemisinin Combination Therapy

Aniline: 3-Chloro-4-(4-chlorophenoxy) aniline

ATCase: Aspartate Carbomyl-transferase

CC_{50}: Cytotoxicity Concentration at 50%

CDC: Center for Disease Control and Prevention

CQ: Chloroquine

DMSO: Dimethyl sulfoxide

ED50: Effective Dose at 50%

ENR: Enoyl Acyl Carrier Protein Reductase

IC_{50}: 50% inhibitory concentration

IP: Intraperitoneal

IC_{50}: Inhibition Concentration at 50%

JKUAT: Jomo Kenyatta University of Agriculture and Technology

KEMRI: Kenya Medical Research Institute

MKU: Mount Kenya University

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MEM: Minimum Essential Eagle's Medium

OD: Optical Density

OECD: Organization for Economic Co-operation and Development

PAUSTI: Pan African University of Basic Sciences Technology and Innovation

***P. f* ATPase:** *P. falciparum* sarco-endoplasmic reticulum calcium ATPase

***P. f* ENR:** (Enoyl Acyl Carrier Protein Reductase)

PMI: President's Malaria Initiative

RBCs: Red Blood Cells

SERU: Scientific and Ethics Review Unit

WHO: World Health Organization.

ABSTRACT

Malaria is a disease caused by protozoan plasmodium transmitted to humans by infected female *Anopheles* mosquitoes. According to WHO report of 2015, there were 214 million worldwide cases of malaria with 438,000 deaths. Ninety percent of world's malaria cases occur in Africa, where the disease is recognized as a serious impediment to economic and social development. Despite advancement in malaria research, the disease continues to be a global problem especially for developing countries. Currently, there is no effective vaccine for malaria control. In addition, though there are effective drugs for treatment of malaria, this could be lost to the drug resistance in different *Plasmodium species*. The most lethal form is caused by *P. falciparum* which has developed resistance to many chemotherapeutic agents and possibly to the current drugs of choice. Reducing the impact of malaria is a key to achieving the sustainable development goals which are geared towards eradicating the disease. Covalent bitherapy is a rational and logical way of drug design which entails joining a couple of molecules with individual intrinsic action into a unique agent, hence packaging dual-activity into one hybrid molecule. The main objective of this study was to synthesize, determine the efficacy and safety of antiplasmodial hybrid drug comprising of sarcosine and 3-Chloro-4-(4-chlorophenoxy) aniline for management of plasmpodial infections. The hybrid drug was synthesized by adding thionyl chloride to sarcosine in order to form acyl chloride which was added to aniline to form sarcosine-aniline hybrid drug. *In vivo* efficacy was done using mice and *in vitro* cytotoxicity assay was done using vero cells. Data for *in vivo* drug assays and cytotoxic assays were analyzed using Microsoft excel software from which ED_{50} and CC_{50} of each drug concentration was determined. Using SPSS software version 20, one way ANOVA was applied for comparing *in vivo* efficacy and cytotoxicity of hybrid drug against control drugs. *In vivo* evaluation was carried out using *P. berghei ANKA* strain sensitive to quinolone. The ED_{50} of sarcosine-aniline hybrid drug was 6.49mg/kg compared to that of aniline derivative which was 3.61mg/kg. The ED_{50} of control drugs were 3.56mg/kg, 2.94mg/kg and 1.78 mg/kg for artesunate-aniline hybrid drug, artesunate and CQ respectively. There was a significant difference ($P<0.05$) between ED_{50} of sarcosine-aniline hybrid and both controls including 3-Chloro-4-(4-chlorophenoxy) aniline, artesunate, artesunate-aniline hybrid and chloroquine. Cytotoxicity results revealed that hybrid drug was safe to vero cells with a CC_{50} of 50.18±3.53µg/ml, whereas the CC_{50} of artesunate, chloroquine and doxorubicin were 19.69±3.26, 57.96±3.85 and 1.96 ± 0.59 µg/ml respectively. Sarcosine-aniline hybrid was significantly less toxic compared to artesunate and doxorubicin ($P < 0.05$). Sarcosine-

aniline hybrid drug was also safe to mice, as no mice died and no significant loss of weight after administering them with 2000mg/kg of the hybrid for 14 days. Therefore, covalent bitherapy should be used in drug development for drug resistance mitigation.

KEY WORDS: Chemical synthesis, ED_{50}, CC_{50}, Antimalarial resistance, Sarcosine-aniline hybrid.

CHAPTER ONE

INTRODUCTION

1.1 Background

Chemotherapy has been the backbone of malaria control strategy. The plasmodia species that are considered responsible for malaria in humans are: *P. vivax, P. falciparum, P. ovale, P. malariae* and *P. knowlesi* (CDC, 2015). However, *P. falciparum,* the parasite that causes over 90% of all global malaria cases is more frequently becoming resistant to classical antimalarials, necessitating an urgent need for research and synthesis of new antimalarial agents, preferably with novel mode of action (Rosenthal, 2003). In the past two decades, only a few compounds belonging to a new class of antimalarial drugs, including amino-alcohols such as mefloquine, halofantrine and lumefantrine, sesquiterpene trioxanes such as artemisinin derivatives and naphthoquinones such as atovaquone were developed for clinical usage (Saifi *et al.,* 2013b). Currently, artemisinin based combination therapy (ACT) is considered as the gold standard against *P. falciparum,* in which the regimen uses a double combination therapy geared towards delay of resistance, or circumventing it altogether (Jadhav *et al.,* 2015). Many approaches to antimalarial drug discovery deployed include optimization of therapy with available drugs such as: combination therapy, developing analogs of the existing drugs, evaluation of potent agents from natural products especially plants, use of compounds originally developed against other diseases, evaluation of drug-resistance reversers (chemosensitizers) as well as new chemotherapeutic targets (Rosenthal, 2003).

Currently, chemotherapy is the mainstay of malaria control strategy. However, reports from South-East Asia, indicate parasite resistance to artemisinin based drugs which are considered as gold standard in treatment of malaria according to WHO (Wells *et al.,* 2015). This suggests the need to develop new antimalarial drugs that are effective against malaria parasite based on new mode of action, molecular targets and chemical structure. *In silico* studies have shown that sarcosine (N-methyl glycine) is able to bind to Asparatate Carbomyl Transferase, an enzyme involved in PYRIMIDINE biosynthesis of the parasite. In addition, it was shown that 3-Chloro-4-(4-chlorophenoxy) aniline (aniline derivative) can bind to *P. f* ENR (Enoyl Acyl

Carrier Protein Reductase), an enzyme which catalyze the last step of the elongation cycle in the biosynthesis of fatty acids (Cronan, 2016) thereby inhibiting the parasite growth. Nevertheless, no experimental study has been conducted to confirm this aspect of the two to have antiplasmodial activity singly or in a hybrid molecule. Globally, malaria transmission occurs in three main WHO regions, where African region accounts for the most global cases(88%),followed by South-East Asia region (10%) and the Eastern Mediterranean region (2%) (WHO, 2015).

It is estimated that 3.2 billion people in 95 countries and territories are at risk of being infected with *plasmodium* species and developing disease (Figure 2.1), and 1.2 billion are at high risk, where more than1 in 1000 people have chance of getting malaria in a year. Results from the World Malaria Report of 2015 show that the malaria cases were ranging between 149 to 303 millions or an average of 214 million cases of malaria globally. The African region accounted for most global cases of malaria (88%), followed by the South-East Asia region (10%) and the Eastern Mediterranean region (2%). In addition, the number of people that died of malaria was in the range of 236, 000 to 635, 000, representing an average of 438 000 malaria deaths. The high burden was heaviest in the WHO African Region (Figure 2.1), where an estimated 90% of all malaria deaths usually occur (Figure 2.1), and in children aged under 5 years, who accounted for more than two thirds of all deaths (WHO, 2015a). This is because children of this age group are highly susceptible to infection, illness than adults (Snow *et al.,* 2005). From the year 2000, malaria incidence rates fell by 37% globally, and by 42% in Africa whereas the malaria mortality rates went down by 66% in the African region and by 60% globally (WHO, 2015).

1.2 Problem Statement

According to WHO report of 2015, almost ninety percent of world's malaria cases occur in Africa, especially in endemic regions (WHO, 2015b), where the disease is recognized as a serious impediment to economic and social development (Ohashi *et al.,* 2002). The high rate of the global malaria burden is found in sub-Saharan Africa. In 2015, 88% of global malaria cases and 90% of global malaria deaths were reported in sub-Saharan Africa, bearing the highest global burden. Up to seventy percent (70%) of Kenyans are at risk and experts argue that climate change is fueling malaria cases in Kenya (Elizabeth *et al.,* 2008). According to WHO, World Malaria Report of 2014, it showed that 100% of the Rwandan population is at risk of being

infected with malaria parasites as confirmed by the President's Malaria Initiative (PMI) in Rwanda survey reported in 2015 (PMI, 2015). Malaria cases rose from 514,173 in 2014 to 1,957,000 as confirmed by the report from Ministry of Health in Rwanda (Rwanda Ministry of Health, 2016). Currently, chemotherapy is the mainstay in malaria control and artemisinin based combination therapies (ACTs) have been advocated as the therapy of choice to handle widespread drug resistance in *P. falciparum* malaria (Jadhav *et al.,* 2015). However, reports from Southeast Asia, indicate possibility of parasite resistance to artemisinin based drugs, the current drug of choice in malaria chemotherapy, thus suggesting a novel development to malaria therapies. Many other antimalaraial drugs of choice have developed resistance over time, for instance chloroquine resistance was documented on every continent with malaria in 1972-1990. In the late 1990s, mefloquine resistance emerged in malaria from Southeast Asia (Wells *et al,* 2015). Using *in silico* drug design approach, it was shown that sarcosine and 3-Chloro-4-(4-chlorophenoxy) aniline can inhibit enzymes involved in pyrimidine and lipid membrane biosynthesis of the parasite, thereby blocking its growth and multiplication. Malaria remains a challenging disease in its diagnosis and control. Even though, there are some effective drugs for treatment of malaria, this could in the near future be lost to drug resistance in *Plasmodium species.* Furthermore no effective vaccine has been successful so far (Doolan *et al.,* 2003). The rapid emergence of *P. falciparum* strains resistance to the available antimalarial drugs and cross-resistance of parasites against antimalarial agents has resulted in the real need for new drugs to control malaria (Wells *et al.,* 2015). Despite improvement in the malaria diagnosis and control, parasite resistance to antimalarial drugs continues to be a serious threat, hence the need for new antimalarial drugs.

1.3 Justification of the study

In search for new drugs based on how the antimalarial drug discovery pipeline has changed over the past 10 years, it is urgent to look for new biological molecules that are effective against malaria parasite based on new mode of action, molecular targets and chemical structure. Sarcosine and 3-Chloro-4-(4-chlorophenoxy) aniline were suggested to have antiplasmodial activity based on bioinformatics studies. Sarcosine acts by binding to *P. falciparum* ATCase (Florence *et al.,* 2012) , an enzyme involved in pyrimidines biosynthesis for the parasite survival, whereas 3-Chloro-4-(4-chlorophenoxy) aniline can inhibit the Enoyl-acyl carrier protein (ACP) reductases (ENRs), which catalyzes the last step of the elongation cycle in the biosynthesis of

fatty acids (Cronan, 2016). Covalent linking of sarcosine and 3-Chloro-4-(4-chlorophenoxy) aniline can carry a dual activity with different mode of action for inhibiting plasmodial growth. Based on the urgent need in drug development, it was necessary to validate this claim in this study. The expected outcome was that sarcosine-aniline hybrid could contain the dual activity of inhibiting the pyrimidine and fatty acids biosynthesis which might circumvent the parasite resistance to ACTs. Sarcosine-aniline hybrid was envisaged to be a safe and affordable antimalarial molecule in treatment of plasmodial infections. Sarcosine-aniline hybrid Sarcosine can be cheap compared to artesunate and lumefantrine combination (coartem), a current drug of choice for treating malaria. Sarcosine-aniline hybrid can be considered as a safe drug by the fact that it targets parasites proteins involved in nucleic acid synthesis without affecting host cells.

1.4 Research questions

a. Does sarcosine-aniline hybrid drug have any bioactivity on plasmodial cells?

b. Is sarcosine-aniline hybrid safe to mice and vero cells?

1.5 Hypothesis

Sarcosine-aniline hybrid has no antiplasmodial activity.

1.5 General objective

To synthesize, determine the efficacy and safety of antiplasmodial hybrid drug comprising of sarcosine and 3-Chloro-4-(4-chlorophenoxy) aniline for management of plasmodial infections.

1.6 Specific objectives

1. To synthesize antimalarial hybrid comprising of sarcosine and 3-Chloro-4-(4-chlorophenoxy) aniline.
2. To determine *in vivo* antiplasmodial activity of sarcosine-aniline hybrid.
3. To determine *in vivo* toxicity of the hybrid using mice and cytotoxicity using vero cells.

CHAPTER TWO

LITERATURE REVIEW

2.1 Epidemiology of malaria

Malaria is a mosquito-borne tropical disease caused by *Plasmodium species* (Aminake & Pradel, 2013). The protozoan *Plasmodium* is a eukaryotic unicellular pathogen belonging in apicomplexa group), which is transmitted to humans by infected female *Anopheles* mosquitoe (Aminake & Pradel, 2013). The *Plasmodia species* that are considered responsible for malaria disease in humans are: *P. vivax***,** *P. falciparum***,** *P. ovale***,** *P. malariae* and *P. knowlesi* (CDC, 2015)**.** It is highly prevalent in developing countries, especially in sub-Saharan Africa (WHO, 2015). It is also found in Central and South America, Asia and Oceania (Hay *et al.,* 2004)**.** *P. falciparum* is the most prevalent species than others (Howes *et al.,* 2011). *P. vivax* is the species which is difficult to control due to its biological features that distinguish it from *P. falciparum* (Moyes *et al.,* 2012). *P. vivax* sporozoites develop faster than *P. falciparum* within the mosquito, and with wider temperature ranges, which allows it to be greatly distributed geographically (Gething *et al.,* 2010). The other most crucial feature of *P. vivax* biology is its capacity to relapse in the weeks or months through a dormant liver stage called hypnozoite (White, 2011). *P. vivax* endemicity is based on the distribution of the Duffy-negative phenotype (Dean, 2005). Duffy glycoprotein is a receptor that binds chemicals that are secreted by blood cells (cytokines) during inflammation (Dean, 2005). it also a receptor for *Plasmodium vivax,* a protozoan parasite that invades erythrocytes and causes malaria (Dean, 2005). In most cases, Duffy negative or Duffy null phenotype (FY a-b-) individuals are more resistant to infection by *P. vivax* because they do not express duffy antigen on their RBCS, and this phenotype is mostly found in Africa at highest frequencies (Howes *et al.,* 2011). *P. malariae* infections are commonly found in sub-Saharan Africa and the southwest Pacific. *Plasmodium ovale* prevalence is more limited to areas of tropical Africa, Guinea, the eastern parts of Indonesia and the Philippines and in different parts of Southeast Asia (Mueller *et al.,* 2007).

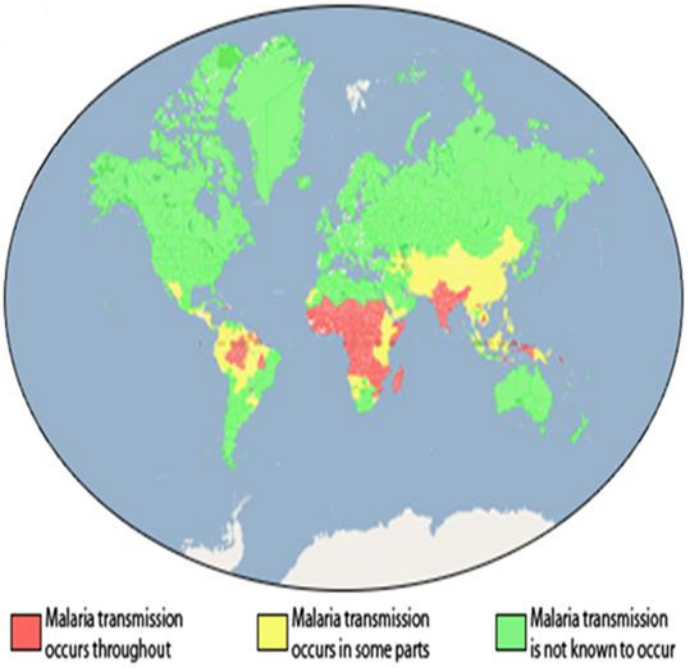


Figure 2.1: Global distribution of Malaria throughout the world (WHO, 2015)

Figure 2.1 shows that most of the malaria cases occur in Africa followed by Southeast Asia and Latin America. From the same figure, it is also shown that malaria has been eradicated in USA and Europe.

2.2 Biology of malaria infection

2.2.1 Mode of transmission

The primary transmission of malaria occurs when an infected female anopheles mosquito (vector) feeds from human host. The other rare ways of transmission include transfusion of blood from infected persons, use of contaminated sharp materials such as: needles and syringes, and from mother to child transmission during delivery (WHO, 2013).

There are about 460 species of Anopheles mosquito genus that have been identified up to date, and over 100 can transmit malaria (Dash *et al.*, 2007). Many mosquitoes were found in India with 58species (Jadhav *et al.*, 2015). The examples of these include *anopheles gambiae, anopheles funestus* and anopheles *stephensi, Anopheles culicifacies, Anopheles fluviatilis, Anopheles minimus, Anopheles sundaicus, Anopheles dirus* (Shiff, 2006) and (Jadhav *et al.*, 2015).

2.2.2 The life cycle of *P. falciparum* in the human host and the anopheles mosquito vector

The life cycle is approximately identical for all *plasmodium* species that may infect humans. The infection undergoes three major stages: the infection of a human with sporozoites, the asexual reproduction and the sexual reproduction. The two first stages take place exclusively in the human body (Figure 2.2), while the third stage starts in the human body and it is completed in the female anopheles mosquito (Weekley & Smith, 2013).

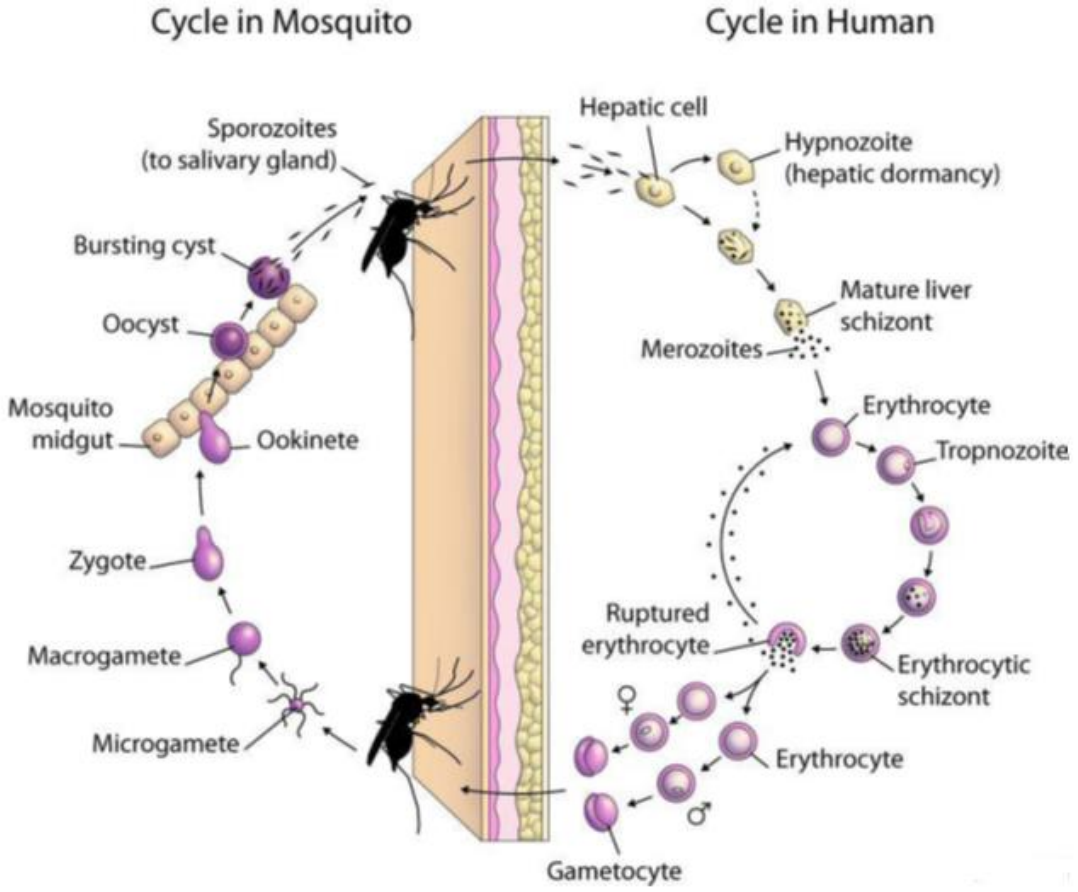


Figure 2.2: Life cycle of *Plasmodium species* (Srinivas, 2015)

As the above figure (Fig 2.2) shows, the human infection starts when the already infected female *Anopheles* feeds from a person, injecting Plasmodium parasites, in sporozoites stages, into the bloodstream. The next stage is the asexual reproduction that is further divided into two phases: the exoerythrocytic phase which occurs outside the erythrocyte and the erythrocytic which takes place inside the red blood cell. After 60 minutes the sporozoites pass into the first target which is human liver. Inside liver tissue, the sporozoites begin subsequent division leading to schizonts

formation between 6 to 7 days. Every schizont develops into merozoites, an exoerythrocytic schizogony that is released into the blood circulation which marks the final step of the exoerythrocytic phase during the asexual reproductive step (Figure 2.2). However, *P. ovale* and *P. vivax* sporozoites might not follow the reproduction process and remain dormant for weeks or even years in the liver, the state in which they are called hypnozoites (Jadhav *et al.,* 2015). They might be stimulated after a long time such as weeks, months or years, which lead to relapses of merozoites and their penetration in the blood stream. The exoerythrocytic period is not pathogenic and might not show symptoms or clinical signs of the disease (Srinivas, 2015).

Merozoites liberated into the blood stream, invade the erythrocytes. This invasion is the starting of the erythrocytic phase. After invasion, the first stage is the formation of a ring stage that transforms into a trophozoite. The next stage is the formation of erythrocytic schizont which consists of forming immature and mature schizont. Every mature schizont grows and give to merozoites, an erythrocytic schizogony, which after Red Blood Cells rupture, are released in the blood stream, so that they can invade other erythrocytes. This is when clinical signs are noticed (Jadhav *et al.,* 2015). Inside RBCs, there is also differentiation of the parasite into gametocytes, forms that do not cause pathogenicity. Once the female anopheles mosquito takes a blood meal from infected person, it picks up these gametocytes. The gametocytes grow into microgametes, which are male gametocytes and macrogametes as the female gametocytes during gametogenesis (Figure 2.2). The period required for the gametocytes to become mature varies for each *Plasmodium* species. For example, the gametocytes of *P. vivax* and *P. ovale* take 3 to 4 days to reach maturity, but it is 6 to 8 and 8 to 10 days for *P. malariae* and for *P. falciparum* respectively (Weekley & Smith, 2013).

Inside the mosquito gut, the nucleus of microgamete undergoes subsequent divisions to produce eight nuclei. Every nucleus fertilizes a macrogamete which results in formation of a zygote, which further becomes the ookinete. Then, ookinete enters inside the wall of the mosquito midgut, in which it encysts into oocyst. Within the oocyst, the ookinete nucleus continues to divide and produce a lot of sporozoites (sporogony). This shows the termination of the third stage of sexual reproduction or sporogony which lasts 8 to 15 days. This is followed by rupture of oocyst in which the sporozoites are deposited within the mosquito cavity and get their direction to its salivary glands (Jadhav *et al.,* 2015). When the infected mosquito feeds again on

a host, it inoculates the sporozoites into the human blood circulation, which starts a new cycle (Figure 2.2).*P. falciparum* is a protozoan parasite which multiplies in different stages from ring stage to gametocytes stage (Figure 2.3). Under microscope, parasites can be observed with 100x objective after staining it with giemsa stain. On figure 2.3, 1 represents normal red blood cell, 2-10 correspond to ring-stage trophozoites, 10-18 are mature trophozoites, 19-26 are schizonts, 27-28 are mature macrogametocytes (female) and 29-30 are mature microgametocytes (male).

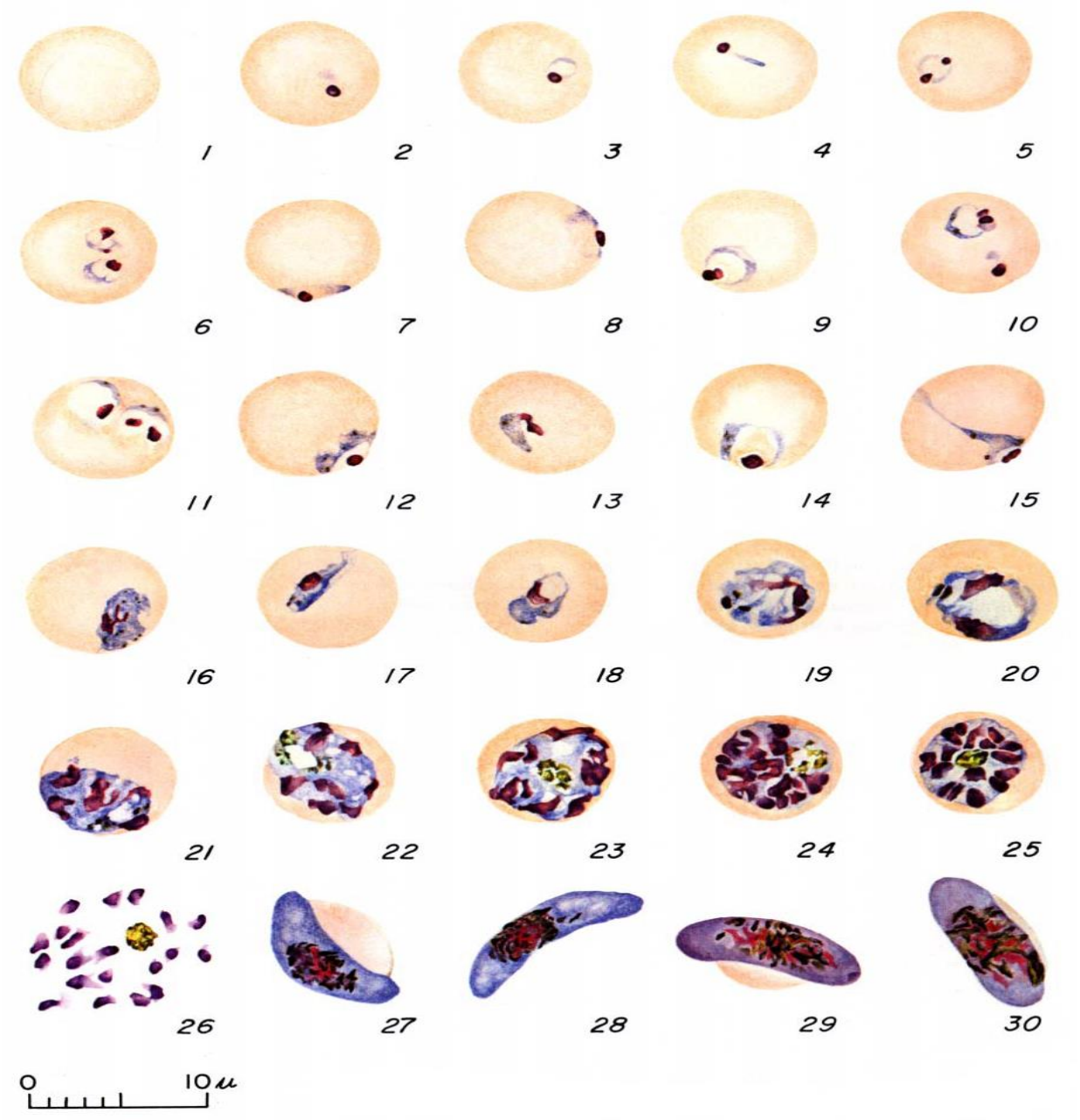


Figure 2.3: Appearance of *P. falciparum* blood stage parasites on thin blood smears (Coatney *et al.*, 1971)

2.3 Classification and mode of action of antimalarial drugs

The antimalarial compounds are classified based on the stage of the parasite to be targeted and their mode of use, either as prophylaxis or treatment. There are different stages of malaria life cycle and each stage differs by morphology, metabolism and antimalarial drug sensitivity (Basco *et al.*, 2001).

a) The best classification of antimalarial drugs is based on their life cycle (Jadhav *et al.*, 2015). The possible stages of drug therapy known so far are categorized into 4 different groups:

1) Drugs that kill the sporozoites injected by the mosquito or prevents the sporozoites from entering the liver. Examples: Pyrimethamine, lumefantrine and mefloquine (Delves *et al.*, 2012).

2) Compound that destroy the hypnozoites residing in hepatocytes or prevent them from becoming merozoites. Examples: Pyrimethamine, artemether, artesunate, Dihydroartemisinin, atovaquone and Primaquine (Saifi *et al.*, 2013b).

3) Drug that can eliminate the merozoites in the blood or prevent them from developing into further stages until they reach gametocytes. Examples: Pyrimethamine and atovaquone, chloroquine, artemether *(*Delves *et al.*, 2012)

4) Antimalarial drugs that can kill the gametocytes before they can enter the mosquito, therefore preventing development into zygotes. Examples: Mefloquine and amodiaquine (Kiszewski, 2011).

b) There are four major types of antimalarials drugs that have been used so far: the quinolines (chloroquine, quinine, mefloquine, amodiaquine, primaquine), the antifolates (pyrimethamine, proguanil and sulfadoxine), the artemisinin derivatives (artemisinin, artesunate, artemether, arteether) and hydroxynaphthaquinones such as atovaquine (Saifi *et al.*, 2013).

2.3.1 Quinolines

The group of quinolone drugs include: chloroquine, amodiaquine, quinine and mefloquine (Figure 2.4). Chloroquine is an alkaline drug which accumulates in high concentration in vacuoles of the parasite and raises its pH (Aminake & Pradel, 2013). Actually, malaria parasite digest host cell's hemoglobin to obtain amino acids and the free heme is released. This heme has capacity to create oxidative damage to cell membranes and parasite proteins through redox-

active free heme (ferriprotoporphyrin IX [PPIXFe]) which is toxic (Loup *et al.*, 2007). Then, the parasite detoxifies it by using enzyme: haeme polymerase which converts toxic haeme into hemazoin compound which is non-toxic to the parasite. Chloroquine inhibits the parasitic enzyme haeme polymerase, thereby resulting in the aggregation of toxic haeme within the parasite, thereby causing the death of the parasite. In fact, Chloroquine stacks with heme to form a stable π-π complex, PPIXFe-chloroquine, which is not incorporated into hemozoin and which leads to the death of the parasite via a redox process (Loup *et al.*, 2007). Quinine has the same mode of action as chloroquine but with some differences, chloroquine causes clumping of the malaria pigment, whereas quinine antagonizes this process (Saifi *et al.*, 2013b). Mefloquine drug also acts via formation toxic complexes with free haeme which in turn damage membranes and interact with other plasmodial components (Dassonville *et al.*, 2011).

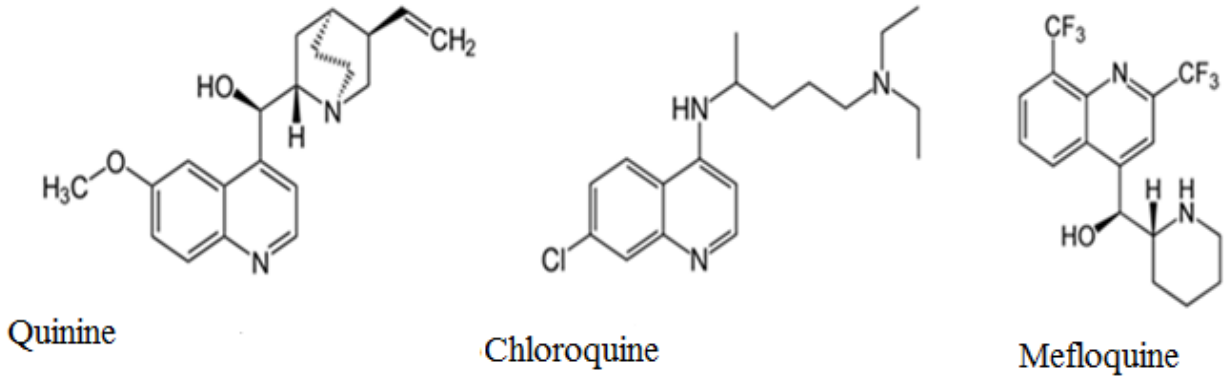


Figure 2.4: Examples of quinoline drugs (Aminake & Pradel, 2013)

2.3.2 Antifolates

Sulfadoxine andpyrimethamine are examples of antifolate (Figure 2.5). Pyrimethamine inhibits the dihydrofolate reductase (DHFR) of Plasmodium whereas Sulfadoxine inhibits dihydropteroate synthase (DHPS) (Aminake & Pradel, 2013), thereby blocking the biosynthesis of pyrimidines and some amino acids which are so essential for DNA synthesis and cell multiplication (Saifi *et al.*, 2013a). This leads to failure of nuclear division at the time of schizont formation in erythrocytes and liver (Bloland, 2001).

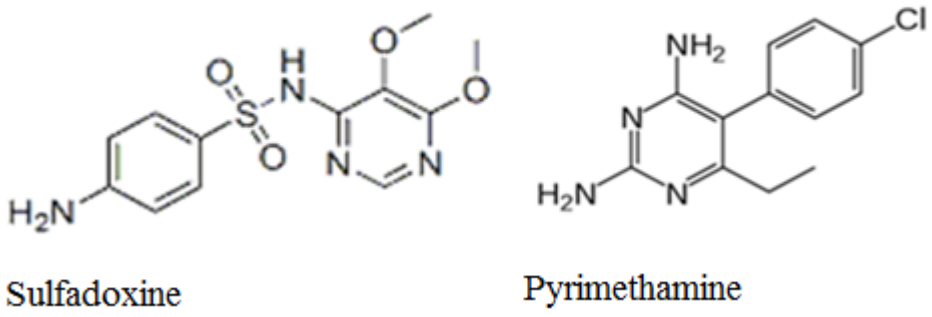


Figure 2.5: Structures of Sulfadoxine and Pyrimethamine (Aminake & Pradel, 2013)

2.3.3 Artemisinin and its derivatives

Artemisinin and its derivatives: artesunate, artemether and arteether (Figure 2.6) are the drugs of choice that are currently being used and their mode of action is based on the production of free radicals within the plasmodium vacuole (Aminake & Pradel, 2013). Artemisinin alkylates heme and forms a well-defined heme-artemisinin adduct mixture (Loup *et al.,* 2007). These adducts cannot be able to crystallize into hemozoin, therefore inducing oxidative damages to the parasite biomolecules (Loup *et al.,* 2007). These free radicals can damage the lipids, membranes and the structures of the organism, and inhibit its growth by inhibiting some enzyme such as sarcoendoplasmic reticulum Ca^{2+}ATPase (Jadhav *et al.,* 2015). In severe malaria, artemisinin and its derivative compounds produce faster parasite clearance and resolution of fever than quinine. Artemisinins also reverse coma more quickly than quinine (Delves *et al.,* 2012).

Artesunate

Artesunate is the sodium salt of the hemisuccinate ester of artemisinin. It is soluble in water but has poor stability in aqueous solutions at neutral or acid pH. It can be given orally, rectally or by the intramuscular or intravenous routes (Krishna, Uhlemann, & Haynes, 2004).

Artemether

Artemether is the methyl ether of dihydroartemisinin. It is more lipid soluble than artemisinin or artesunate. It can be given as an oil-based intramuscular injection or orally. It is also coformulated with lumefantrine (previously referred to as benflumetol) for combination therapy (Krishna *et al.,* 2004).

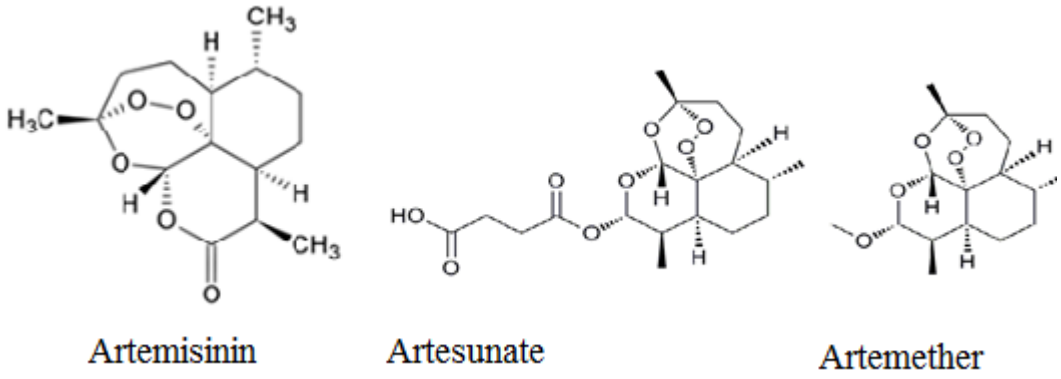


Figure 2.6: Structures of artemisisnin, artesunate and artemether (Aminake & Pradel, 2013).

2.3.4 Hydroxynaphthaquinones

Atovaquone is a hydroxynaphthaquinone which inhibits cytochrome c reductase activity in *P. falciparum* by binding to the cytochrome bc1 complex of the parasite mitochondrial electron transport chain which results in a loss of mitochondrial function (Aminake & Pradel, 2013). The malaria mitochondria electron transport chain disposes of electrons generated by dihydroororate dehydrogenase during the synthesis of pyrimidines. The inhibition of this process by atovaquone leads towards the death of the parasite (Bloland, 2001). Atovaquone can also dissipate the mitochondrial membrane potential of the malaria parasite which might kill the parasite by initiating a process similar to apoptosis (Delves *et al.*, 2012).The molecular weight of atovaquone is 366.837 g/mol and its structure is $C_{22}H_{19}ClO_3$ (Figure 2.7)

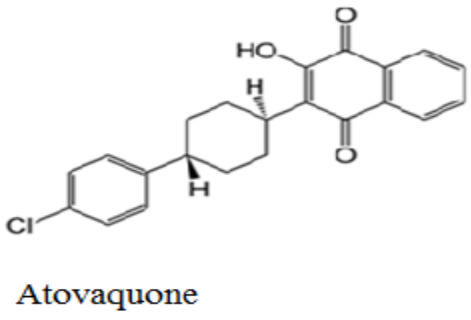


Figure 2.7: Structure of atovaquone (Aminake & Pradel, 2013)

2.4 Resistance to current drugs of choice

Artemisinin resistance is explained as a delay in parasite clearance following the treatment by an artesunate as a solely monotherapy drug, or after treatment with an artemisinin-based combination therapy. Such resistance represents partial resistance. The high resistance rate have been observed in Cambodia, Thailand and Greater Mekong sub-region (WHO, 2015c). In the study done in GMS, a molecular marker of artemisinin resistance was identified and it was proved that the mutations in the Kelch 13 (K13) propeller region are linked to the delayed parasite clearance for both *in vitro* and *in vivo* experiments (WHO, 2015c).

2.5 Antimalarial drug toxicity

Primaquine was proved to cause the toxic reactions in blood, such as: Leucopenia, granulocytopenia, formation of methahaemoglobin (cyanosis), and acute intravascular haemolysis (Ogutu *et al.,* 2014). Quinine was found to be a good drug for treating severe malaria because of its rare cardiovascular or Central Nervous System (CNS) toxicity, but it was shown to cause hypoglycemia, fever, confusion and respiratory arrest (Hussien, 2007). Artemether-lumefantrine showed the capability of fast parasite clearance in case of *P. malariae*. However it has some adverse effects such as: abdominal pain, anorexia, nausea, vomiting, diarrhea and central nervous system disorder including headache and dizziness. In addition, mefloquine can cause serious neuropsychiatric whereas pyrimethamine is able to cause agranulocytosis if the recommended dose is exceeded (Saifi *et al.,* 2013b). Chloroquine showed various adverse reactions such as neurotoxicity resulting in headache, confusion, depression, leukopenia, granulomatous hepatitis, retinopathy, rashes, pruritus and cardiovascular toxicity (Bosman, 2003). Amodiaquine was revealed to cause hepatotoxicity, especially leading to liver damage through formation of an electrophilic metabolite, which in turn cause hypersensitivity reactions (Alkadi, 2007).

2.6 *In silico* studies of sarcosine and aniline

Sarcosine was found to bind the parasite target such as proteins that are involved in parasite survival. For instance, *in silico* studies by using molecular docking, suggested that sarcosine can bind to *P. falciparum* ATCase (Florence *et al.,* 2012). *P. falciparum* ATCase is a highly regulated enzyme that catalyzes the first committed step in pyrimidine biosynthesis, the condensation of aspartate and carbomoyl phosphate to form N-carbomoyl-L-aspartate and

inorganic phosphate. The malaria parasite obtains the preformed purines by the salvage pathway and synthesizes pyrimidines de *novo.* Parasite cannot utilize preformed pyrimidines and must synthesize them from biocarbonate and glutamine (Bloland, 2001).

Enoyl-acyl carrier protein (ACP) reductases (ENRs) catalyze the last step of the elongation cycle in the biosynthesis of fatty acids (Cronan, 2016). Enoyl acyl carrier protein reductase catalyses the NAD (P)-dependent reduction of a trans-2,3 enoyl moiety into a saturated acyl chain, the second reductive step in the fatty acid biosynthesis pathway (Cronan & Massengo, 2009). In different studies done, it was shown that compounds such as the diazaborines and triclosan are able to inhibit the ENR enzyme(Rima *et al.,* 2002). Using bioinformatics studies, *P. falciparum* genome was screened and it was found that *P. f* ENR (Enoyl Acyl Carrier Protein Reductase) can be a drug target in order to inhibit the parasite survival. Using docking tool (Arguslab software),it was found that aniline can be a ligand to target protein (ENR), thereby inhibiting its activity (Florence *et al.,* 2012).

2.7 Sarcosine

Sarcosine is a molecule which is an intermediate compound and byproduct formed during glycine synthesis and degradation. It is converted into glycine with the aid of the enzyme called sarcosine dehydrogenase. On the other hand, the glycine-N-methyl transferase transforms glycine into sarcosine. Sarcosine is a fundamental amino acid encountered in body tissues, especially in muscles (NIH, 2015). Furthermore, it is synthesized from the nutritious intake of Choline and during methionine metabolism, in which it acts as an intermediate compound during the conversion of choline to glycine. It is then transformed into glycine, which is a crucial component of protein and it has a function in many physiological processes as an essential metabolic source of living cells components such as creatine, glutathione, serine and purines. In the blood serum, sarcosine concentrations are standardized to be 102.3ng/mL in men and 80.8ng/mL in women. Reference amount of sarcosine found in urine are 138.5ng/mL and 94.8ng/mL in men and women respectively. Urinary sarcosine was found to be correlated with urinary creatinine (Cernei *et al.,* 2013).

The formula of sarcosine is $C_3H_7NO_2$ (Figure 2.8), with net charge equal to zero (NIH, 2015). Its average Mass is 89.09322, ionization energy (IE) is 9.20 eV, Critical Pressure ($\mathbf{P_c}$) is 5585.83kPa

and Critical temperature is 643.02 K. Sarcosine is soluble in water and it reacts with acids, carboxylic; amides and imides (NIH, 2015).

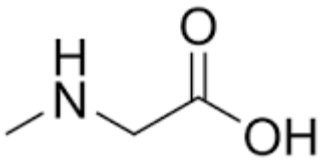


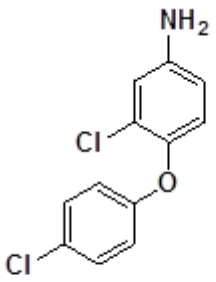
Figure 2.8: Structure of sarcosine (NIH, 2015)

2.8 Sarcosine in clinical diagnosis and treatment

Sarcosine is used as an antipsychotic ability in treatment of schizophrenia. It reduces neurocognitive and general psychopathological symptoms. In addition, it was proved to act as a type 1 glycine transporter inhibitor and a glycine agonist. It increases glycine concentrations in the brain, thus causing the increase of NMDA receptor activation and a reduction in symptoms (Cernei *et al.,* 2013). Actually, N-methyl-D-aspartate (NMDA), one subtype of glutamate receptors, plays an important role in learning and memory. Sarcosine was found to be an enhancing agent of N-methyl-D-aspartic acid (NMDA) and therefore can be used adjunctive therapy in treating schizophrenia and depression. Sarcosine was shown to play a great role in patients suffering from schizophrenia and no toxicity was observed (Doolan *et al.,* 2003).

2.9 3-Chloro-4-(4-chlorophenoxy) aniline

It is a chemical compound with molecular weight is 254.11g/mol, with the formula: $C_{12}H_9Cl_2NO$,(Figure 2.9), CAS: 24900-79-6 (NIH, 2015). It is used for research purposes (NIH, 2015).



3-Chloro-4-(4-chlorophenoxy) aniline

Figure 2.9: Structure of 3-Chloro-4-(4-chlorophenoxy) aniline (NIH, 2015)

2.10 Use of hybrid molecules in malaria chemotherapy

Hybrid molecules are promising antimalaraial drug candidate as it entails covalently linking molecules into one molecule. Hybrid molecules can be classified into four classes: Conjugate hybrids, in which both the pharmacophores are joined by stable linker which is not a part of either of the individual pharmacophores. Cleavage conjugate hybrids, in which molecules are joined by a linker designed to be metabolized inside the biological system in order to release the two independent pharmacophores that interact at different targets. Fused hybrids, in which the size of the linker is decreased or removed such that the framework of the pharmacophores is in close contact. Merged hybrids, have their frameworks merged by taking advantage of the common pharmacophore in the structures of the starting compounds, which give rise to smaller and simpler molecules (Chauhan & Sharma, 2010). Hybrid drugs have many advantages over single or combined drugs with improved pharmacokinetic profiles and potential activity against resistant strains (Lödige & Hiersch, 2015). Hybrid drugs are absorbed, distributed, metabolized, and excreted at one single rate. With hybrid drugs there is no competition for plasma protein binding as in the case of single drugs (Lödige & Hiersch, 2015). Trioxaquine is one example of conjugate hybrid, made by covalently linking a trioxane to a quinolone. The endoperoxide ring is essential for the antimalarial activity of artemisinin. Trioxane has alkylating ability from endoperoxide which facilite the drug's penetrarion into infected erythrocytes and an aminoquinoline moiety which facilitates the accumulation in the parasite vacuole and for the interaction with free heme (Garah *et al.*, 2008).

2.11 Use of model organisms in drug discovery and development

Model organisms have been used for long time as a mainstay of basic and applied researches in the life sciences era (Greek, 2013). In all models developed, only the animal model became the central model in medical research, pharmaceutical and biotechnology researches, including drug discovery, preclinical studies, and toxicology (Greek, 2013). Animals models that have long been used are mammalian species such as mice, rats, dogs, cats, pigs and primates (Haberman, 2010). In recent studies, employment of other species in animal mode have emerged in research studies. These include many invertebrate and lower vertebrate animal models such as *Caenorhabditis elegans*, fruit fly Drosophila, and zebrafish. The adoption of using such invertebrates and zebrafish as animal models was driven by the era of advances in genomics, where the different findings pointed out that not only genes but also some pathways, tend to be conserved during evolution (Haberman, 2010).

Nowadays, researchers use animal models for basic research, in developing new therapeutic strategies to treat human diseases, in drug discovery and preclinical studies of drug safety and efficacy. The drug discovery and development research entails target identification and validation, drug screening and lead optimization, toxicity and safety screening, thus it can provide the information of pharmacokinetic patterns of a drug such as absorption, distribution, metabolism, elimination and toxicity (ADMET) of a given drug or chemical compound (Greek, 2013). The approach of creating the new animal models is very crucial because of the poor predictive values in some therapeutic areas of oncology and central nervous system diseases which in turn cause major drug attrition during drug development (Haberman, 2010). Thus the introduction of human model based on induced pluripotent stem (iPS) cells can serve as a cutting-edge in organisms model researches with improved prediction in understanding physiology and pathway of many human diseases (Haberman, 2010). Mouse models are suitable sustems to study human diseases because their genetic makeup is closed to humans, easy to handle, accurate histological results generated which help in predicting treatment for human diseases including cancer (Kim & Sharpless, 2012).

2.12 Use of cell lines to study drugs toxicity

Cell lines used in experimental research are developed either in-house, purchased from cell bank or it can be acquired from other Laboratories (Geraghty *et al.,* 2014). Cell lines can be classified

into three categories such as normal cell lines (taken from a tumor tissue and culture as a single cell type), transformed cell lines (Normal cells that have undergone a genetic change to be tumor cells) and stem cell lines (master cells that generate other differentiated cell types) (Eltayeb, 2006). Many cell lines have been used for long time ago, U937 is a cell line isolated from a human with leukemia and it has been used for forty years ago in research studying mostly the function of genes and their defects in encoding protein especially in cancer development (Roy *et al.,* 2008). Hela cell lines have been isolated from a 31years old woman in Baltmore, US and they are still used nowadays in cervical cancer studies *(*Lucey *et al.,* 2009). C2C12 and the rat cell line L6 are used to assess and investigate the growth of muscle cells (Lawson, 2000). L6 Rat Myoblast Cell Line expresses GLUT4 protein containing a fourteen amino acid epitope of human c-myc, therefore it can be used to study glucose uptake and GLUT4 translocation (Coppock *et al.,* 1996). L6 cell lines can be used also to screen new compounds against diabetes (Coppock *et al.,* 1996). Vero cells are extracted from the kidney of the African green monkey, *Cercopithecus aethiops (*Levenbook *et al.,* 1984). They are quite homologous with human body cells. According to the British Standard 6920-2.5:2000, healthy Vero cells are triangular in shape (Liao *et al.,* 2010). Different studies done on viral vaccine production, showed that vero cells are suitable systems for isolation and growing influenza A and B viruses for vaccine production (Govorkova *et al.,* 1996).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

This project was a prospective lab based experimental study carried out at Kenya Medical Research Institute (KEMRI), Centre for Biotechnology Research and Development (CBRD), Malaria unit.

3.2 Materials

3.2.1 Sources of chemicals

Sarcosine, aniline, thionyl chloride, Dichloromethane, Magnesium sulfate and ammonium chloride, and Thin Layer Chromatogaraphy (TLC) plates provided by Sigma Aldrich, Kenya.

3.2.2 Experimental animals and parasites

Female Swiss albino mice 50 mice were got from the animal house of the Center for Biotechnology and Research Development at Kenya Medical Research Institute (KEMRI) where the animal experiments were carried out. The animals were six weeks old, weighing 20 to 22g, and they were kept in mice cages and were allowed to access mice pencils (Ungafeed Company Ltd) and water prior experimental testing. *Plasmodium berghei* ANKA strains, sensitive to quinolone and artemisinin based drugs were obtained from the Unit of Malaria, Centre for Biotechnology and Research and Development, Kenya Medical Research Institute. Parasites were maintained and cryopreserved in freezer (-80^{o}C). The parasite was subsequently maintained in the laboratory by serial blood passage from mouse to mouse on weekly basis.

3.2.3 Vero Cells

Vero cells were obtained from Center for Traditional Medicine Drugs and Research (CTMDR), KEMRI. Vero cells are kidney cells extracted from an African green monkey (*Cercopithecus aethiops*). They were stored in nitrogen tank (-191^{o}C) in Biological department, CTMDR, KEMRI.

3.3 Coupling aniline to sarcosine

3.3.1 Procedure for coupling aniline to sarcosine.

Thionyl chloride (1.5 mL, 20 mmol) was added to sarcosine (0.09 g, 1.0 mmol) and the resulting suspension was refluxed for 6 hours to give a clear yellow solution. Excess thionyl chloride was removed *in vacuo* and the acid chloride was dissolved in dry dichloromethane (CH_2Cl_2) (10 mL) and cooled to 0°C. A solution of 3-Chloro-4-(4-chlorophenoxy) aniline (4.0 mmol) and triethylamine (0.20 mL, 2.0 mmol) in dry CH_2Cl_2 (2.5 mL) was added via cannula. The resulting mixture was stirred at room temperature for 16 hours during which time a white precipitate was formed. The suspension was washed with half-saturated aqueous ammonium chloride solution (2 × 6 mL) and water (2 × 3 mL), then dried over anhydrous magnesium sulphate ($MgSO_4$) and concentrated *in vacuo*. The formation of hybrid molecule was monitored by Thin Layer Chromatography (TLC).

3.3.2 Thin Layer Chromatography procedure

The TLC was run by spotting the sarcosine-aniline hybrid on TLC plate in a solvent system of ethyl acetate (2ml).The plate was dried in order to observe and take a photo of the spot, in a small container that has a lid mix of about 4g of silica gel and 1g of iodine crystals. The TLC plate was placed in the mixture and shaked gently in order to get in contact with the TLC plate for 5minutes. The plate was taken to fluorescent machine under UV to observe dark spots of aniline and sarcosine-aniline hybrid. After TLC experiment, the location of each spot on the plate was represented by calculating its Retention factor (Rf). The Retention factor (Rf) was calculated by dividing the distance travelled by the compound by the distance from the baseline to the solvent front (Spangenberg *et al.,* 2011).

$$Rf = \frac{\text{distance spot moved}}{\text{distance solvent moved}}$$ (Vissers *et al.,* 1995)

3.4 *In vivo* efficacy of sarcosine-aniline hybrid

3.4.1 Procedure for *in vivo* efficacy

This consisted of *in vivo* evaluation of hybrid drug against *P. berghei* ANKA, a rodent parasite which is commonly used in antimalarial studies (Fidock *et al*., 2004). Swiss albino donor mouse was inoculated with *P. berghei* ANKA and parasitaemia was assessed after 5 days under

microscope using giemsa staining technique. The donor mouse was sedated using carbon dioxide in order to collect blood via cardiac puncture in heparinized tube using a syringe and a needle. Carcasses were pooled in a bio-hazard container and stored at room temperature while waiting to be incinerated. Afterwards, the experimental mice were infected with inoculums of $1x10^7$ parasitized erythrocytes using intraperitoneal method. The mice were grouped into five groups consisting of 5 mice per group. The sarcosine-aniline hybrid was dissolved in 10% of tween-80 and daily oral drug administration was done for 4 days. Blood for making thin blood smear was collected from the tip of the mouse tail. The efficacy of the drug was measured by comparison of blood parasitaemia after the four days of therapy (that is on day 5 post-infection). The ones still alive at the end of the experiments were killed by sacrificing them using carbon dioxide gas followed by incineration.

3.4.2 Determination of the dose

The determination of the dose to be administered was calculated individually according to each mouse weight. In this regard, these formulas were applied:

HED (mg/kg) = Animal Dose (mg/kg)*[Animal Km / Human Km], Where HED stands for Human Equivalent Dose and Km which is a conversion factor.

With Km (a constant) = Weight/Body surface area

For an adult human of 60kg, with body surface area of 1.6, Km= 60/1.6=37

For mice of 0.02kg, with body surface area of 0.007, Km= 0.02/0.007=3

(Shannon *et al.*, 2007).

$$\text{Dosage (mg/kg)} = \frac{Drug\ concentration\left(\frac{mg}{ml}\right) * Volume\ (ml)}{Body\ weight\ (Kg)}$$

From the above second formula:

$$\text{Volume (ml) of drug to be given to each mouse} = \frac{Body\ weight\ (Kg) * Dosage\ (mg/kg)}{Drug\ concentration\ (mg/ml)}$$

(Research Animal Resources Center, 2016)

3.4.3 Administration of the drug

Administration of the dose for testing drugs and reference drugs (artesunate and chloroquine) to assess the treatment of the experimental groups had been done using oral method, with varying dosages of 10, 5, 2.5, 1.25 and 0.625mg kg^{-1}. The control groups had been given the normal saline alongside testing using the same procedure. The parasitaemia was checked for every 24 hours from time of infection for 4 days. Thin blood smears were prepared from each mouse tail

venous blood. The smears were prepared by giemsa staining technique and the parasitaemia of individual mouse had been checked using light microscope (Kalra *et al.*, 2006).

3.4.4 Procedure for giemsa staining

Prepared thin blood smears were fixed with methanol for 1-2 minutes after placing them on the staining rack at horizontal flat position. Smears were then flooded with Giemsa working solution and allowed to be stain for 10 to 20 minutes. After this, slides were rinsed with tap water and then dried on air dryer (Ogutu *et al.*, 2014). Microscopy examination was done using light microscope for observation of parasitized red blood cells. The following formula was used to calculate the percentage parasitemia:

$$\text{The Percentage of parasitaemia} = \frac{\text{Number of parasitized RBCs/field}}{\text{Total number of RBCs/field}} * 100$$

(Kalra *et al.*, 2006)

After 4 days, the percentage (%) chemosuppression of each drug was determined using the formula:

$$\text{Percentage chemosuppression} = 100 - \left[\left(\frac{A-B}{A}\right) \times 100\right]$$ (Peters *et al.*, 1975)

Where A is the mean parasitaemia in the negative control group and B is the parasitaemia in the test group. The dose that cured 50% of infected animals had been determined as Effective dose (ED_{50}) using a non-linear regression logistic dose-response model.

3.5 Cytotoxicity assays

3.5.1 Preparation of drugs solutions

To conduct *in vitro* toxicity, stock solutions of the test compound and reference drugs were prepared with sterile water. The drugs which were insoluble in water had their solubility been enhanced by first dissolving 10mg in 100µl of 100% dimethyl-sulfoxide. The test samples were prepared as a 1mg ml^{-1} stock solution. Further dilutions were prepared on the day of biological assays. All the drug solutions were stored at 4°C for later use.

3.5.2 Culture of the vero cells

Cells were maintained in Minimum Essential Eagles Medium (MEM) containing 10% fetal bovine serum (FBS), penstrep, and glutamine. Vero cells were cultured using T-75 culture flasks. The flasks were kept at 37ºC in 5% CO_2 and the cells passaged every 2 to 3 days in order to keep

the cells alive. Trypsinization was carried out to detached cells which overlap each other and counted using the hemocytometer counter (New bauer).

3.5.3 Procedure for cytotoxicity assay

Using a 96 micro well plate, a cell density of 20,000 vero cells were seeded and incubated for 24 hours at 37 °C under 5% CO_2 to allow cells to attach to the surface/base of the plate.

Sarcosine-aniline hybrid drug as the test drug and controls were added in triplicate to the cultured cells using a concentration range 100ug/ml and subsequent dilutions were carried out over 7 folds, from 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 to 0.78125 µg/ml. The plates were incubated for 48 hours at 37°C under 5% CO_2 to allow the reaction to occur. After 48 hours, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) was added for calorimetric measurement of the ability of a drug to kill the vero cell lines. DMSO was added to each well and mixed to ensure cell lysis and dissolving of the formazan crystals.

Optical density was read using Multi Skan Ex reader machine 48X, from Thermo Fisher Scientific company in a UV–visible spectrophotometry at 562 and 690 nm. The amount of formazan measured was directly proportional to the number of viable cells. The results were recorded as optical density (OD) per well at each drug concentration and analyzed using Microsoft Excel software 2010, from which the percentage of cytotoxicity (PC) was calculated using the following equation:

Percentage cytotoxicity = $[(A - B)/A] \times 100$ (Mosmann, 1983).

Where A is the mean OD of untreated cells and B is the mean OD at each drug concentration. The drug concentration that leads to 50% inhibition of cell growth (CC_{50}) was determined by non-linear regression logistic dose-response model.

3.6 *In vivo* toxicity studies

In this step, acute toxicity was done. Three dosages, such as 2000 mg kg^{-1} and 300 mg kg^{-1} and 50mg/kg were administered orally to mice in three groups and the fourth group which served as control received water as a placebo. Each group consisted of 3 mice and the monitoring of weight and number of dead mice was monitored and recorded after each 4 days over an interval

of 14 days. Clinical signs were also recorded during the study period. At the end of experiment, the mice were killed by sacrificing them using concentrated carbon dioxide gas followed by incineration.

3.7 Ethical considerations

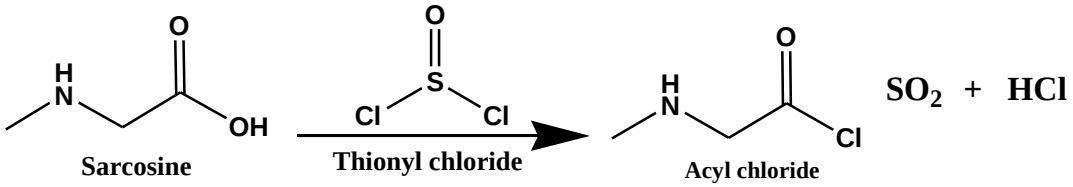
Handling of animals was done in accordance to the KEMRI Guide for Care and Use of Laboratory animals. Ethical clearance was sought from Scientific Ethic Review Unit (SERU), in KEMRI (Protocol number: KEMRI/SERU/CBRD/PROP155/3324). Mice were handled with a lot of care as stipulated in guidelines set by KEMRI Animal Care and Use Committee (Ref Number: KEMRI/CBRD/MP/AH/01). The end points of the mouse experiment in the *in vivo* assays were set to consider with the development of the clinical signs such as impaired ambulation which prevents animals from reaching food or water, excessive weight loss and extreme emaciation, lack of physical or mental alertness, difficult labored breathing and prolonged inability to remain upright. To avoid severe and enduring distress mice that showed those clinical signs were killed through euthanasia using carbon dioxide gas into a closed chamber and then incinerated.

CHAPTER FOUR

RESULTS

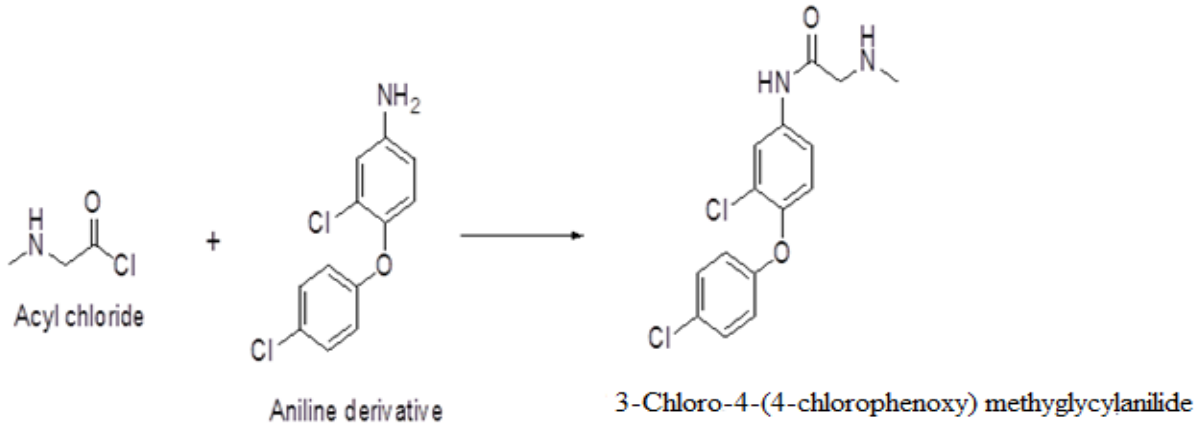
4.1 Sarcosine-aniline hybridization

The sarcosine-aniline hybrid drug was synthesized by coupling sarcosine to 3-Chloro-4-(4-chlorophenoxy) aniline as shown in the following reactions:



Reaction 4.1: Reaction between sarcosine and thionyl chloride

Reaction 4.1 shows the reaction between sarcosine and thionyl chloride in dicloromethane solvent in order to form acyl chloride. This reaction is irreversible because SO_2 and HCl gasses that are lost in reaction mixture.



Reaction 4.2: Coupling acyl chloride to 3-Chloro-4-(4-chlorophenoxy) aniline

Reaction 4.2 illustrates the reaction between acyl chloride and 3-Chloro-4-(4-chlorophenoxy) aniline to form 3-Chloro-4-(4-chlorophenoxy) methyglycylanilide at room temperature. In this reaction, there is a nucleophilic substitution of Cl by NH_2. The formation of sarcosine-aniline

hybrid was spotted on thin layer chromatography and observed as band under UV light (Plate 4.1).

Plate 4.1: Thin Layer Chromatography results

Plate 4.1, shows the results of sarcosine-aniline hybrid formation on Thin Layer Chromatography plate. EtOAc stands for ethyl acetate and solvent system used to run this TLC consisted only of EtOAc (100% concentrated). Aniline was used as a control in the middle of the plate (second column). From left to right, for the first and third column, the first spots closer to the base line of the columns is 3-Chloro-4-(4-chlorophenoxy) aniline, then the spots closer to the top are Sarcosine-aniline hybrid. The sarcosine-aniline hybrid is less polar than 3-Chloro-4-(4-chlorophenoxy) aniline, so it would elute faster than 3-Chloro-4-(4-chlorophenoxy) aniline. The Retention factor (Rf) value for the sarcosine aniline hybrid (S-A) was 4.4/5.8=0.76 and the Rf of aniline was 4/5.8=0.69.

4.2 *In vivo* efficacy studies

Table 4.1: ED_{50} of sarcosine-aniline hybrid versus control drugs on *P. berghei* ANKA

	DRUGS	ED_{50}	P value					
			1	2	3	4	5	6
1	Sarcosine-aniline hybrid drug	6.49mg/kg		0.000*	0.000*	0.000*	0.000*	0.000*
2	Aniline	3.61mg/kg	0.000*		0.000*	0.000*	0.000*	0.000*
3	Artesunate-aniline hybrid	3.56 mg/kg	0.000*	0.1		0.000*	0.000*	0.000*
4	Artesunate	2.94mg/kg	0.000*	0.424	0.000*		0.000*	0.000*
5	Chloroquine	1.78 mg/kg	0.000*	0.000*	0.000*	0.000*		0.000*
6	Sarcosine	No activity	0.000*	0.000*	0.000*	0.000*	0.000*	

*significant: P< 0.05,

Table 4.1 shows the effective dose of the drug which inhibits the growth of *P. berghei* ANKA sensitive strain to quinoline and artemisinin based drugs *parasites* by 50% after four days of oral treatment. The ED_{50} of sarcosine-aniline hybrid was 6.49mg/kg. The other drugs and compounds such as artesunate, artesunate-aniline hybrid, chloroquine and sarcosine were used as controls. Sarcosine did not show *in vivo* activity.

Table 4.1 also describes the comparisons of ED_{50} of sarcosine-aniline hybrid drug against artesunate, artesunate-aniline hybrid, 3-Chloro-4-(4-chlorophenoxy) aniline and chloroquine. There was a significant difference between the ED_{50} of sarcosine-aniline hybrid drug against artesunate, 3-Chloro-4-(4-chlorophenoxy) aniline, and chloroquine (P value <0.05).

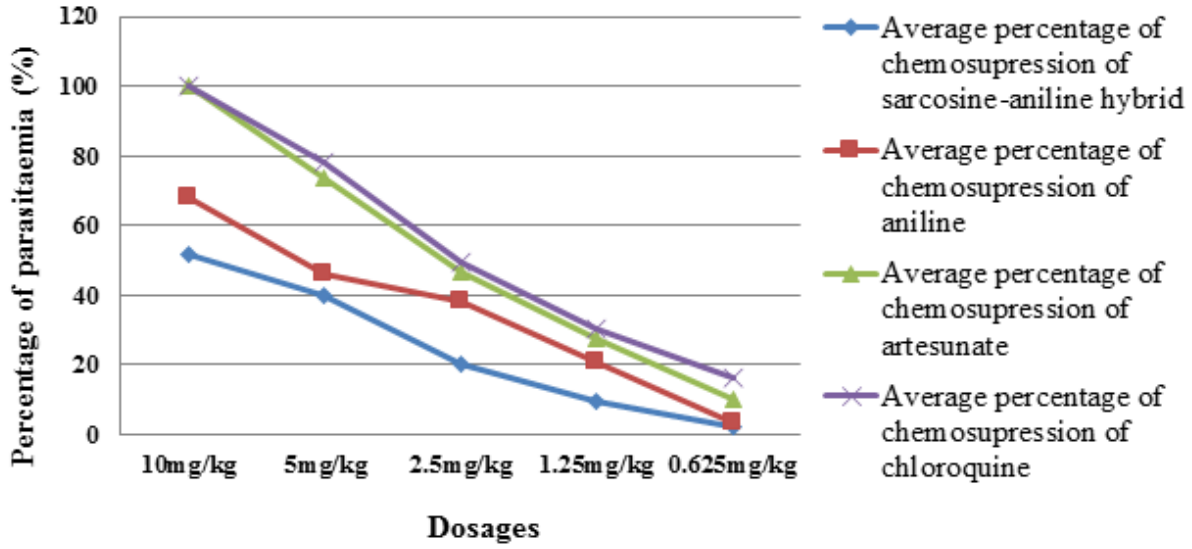


Figure 4.1: Percentage of parasitemia chemosupression of sarcosine-aniline hybrid

Figure 4.1 shows the percentage of chemosupression of parasitaemia for sarcosine-aniline hybrid, aniline derivative, artesunate, artesunate-aniline hybrid and chloroquine after 4days of oral treatment. The percentage of chemosupression for mice treated with with 10mg/kg of sarcosine aniline hybrid was 52% whereas those treated with 3-Chloro-4-(4-chlorophenoxy) aniline, it was 68%. No parasites observed under microscope for the mice treated with 10mg/kg of artesunate and that of chloroquine.

Table 4.2: Parasitemia in mice growth of sarcosine-aniline hybrid after stopping treatment

Drugs	Day 7	Day 9	Day 11
Sarcosine-aniline hybrid	9.74	12.98	15.61
Aniline	6.97	9.03	11.84
Artesunate	0.00	0.2	0.67
Chloroquine	0.00	0.03	0.42

Table 4.2 shows the actual average of parasitemia at day 7, 9 and 11 after stopping the treatment with 10mg/kg. The parasitemia of sarcosine-aniline hybrid was 9.74%, 12.98% and 15.61% on

day 7, 9 and 11 respectively. The recrudescence of CQ and artesunate reappeared on day 9 with 0.03% and 0.2% respectively.

Table 4.3: Survival rate of mice after 11 days of *in vivo* efficacy testing

Dosage	Sarcosine-aniline hybrid	Sarcosine	3-Chloro-4-(4-chlorophenoxy) aniline	Artesunate	Artesunate-aniline hybrid	Chloroquine
10mg/kg	3	1	4	5	3	5
5mg/kg	2	2	2	3	2	4
2.5mg/kg	1	1	3	4	3	4
1.25mg/kg	1	1	3	3	3	3
0.625mg/kg	1	0	1	2	1	3

Table 4.3 shows the number of mice which survived up to 11days of experiment, which is 7days after stopping oral drug administration. For sarcosine-aniline hybrid, 3 mice among 5 survived for the groups which received 10 mg/kg, and 2 mice survived in group which received 5 mg/kg whereas only 1mouse survived for the groups which received 2.5 mg/kg, 1.25 mg/kg and 0.625mg/kg. Up to day 11, all mice which received 10mg/kg of artesunate and chloroquine survived along *in vivo* efficacy testing.

4.3 *In vivo* toxicity

Acute toxicity

Table 4.4: Number of dead mice following different dosage administrations

Group	**Dosage of hybrid drug administered orally**	**Number of mice per group**	**Number of dead mice after 14 days**
Group 1	2000mg/kg	3	0
Group 2	300mg/kg	3	0
Group 3	50mg/kg	3	0
Control group	0mg/kg	3	0

Table 4.4 illustrates the number of dead mice during acute toxicity testing for 14 days.The three groups of mice received different dosage (2000mg/kg, 300mg/kg, 50mg/kg) of sarcosine-aniline hybrid drug and the control group did not receive any dosage rather water. For all groups, the initial weights were ranging from 20 to 22g and they were all females. No mice died in all groups for 14 days.

Table 4.5: Weights of mice according to hybrid dosage during acute toxicity experiment

Groups	Dosage	Mice	Day 1	Day 5	Day 9	Day 14	Mean	SD	Mean average
Group 1	2000mg/kg	M1	20	19.4	20.1	21.5	20.25	0.88	21.34
		M2	21	20.5	22.0	24	21.87	1.54	
		M3	22	21	21.5	23.1	21.90	0.89	
Group 2	300mg/kg	M1	20	19.7	20.6	21.7	20.50	0.88	21.58
		M2	21	20.3	21.9	23.1	21.57	1.20	
		M3	22	21.6	22.7	24.3	22.69	1.19	
Group 3	50mg/kg	M1	20	20.6	21.2	23.4	21.3	1.48	22.24
		M2	21	21.4	22.5	24.2	22.27	1.43	
		M3	22	21.5	24.0	25.1	23.15	1.69	
Control group	Placebo	M1	20	20.7	21.0	23.5	21.17	1.28	22.33
		M2	21	21.6	22.9	24.1	22.4	1.38	
		M3	22	22.1	24.2	25.4	23.42	1.66	

Table 4.5 describes the weight of each individual mouse in each group and the weight variation in for 14 days of acute toxicity testing. The three groups of mice received different dosage (2000mg/kg, 300mg/kg, 50mg/kg) of sarcosine-aniline hybrid drug and the control group did not receive any dosage rather water. The initial weights for all groups were ranging from 20 to 22g and they were all females. The weights were recorded after 4 days of intervals. The mean average of weight for the mice which received 2000mg/kg was 21.34g after 14days. The mean average of weights for the mice which received 300mg/kg, 50mg/kg and control group were 21.58g, 22.24g and 22.33g respectively after 14 days of acute toxicity testing (table 4.5).

Table 4. 6: Multiple comparison of mice's weights among groups after acute toxicity testing

Groups of mice to be compared according to dosage	**Mean difference**	**Significance**
Group1 (2000mg/kg)VS Group2(300mg/kg)	-0.24667	0.990
Group1 (2000mg/kg)VS Group3(50mg/kg)	-0.90000	0.714
Group1 (2000mg/kg)VS Control group(placebo)	-0.99000	0.654
Group2 (300mg/kg)VS Group3(50mg/kg)	-0.65333	0.862
Group2 (300mg/kg)VS Control group(placebo)	-0.74333	0.812
Group3 (50mg/kg)VS Control group(placebo)	0.09000	1.000

*significant: P value < 0.05

Table 4.6 shows comparisons between groups of mice which received different dosages from the highest (2000mg/kg) to the lowest (50mg/kg) as well as control group which did not receive drug. There was no significant difference between mice which received 2000mg/kg and mice which received 300mg/kg (P value of 0.990), 50mg/kg (P value of 0.714) and control group (P value of 0.654), the drug and between the control group.

Table 4.7: Records of clinical signs in mice during acute toxicity

Group	**Dosage of hybrid drug administered orally**	**Number of mice per group**	**Number of mice which developed clinical signs**
Group 1	2000mg/kg	3	0
Group 2	300mg/kg	3	0
Group 3	50mg/kg	3	0
Control group	0mg/kg	3	0

Table 4.7, shows that no mice developed physical clinical signs for all dosages used in this study. The assessed signs were impaired ambulation, excessive weight loss, extreme emaciation, lack of physical or mental alertness, difficult breathing and prolonged inability to remain upright.

4.4 Cytotoxicity results

Table 4.8: CC_{50} values of the different antimalarial drugs against vero cells

SN	DRUGS	Mean CC_{50} in µg/ml	SD
1	Sarcosine-aniline hybrid	50.18	3.53
2	Chloroquine	57.96	3.85
3	Artesunate	19.69	3.26
4	Doxorubicin	1.96	0.59

Table 4.8 shows the $CC_{50,}$ Standard deviation (SD) of the mean of sarcosine-aniline hybrid drug against vero cells after triplicate experiments. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed which is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of pale yellow MTT and thereby form dark blue formazan crystals which are largely impermeable to cell membranes, resulting in their accumulation within healthy cells'cytoplasm. Chloroquine and artesunate were used as standard drugs which are used to treat malaria whereas doxorubicin was used as a known cytotoxic drug. During experiment, the starting concentration (highest) for both drugs was 100µg/ml. Sarcosine-aniline hybrid showed a CC_{50} of 50.18µg/ml while the CC_{50} of doxorubicin was 1.96µg/ml.

If CC_{50} of a drug is lesser than 2 µg/ml, a drug is considered as cytotoxic, when **CC_{50}** is 2-89 µg/ml, a drug is considered as moderately cytotoxic and when it is above 90 µg/ml, a drug is considered as not cytotoxic (safe) (Engineering, n.d. Retrieved from http://www.who.int/tdr/grants/workplans/en/cytotoxicity_invitro.pdf, on 10th June 2016)

Table 4.9: CC_{50} values of sarcosine-aniline hybrid versus other antimalarial drugs

SN	Cell type to be compared	Mean Difference	P value
1	Vero Sarcosine-aniline hybrid VS Vero Chloroquine	-7.78	0.006*
2	Vero Sarcosine-aniline hybrid VS Vero Artesunate	30.48	0.000*
3	Vero sarcosine-aniline hybrid VS Vero Doxorubicin	48.21	0.000*

*significant: P value < 0.05

Table 4.9 describes the comparisons of CC_{50} of sarcosine-aniline hybrid drug against artesuante, chloroquine and doxorubicin. There was a significant difference between the CC_{50} of sarcosine-aniline hybrid drug against chloroquine, artesunate and doxorubicin. The highest mean difference was observed between the CC_{50} of sarcosine-aniline hybrid and doxorubicin (48.21) whereas the lowest mean difference was observed between sarcosine-aniline hybrid and chloroquine (-7.78).

Therapeutic index of sarcosine-aniline hybrid

Therapeutic index (TI) of drug is equal to its lethal dose concentration divided by its effective dose (LD50/ED50). The LD_{50} of sarcosine-aniline hybrid was estimated to be greater than 5000mg/kg and its ED50 was 6.49mg/kg. Therefore the Therapeutic Index of sarcosine-aniline hybrid drug was greater than 770.41(Muller & Milton, 2012).

CHAPTER FIVE

DISCUSSION

5.1 Sarcosine-aniline hybridization

In this study,the two molecules were used to synthesize a hybrid: 3-Chloro-4-(4-chlorophenoxy) aniline and sarcosine. The molecular purity of 3-Chloro-4-(4-chlorophenoxy) aniline and sarcosine was 97% and 98% respectively as confirmed by the manufacturer labels upon their delivery in Kenya by Sigma Aldrich. The sarcosine was completely soluble in water but the solubility of 3-Chloro-4-(4-chlorophenoxy) aniline and the hybrid in water was very low. They were soluble in dimethyl sulfoxide (DMSO), and slightly soluble in Tween-80. It is clear that the low solubility of sarcosine-aniline hybrid affected the drug absorption, distribution and its metabolism. Confirmed by thin layer chromatography, the hybrid (was successfully synthesized using 3-Chloro-4-(4-chlorophenoxy) aniline and sarcosine pharmacophores.

5.2 *In vivo* efficacy of sarcosine-aniline hybrid

Results from this study showed that sarcosine-aniline hybrid drug has *in vivo* antimalarial activity. However, sarcosine, when used singly was not having inhibition activity against *P. berghei* ANKA sensitive strain (ED_{50} not detectable). This was due to different reasons, such as sarcosine being transformed into glycine by glycine-N-methyl transferase enzyme. The ED_{50} of sarcosine-aniline hybrid drug was 6.49mg/kg whereas that of 3-Chloro-4-(4-chlorophenoxy) aniline alone was 3.61mg/kg. There was a significant difference between ED50 of sarcosine-aniline hybrid and both controls such as 3-Chloro-4-(4-chlorophenoxy) aniline, artesunate, artesunate-aniline hybrid drug and chloroquine. On the hand, there was no significant difference between ED_{50} of artesunate-aniline hybrid drug and 3-Chloro-4-(4-chlorophenoxy) aniline as well as between 3-Chloro-4-(4-chlorophenoxy) aniline and artesunate. In the study done on *in vivo* antiplasmodial potentials of the combinations of four Nigerian antimalarial plants extracts, the ED_{50} of CQ using oral administration pathway was 2.2 mg/kg *(Adebajo et al.,* 2014), and this is in agreement with current study where the ED_{50} of chloroquine was 1.78 mg/kg.

The *in vivo* activity of sarcosine-aniline hybrid drug was less than that of other control drugs used in this study. This may be due to other conversions of the hybrid drug which may occur along oral administration pathway such as in the bowels. As a result this can prevent hybrid

molecule from reaching the target sites. The type of drug administration method for effectiveness of a drug can also be of a great concern. Therefore, if sarcosine-aniline hybrid drug can be administered intravenously or subcutaneously, it might also improve its efficacy as well as its bioavailability. It is known that the artesunate has a shorter half-life and its combination with other drugs increases its bioavailability. The half-life of both 3-Chloro-4-(4-chlorophenoxy) aniline and sarcosine is not yet known in order to determine their stability while combined or hybridized with other compound or drugs. Artesunate-aniline hybrid drug was active than both 3-Chloro-4-(4-chlorophenoxy) aniline and artesunate when used singly. This might be because artesunate is a semi-synthetic derivative of artemisinin whose water solubility facilitates absorption and provides an advantage over other artemisinins. In addition, artesunate is rapidly hydrolyzed to dihydroartemisinin, which is the most active schizonticidal metabolite (Li & Weina, 2010). In contrast, 3-Chloro-4-(4-chlorophenoxy) aniline being a low water soluble compound might affect the artesunate solubility in resultant hybrid drug.

A study done on potent *in vivo* anti-malarial activity and representative snapshot pharmacokinetic evaluation of artemisinin-quinoline hybrid, the three synthesized artemisinin-quinoline hybrids were differing from each other by one methyl group in the linker and position in the chain, their ED_{50} were 1.1, 1.4 and <0.8 mg/kg respectively using intraperitoneal route and 12, 16 and 13 mg/kg respectively using oral route (Lombard *et al.*, 2013). The ED_{50} of artesunate was 1.8 mg/kg using oral route with 80mg/kg as highest dosage and it was <1mg/kg using intraperitoneal route (Lombard *et al.*, 2013), these results differ slightly with my results because of the dosages and methods used, in current study, no intraperitoneal method used rather oral method. In another study done on oral artesunate dose-response relationship in acute Falciparum malaria, the effective doses were determined after treating patients from whom the infections were detected with a dose varying from 0 to 250 mg of artesunate together with a curative dose of mefloquine, the resultant ED_{50} was 1.6 mg/kg (Angus *et al.*, 2002), this result differ with current study as the artesunate here was combined with mefloquine and the study used the patients while in my study I used *P.berghei* in mice.

The percentage of chemosupression for sarcosine-aniline hybrid was around 55% whereas that of aniline was 68%, the percentage of chemosupression was 100% for mice treated with 10mg/kg of artesunate and chloroquine. However, there was a recrudescence after 5 days post treatment for both mice treated with artesunate and chloroquine whereas for other drugs, there was a

continuous increase of the parasitemia after stopping the treatment (Table 2). The recrudescence in mice treated with artesunate and chloroquine, might be due to some remnant parasites which survived after stopping the treatment. In the study done on antimalarial activity of methanolic leaf extract of *piper betle* L, there was 100% of chemosupression for the mice treated with chloroquine with 20 mg/kg using oral route (Al-adhroey *et al.,* 2011), which agrees with current study where the percentage of chemosupression was 100% at day four following oral treatment at 10 mg/kg. In the study conducted on antiplasmodial activity of methanol root bark extract of Alstonia Boonei against *Plasmodium berghei* infection in mice, chloroquine showed a chemosupression of 91.7 at 5 mg/kg administered orally to mice at day 4 of experiment (Onwusonye & Uwakwe, 2014). The difference in values with this study was due to the different dosages used.

The number of mice which survived up to 11days of experiment during in vivo efficacy testing was recorded. Actually after 7days when the drug administration was stopped, the number of mice which were still alive was different according to the type of drug and its dosage. This is because at this dosage there was a 100% of parasites' growth inhibition. According to current study, there was where the low drug dosage showed a high number of survived mice compared to the higher dosage concentration of the same drug.

5.3 *In vitro* toxicity of sarcosine-aniline hybrid

In-vitro toxicity tests are the alternative method approaches to animal acute toxicity evaluation (Frión-herrera *et al.,* 2014). Vero cell lines are usually employed in prospective studies to determine the cytotoxic effect of different natural and artificial products (Frión-herrera *et al.,* 2014). Cytotoxicity results from this study, showed that the hybrid drug was safe to vero cells. When compared to other standard drugs, sarcosine-aniline hybrid was significantly less toxic compared to artesunate. There was also a significant difference between cytotoxicity of hybrid drug and that of CQ against mammalian cell lines. In the study done on effects of chloroquine to inhibit dengue virus type 2 replication in vero cells but not in C6/36 cells, the concentrations equal or greater than 500 µg/ml showed major cytotoxicity but the the concentrations equal or lesser than 50µg/ml did not reveal cytotoxicity effects on vero cells (Juvenal *et al.,* 2013). These results agree with study, where the CC_{50} of chloroquine was 57.96µg/ml.

Therefore, the side effects caused by CQ such as neurotoxicity, leukopenia, retinopathy and cardiovascular toxicity might be absent, reduced or increased for sarcosine-aniline hybrid drug, but this requires chronic toxicity to be done using mice so that blood parameters and histopathology of liver, kidney, heart and brain dysfunction might be elucidated. Doxorubicin was significantly more toxic than hybrid drug to vero cells. Doxorubicin served as a control in cytotoxicity studies as it was revealed that it is more toxic to cancerous cells and even to normal cells including vero cells which had been used in this study. Doxorubicin's cytotoxicity is based on its capacity to bind to DNA-associated enzymes (topoisomerases), intercalate with DNA base pairs, and target multiple molecular targets in order to produce a wide range of cytotoxic effects. It also activates the Bcl-2/Bax apoptosis pathway when it interacts with cells' membranes (Tacar, Sriamornsak, & Dass, 2013).

5.4. Acute toxicity of sarcosine-aniline hybrid

Acute toxicity showed that the hybrid drug was safe to mice. There was no dead mice observed with 2000mg/kg, so the LD_{50} is expected to be above 5000mg/kg, thus this drug is classified in category 5 according to OECD/OCDE guideline for testing chemical compounds (Guideline, Testing, & Chemicals, 2001).The weights measured during acute toxicity testing showed that the weight loss was observed in first week of experiment and increased in the second week of experiment for the group which received 2000 mg/kg and 300mg/kg. There was a continuous gain in weights for the group which received a single dose of 50mg/kg and control group. There was no significant difference in weights within and between groups at the end of acute toxicity experiment. No clinical physical signs of discomfort such as impaired ambulation, excessive weight loss, lack of physical or mental alertness, difficult breathing, prolonged inability to remain upright and extreme emaciation observed during acute toxicity experiment. Therapeutic index of a drug is the ratio that compares the blood level concentrations at which a given drug is toxic (lethal dose) and the concentration at which the drug is effective (effective dose), which is a vital criteria for drug selection (Xie *et al.*, 2006).

Therapeutic index of sarcosine-aniline hybrid drug is greater than 770.41, which confirms its safety as the concentration required to cause toxicity (>5000mg/kg) is far greater than that of required to kill the parasites (6.49 mg/kg) (Muller & Milton, 2012). The closer the TI is to 1, the more dangerous is and the larger the therapeutic index (TI) the safer the drug is (Muller & Milton, 2012). In fact, if the TI is small, the drug might be dosed thoroughly and the patient

receiving the drug should be monitored carefully for any clinical signs of drug toxicity (Muller & Milton, 2012). The study done by Xie *et al., (*2006), on new potential antimalarial agents: Therapeutic-Index evaluation of Pyrroloquinazolinediamine and its prodrugs in a rat model of severe malaria, the therapeutic index of artesunate was 4 (Xie *et al.,* 2006). When compared the results from Xie *et al., (*2006), the therapeutic index of sarcosine-aniline hybrid is 192.6 folds greater than that of artesunate which implies that the sarcosine-aniline hybrid is safer than artesuante.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The following were the conclusion of this study:

Sarcosine-aniline hybrid has been synthesized using sarcosine and 3-Chloro-4-(4-chlorophenoxy) aniline pharmacophores and the product formation was monitored and confirmed by Thin Layer Chromatogaraphy.

Sarcosine-aniline hybrid drug is a promising antiplasmodial prodrug as it showed activity for *in vivo* studies with an ED_{50} of 6.49 mg/kg, which is within acceptable ranges of drugs used to treat severe malaria (WHO. (2015d)).

The findings of this study point out that sarcosine-aniline hybrid drug is safe to vero cells with CC_{50} of 50.18±3.53µg/ml compared to doxorubicin which is most toxic with CC_{50} of 1.96±0.59 µg/ml. The acute toxicity results showed no dead mice up to the dosage of 2000mg/kg using oral administration for 14 days, and there was no significant loss of weight in mice within and between groups with different dosages of sarcosine-aniline hybrid as well as control group.

6.2 Recommendations

1. There should be use of covalent bitherapy in drug development.

2. The use of covalent biotherapy in drug resistance mitigation is recommended.

3. It is recommended that other preclinical trial testing such as *in vitro* activity and chronic toxicity are carried out prior passing to clinical trial phase.

4. The solubility of sarcosine-aniline hybrid drug was low, so new ways of improving its solubility can serve as great advantages for better *in vivo* and *in vitro* activity.

5. The intravenous, subcutaneous as well as other mode of drug administration can be tried for further improvement of hybrid drug uptake in the targeted sites.

REFERENCES

Adebajo, A.C.; Odediran, S.A.; Aliyu, F.A.; Nwafor, P.A.; Nwoko, N.T.; Umana, U.S. (2014). *In vivo* antiplasmodial potentials of the combinations of four Nigerian antimalarial plants. *Molecules*, 2014(19), 13136-13146.

Abdulelah, H. A., Zurainee M.N., Hesham M. M., Adel A. Amran., Rohela M. (2011). Antimalarial activity of methanolic leaf extract of Piper betle L. *Molecules*, 16 (1), 107-18.

Alkadi, H. O. (2007). Antimalarial drug toxicity : A Review. *Chemotherapy*, 53, 385–391. http://doi.org/10.1159/000109767

Aminake, M. N., & Pradel, G. (2013). Antimalarial drugs resistance in *Plasmodium falciparum* and the current strategies to overcome them. Microbial pathogens and strategies for combating them: science, technology and education, pp. 269–282.

Angus, B. J., Thaiaporn, I., Chanthapadith, K., Suputtamongkol, Y., & White, N. J. (2002). Oral artesunate dose-response relationship in acute Falciparum malaria. *Antimicrobial Agents and Chemotherapy*, 46(3), 778–782. http://doi.org/10.1128/AAC.46.3.778

Basco, L.K., Dechy-Cabaret, O., Ndounga, M., Meche, F.S., Robert A., M. B. (2001). In vitro activities of DU-1102, a new trioxaquine derivative against *Plasmodium falciparum* isolates. *Antimicrobial Agents Chemotherapy*, 45(6), 1886–1888.

Bosman, A.,WHO/MAL. (2003). WHO Training Workshop on Pharmacovigilance: Basic Introduction and Specifics for Malaria Programmes 23 March - 2 April 2003, Lusaka, Zambia. Accessed from http://www.who.int/medicines/areas/quality_safety/safety_efficacy/trainingcourses/safety.pdf, on 27th February 2017,

CDC. (2015). About malaria biology. Malaria parasites. Retrieved February 15, 2017, from http://www.cdc.gv/malaria/about/biology/parasites.html

Cernei, N., Heger, Z., Gumulec, J., Zitka, O., Masarik, M., Babula, P., Adam, V. (2013). Sarcosine as a potential prostate cancer biomarker-a review. *International Journal of Molecular Sciences*, *14*(7), 13893–13908. http://doi.org/10.3390/ijms140713893

Chauhan, S. S., Sharma, M., & Chauhan, P. M. S. (2010). Trioxaquines : hybrid molecules for the treatment of malaria. *Drug News Perspective*, 23(10), 632–646.

Clive Shiff. (2006). Malaria Epidemiology. Accessed from http://ocw.jhsph.edu/courses/Malariology/PDFs/lecture3.pdf, on 18th February 2017.

Coatney GR, Collins WE, Warren M, C. P. T. P. M. (1971). *Plasmodium falciparum b*lood Stage Parasites , Thin Blood Smears *Plasmodium malariae* blood stage parasites , thin blood smears. U.S. Bethesda.

Coppock, H. A., Owji, A. A., Bloom, S. R., & Smith, D. M. (1996). A rat skeletal muscle cell line (L6) expresses specific adrenomedullin binding sites but activates adenylate cyclase via calcitonin gene-related peptide receptors. *Biochemistry Journal,* 318, 241–245.

Cronan, J.E., Massengo, T. R. (2009). Diversity in enoyl-acyl carrier protein reductases. *Cellular and Molecular Life Sciences,* 66(9), 1507-1517.

Dassonville-Klimpt, A., Jonet, A., Pillon, M., Mullié, C., & Sonnet, P. (2011). Mefloquine derivatives: synthesis, mechanisms of action, antimicrobial activities. Science against Microbial Pathogens: Communicating Current Research and Technological Advances. A. Méndez-Vilas (Ed.). pp. 23–35. Accessed from http://www.formatex.info/microbiology3/book/23-35.pdf,on March 3rd, 2017.

Dean, L. (2005). Blood groups and red cell Antigens. National Center for Biotechnology Information (NCBI), National Library of Medicine, National Institutes of Health, Bethesda, MD 20892-6510. https://www.ncbi.nlm.nih.gov/books/NBK2261/

Delves M, Plouffe D, Scheurer C, Meister S, Wittlin S, Winzeler EA, *et al.* (2012).The activities of current antimalarial drugs on the life cycle stages of *Plasmodium*: A Comparative Study with Human and Rodent Parasites. *PLoS Medicine* 9(2): e1001169. doi:10.1371/journal.pmed.1001169

Doolan, D. L., Aguiar, J. C., Weiss, W. R., Sette, a, Felgner, P. L., Regis, D. P., Carucci, D. J. (2003). Utilization of genomic sequence information to develop malaria vaccines. *The Journal of Experimental Biology*, 206(Pt 21), 3789–3802. http://doi.org/10.1242/jeb.00615

Elizabeth, J., Charles, O., Willis, A., Bernhards, R. (2008). A randomized, open-label, comparative efficacy trial of artemether-lumefantrine suspension versus artemether-lumefantrine tablets for treatment of uncomplicated *Plasmodium falciparum* malaria in children in western Kenya. *Malaria Journal*, 7, 262.

Eltayeb, M. (2006). Theoretical and practical approaches of hepatocyte primary culture.

Accessed from http://www.aun.edu.eg/gembc/Liver%20cell%20culture/intrudctory%20lect..pdf, on 2nd March 2017.

Engineering, D. natural sciences. (n.d.). *In vitro* determination. Retrieved from http://www.who.int/tdr/grants/workplans/en/cytotoxicity_*in vitro*.pdf, on 13th February 2017.

Florence, A., Johnson, K., Daniel, K., Nganga, J. (2012). Experimental and *in silico* drug design against *P. falciparum*. Thesis submitted to Jomo Kenyatta University of Agriculture and Technology.

Frión-herrera, Y., Díaz-garcía, A., Rodríguez-sánchez, H., Ruiz-fuentes, J. L., Fidalgo, L. M., & Setzer, W. N. (2014). Cytotoxic effect of Cuban propolis extracts on normal cells and *in-vitro* basal toxicity assay to estimate acute oral toxicity. *American Journal of Essential Oils and Natural Products*, 2(1), 19–23.

Garah, F. B., Claparols, C., Meunier, B., & Robert, A. (2008). The antimalarial trioxaquine DU1301 Alkylates Heme in malaria-infected mice. *Antimicrobial Agents and Chemotherapy*, 52(8), 2966–2969. http://doi.org/10.1128/AAC.00165-08

Geraghty, R. J., Davis, J. M., Downward, J., Freshney, R. I., & Knezevic, I. (2014). Guidelines for the use of cell lines in biomedical research. *British Journal of Cancer*, 111(6), 1021–1046. http://doi.org/10.1038/bjc.2014.166

Gething, P.W., Smith, D.L., Patil, A.P., Tatem, A.J., Snow, R.W., Hay, S. I. (2010). Climate change and the global malaria recession. *Nature*, 465(7296), 342–345. doi: 10.1038/nature09098.

Govorkova, E. A., Murti, G., & Meignier, B. (1996). African green monkey kidney (Vero) cells

provide an alternative host cell system for influenza A and B Viruses, *Journal of Virology*, 70(8), 5519–5524.

Greek, R. (2013). Animal models in drug development. *New insights into toxicity and drug testing.* World's largest Science, technology & medicine. Open Access book publisher. Accessed from http://cdn.intechopen.com/pdfs-wm/42018.pdf , on February 26th, 2017

Haberman, A. B. (2010). Animal models for therapeutic strategies. Cambridge Healthtech Institute. Retrieved from http://www.insightpharmareports.com/uploadedFiles/Reports/Reports/Animal_Models/Animal_Models_Sample_Report.pdf?n=9203, on February 22nd, 2017.

Hay, S. I., Guerra, C. A., Tatem, A. J., Noor, A. M., & Snow, R. W. (2004). Reviews The global distribution and population at risk of malaria : past , present , and future. *Lancet Infectious Disease*, 4(6), 327–336.

Howes, R.E., Patil, A.P., Piel, F.B., Nyangiri, O.A., Kabaria, C.W., Gething, P.W., Z., P.A., Barnadas, C., Beall, C.M., Gebremedhin, A., Menard, D., Williams, T.N., W., & D.J., Hay, S.I., 2011. (2011). The global distribution of the Duffy blood group. *Nature*, (2), 266.

Hussien, O. A. (2007). Antimalarial drug toxicity. *Chemotherapy*, 53(6), 385–391. DOI: 10.1159/000109767

Jadhav, S., Shinde, N. (2015). A review about malaria and its treatment. *World Journal of Pharmacy and Pharmaceutical Sciences*, 4(3), 405–431.

Juvenal, K., Farias, S., Renata, P., Machado, L., & Antônio, B. (2013). Chloroquine Inhibits Dengue Virus Type 2 Replication in Vero Cells but Not in C6 / 36 Cells. *The Scientific World Journal*, 2013 (2013), Article ID 282734, 5 pages. http://dx.doi.org/10.1155/2013/282734

Kalra, B., Chawla, S., Gupta, P., & Valecha, N. (2006). Screening of antimalarial drugs: An overview. *Indian Journal of Pharmacology*, 38(1), 5. http://doi.org/10.4103/0253-7613.19846

Kim, W. Y., & Sharpless, N. E. (2012). Drug Efficacy Testing in Mice. *Current Topics in Microbiology Immunology*, 355: 19–38. DOI: 10.1007/82_2011_160

Kiszewski, A. E. (2011). Blocking Plasmodium falciparum Malaria Transmission with Drugs: The Gametocytocidal and Sporontocidal Properties of Current and Prospective Antimalarials. *Pharmaceuticals,* 4, 44-68. http://doi.org/10.3390/ph4010044

Krishna, S., Uhlemann, A., & Haynes, R. K. (2004). Artemisinins : mechanisms of action and potential for resistance. Drug resistance updates, 7, 233–244. http://doi.org/10.1016/j.drup.2004.07.001

Lawson MA, P. P. (2000). Differentiation of myoblasts in serum-free media: effects of modified media are cell line-specific. *Cells Tissues Organs,* 167(2-3):130-7. http://doi.org/16776

Levenbook, I. S., Petricciani, J.C., Elisbergt, B. L. (1984). Tumorigenicity of vero cells. *Journal of Biological Standardization,* 12(4):391-8.

Li, Q., & Weina, P. (2010). Artesunate: The Best Drug in the Treatment of Severe and Complicated Malaria. *Pharmaceuticals*, 3(7), 2322-2332; doi: 10.3390/ph3072322

Liao, T. I. N. G. I. N. G., Shi, Y. A. N. I. N. G., Jia, J. I. A. N. E. I., & Wang, L. E. I. (2010). Sensitivity of Different Cytotoxic Responses of Vero Cells Exposed to Organic Chemical Pollutants and their Reliability in the Bio-toxicity Test of Trace Chemical Pollutants. *Biomedical and Environmental Sciences,* 23(3):219-229

Lödige, M., & Hiersch, L. (2015). Design and synthesis of novel hybrid molecules against malaria. *International Journal of Medicinal Chemistry,* 2015 (2015), Article ID 458319, 23 pages. http://dx.doi.org/10.1155/2015/458319

Lombard, M. C., Da, D. N., Ba, C. T. Van, Wein, S., & Norman, J. (2013). Potent *in vivo* anti-malarial activity and representative snapshot pharmacokinetic evaluation of artemisinin-quinoline hybrids. *Malaria Journal,* 12:71. doi: 10.1186/1475-2875-12-71.

Loup, C., Lelie`vre, J., Vical B.F., Meunier, B. (2007). Trioxaquines and Heme-Artemisinin Adducts Inhibit the In Vitro Formation of Hemozoin Better than Chloroquine. *Antimicrobial Agents and Chemotherapy,* 51(10), 3768–3770. http://doi.org/10.1128/AAC.00239-07

Lucey, B. P., Nelson-rees, W. A., & Hutchins, G. M. (2009). Henrietta Lacks , HeLa Cells , and Cell Culture Contamination. *Archives of Pathology & Laboratory Medicine*, 133(9):1463-7. doi: 10.1043/1543-2165-133.9.1463.

Ministry of Health, R. (2016). Rwanda focuses 2016 first quarter on fighting malaria. Retrieved from http://www.moh.gov.rw/index.php?id=34&tx_ttnews%255Btt_news%255D=622&cHash=3e867eeb6c5a4af5a891d6294eddc4f8,on 14th February 2017.

Mosmann, T. (1983). Rapid colometric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65, 55–63.

Moyes, C. L., Sinka, M. E., Howes, R. E., Guerra, C. A., Price, R. N., Baird, J. K., & Hay, S. I. (2012). *Significance of Plasmodium vivax*. *BS:APAR* (Vol. 80). Elsevier. http://doi.org/10.1016/B978-0-12-397900-1.00001-3

Mueller, I., Zimmerman, P. A., & Reeder, J. C. (2007). *Plasmodium malariae* and *Plasmodium ovale* - the 'bashful' malaria parasites. *Trends in Parasitology*, 23(6), 278-283. DOI: 10.1016/j.pt.2007.04.009

Muller Y. Patrick & Milton N. Mark. (2012). The determination and interpretation of the therapeutic index in drug development. *Nature Reviews. Drug Discovery*, 11, 751–761. http://doi.org/10.1038/nrd3801

NIH. (2015). National Centre for Biotechnology Information. Retrieved February 28th, 2017, from https://pubchem.ncbi.nlm.nih.gov/compound/sarcosine#section=Top

OECD/OCDE. (2001). OECD guideline for testing of chemicals. Accessed from https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecd_gl423.pdf, on 2nd March 2017.

Ogutu, B., Oloo, F., Swai, H., Melariri, P., Mahanga, G. M., & Waweru, J. (2014). Preparation, characterization, and optimization of primaquine-loaded solid lipid nanoparticles. *International Journal of Nanomedicine*, 9(1), 3865–3874.

Ohashi, J., Naka, I., Looareesuwan, S., Tokunaga, K. (2002). Malaria. *Journal of Tropical*

Medicine and Public Health, 33(3), 5–7.

Onwusonye, J. C., & Uwakwe, A. A. (2014). The antiplasmodial activity of methanol root bark extract of Alstonia Boonei against *Plasmodium berghei* infection in mice. *International Journal of Science and Research*, 3(7), 2199–2201.

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

PMI. (2015). Fighting Malaria and Saving Lives. Accessed from http://reliefweb.int/sites/reliefweb.int/files/resources/rwanda_profile.pdf, on 4th March 2017.

Research Animal Resources Center. (2016). Calculating injection doses for rodents. Retrieved February 28th , 2017, from https://www.rarc.wisc.edu/animal_health/experimental_techniques/calculating_injection_dose_rodent.html

Rima McLeod, Stephen P. Muench. John B. Rafferty, Dennis E. Kyle, Ernest J. Mui, Michael J. Kirisits, Douglas G. Mack, Craig W. Roberts, Benjamin U. Samuel, Russell E. Lyons, Wilbur K. Milhous, S. P. and D. W. R. (2002). Fab i and inhibition of apicomplexan parasites. CA2432992 A1. http://www.google.ee/patents/CA2432992A1?cl=en&hl=et

Rosenthal, P. J. (2003). Antimalarial drug discovery: old and new approaches. *Journal of Experimental Biology*, 206(21), 3735–3744. http://doi.org/10.1242/jeb.00589

Roy, S., Besra, S. E., De, T., Banerjee, B., & Mukherjee, J. (2008). Induction of apoptosis in Human Leukemic Cell Lines U937 , K562 and HL-60 by litchi chinensis leaf extract Via Activation of mitochondria mediated caspase cascades. *The Open Leukemia Journal*, 1–14. DOI: 10.2174/1876816400901010001

Rwanda Ministry of Health. (2016). Rwanda focuses 2016 first quarter on fighting malaria.

Accessed from http://www.moh.gov.rw/index.php?id=34&tx_ttnews%5Btt_news%5D=622&cHash=3e867eeb6c5a4af5a891d6294eddc4f8, on 7th March 2017.

Saifi, M. A., Beg, T., Harrath, A. H., Suleman, F., Altayalan, H., & Quraishy, S. Al. (2013a). Antimalarial drugs : Mode of action and status of resistance. *African Journal of Pharmacy and Pharmacology*, 7(5), 148–156. http://doi.org/10.5897/AJPPX12.015

Saifi, M. A., Beg, T., Harrath, A. H., Suleman, F., Altayalan, H., & Quraishy, S. Al. (2013b). Antimalarial drugs: Mode of action and status of resistance. *African Journal of Pharmacy and Pharmacology*, 7(5), 148–156. http://doi.org/10.5897/AJPPX12.015

Shannon Reagan-Shaw, Minakshi Nihal, and N. A. (2007). Dose translation from animal to human studies Revisited. *Journal of the Federation of American Society for Experimental Biology*, 22(3), 659-661. http://www.fasebj.org/content/22/3/659.full.pdf+html

Snow, R. W., Guerra, C. A., Noor A. M., Myint H. Y., and H. S. I. (2005). The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature*, 434, 214–217.

Spangenberg, B., Poole, C. F., Weins, C. (2011). Quantitative Thin-Layer Chromatography*(TLC)*. ISBN: 978-3-642-10727-6. http://www.springer.com/gp/book/9783642107276, accessed online on 19th February 2017.

Srinivas. (2015). Transmission of malaria-Life cycle of malaria. Retrieved from http://www.malariasite.com/life-cycle/, on February 16th , 2017.

Tacar, O., Sriamornsak, P. and Dass, C.R. (2013) Doxorubicin: An update on anticancer molecular action, toxicity and novel drug delivery systems. *Journal of Pharmacy and Pharmacology*, 65, 157-170. http://dx.doi.org/10.1111/j.2042-7158.2012.01567.x

Vissers, J.P.C.; Claessens, H..A.; Coufal, P. (1995). Calculation of retention factors in capillary electrochromatography from chromatographic and electrophoretic data. *Journal of Separation Science*, 18(9)**,** 540–544. http://doi.org/10.1002/jhrc.1240180906

Weekley, C., & Smith, D. S. (2013). *Malaria : the clinical basics Learning Overview (Clinical*

). Accessed from http://documentslide.com/documents/50-malaria-the-clinical-basics-final.html, on March 2nd, 2017.

Wells, T. N. C., van Huijsduijnen, R. H., & Van Voorhis, W. C. (2015). Malaria medicines: a glass half full? *Nature Reviews. Drug Discovery*, 14(6), 424–442. http://doi.org/10.1038/nrd4573

White, N. J. (2011). Determinants of relapse periodicity in *Plasmodium vivax* malaria. *Malaria Journal*, (10), 297.

WHO. (2001). Drug resistance in malaria. *Geneva: World Health Organization*. Accessed from http://www.who.int/csr/resources/publications/drugresist/malaria.pdf, 28th February 2017

WHO. (2015a). Malaria. Retrieved from http://www.who.int/gho/malaria/en/ on 26th February 2017

WHO. (2015b). Malaria world report. Disease burden in 2015. Retrieved February 12, 2016, from http://www.who.int/mediacentre/factsheets/fs094/en/

WHO. (2015c). Status report on artemisinin and ACT resistance September 2015, (September), 1–8. http://doi.org/10.1017/CBO9781107415324.004, accessed on 29th January 2017.

WHO. (2015d). Guidelines for the treatment of malaria. Accessed from http://apps.who.int/iris/bitstream/10665/162441/1/9789241549127_eng.pdf, on 19th February 2017.

Xie, L. H., Li, Q., Lin, A. J., Smith, K., Zhang, J., & Skillman, D. S. (2006). New potential antimalarial agents : therapeutic-index evaluation of pyrroloquinazolinediamine and its prodrugs in a rat model of severe malaria. *Antimicrobial Agents and Chemotherapy*, 50(5), 1649–1655. http://doi.org/10.1128/AAC.50.5.1649

APPENDICES

Appendix 1: Ethical approval from KEMRI/Scientific and Ethics Review Unit

KENYA MEDICAL RESEARCH INSTITUTE

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

KEMRI/RES/7/3/1 **December 15, 2016**

TO: **JEAN BAPTISTE NIYIBIZI,**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. KIMANI GACHUHI**
THE DIRECTOR, CBRD,
NAIROBI

forwarded 21/12/16

Dear Sir,

RE: **PROTOCOL NO. KEMRI/SERU/CBRD/PROP155/3324 (*RESUBMISSION OF INITIAL SUBMISSION*): VALIDATION OF SARCOSINE AS A POTENTIAL ANTI-MALARIAL DRUG CANDIDATE WHEN USED SINGLY AND IN COMBINATION WITH QUINOLINE AND ARTEMISININ BASED DRUGS (*VERSION 2.0 DATED 16TH JUNE 2016*)**

Reference is made to your letter dated 5th December, 2016. The KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on the 15th December, 2016.

This is to inform you that the Committee notes that the issues raised during the 254th Committee B meeting of the KEMRI/SERU held on 17th August, 2016 have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **15th December, 2016** for a period of one year. Please note that authorization to conduct this study will automatically expire on **December 14, 2017.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **31st October, 2017**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

FOR: **DR. EVANS AMUKOYE,**
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT

In Search of Better Health