# EVALUATION OF EFFICACY AND PHARMACOKINETIC PROFILE OF A NOVEL TRIOXAQUINE IN MANAGEMENT OF CEREBRAL MALARIA IN MICE

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# Evaluation of Efficacy and Pharmacokinetic Profile of a Novel Trioxaquine in Management of Cerebral Malaria in Mice

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## DECLARATION

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

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## DEDICATION

This thesis is dedicated to my family, especially to my wife Linet for her encouragement and support at the time I decided to get back to study. You assumed the fatherly responsibility and provided for our family throughout this duration. May God bless you.

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## ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **ACTs** | Artemisinin Combination Therapies |
| **ACUC** | Animal Care and Use Committee |
| **ART** | Artemisinin |
| **AUC** | Area Under Curve |
| **BBB** | Blood-Brain Barrier |
| **CBRD** | Centre for Biotechnology Research and Development |
| **CL** | Plasma Clearance |
| **CM** | Cerebral Malaria |
| **CQ** | Chloroquine |
| **CQR** | Chloroquine Resistant |
| **CTMDR** | Centre for Traditional Medicine and Drug Research |
| **DCQ** | Dichloroquinoline |
| **ECM** | Experimental Cerebral Malaria |
| **ECs** | Endothelial cells |
| **$ED_{50}$** | Effective dose of the drug leading to reduction of parasite population by 50 % |
| **HPLC** | High Performance Liquid Chromatography |
| **ICAM-1** | Intracellular Adhesion Molecules 1 |
| **ILRI** | International Livestock Research Institute |
| **IP** | Intraperitoneally |
| **iRBCs** | Infected Red Blood Cells |
| **IV** | Intravenous |
| **KEMRI** | Kenya Medical Research Institute |

| | |
|---|---|
| **$LD_{50}$** | Lethal dose of the drug leading to mortality of 50% of the dosed animals |
| ***PfCRT*** | *Plasmodium falciparum* Chloroquine Resistant Trait |
| ***Pf*EMP1** | *Plasmodium falciparum* Erythrocyte Membrane Protein 1 |
| **PI** | Post-infection |
| **PK** | Pharmacokinetics |
| **pRBC** | Parasitized Red Blood Cells |
| **SERU** | Scientific Ethics and Review Unit |
| **SI** | Selectivity index |
| **SP** | Sulfadoxine-Pyrimethamine |
| **TI** | Therapeutic index |
| **TNF** | Tumour Necrosis Factor |
| **VCAM-1** | Vascullar Cellular Adhesion Molecules 1 |
| **Vd** | Volume of Distribution |
| **WHO** | World Health Organisation |

## ABSTRACT

The emergence of multidrug-resistant strains of *Plasmodium falciparum*, stalled efforts in malaria control coupled with low success rates for new chemotherapies to proceed into clinical trials pose a great threat of increased fatalities in cases of cerebral/ severe malaria infections in which parenteral artesunate monotherapy is the current drug of choice. This calls for preservation of efficacy of available effective drugs and to protect them from development of parasite resistance. The study aimed to investigate efficacy, safety and pharmacokinetic profile of a novel trioxaquine in a mouse model of human cerebral malaria whether this compound chemically synthesized by covalent linking of a 4,7-dichloroquinoline pharmacophore to artesunate through drug development approach termed 'covalent bitherapy' could improve the curative outcomes in cerebral malaria infections. *In vivo* acute oral toxicity for the trioxaquine was done according to Organization for Economic Co-operation and Development (OECD) guidelines 423, where female Swiss albino mice were orally administered with either 300 or 2000 mg/kg of the trioxaquine and monitored for signs of severity, and or mortality for 14 days post-treatment. Human cerebral malaria rodent model, the C57BL/6 male mice were infected intraperitoneally (ip) with *Plasmodium berghei* ANKA and intravenously (iv) treated with the trioxaquine from day 8 post-infection (pi) at 12.5 and 25 mg/kg, respectively, twice a day for 3 days. Treatments with the trioxaquine precursors (artesunate and 4,7-dichloroquine), and quinine were also included as controls. 20mg/kg of the trioxaquine was administered iv and plasma samples obtained at various time intervals (0 -12hrs) for pharmacokinetic analysis. The trioxaquine showed a good safety profile with 67% animals survival at the highest dose administered and a potent antiplasmodial activity with 80% parasite clearance in the first 24 hrs for the two dosages used with no recrudescence observed even beyond 60 days post-treatment. An apparent half-life of 3.52hrs was also reported. The curative effect together with the good safety, and pharmacokinetic profiles observed with the trioxaquine clearly demonstrated its potential as a drug candidate for management of CM, especially in a time of shrinking antimalarial armamentarium for management of CM.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background of the Study

Despite many years of research and great progress in line with the goal of malaria eradication (Wells *et al*., 2015), Cerebral malaria (CM) has remained the most dreadful severe complication of *P. falciparum* infection, with a higher burden greatly felt in the larger sub-Saharan Africa (Desruisseaux *et al*., 2010), a continent already under immense pressure and effects of other diseases as Tuberculosis and HIV/AIDS (Mock *et al*., 2004). Initial attempts towards the fight against malaria greatly involved the use of insecticides and DDTs to which mosquito vectors developed metabolic resistance (Hemingway and Ranson, 2000). With regard to vaccine development, all vaccine approaches so far have been unsuccessful (Girard *et al*., 2007). Reviews on the most advanced and the most promising of the vaccine candidates RTS, S, at phase III of the clinical trials showed lack of enduring protection indicating that malaria disease eradication would not be achieved with this vaccine (Lorenz *et al*., 2014). Review reports on the vaccine showed imperfect and short lived immunological and clinical response reflecting a deteriorating efficacy of the vaccine over time (Lorenz *et al*., 2014, Girard *et al*., 2007). With these results therefore, it is assumed that vaccine trials are still in the pipeline and thus an effective vaccine against malaria is yet to be availed (Girard *et al*., 2007).

Chemoprophylaxis and chemotherapy has therefore remained the mainstay strategy in fighting malaria (Reyburn, 2010, Schlitzer, 2008). Since the invention and introduction of the use of synthetic antimalarial drugs, just but a limited number of these therapeutic agents have been put to clinical application (White, 2008; Schlitzer, 2008). This number is however extremely limited when it comes to cases of cerebral malaria and other forms of severe malaria infections and disease (Reyburn, 2010). For decades, quinine has been the drug of choice for management of CM (Achan *et al*., 2011). However, concerns over increasing resistance to the quinoline-based drugs such as chloroquine (CQ) prompted World Health Organization (WHO) policy change to use of artemisinin-based drugs such as artemether and artesunate. In management of CM, preference has been given to parenteral artesunate due to its superior efficacy and survival outcome in comparison to intramuscular artemether (Phu *et al*., 2010; Pasvol, 2005).

Despite effective CM chemotherapy with artesunate, mortality rate still remain as high as 15-30% among the treated individuals while those that survive the acute episodes of CM suffer long term neurological sequelae including epilepsy specifically in children (Idro *et al.*, 2010; 2005).

Close to 90% of global malaria related deaths occur in sub-Saharan Africa of which *P. falciparum* claims responsibility. Unfortunately this lethal pathogen within the *Plasmodia* population has shown multidrug-resistance (Noedl *et al.*, 2010; Phyo *et al*., 2012). Reports of the parasite resistance against the WHO-recommended artemisinin (ART) derivatives- the first-line antimalarials have already been documented,

prompting an urgent need for new drugs (Dondorp *et al*., 2009). Extremely low success rates for new chemotherapies in the development pipeline to proceed to clinical trials (Olliaro and Wells, 2009), has necessitated a strategy of redeploying the available effective drugs, to explore full potential of their therapeutic life and also to protect them from development of parasite resistance (Muregi and Ishih, 2010). Currently, ART derivatives which are the WHO gold standard for management of *falciparum* malaria are prescribed in combination with longer acting antimalarials, in what is termed as artemisinin-based combination therapy (ACT), with a goal to protect them against parasite resistance (Reyburn, 2010). Unfortunately in CM, artesunate has often been used as a monotherapy (Pasvol, 2005), thus calling for a polypharmacology treatment approach in such a scenario especially in sub-Saharan Africa.

Latest advances and approach in drug design and development is embracing the use of hybrid drugs/ novel agents that have dual targets and synergistic functionality through the concept of covalent bitherapy (Muregi and Ishih, 2010). This technology is believed to be cheaper since only the active moieties are joined thus avoiding the long, laborious, and expensive drug development procedures before co-formulation.

This study therefore sought to demonstrate the potential of a novel trioxaquine (a hybrid drug arising from conjugation of 1,2,4-endoperoxide bridge of the artemisinins and a quinoline pharmacophore) (Wamakima *et al*., 2015), for future development and clinical application in management of cerebral malaria. The acute oral toxicity, efficacy and pharmacokinetic profile of the compound were evaluated in a mouse model.

This trioxaquine, termed as N-(7-chloroquinolin-4-ylamino)-ethyl-artesunate-19-carboxamide (Figure 1.1) was previously synthesized at the Centre for Traditional Medicine and Drug Research (CTMDR – KEMRI) and demonstrated to possess remarkable antiplasmodial effect against blood stage rodent malaria parasite in a mouse model ($ED_{50}$ and $ED_{90}$ of 5.5 and 13.5mg/kg respectively) including a remarkable *in vitro* antiplasmodial activity against CQ-sensitive (D6) ($IC_{50}$, 6.89ng $mL^{-1}$) and CQ-resistant (W2) ($IC_{50}$, 3.62ng $mL^{-1}$) *P. falciparum* isolates (Wamakima *et al.*, 2015).

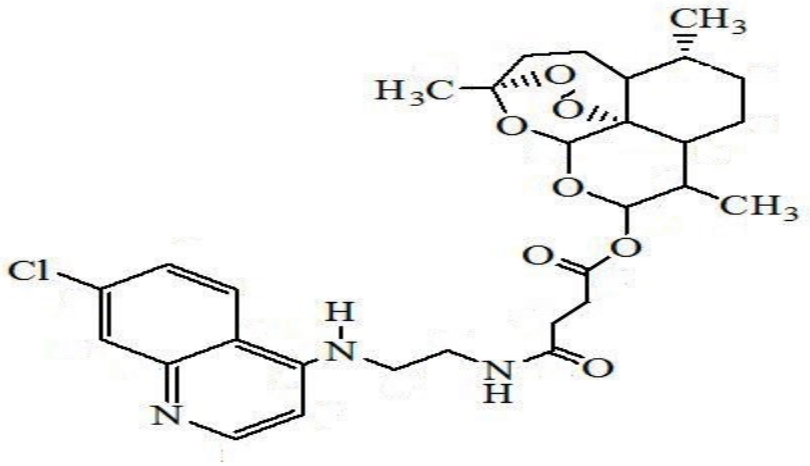


**Figure 1.1** The trioxaquine structure

**1.2 Problem Statement**

Apart from posing a serious threat to human health worldwide, malaria still stands out among the parasitic diseases leading with high infections and deaths especially in developing countries, (D'Alessandro, 2009). Cerebral, and or severe forms of malaria record unacceptably high mortality (Snow *et al.*, 2005). In the absence of a potential

vaccine, chemotherapy remains the mainstay strategy in combating malaria (Reyburn, 2010). Chemotherapy is however compromised by the few options available especially for severe malaria as quinine and artesunate (Reyburn, 2010). With reported increasing tolerance by *P. falciparum* even to artesunate, it is critical that new strategies are developed for new antimalarial drugs (Morphy and Rankovic, 2005). The process of developing and discovering new antimalarials however, is extremely expensive. The cost of research and developing new drugs surpass $1.3 billion for any possible new drug candidate thus affecting investments in pharmaceutical research considering the high burden facing developing countries (Weisman *et al*., 2006). Extremely low success rates for new chemotherapies to proceed to clinical trials have also been a hindrance, even a decade can elapse before a single molecule reaches clinical application (Olliaro and Wells, 2009). This calls for deploying the currently remaining effective drugs in clinical use for maximization of their therapeutic life (Muregi and Ishih, 2010).

### 1.3 Justification

Current approach in drug design known as "covalent bitherapy" in which dual activity is presented in a hybridized molecule is believed to be a cheaper and faster option to provide the urgently needed solution of new antimalarials (Muregi and Ishih, 2010). The covalent linking is believed to present several merits over the co-formulated constituents including increased cellular uptake than the individual components, and a stronger synergism due to their proximity thus having increased potency (Walsh *et al*., 2007).

The products of this technology have often shown more efficacy than the individual components alone, or in co-formulation and also reduces the risk of treatment failure by protecting the partner drug from development of resistance (Grellepois *et al*., 2005). A number of research groups have embraced and adopted the concept of covalent bitherapy in the treatment of malaria (Grellepois *et al*., 2005; Benoit-Vical *et al*., 2007; Coslédan *et al*., 2008). Some of these hybrid drugs (artesunate/aminoquinoline, imipramine/aminoquinoline) have proved to be potent antimalarial agents with high safety profiles (Benoit-Vical *et al*., 2007). However, despite the potency and safety profiles exhibited by these molecules, none has reached clinical application (Benoit-Vical *et al*., 2007; Coslédan *et al*., 2008). Lack of information on the pharmacokinetic profile of these molecules still hinders any further development and clinical application (Benoit-Vical *et al*., 2007; Coslédan *et al*., 2008), thus justifying this study.

### 1.4 Research Questions

1 What could be the acute oral toxicity of the trioxaquinne in untreated mice?

2 Could the trioxaquine be effective in the management of cerebral malaria in mice?

3 What plasma concentration would be necessary to achieve the desired clinical outcome using parenteral route of administration?

### 1.5 Null hypothesis

The novel trioxaquine has no *in vivo* antimalarial activity in severe malaria infections.

## 1.6 Objectives

### 1.6.1 General objective

To evaluate the efficacy of a novel trioxaquine for future development and clinical application in management of cerebral malaria.

### 1.6.2 Specific objectives

1. To evaluate the *in vivo* acute oral toxicity of the trioxaquine in uninfected mice using the OECD guidelines 423.
2. To establish the efficacy of the trioxaquine in treatment of cerebral malaria using human cerebral malaria rodent model.

3. To evaluate the pharmacokinetic profile (PK) of the trioxaquine in mice, using High Performance Liquid Chromatography, and pharmacokinetic software tool.

### 1.6.3 Expected outputs

1. The establishment of the acute oral toxicity of the trioxaquine in mice.
2. The establishment of the efficacy of the trioxaquine in management of cerebral malaria in mice.
3. The establishment of intravenous pharmacokinetic profile of the trioxaquine in mice.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Malaria

Malaria, one of the world's most devastating parasitic diseases is caused by protozoan parasites of the genus *Plasmodium* which is transmitted to man by the *Anopheline* mosquito. Five *Plasmodia* species are known to infect humans namely, *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and currently, *P. knowlesi* (Sutherland *et al*., 2010). *P. knowlesi*, a primate parasite capable of causing *zoonotic* malaria (Cox-Singh, 2010). Among the five malaria parasite species, *P. falciparum* claims 90% of all malaria related deaths in sub-Saharan Africa and responsible for cerebral malaria though infections with *P.vivax*, and *P. knowlesi* can also lead to severe malaria (Reyburn, 2010).

Even though great progress has been made in the fight against malaria over the last decades, the disease is still key among the global health concerns (Reyburn, 2010). Almost half of the world's population inhabit malaria endangered areas in which every year, close to two hundred and fifty million people experience clinical malaria outbreak of which, 584,000 – 755,000 people die of malaria (Hay *et al*., 2004). However, this number could be twice as high on inclusion of cases of the disease that go undiagnosed or unreported at the health facilities (Murray *et al*., 2012). About 83% of these deaths are recorded in sub-Saharan Africa with most of the casualties being children below 5 years old and expectant women in their first trimester (Reyburn, 2010).

In Kenya, malaria still leads in morbidity and mortality with over 25 million people out of the country's population at risk of malaria (Noor *et al*., 2007). Deaths due to malaria still records the highest among the reported cases even with the decreasing trend of 16.5% in 2010 to 12.2% in 2013 as reported in Kenya malaria indicator survey 2015.

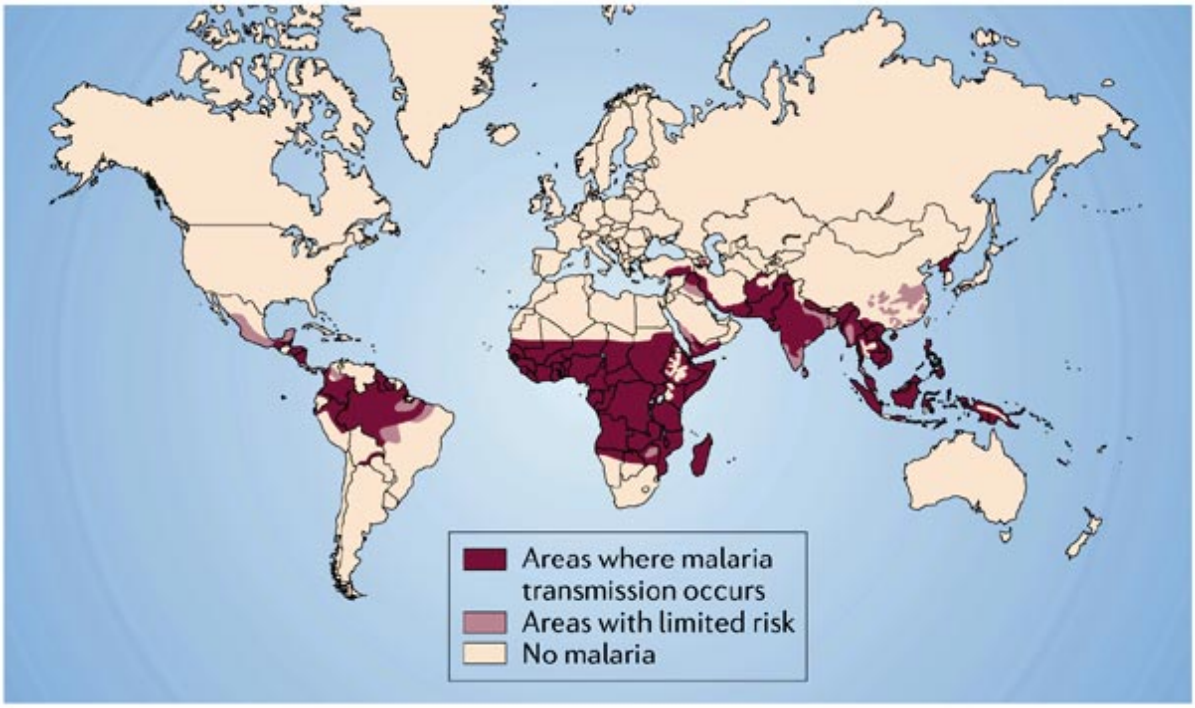



**Figure 2.1** Global distribution of countries and areas at risk of malaria transmission (Hay *et al.*, 2004).

With no vaccine currently available, malaria control strategies has greatly relied on preventive measures including insecticidal treated bed nets, indoor residual sprayings, environmental controls such as swamp drainage, and personal protective methods such as using mosquito repellent creams (Reyburn, 2010). Artemisinins in combination therapy (ACT) on recommendation of World Health Organization is the first choice of treatment in uncomplicated *P. falciparum* infection (Dondorp *et al.*, 2009). In this

scenario, more than one anti-malarial agents are often used in co-formulation, a fast acting but short lived artemisinin (ART) derivative co-formulated with a less effective but a longer acting partner drug (Reyburn, 2010).

On entering the blood stream, the malarial parasites attack and damage the red blood cells resulting to observed symptoms of fever chills, headache, muscle aches, tiredness, nausea, vomiting among others. However, the above symptoms are self-reported and nonspecific, not indicating any specific disease process (Reyburn, 2010).

### 2.2 Cerebral Malaria

Microscopic confirmation of *P. falciparum* parasites in blood smears associated with cerebral manifestations especially a coma that can last for more than 30 minutes following a seizure defines cerebral malaria (CM) (Idro *et al*., 2010). This condition is regarded as one of the most dramatic presentations of severe *falciparum* infection among other manifestations as kidney failure, pulmonary oedema, respiratory distress, and chronic anaemia in infected patients (Robinson *et al*., 2006) although *P. vivax* and *P. knowlesi* infections can also result to CM (Cox-Singh *et al*., 2008; Kantele and Jokiranta, 2011). CM is thus regarded as a life-threatening medical emergency with patients presenting with this condition being in dire need of highest possible level of clinical attention (Pasvol, 2005; Maitland *et al*., 2005). Delayed management of uncomplicated *falciparum* malaria further increases the risk of CM thus clinical diagnosis and prompt treatment of uncomplicated malaria is of great importance (Pasvol, 2005; Maitland *et al*., 2005). However, severe *falciparum* malaria may develop

so fast in children that even prompt management of uncomplicated malaria would not be feasible (Idro *et al*., 2010).

### 2.2.1Clinical manifestation

Cerebral malaria often has variable clinical progress which would include sudden or gradual onset of coma (Walker *et al*., 1992). Infected individuals present with drowsiness, disorientation, delirium or agitation, respiratory distress with frequent focal seizures particularly in children (Brewster *et al*., 1990; Marsh *et al*., 1995). However, the observed coma would be resolved within 48-72 hours post-treatment (Walker *et al*., 1992). Patients with CM sometimes are open-eyed but non-seeing, presenting with disconjugate gaze and nistagmus (Brewster *et al.*, 1990). Sustained upward or lateral ocular deviations are often observed, with the patients exhibiting abnormal posturing (arms and legs extended or flexed arms and extended legs) (Idro *et al*., 2005). In-depth analysis to establish the presence of cerebral malaria and no other forms of encephalopathy has shown a clear Cerebral Spinal Fluid (CSF) with mild lymphocyte pleocytosis < 10 cells/µl in addition to a raised protein levels not exceeding 150 mg/dL (Beare *et al*., 2006).

Other commonly observed manifestations include psychosis, cranial nerve lesions, extrapyramidal tremor, ataxia, and polyneuropathy though these signs are majorly transient neurologic disturbances resolving in a few days to several weeks after their onset (Carter *et al*., 2004). Vital organs dysfunction is often noted in CM and this would determine the mortality rate in both adults and children (Brewster *et al*., 1990). Even though recovery could be relatively rapid with the observed neurological signs being

completely reversed (Brewster *et al.*, 1990; Idro *et al*., 2010), residual neurological deficit which include language disorders, motor, cognitive dysfunctions, and epilepsy do occur in children recovering from acute episodes of cerebral malaria (Carter *et al*., 2004; 2006; Holding *et al*., 1999).

### 2.2.2 Determining epidemiological data on cerebral malaria and other forms of severe malaria in Africa and Kenya

Specific case identification is necessary for an accurate determination of the incidences and distribution of severe malaria though this has been a great challenge in Africa (Korenromp *et al*., 2003; Hay *et al*., 2010). Malaria prevalence is highly recorded in poverty stricken habitats where proper methods of disease identification (microscopy), documentation and reporting are lacking (Mudenda *et al*., 2011). Many illnesses and deaths due to cerebral or severe malaria occur at homes with case fatality rate assumed at 90% and such incidences never come to the attention of a formal health facility (Mudenda *et al*., 2011). In most of the epidemiological studies, 'Verbal autopsies' have been employed in identifying causes of death in communities (Korenromp *et al*., 2003). However, in malaria the accuracy of verbal autopsy is quite poor due to lack of distinct features to differentiate severe malaria from many other fatal febrile conditions without a laboratory test (Mudenda *et al*., 2011). Cases of missed diagnosis are also likely to occur even in scenarios where severe malaria is reported in a health facility (Reyburn *et al*., 2004; Taylor *et al*., 2004). In some instances, clinicians have ended up treating for malaria in situations where an accurate test would have been negative and failing to investigate or treat other possible cases due to lack of confidence in laboratory

diagnosis and consequent invisibility of other etiologies (Reyburn *et al*., 2004). Data generated by the verbal autopsies are not supported by any clinical diagnosis (White *et al*., 2012). Such non reliable and incomprehensive vital registration systems have therefore been a great hindrance to measuring specific mortality rate of cerebral malaria in sub-Saharan African countries (Korenromp *et al*., 2003). In countries where the vital registration systems are comprehensive, events of underreporting do occur and thus the reported figures seen as minimum estimates (Korenromp *et al*., 2003).

### 2.3 *Plasmodium* life cycle

The malaria parasite has a complex life cycle spanning both humans and *Anopheles* mosquito host, differentiating several times during its transmission and infection process (Reyburn, 2010). This complexity in the life cycle has been the greatest tool the parasite has employed in evading all the possible strategies to developing an effective malaria vaccine towards it (Greenwood *et al*., 2008; Volkman *et al*., 2012).

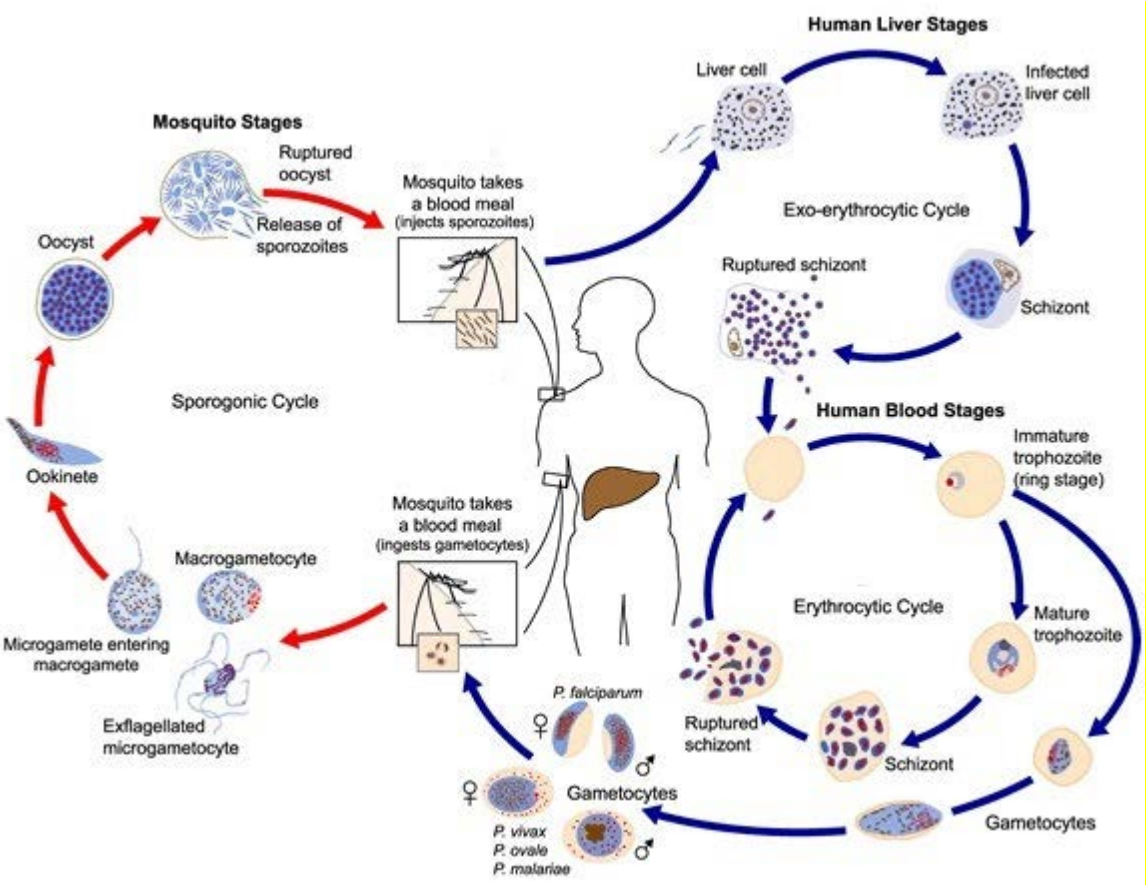



**Figure 2.2:** *Plasmodium* life cycle (CDC, 2012)

### 2.3.1 Hepatocyte stage

In the process of blood feeding, a female *Anopheles* mosquito infected with malaria parasites will injects sporozoites into the human host circulatory system. The sporozoites infect hepatocyte cells where they mature into schizonts (Vaughan *et al*., 2008).

### 2.3.2 Blood stage

Merozoites invade and infect red blood cells. Some parasites at this point differentiate into gametocytes. Clinical sign of the disease are manifested at this stage (Baum *et al*., 2008).

### 2.3.3 Gametocyte stage

Both male and female gametes are ingested by *Anopheles* mosquito during blood feeding. Gametogenesis process occurs during this stage where the male microgametes penetrate the female macrogametes forming zygotes in the mosquito's stomach (Cowman and Crabb, 2006).

### 2.3.4 Sporogonic stage

The products of gametogenesis (zygotes) transform and elongate into ookinetes. These move to the midgut wall of the mosquito where they mature into oocysts. The oocysts release sporozoites which are injected into another new human host thus propagating the malaria life cycle (Boëte, 2009).

## 2.4 Chemotherapy in cerebral and other forms of severe malaria

Antimalarial therapies play a critical role in the control, prevention and ultimately elimination of malaria. However, a greater challenge to chemotherapy remains the increasing tolerance and development of resistance by the malarial parasites to most antimalarial agents currently in the market (Reyburn, 2010).

According to their mode of action, four major classes of antimalarials are currently in clinical use namely, quinoline-related compounds, antifolates, artemisinin-based drugs, and antimicrobials (Reyburn, 2010). Since none of these drugs can single handedly get

rid of all forms of the malarial parasite's life cycle, one or more classes of these antimalarials are often given in a combination therapy to synergistically combat malaria (Reyburn, 2010). In cerebral or severe malaria however, parenteral monotherapy forms of these drugs of which only the quinoline related, quinine and the ART derivative, artesunate have been preferred (Reyburn, 2010).

### 2.4.1 Chloroquine

Chloroquine (5) (CQ), a 4-amino-quinoline with structural similarities to quinine has been greatly and widely indicated both for malaria treatment and prevention since its discovery in 1946 (Baird, 2004). It was effective in curing all forms of malaria with few side effects when taken in the prescribed dose however, a few side effects including itching, gastrointestinal disturbances were observed with acute overdose being life threatening because of the fatal cardiac arrhythmias (Baird, 2004).

Mode of action of CQ (5) is believed to be through its accumulation in the parasite food vacuoles leading to increased concentrations of the drug in the parasite food vacuole. This subsequently increases the food vacuole pH thereby controlling the transformation of toxic haem to hemozoin as the hemozoin biocrystallization process is prevented. The increased toxicity levels inside the parasite’s food vacuole leads to its poisoning (Hempelmann, 2007).

The extensive use of CQ (5) led to widespread development of resistance and currently, it is virtually rendered useless in treatment of *P. falciparum* infections. Reports on

increasing cases of CQ (5) resistance especially in infections of *P. vivax* are on the rise (Baird, 2004).

### 2.4.2 Quinine and related agents

These are classified as arylaminoalcohols which include quinine, quinidine, and mefloquine. Quinine was among the first drugs of plants origin (cinchona bark) for treatment of malaria in the early 1800s. Quinidine, a dextroisomer to quinine was also discovered around the same time (Kumar *et al*., 2009).

Quinine acts as a blood schinzonticide with a weak gametocidal activity against *P. vivax* and *P. malariae* (Carrara *et al*., 2006). In *P. falciparum*, quinine accumulates in the food vacuoles where it acts by preventing the process of hemozoin biocrystallization leading to accumulation of cytotoxic haem in the food vacuole and eventual poisoning of the parasite (Hempelmann, 2007). Both quinine and quinidine has been used in treatment of cerebral or severe malaria with quinine still being the last resort drug especially in the larger sub-Saharan Africa (Carrara *et al*., 2006).

#### 2.4.2.1 Challenges facing the use of quinine in management of malaria

Quinine has been the key drug in the treatment of severe or cerebral malaria and still remains the first-line drug in such cases even though preference is changing to artemisinin derivative artesunate (Pasvol, 2005). Parasite displaying increased tolerance and development of resistance to commonly used drugs is the greatest problem thwarting all the efforts geared towards malaria control worldwide (Reyburn, 2010). Cases of quinine resistance emerged in 1910 (Peters, 1982) with more reports on decreased sensitivity of *P. falciparum* to quinine being widely documented in Asia and

South America (Mayxay *et al*., 2007; Legrand *et al*., 2008). Though such reports are still silent in Africa, a significant reduction in efficacy of quinine has been documented (Tinto *et al*., 2006; Toure *et al*., 2007; Pradines *et al*., 1998).

A varying pharmacokinetic profile of quinine is believed to be among the major avenues behind the treatment failures being reported with the drug (Pasvol, 2005). Factors as age, pregnancy, immunity, and disease severity are believed to determine quinine therapeutic responses and pharmacokinetic properties (Chongsuphajaisiddhi *et al*., 1981). Recovering patients have often exhibited a rise in systemic clearance rate and expanded volume of distribution, factors that could lead to low plasma concentration of the drug subsequently resulting to incomplete clearance of infection hence an avenue for treatment failure (Looareesuwan *et al*., 1990).

A smaller value of therapeutic index has been observed with quinine use, with a number of adverse effects accompanying its use (Pasvol, 2005). These ranges from the mild forms as tinnitus, slight hearing impairment, headache, nausea to serious forms as vomiting, abdominal pains, vision loss, hypotension, vertigo, hypoglycaemia, including life threatening episodes of cardiac arrhythmias (Bonington *et al*., 1996).

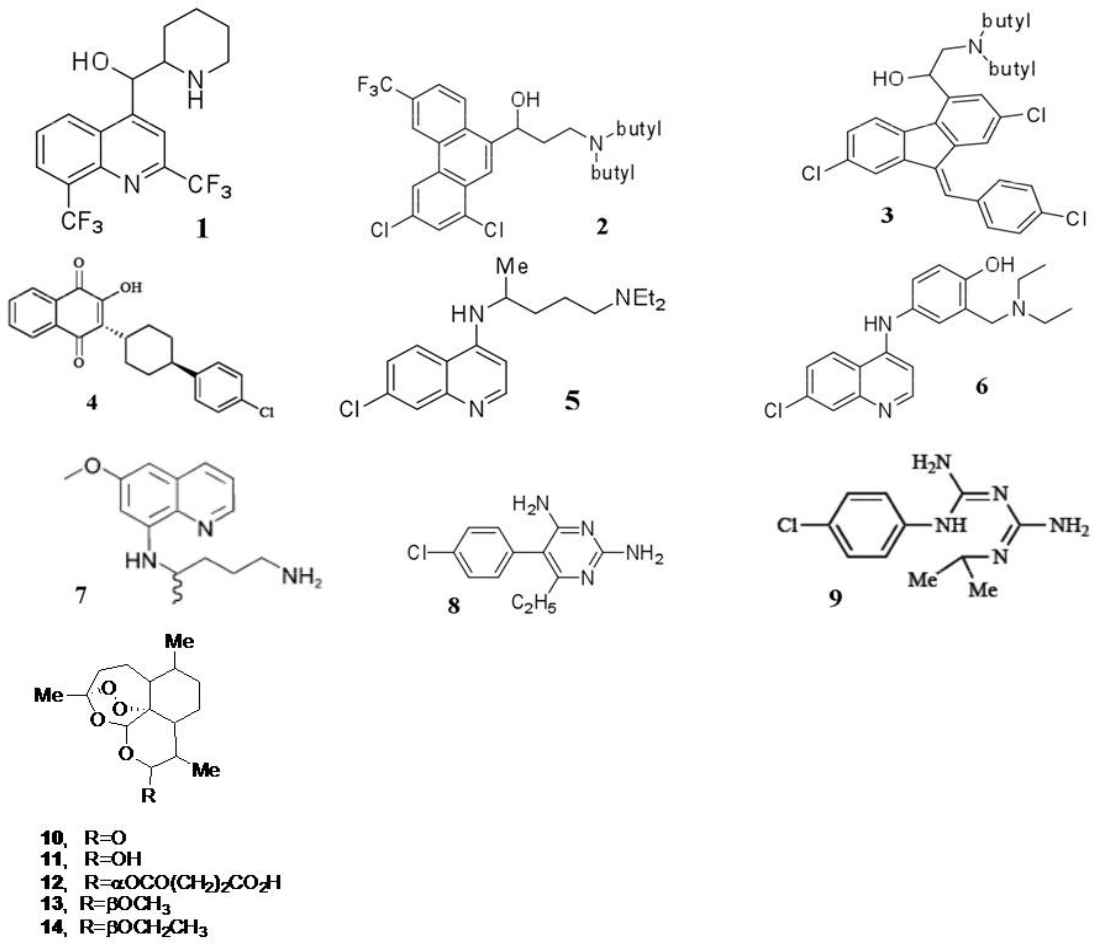
### 2.4.3 Artemisinin and its derivatives

Originally known as qinghaosu, a drug of plant origin qinghao (*Artemisia annua*; sweet wormwood), came into public domain as potential drug to treat malaria in the year 1979 (Hyde, 2007). Use of qinghaosu, later named artemisinin **(10)** (ART), gained momentum when treatment failures with CQ, Sulfadoxine-Pyrimethamine (SP) and

Amodiaquine (ADQ) were on the rise and consequently, infections and deaths reaching extremely high levels (Warsame *et al.*, 2010). Current clinical practise in cases of uncomplicated *falciparum* malaria relies entirely on ART derivatives including artemether artesunate, arteether , and dihydroartemisinin with a partner drug being either Lumefantrine (LMF) **(3**), ADQ, Piperaquine (PPQ) or MQ **(1)** in ACT as recommended by WHO (Reyburn, 2010).

ART though a prodrug, the resulting pharmacological activity is due to the modification of its sesquiterpene lactone endoperoxide bridge (Eastman and Fidock, 2009). Mode of action of ARTs is still unclear though in the process of haemoglobin digestion in the parasite food vacuole, the lactone Endoperoxide Bridge is believed to interact with reduced iron in the haem residue together with the free intracellular reduced iron species leading to the bioactivation of ART (O'neill *et al*., 2010). The bioactivated ART then covalently bind to macromolecules throughout the whole parasite (O'neill *et al*., 2010). The ART is also believed to interact with specific proteins and phospholipids, resulting to oxidative membrane and DNA damage in the parasite (Hartwig *et al*., 2009).

**Figure 2.3** Structural representation of some of the discussed classes of antimalarial drugs




Mefloquine **(1),** halofantrine **(2),** lumefantrine **(3),** atovaquone**, (4),** chloroquine **(CQ, 5),** amodiaquine **(6),** primaquine **(7),** pyrimethamine **(8),** proguanil **(9),** artemisinin **(10)** and its semisynthetic derivatives dihydroartemisinin **(11),** artesunate **(12),** artemether **(13)** and arteether **(14).**

## 2.5 Hybrid drugs

It has been a long history in the practice of many clinicians to use the tactic of simultaneous treatment through multiple drug targets to achieve an optimal clinical outcome (Singh *et al*., 2013). Combination of drugs from complementary classes has been found to be five times more effective than even increasing the dose of one component drug (Gradman *et al*., 2011). Combination therapy where ARTs based compounds are co-formulated with other classes of antimalarials namely Duocotexin® (dihydroartemisinin/ piperaquine phosphate), Mephaquine® (artesunate/ mefloquine), Lurmartem® (artemether/ lumefantrine), to form ACTs is the currently available method of polypharmacology in treatment of uncomplicated malaria in kenya (Nosten and Brasseur, 2002).

Covalent bitherapy which involves the covalent linking of different active ingredients to form a single chemical entity also known as a hybrid/ dual drug is the most recent approach towards polypharmacology (Morphy and Rankovic, 2005).

Meunier and co-workers described such compounds as having two or more structural domains with two or more distinct pharmacophores possessing different biological functions or broad spectrum of activity (Meunier, 2007).

Morphy and Rankovic in the year 2005 gave classification of hybrid/ dual drugs as:

i) **Conjugates hybrids**, comprising of two pharmacophores joined by a stable linker group that is unique to each of the individual pharmacophore.

ii) **Cleavage conjugate hybrids**, in which the separate pharmacophores are joined together with metabolizable linkers leading to release of individual pharmacophore to interact independently.

iii) **Fused hybrids**, where the frame works of the different pharmacophores touch each other as the size of the linker is extremely reduced to allow this.

iv) **Merged hybrids**, where the different pharmacophores share a common pharmacophoric structure through which they are linked together.

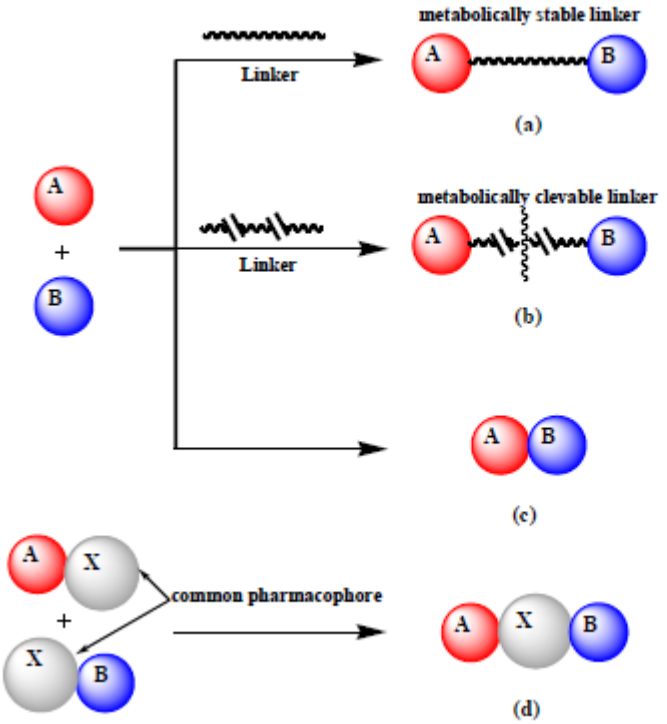



**Figure 2.4** Different hybridization approaches

(a) Conjugate hybrid, (b) Cleavage conjugate hybrid, (c) Fused hybrid, (d) Merged hybrid (Chauhan *et al*., 2010).

### 2.5.1 Artemisinin-based hybrids

In combination with an amionoquinoline moiety, the 1,2,4-trioxane and 1,2,4-trioxalane endoperoxide analogs of ART have resulted to trioxaquines, and trioxalanes respectively (Loup *et al*., 2007). In these hybrid molecules, dual activity is packaged by incorporation of the haem alkylating features of the endoperoxide pharmacophore and the anti-hemozoin biocrystalization properties of the quinoline pharmacophore (Muregi and Ishih, 2010). The trioxaquines have been found to have a superior antimalarial activity on both wild and resistant *Plasmodium* strains in comparison to the individual components symbolizing a synergy in this class of antimalarials (Araújo *et al*., 2009). More ART-based hybrids are still in the pipeline including trifluoromethyl-artemisinin, ART- mefloquine (Grellepois *et al*., 2005), and ART- quinine (Walsh *et al*., 2007). A novel artemisinine-quinine hybrid synthesized by Walsh and his team by esterification of dihydroartemisinine to quinine through the latter's carboxylic acid derivative (Walsh *et al*., 2007), showed a greater *in vitro* antiplasmodial activity to both the wild and resistance strains of *P. falciparum* in comparison to quinine and, artemisinine singly and even to a 1:1 co-formulation of both drugs, an evidence of beneficial synergy from covalent linking of the two drugs (Walsh and Bell, 2009).

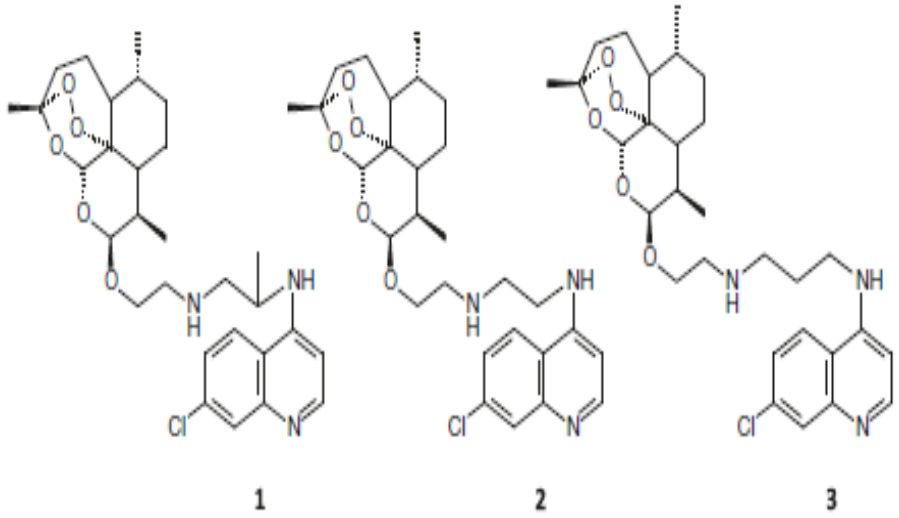



**Figure 2.5** The structures of artemisinin-quinoline hybrids. The chemical structures of C-10 acetal artemisinin-aminoquinoline hybrids 1 – 3 only differs with one methylene group in the linker and its position in the chain (Lombard *et al*., 2013).

### 2.5.2 Quinoline-hybrids

Reversal of the resistance that is associated with the *P. falciparum* CQ resistance (*Pf*CRT) that involved the exportation or pumping of this class of antimalarial out of the parasite's food vacuole has been the major strategy behind the quinoline hybrids (van Schalkwyk and Egan, 2006).

Burgess *et al*., (2006) synthesized a quinoline hybrid comprising of a 7-chloroquinoline pharmacophore with an imipramine pharmacophore that was able to undo the efflux-driven resistance at $IC_{50}$ value lower in comparison to $IC_{50}$ of CQ alone in both *P. falciparum* CQ-sensitive and resistant strains.

Due to their advanced mode of action and high selectivity, the hybrid molecules based chemotherapy is becoming a beneficial tool in contemporary trend of novel antimalarial

discovery (Muregi and Ishih, 2010). Several developments towards this direction of covalent bitherapy have given a new impetus to antimalarial chemotherapy and have demonstrated that this approach as a new strategy in antimalarial discovery is feasible (Burgess *et al*., 2006). A number of research groups are reviewing the potentiality of hybrid molecules as antimalarials especially the Artemisinin and quinoline-based hybrid which is currently the most predominantly researched entities (Muregi and Ishih, 2010; Lombard *et al*., 2010; 2011).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Study site and drugs

The project work was conducted both at the Centre for Biotechnology Research and Development (CBRD), malaria laboratory of Kenya Medical Research Institute (KEMRI), Nairobi, Kenya and Food Biochemistry Laboratory JKUAT.

The test drugs, namely artesunate, 4,7-dichloroquinoline, and quinine were purchased from Sigma Aldrich® India , while the trioxaquine, a kind gift from Prof. Francis W. Muregi, was previously synthesized by linking the 4,7-dichloroquinoline to a linker and coupling the product to artesunate at the Centre for Traditional Medicine and Drug Research-KEMRI (Wamakima *et al*., 2015).

## 3.2 *In vivo* studies

### 3.2.1 Ethical Clearance

The use of mice in this research project was permitted by KEMRI'S Animal Care and Use Committee (ACUC), and the Scientific Ethical Review Unit (SERU), approval number KEMRI/ACUC/01.04.16, and Permit Number: KEMRI/RES/7/3/1 respectively. All the experimental animals that were deemed to have accomplished their intended role in the study were euthanized using 150mg/kg sodium pentobarbital solution injected ip, placed in biohazard bags and incinerated.

### 3.2.2 Evaluation of acute oral toxicity of the trioxaquine

To evaluate the safety of the trioxaquine, the Organization for Economic Co-operation and Development (OECD) Test Guidelines 423 (Acute Toxic Class Method, adopted on

17th December, 2001) were adopted. This is a protocol that make use of three uninfected animals of same sex especially females at every dosage category and assessment based on death and or signs of severity observed with the dosed animals. Averagely, 2-4 dosage steps in the range of 5mg/kg, 50mg/kg, 300mg/kg, and 2000mg/kg are considered enough in making judgment on the acute oral toxicity of the test compound (Schlede *et al*., 2005).

Two fixed dose levels of 300 and 2000 mg/kg were used and dosing was done in a stepwise manner starting with the lowest dose upwards. Swiss albino female mice weighing 20 ± 2g were used where the animals were first fasted for four hours after which they were dosed orally with the above dosages. The start, duration, and severity of toxic signs of the test compound such as changes in skin and fur, eyes, mucous membranes, tremors, convulsions, salivation, diarrhea, coma, and even death were monitored. The animals were observed individually at least once during the first 30 minutes, periodically during the first 24 hours with special attention given during the first 4 hours, and daily thereafter for the next fourteen days.

### 3.2.3 Parasite and Animal Models

*Plasmodium berghei*-ANKA (clone 676m1cl1), an isolate derived from clone 1.49L *Plasmodium berghei* ANKA (Janse *et al*., 2006) was used in the study. This parasite maintained at −80°C in Kenya Medical Research Institute (KEMRI) at the Centre for Biotechnology, Research, and Development (CBRD), was thawed and revived by intraperitoneal (ip) inoculation into male Swiss albino mice, 8 weeks old weighing 20 ± 2 g. The mice served as parasite donor for passaging into C57BL/6 male mice 8 weeks

old weighing 25 ± 2 g, being the mouse model of CM. Both the donor mice and the experimental mice were randomly bred at the KEMRI's animal facility, with the experimental mice parental stock having been sourced from International Livestock Research Institute (ILRI-Nairobi). On the days of experiment, mice were subsequently randomized into groups of five per cage and fed with pellets and water, *ad libitum* (Gimode *et al*., 2015).

On the day of administration, artesunate, the dichloroquinoline, and the trioxaquine were each dissolved in a solution of 70% Tween-80 (d = 1.08 g/ml) and 30% ethanol (d= 0.81 g/ml) at a final diluted concentration of 7% (v/v), and 3% (v/v) for tween 80 and ethanol respectively (Gimode *et al*., 2015). Quinine was dissolved in normal saline.

### 3.2.4 Infection of experimental mice

The donor mice were anaesthetized with sodium pentobarbital and the infected red blood cells collected using heparinized syringe via cardiac puncture. The parasitaemia was determined through Giemsa stained thin blood smears and then adjusted downwards using sterilized phosphate-buffered saline (PBS). Male C57BL/6 mice were then inoculated ip with approximately $1 \times 10^5$ parasitized erythrocytes (pRBCs) at inoculum of 0.2 ml. The infected mice were then randomized in plastic cages in groups of five and observed daily for signs of experimental cerebral malaria (ECM), including rough coat, limb paralysis, ataxia, convulsions, and or coma (Ghosh *et al*., 2011; Wang *et al*., 2014). In C57BL/6, ECM signs usually occur between day 6 and 9 post-infection (pi) (Ghosh *et al*., 2011; Wang *et al*., 2014). Giemsa-stained thin-blood smear slides were observed under microscope (×1000) to establish infection.

### 3.2.5 *In vivo* evaluation of the trioxaquine in cerebral malaria rodent models

The treatment of CM in experimental mice with established infection was carried out by the approach of Ryley and Peters (Ryley and Peters, 1970) in which treatment with a drug was initiated when the infection was already well established and the mice had exhibited clear signs for cerebral malaria. Based on the previous results of the four-day suppressive test that had been used to evaluate chemo-suppression ability of the trioxaquine *in vivo*, (Wamakima *et al*., 2015), a dosage of 12.5mg/kg and 25mg/kg were administered intravenously (iv) twice daily for three days through the lateral tail veins from day 8 pi. Three positive control groups were also similarly treated with artesunate (12.5 mg/kg), quinine (60 mg/kg), and 4,7-dichloroquinoline (12.5mg/kg). A negative control group that received only the drug vehicle was also included. Parasitaemia of each mouse was determined by microscopic examination of Giemsa-stained thin-blood smears prepared from mouse tail blood. Slides were coded and infected red blood cells (iRBCs) count was done microscopically in at least five microscopic fields, each with an approximation of 200-400 (iRBCs). Smears were done at 24hrs post-treatment, and 48hrs post-treatment later to monitor progression of parasitaemia. Parasitaemia counts of blood films from each mouse were processed using Microsoft® Excel (Microsoft Corp.). Percentage (%) parasite suppression (chemo-suppression) at each drug dose was determined as described by Muregi *et al*., (2011).

Percentage suppression = 100- ([mean parasitaemia treated ÷ mean parasitaemia untreated] × 100).

The survival of mice was monitored for up to 60 days after the end of the three-day treatment. All RBC counts and parasitaemia levels were expressed as mean values ± standard deviations and the parasitaemia data were processed using the one way analysis of variance (ANOVA). Mean parasitaemia between each experimental group relative to controls was compared using Student's t-test with a probability of 5% ($p < 0.05$) considered significant.

### 3.2.6 Evaluation of the integrity of mouse blood-brain barrier

C57BL/6 mice were injected iv with 200µl of 2% (w/v) Evans Blue dye (Fischer Scientific®-USA) on day 8 pi after the experimental animals had exhibited clear signs for cerebral malaria. The animals were euthanized and sacrificed after one hour for brain extraction to assess the extent of brain staining with the dye and the images documented. The principle behind this method is based upon the formation of bonds between the dye and plasmatic albumin and, in the event of brain blood vessels leakage due to compromised blood-brain barrier (BBB), the dye-protein complex migrates to the tissue, impregnating it in a tone of blue visible to the naked eye Da Silva Barbosa *et al*., (2016).

### 3.2.7 Evaluation of pharmacokinetic profile of the trioxaquine

The *in vivo* pharmacokinetic profile of the trioxaquine was evaluated following intravenous (iv) administration of 20mg/kg of the compound in tween 80/ ethanol solution to male C57BL/6 mice in groups of five. Blood samples were collected via tail bleeding in heparinized syringes at predetermined sampling times within (0hr to 12hrs) post-dosing.

Sample volumes at every sampling time were targeted at a lowest volume of (100µl) per mouse.

### 3.2.7.1 Extraction of drugs from the plasma

The whole blood was separated by centrifugation (Micron 200® -Germany) at 1500rpm for 10min, and the plasma layer transferred to 1.5ml micro centrifuge tubes. For effective separation of drugs from plasma components the deprotonization method was used (Humberstone *et al*., 1995). In brief, 500µl of acetonitrile was added to 500µl of plasma, mixture vortexed for 1min and centrifuged for 15mins at 1500rpm.

### 3.2.7.2 HPLC analysis

Approximately 20µl of the Supernatant was then injected onto the UV-VIS High Performance Liquid Chromatography column for pharmacokinetic analysis.

Concentrations of the trioxaquine in plasma were determined by UV-VIS High Pressure Liquid Chromatography (Shimadzu –Japan), pump - LC10AS, column oven – CTO10A, detector – SPD10AV, recorder – CR8A) under isocratic conditions as described by (Mberu *et al*., 1992). In brief, the column effluent was monitored with a variable wavelength absorbance detector set at 254 nm. The mobile phase was acetonitrile, methanol (60:40 vol /vol) containing 1% potassium dihydrogen phosphate. The flow rate was determined at 1ml/min and all the chromatograms obtained at room temperature. Dihydroartemisinin® Sigma Aldrich India was used as control and the chromatograms were obtained as described by Adegoke *et al*., (2012). Also 4,7-dichloroquinoline and parent trioxaquine were included.

### 3.2.7.3 Pharmacokinetic analysis

The mean area under the curve (AUC) for the concentrations of the trioxaquine in plasma was calculated by the trapezoidal rule during the experiment period from 0hr to 12hrs ($AUC_{0-12}$) and thereafter the following pharmacokinetic parameters were reported: an apparent terminal half-life (t½ [hrs]), plasma exposure Area under the Curve ($AUC_{0-\infty}$ [μg.hr/ mL]), volume of distribution Vd [L/kg], and plasma clearance CL [L/ hr].

## 3.3 Data analysis

Data analysis was performed using SPSS version 16. All values are expressed in means (± SDs). Cumulative survival rates were calculated according to Kaplan-Meier method and groups were compared using the log-rank test $P$ value < 0.05. Parasitaemia courses were compared by analysis of variance (ANOVA). Parasitaemia before and at 24 hours after treatment were compared by paired student t-test. $P$ value < 0.05 was considered to be statistically significant.

Pharmacokinetic parameters for the trioxaquine were analyzed through non-compartmental analysis. Pharmacokinetic software in excel-based programme (Summit pharmacokinetic software, version 2.0) was used to calculate the reported pharmacokinetic data.

# CHAPTER FOUR

# RESULTS

## 4.1 Evaluation of acute oral toxicity of the trioxaquine in mice

Acute oral toxicity of the trioxaquine was evaluated by using uninfected female mice 8 weeks old weighing 20 ± 2 g and had been starved for four hours, administered with oral dosages of 300mg/kg and 2000mg/kg once. The test animals were then observed for fourteen days post-dosing, basing the assessment on signs of severity, and or death observed with the test animals. All the animals did not show any sign of severity or toxicity as no changes in skin and fur, eyes and mucous membranes, as well as aberrant respiratory activities were observed. Also no tremors, convulsions, salivation, diarrhea, sleep and coma were observed (Table 4.1). The mice survived past the fourteen days of observation except for a delayed death of one animal on day 7 post-dosing recorded with the dose of 2000mg/kg, translating to 67% survival. This is an indication that the exact lethal dose-50 ($LD_{50}$) could be within 2000mg/kg resulting to a possible Therapeutic Index (TI) value of > 400 considering the ratio of oral lethal dose-50 to that of effective dose-50 $ED_{50}$ (i.e $LD_{50}$/ $ED_{50}$) ratio. The $ED_{50}$ had previously been determined orally during *in vivo* antiplasmodial evaluation for the trioxaquine using the 4-day suppressive test (Wamakima *et al.*, 2015). This relatively weak toxicity indicates a very wider margin of safety for the test compound implying that the compound could be a promising drug candidate for further exploration.

Table 4.1: Parameters of safety for the trioxaquine using the acute oral toxicity test based on OECD guidelines 423. **The test animals were fasted for four hours, dosed orally with either 300mg/kg or 2000mg/kg of the trioxaquine with each dose repeated once and the animals for 14 days post-dosing**

| | | SAFETY PARAMETERS | | | | | |
|---|---|---|---|---|---|---|---|
| **DOSE LEVEL** | | **Fur changes** | **Mucus membranes** | **Respiratory distress** | **Tremors / convulsions** | **Diarrhea** | **Death** |
| **300mg/kg** | MOUSE 1 | N/O | N/O | N/O | N/O | N/O | N/O |
| | MOUSE 2 | N/O | N/O | N/O | N/O | N/O | N/O |
| | MOUSE 3 | N/O | N/O | N/O | N/O | N/O | N/O |
| **2000mg/kg** | MOUSE 1 | N/O | N/O | N/O | N/O | N/O | N/O |
| | MOUSE 2 | N/O | N/O | N/O | N/O | N/O | YES |
| | MOUSE 3 | N/O | N/O | N/O | N/O | N/O | N/O |

*Healthy Swiss albino female mice in experimental groups of three were first fasted for four hours and then dosed orally with either 300mg/kg or 2000mg/kg in a step wise manner whereby the first dose was done twice before proceeding to the next dose. The animals were then observed keenly in the first 30 minutes and then monitored for a period of 14 days post-dosing. Assessment was based on moribund status of the test animals, and or mortality. N/O; Not Observed. YES; Parameter observed.

**Table 4.1.1 $IC_{50}$ values for trioxaquine, artesunate, 4,7-dichloroquinoline and chloroquine (CQ) against Hep2 cell line, CQR* parasites (W2) and the selectivity index (SI) Adapted from wamakima *et al.*, (2015)**

| Drug | $IC_{50}$ (ηg $ml^{-1}$) | | |
|---|---|---|---|
| | Hep2 cells | CQR* parasite (W2) | Selective Index ( |
| Dual drug | >10,000 | 3.62 | >2762 |
| Artesunate | >10,000 | 4.04 | >2445 |
| 4,7-dichloroquinoline | >10,000 | >1000 | NA |
| Chloroquine | >10,000 | 65.35 | >153 |

*CQR, chloroquine resistant *P. falciparum* isolate (W2); NA, not applicable

### 4.2 Assessment of the therapeutic effect of the trioxaquine in CM

Treatment was initiated on day eight post-infection (pi) after the experimental animals were diagnosed with ECM or having presented with at least one or both well-recognized signs of ECM in animal models including neurological perturbations as ataxia, rough coat, limb paralysis, *inter aria*. Parasitaemia load reduction for each test drug within the first 24 hours post-treatment is presented in Table 4.2. The parasitaemia was monitored for 60 days post-treatment.

**Table 4.2: Parasite load reduction percentage of each drug within 24 hours after the first day of treatment with *the trioxaquine, artesunate, and 4,7-dichloroquinoline against *P. berghei* ANKA in C57BL/6 mice.**

| Drug/ dosage (mg/kg) | Day 8 pi mean parasitaemia ± SD | 24hrs post-treatment mean parasitaemia ± SD | 48hrs post-treatment mean parasitaemia ± SD | Percentage parasitaemia clearance in 24hrs | Recrudescence |
|---|---|---|---|---|---|
| **Trioxaquine** | | | | | |
| 25mg/kg | 11.73 ± 0.06 | 0.42 ± 0.11 | 0.00 | 96.4 | Not observed |
| 12.5mg/kg | 11.69 ± 0.03 | 1.56 ± 0.08 | 0.00 | 86.6 | Not observed |
| **Artesunate** | | | | | |
| 12.5mg/kg | 11.75 ± 0.05 | 1.77 ± 0.04 | 0.00 | 84.8 | Observed |
| **4,7-Dichloroquinoline** | | | | | |
| 12.5mg/kg | 11.55 ± 0.07 | 11.45 ± 0.04 | 11.41 ± 0.02 | 1.89 | ND |
| **Quinine** | | | | | |
| 60mg/kg | 11.65 ± 0.03 | 11.79 ± 0.01 | 6.52 ± 0.02 | +1.03 | Observed |
| **Untreated Control** | 11.67 ± 0.05 | 11.83 ± 0.04 | 100% Mortality recorded | - | - |

*Treatment was initiated on day 8 post-infection. Drugs were administered iv twice a day, for 3 days within 12 hours interval and parasitaemia data before and at 24 hours post-treatment compared using paired student t-test ($p<0.05$). Parasitaemia levels were similar in all the experimental groups before treatment ($p > 0.05$). ND; Not Determined (100% mortality occurred).

At the two dosages used, 12.5 mg/kg and 25 mg/kg iv, the trioxaquine showed a high antiplasmodial activity. At 12.5mg/kg, parasitaemia decreased from 11.7 to 1.6 (86.6% clearance) and from 11.7 to 0.4 (96.4% clearance) at 25mg/kg just after 24hrs post-treatment and no parasite was observable under microscope at 48 hours post-treatment. A potent antimalarial activity was therefore exhibited by the artesunate-quinoline hybrid drug at the two dosages, resulting to a rapid parasite clearance. The treated animals also

survived beyond 60 days post-treatment except for the 12.5mg/kg where death of one animal was recorded on day four post-treatment even after no parasite was observed under microscope.

Artesunate monotherapy at 12.5 mg/kg also showed a rapid parasitaemia clearance of 84.8% within the first 24hrs post-treatment, and no parasite was observable under microscope at 48hrs post-treatment. However, recrudescence was observed on day 8 post-treatment with all the animals succumbing to the infection by day 16 post-treatment (Figure 4.2). Quinine at 60mg/kg and 4, 7-dichloroquinoline at 12.5mg/kg did not show any significant parasitaemia reduction in the first 24hrs post-treatment with all the animals in these experimental groups dying by day 12 and day 7 post-treatment respectively (Figure 4.2). Quinine even after exhibiting a significant parasitaemia reduction at 48 hrs post-treatment (44.9%), further monitoring of parasitaemia showed progression of parasite growth for this group leading to death of mice (Figure 4.2). Mice in untreated control also succumbed to the infection by day 10 pi (Figure 4.2).

Lack of recrudescence observed with the trioxaquine-treated animals could be attributed to the 4,7- dichloroquinoline, the partner drug to artesunate in the trioxaquine.

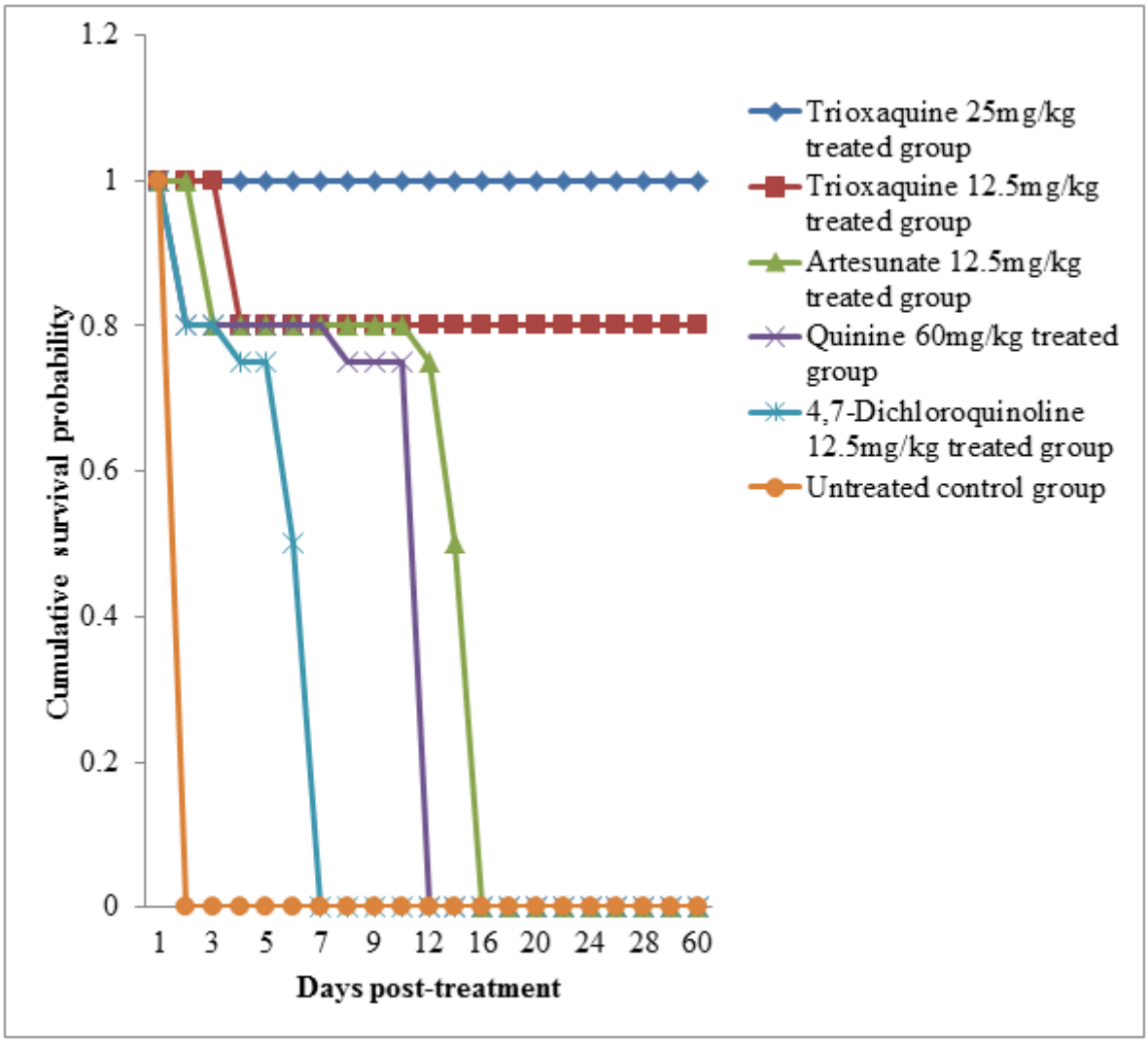



**Figure 4.2 Cumulative Survival Probabilities of the treated animals after having been infected with *P. berghei* ANKA, and treated on day 8 post-infection with the various dosages of the trioxaquine, artesunate, and 4, 7-dichloroquine as indicated below. The animals were then monitored for 60 days post-treatment**

*Survival probability analysis of the treated mice after infection with an inoculum of 0.2ml of approximately $1\times10^5$ *P.berghei* ANKA pRBCs, treated on day 8 pi with various dosages of the trioxaquine (12.5/25mg/kg), artesunate (12.5mg/kg), 4,7-dichloroquine (12.5mg/kg), and quinine 60mg/kg), twice a day for 3 days and their survival monitored for 60 days post-treatment. Untreated control groups were also included. Survival probability analysis using Kaplan-Meier software revealed that there was a significant reduction in mortality in the trioxaquine treated groups ($p < 0.05$) in comparison to the standards (quinine, and artesunate), including the precursor 4,7-

dichloroquinoline. A part from the trioxaquine treatment groups, all the other treatment groups as well as the negative controls had 100% mortality by day 16 post-treatment.

### 4.2.1 Assessment of integrity of mouse blood- brain barrier

Damage to the blood-brain barrier (BBB) has been implicated and is believed to be one of the underlying mechanisms of pathophysiology of CM as the leaky BBB would allow toxic or unwanted compounds to enter the brain, causing neurological dysfunction. Observations of the brain tissue in infected animals that were not injected with the 2% Evans Blue dye on day 8 pi showed a higher degree of brain whitening in C57BL/6 mice (Figure 4.2.1a: brain of *P. berghei* infected C57BL/6 mouse on day 8 post-infection). The massive brain whitening observed in Figure 4.2.1a is believed to be due to reduced or blocked blood flow at multiple sites in the brain (hypoxia), a confirmation of the phenomenon of *p*RBCs sequestration in the brain microvasculature which is one of the major hallmarks in cerebral malaria. Uninfected C57BL/6 mice (Figure 4.2.1b: brain of non- infected C57BL/6 mouse) showed a normal healthy brain with normal blood supply with absence of brain staining with Evans Blue dye indicating an intact BBB integrity. Observation of brains after the injection of the infected experimental animals with 2% Evans Blue dye on day 8 pi showed a higher extent of brain staining in infected C57BL/6 mice (Figure 4.2.1c: brain of *P. berghei* infected C57BL/6 mouse stained with 2% Evans Blue dye on day 8 post-infection), a feature that substantiates the impairment of the integrity of the blood-brain barrier. Observation of brain images of infected C57BL/6 mice at 60 days post-treatment, exhibited a complete recovery, return of normal blood supply and lack of brain staining by the Evans Blue

dye (Figure 4.2.1d: brain of *P. berghei* infected C57BL/6 mouse, trioxaquine treated and stained with 2% Evans Blue dye at 60 days post-treatment), results that are comparable to Figure 4.2.1b of a healthy uninfected animal.

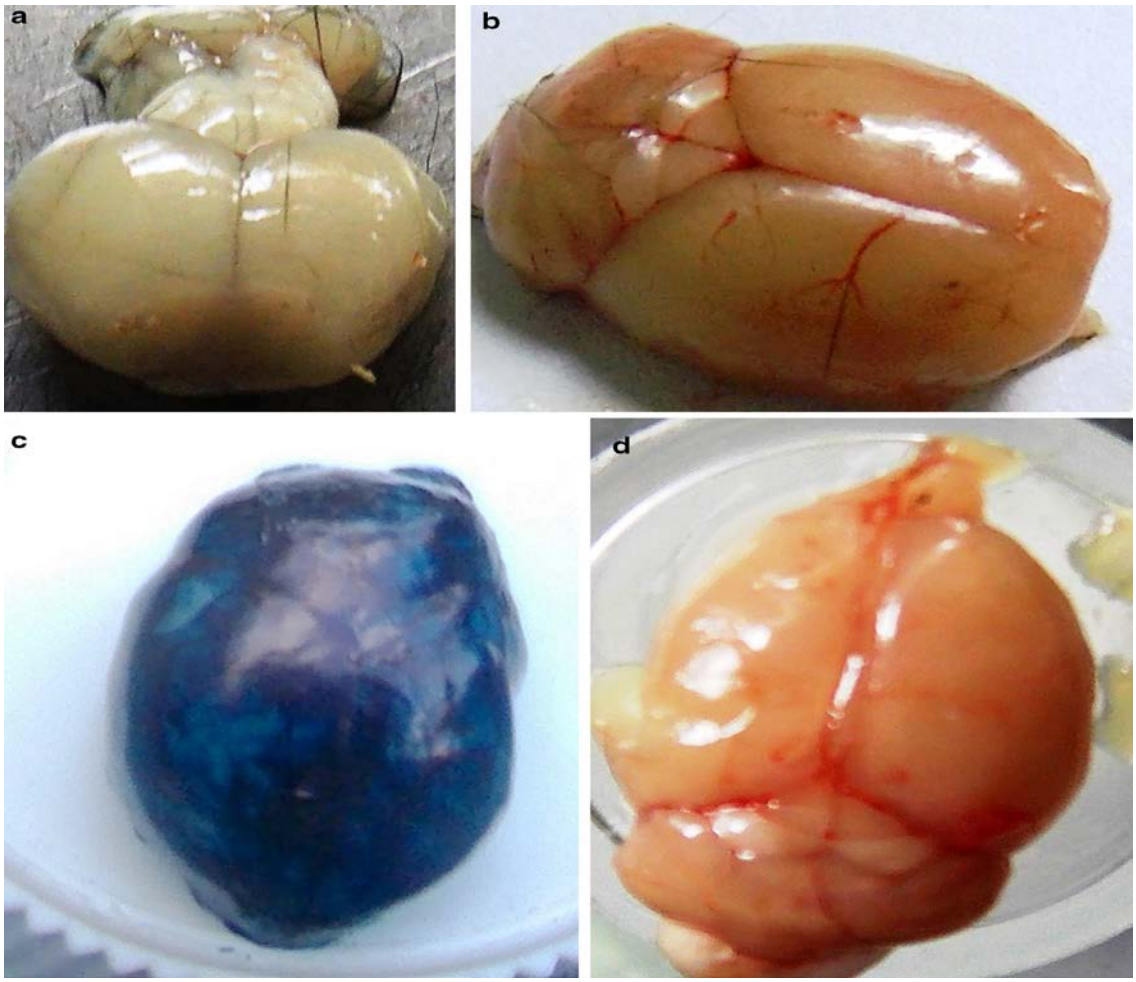



**Figure 4.2.1 Evaluation of blood-brain barrier integrity with Evans Blue dye. The susceptible mice were first infected with *P. berghei* ANKA and the dye administered iv on day 8 pi after the infected mice had exhibited clear signs of ECM. The mice were euthanized one hour later and the brain images documented. This protocol was repeated on mice that had been treated with the trioxaquine on 60 days post-treatment**

*Evaluation of blood-brain barrier integrity with Evans Blue dye. The susceptible mice were first infected with *P. berghei* ANKA and the dye administered iv on day 8 pi after the infected mice had exhibited clear signs of ECM. The mice were euthanized 1 h later

and the brain images documented. The same protocol was repeated on mice that were treated with trioxaquine at 60 days post-treatment. **a** shows non-stained, *P. berghei* ANKA infected C57BL/6 mouse brain on day 8 pi depicting hypoxia; **b** shows non-infected C57BL/6 mouse brain depicting normal blood supply; **c** shows brain staining for C57BL/6 mouse on day 8 pi; **d** depicts brain from the same animal model on day 60 post-treatment exhibiting recovery from ECM, and lack of staining with the Evans blue dye

### 4.3 *In vivo* pharmacokinetic evaluation for the trioxaquine

Plasma samples for the trioxaquine were harvested as outlined in part 3.2.7.1 and analyzed together with the quality controls as recorded in part 3.2.7.2. A summit Pharmacokinetic software tool in excel format through non compartmental model was used to process and analyze the data to give both the Plasma concentration vs time profiles, and Pharmacokinetic parameters as presented in Figure 4.3, and Table 4.3 respectively.

A promising pharmacokinetic profile was exhibited by the trioxaquine following the iv administration of 20mg/kg. An apparent half-life was reported at 3.52hrs with a clearance of 0.01L/hr, and a volume of distribution of 0.06L/kg. The iv area under the curve was reported at 1562.8µg/mL/hr

.

**Table 4.1 *In vivo* pharmacokinetic parameters for the trioxaquine in mice after an iv administration of 20mg/kg of the compound in mice. Blood samples harvested in heparinized syringes within the prescribed sampling time intervals through tail bleeding, plasma separated by centrifugation and analyzed using uv-vis HPLC**

| **Pharmacokinetic parameters** | |
|---|---|
| **Apparent $t_{1/2}$ (h)** | 3.52 |
| **Clearance CL (L/hr)** | 0.0113 |
| **Apparent volume of distribution Vd (L/kg)** | 0.0568 |
| **AUC $_{0-\infty}$ (µg/mL/hr)** | 1562.75712 |
| **$C_{max}$ (µg/mL)** | ND |
| **Bioavailability %** | ND |

*Experimental animals injected with 20mg/kg of the trioxaquine and blood samples withdrawn within a prescribed time period (0-12hrs) post-dosing. Plasma separated through centrifugation and then deprotonized using acetonitrile. 20µl of this supernatant was then subjected for further uv-vis HPLC analysis.

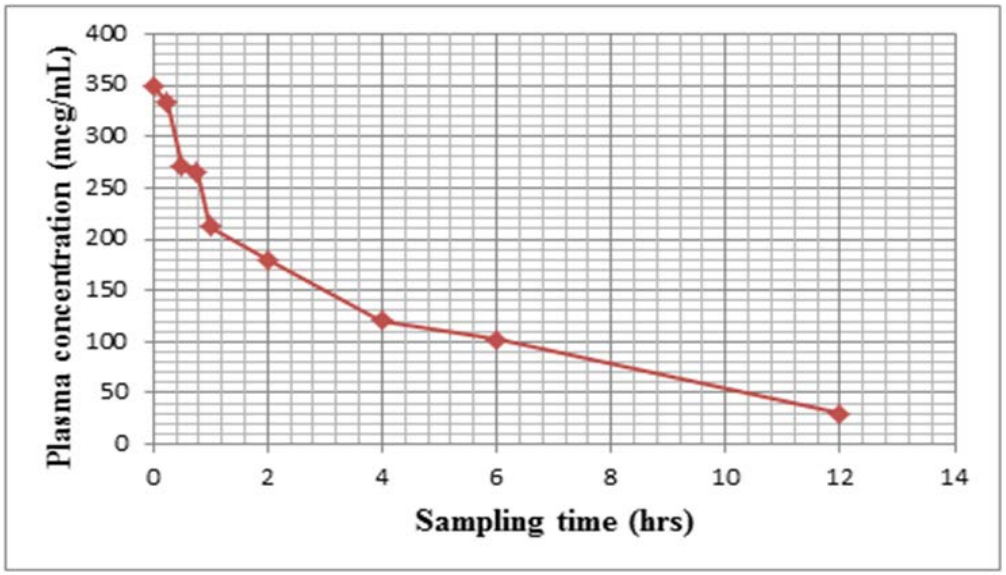



**Figure 4.1 Pharmacokinetic plasma concentration vs time profile for the trioxaquine after an iv administration of 20mg/kg in uninfected C57BL/6 male mice.**

*20mg/kg of the trioxaquine was administered iv through the lateral tail veins to C57BL/6 male mice and plasma samples withdrawn at various time intervals for pharmacokinetic analysis

The plasma concentration time profile (Figure 4:3) depicts two phases during the drug decay process, an initial steeper phase (distribution phase), and a linear (less steep) phase which marks an elimination phase. The result in this case shows the trioxaquine distributes rapidly to the target cells/ tissues in the first 45 minutes of administration but takes relatively longer time to be eliminated as reflected in the reported half-life of 3.52hrs (Table 4:3).

# CHAPTER FIVE

## DISCUSSION

Infection with *Plasmodium falciparum* often has high chances of progressing into CM, a severe form of malaria that contributes to high malaria mortality in sub-Saharan Africa (Idro *et al*., 2010). Children 5 years old and below, expectant women at first trimester, and individuals with naive immunity visiting malaria endemic areas are at increased risk of infection (Reyburn, 2010).

*Plasmodium falciparum*, the most fatal among the *Plasmodia* species has shown a rising trend of evolution and emergence of resistance to the currently used drugs, even to the WHO gold standard antimalarial, the artemisinin combination therapy (ACT) (Reyburn, 2010; Dondorp *et al*., 2009). Also, very few antimalarial drugs are in clinical development pipeline. Optimizing on use of the existing drugs as well as use of rational drug development strategies such as covalent bitherapy could lead to enhanced useful therapeutic lives of existing therapies as well as add new therapies into the existing antimalarial drug repertoire (Muregi and Ishih, 2010; Benoit-Vical *et al*., 2007). In covalent bitherapy, just as in combination therapy, the goal is to either delay or circumvent development of resistance. In trioxaquines, the fast acting precursor (ART pharmacophore) provides rapid clearance of the bulk of the parasite load while the quinoline moiety clears the remnant parasite that survives the effect of the former until complete clearance is achieved (Benoit-Vical *et al*., 2007). This strategy has potential to improve the therapeutic effectiveness as well as delaying or circumventing the emergence of resistance to both individual precursors of the hybrid drug besides

overcoming the challenge of long drug development pipeline of co-formulated ACT drugs (Grellepois *et al*., 2005).

The trioxaquine exhibited a higher efficacy compared to the individual precursors alone. Based on the curative test using established infection, the trioxaquine though exhibiting activities comparable to artesunate, proved to be more effective than both the parenteral artesunate, quinine, and 4,7-dichloroquine. The trioxaquine manifested a rapid parasite clearance of greater than 80% within the 24 hrs post-treatment and no parasite was observable under microscope at the 48 hr post-treatment for both dosages of 12.5mg/kg and 25mg/kg.

Long term monitoring of animals treated with the trioxaquine at both 12.5mg/kg and 25mg/kg showed no recrudescence with the animals surviving beyond 60 days post-treatment. This is a clear indication of complete cure in the treated animals since no recrudescence was observed, an indicator that the trioxaquine could potentially circumvent or delay development of drug resistance.

Though rapid parasite suppression (84.8 %) was observed in the first 24 hrs post-treatment with artesunate and no parasite was observable under microscope 48 hrs post-treatment, recrudescence was observed in this treatment group with all the animals succumbing to the infection by day 16 post-treatment. Even though the quinoline moiety on the other hand did not show any significant parasite reduction in the first 24h post-treatment, its contribution in the trioxaquine was manifested by lack of recrudescence in the trioxaquine treated animals even beyond 60 days post-treatment.

The quinolone pharmacophore (4,7-dichloroquinoline) is believed to promote the dual drug accumulation in the food vacuole, allowing the artemisinin-based partner (artesunate) to have a longer half-life (Kelly *et al*., 2007), a possible explanation for the observed improved treatment outcome in trioxaquine treatment groups in comparison to the artesunate treated group. During treatment with 12.5mg/kg of the trioxaquine, one mouse was lost on day 4 post-treatment. However, it was deemed that the death was not due to drug toxicity since even mice which received 25mg/kg of the trioxaquine had 100% survival. CM leads to a multi-organ dysfunction involving the liver, kidney, and brain with the extent of the organs damage varying from individual to individual. Damage to these major organs are cited as the major reason behind CM deaths in infected individuals even after chemotherapeutic intervention (Nacer *et al*., 2014). This reason could support the loss of one animal in the 12.5mg/kg trioxaquine treatment group even after parasitaemia monitoring had shown complete clearance. Our results are in agreement with other previous related studies using the same experimental animals that noted manifestation of neurological symptoms of CM between day 6-9 pi (Ghosh *et al*., 2011) and death in untreated animals by day 10 pi (Hayano *et al*., 2003). Recrudescence was also reported in artesunate treated animals even at higher doses of 32mg/kg and 64mg/kg with all the treated animals succumbing to the infection and quinine being effective only at higher doses > 120mg/kg that are not well tolerated by the experimental animals (Clemmer *et al*., 2011).

However, albeit in blood stage infection, it was previously reported that trioxaquines were capable of inhibiting parasite in mice and also affording curative effect in both the 4-day suppressive test and in established infection studies (Benoit-Vical *et al*., 2007)

Two components have been implicated to be participating in the development of cerebral malaria namely, the parasite related factors and the host immune factors. Rupture of the hepatocytes leads to accumulation of the infected RBCs (iRBCs) within the brain microvasculature as the parasite invades the blood stream (Carvalho, 2010). On the blood side of the blood-brain barrier (BBB), *P. falciparum* infected RBCs (*Pf*RBCs) cytoadhere to the brain endothelium by binding of *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1), a specific cell-surface ligand expressed by iRBCs (Berendt *et al*., 1994). The sequestration of iRBCs triggers activation of the endothelial cells (ECs) (Rénia *et al*., 2012) which in turn lead to inflammatory responses where there is release of pro inflammatory cytokines tumor necrosis factor-α (TNF– α) (Haldar *et al*., 2007). This cytokine is known to up-regulate endothelial receptor cells resulting to up-regulation of intracellular adhesion molecules 1 (ICAM–1) and vascular cellular adhesion molecules–1 (VCAM–1) (Wassmer *et al*., 2011). All these factors would eventually lead to numerous downstream vascular effects such as increased vasoconstriction, reduced cerebral blood flow to several sites in the brain, vascular obstruction, oxygen starvation, and even disruption of the integrity of the BBB (Coltel *et al*., 2004). The observed whitening of the susceptible strain brains could therefore be attributed to the reduced or the complete blockage of cerebral blood flow to several sites in the brain, a condition termed as hypoxia.

The BBB is comprised of specialized endothelial cells (ECs) that function as a selective permeable membrane to control nutrients and ion transport into the brain and to bar unwanted molecules or compounds into the brain (Abbott *et al*., 2006; Luissint *et al*., 2012). The ECs are sealed together by the presence of tight junctions. However, loss of tight junctions has been observed in areas of iRBCs sequestration leading to creation of openings or gaps within the BBB (Brown *et al*., 2001).

Such gaps or openings could function as a gateway for the entry of toxic molecules or compounds into the brain, thus a pointer to the dysfunctioning BBB which is a major pathological event associated with CM (Coltel *et al*., 2004; Medana and Turner, 2006). This was greatly demonstrated in this work through staining of the susceptible mice strain brains with the 2% Evans Blue dye, an indicator of the impaired BBB integrity in the infected mice. Our data are similar to other previously related studies that confirmed impairment of the integrity of BBB in susceptible animal models and subsequent brain staining with Evans Blue dye (Baptista *et al*., 2010; Morrell *et al*., 2011; Herbas *et al*., 2010; Tamura *et al*., 2011).

Good safety profile was exhibited as the drug was well tolerated in mice even at high dose of 2000mg/kg administered in acute oral toxicity test, with 67% survival. The remarkable mice survival indicates that the exact $LD_{50}$ could be within 2000mg/kg. However, it should be noted that this is a dosage 40 times the curative dose of 25mg/kg. Considering the possible $LD_{50}/ED_{50}$ ratio, a possible TI value > 400 reflects a wider margin of safety for the trioxaquine. The same safety profile was also manifested in *in*

*vitro* cytotoxicity studies (table 4.1.1) where the selectivity index (SI) of the trioxaquine was determined by comparing the $IC_{50s}$ of Hep2 cell line and that of the CQ-resistant parasite (W2) for the trioxaquine (Wamakima *et al*., 2015). The high SI value obtained (>2762) indicates that the high antiplasmodial activity for the trioxaquine observed during the *in vitro* antiplasmodial evaluation was due to its activity and not due to its cytotoxicity (Wamakima *et al*., 2015).

A similar class of trioxaquine was reported to have antiplasmodial activity against all the erythrocytic forms of the parasite including the gametocytes with TI value range of 23 to 100 considered safe (Benoit-Vical *et al*., 2007). The high safety profile exhibited both *in vivo* and *in vitro* by the trioxaquine would therefore be attributed to the fact that one of the precursors for the trioxaquine (artesunate) is already indicated for clinical use in management of malaria.

For a complete cure of any infection to be achieved, adequate blood concentrations of the drugs used in the treatment is crucial (Nishant *et al*., 2011). It is therefore a prerequisite that these drugs reach plasma concentration (Cp) within the therapeutic window thereby achieving efficacy without toxicity (Smith *et al*., 1996). Accurate measurements of plasma concentrations will ensure optimal dosing and also assist in differentiating clinical treatment failure resulting due to inadequate dosing from failure due to drug-resistant pathogens (Nishant *et al*., 2011).

Inappropriate pharmacokinetics (low bioavailability, short elimination half-life, toxicity) has been cited as a major cause perpetuating the ever rising cost and time to

bring new chemotherapies into clinical use (Grabowski *et al*., 2002; Palmer, 2003). These observations therefore highlight the importance of defining pharmacokinetic profile of potential drug candidates, a key step in drug discovery and development for it will speed up the conversion of hits into lead compounds with key goal of focusing on such compounds for further development (Palmer, 2003; Keiser e*t al*., 2009).

With recent reports of tolerance cum development of resistance to most of the chemotherapeutics in clinical practice increasing among the parasite population, the therapeutic blood or plasma concentration ranges would automatically change thus more information needed to improve on the dosage regimens (Dondorp *et al*., 2009; Noedl *et al*., 2010). Recommendation of dosages without clear data on the plasma levels especially in key patient sub-populations, (children and pregnant women) has often resulted in the recommended doses being too low (Ward *et al*., 2007). This is among the factors that have aggravated the problem of resistance observed in individuals with higher pathogen densities and minimal plasma drug levels (Ward *et al*., 2007). Extrapolation of dosage regimens indicated for mild disease conditions to individuals with chronic infections or dosage regimens recommended for adults to children or pregnant women has also been blamed for treatment failures and development of resistance, for example, sulfadoxine–pyrimethamine (SP) is believed to have been partially rendered useless because of its systematic under-dosing in children (Terlouw *et al*., 2003).

A good and superior pharmacokinetic profile was reported by the trioxaquine as compared to reported data on pharmacokinetic profiles of standard antimalarials such as artesunate and dihydroartemisinin following the iv administration of 20mg/kg of the trioxaquine. The determined apparent half-life for the trioxaquine was much better at approximately 3.52 hours as compared to the reported half-lives for both artesunate and dihydroartemisinin at the range of 15 to 40 minutes in both mice and human respectively (Xing *et al*., 2007; Batty *et al*., 1998; 2008), but with lower values of 0.06L/kg for volume of distribution and 0.01L/min/kg for clearance. The observed improvement in the apparent half-life for the trioxaquine would be attributed to the fact that the 4,7-dichloroquinoline is believed to promote the dual drug accumulation (Kelly *et al*., 2007). The trioxaquine was able to sustain a high plasma concentration for up to two half-lives above the minimum inhibitory concentration (MIC) that reduced the parasitaemia load by 50% ($ED_{50}$). The sharp decrease in plasma concentration reflected in the initial phase of plasma concentration time profile shows a rapid distribution of the trioxaquine from plasma volume into the target tissues/ cells which in this case is believed to be the red blood cells and this phase marks the distribution phase for the trioxaquine. However, at the elimination phase the drop in plasma concentration is mainly dependent on the elimination of the drug from the body and the less steep the slope is, the longer it takes to eliminate such a drug from the body resulting to a longer half-life. This last phase is believed to occur after establishment in distribution equilibrium between the target cells and the plasma.

The reported low value for volume of distribution in iv administration in this case would be attributed to the fact that this class of chemotherapeutic targets the blood stage of malarial parasite within the red blood cells therefore the drug won't distribute into deeper body organs and tissues unlike in bacterial infections which are mostly localized deep within body organs and tissues resulting to larger volume of distribution for antibiotics as the drugs must distribute to the site of infection deep within the body (Andes and Craig, 2002; Toutain *et al*., 2002; Frimodt-Møller, 2002).

Half-life being dependent on Volume of distribution and Clearance, the reported values of 0.01L/hr for CL and 0.06L/kg for Vd resulted to a longer half-life of 3.52hrs as it would take longer duration to remove the drug from the body.

These results are in agreement with the findings of Lombard and his team who while evaluating the iv pharmacokinetic profile of the same class of compound (artemisinin-quinoline hybrid drug) also reported similar results including a longer half-life of approximately 4hrs with low values of 0.4L/kg and 0.06L/min/kg for volume of distribution and clearance respectively (Lombard *et al*., 2013).

The remarkable efficacy, good safety, and pharmacokinetic profile of the trioxaquine observed imply that if the findings could be replicated in clinical studies, then the drug has potential for use in management of CM.

# CHAPTER SIX

# CONCLUSION AND RECOMMENDATIONS

## 6.1 CONCLUSION

The remarkable *in vivo* results for the trioxaquine obtained by the established infection test as well as the post-treatment survival data clearly indicate that the trioxaquine is feasible drug for management of CM. Improvement in the outcome exhibited by the experimental mice treated by the trioxaquine clearly validates the viability of covalent bitherapy concept as a proffered source of the urgently needed antimalarial agents for management of CM, and that this polypharmacology approach can be applied to prevent or delay the development of resistant against the partner drugs just like in ACTs.

Weaker toxicity profile observed in the *in vivo* studies by the oral route for the trioxaquine is very promising as the results reflect a wider margin of safety with a therapeutic index (TI) value of > 400 considering the $LD_{50}/ED_{50}$ ratio. Overall therefore, curative effect together with the good safety, and pharmacokinetic profiles observed with the trioxaquine clearly demonstrates its potential as a drug candidate for management of CM, especially in a time of shrinking antimalarial armamentarium for management of CM.

## 6.2 RECOMMENDATIONS

Although findings of *in vivo* studies of the trioxaquine against *P. berghei* ANKA in CM rodent model using the established infection test were promising, inherent interspecies variation has been reported between human malaria parasites with that of their rodent counterparts. It is therefore necessary to conduct a preclinical study on non-human

primates models of human cerebral malaria infected with human malaria parasite *P. falciparum* to find out whether or not the efficacy exhibited by the trioxaquine in mice can be replicated in the higher non-human primates.

An in-depth clinical pharmacokinetics profile of the trioxaquine should be conducted to yield more information on plasma protein binding, kinetic solubility, metabolite identification, permeability, and metabolic stability and even the distribution pattern as these are some of the key factors that influence the pharmacokinetic-pharmacodynamics parameters of not only therapeutic agents already in clinical use but the results of these studies are crucial also in placing priority to lead compounds.

Further studies should also be carried out on the compound to assess its long term safety and suitability for clinical application by conducting histopathology studies on major organs of the body as the liver, kidney, and heart to confirm effects of this compound on these organs.

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# APPENDICES

## APPENDIX 1 Animal Care and Use Committee approval letter

KENYA MEDICAL RESEARCH INSTITUTE

Centre for Virus Research, P.O. Box 54628 - 00200 NAIROBI - Kenya
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**KEMRI/ACUC/ 01.04.16** **18Th April 2016**

Christopher O. Onyango
Jomo Kenyatta University of Agriculture and Technology
C/o CBRD

Onyango,

**RE: Animal use approval for CBRD 144 - 'Evaluation of the efficacy and pharmacokinetic profile of a novel trioxaquine in management of cerebral malaria in mice "protocol**

The KEMRI ACUC committee acknowledges the resubmission of the above mentioned protocol. It has been confirmed that all the issues raised earlier have been addressed appropriately.

The committee grants you the approval to use C57BL/6 and Swiss albino mice in your study but recommends that you proceed with the study only after obtaining the final approval from the KEMRI scientific and ethics review unit (SERU).

Approval is granted for a period of one year starting from when the SERU approval will be obtained. If you still intend to use laboratory animals after the initial approval, you are required to submit an application for continuing approval to the ACUC 1 month prior to the expiry of the initial SERU approval.

The committee expects you to adhere to all the animal handling procedures as described in the protocol.

The committee wishes you all the best in your work.

Yours sincerely,

Dr. Konongoi Limbaso
Chairperson KEMRI ACUC

KENYA MEDICAL RESEARCH INSTITUTE
★ 18 APR 2016 ★
ANIMAL CARE AND USE COMMITTEE
Signature: .....

**APPENDIX 2 Scientific and Ethical Review Unit approval letter**

# KENYA MEDICAL RESEARCH INSTITUTE

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**KEMRI/RES/7/3/1** **May 05, 2016**

**TO:** **CHRISTOPHER O. ONYANGO,**
**PRINCIPAL INVESTIGATOR**

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

RECEIVED
16 MAY 2016

Dear Sir,

**RE:** **PROTOCOL NO. KEMRI/SERU/CBRD/0144/3195 (*RESUBMITTED3 INITIAL SUBMISSION*): EVALUATION OF THE EFFICACY AND PHARMACOKINETICS PROFILE OF A NOVEL TRIOXAQUINE IN MANAGEMENT OF CELEBRAL MALARIA IN MICE-(*VERSION 1.2 DATED 12TH FEBRUARY, 2016*)**

Reference is made to your letter dated 3rd May, 2016. KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on 4th May, 2016.

This is to inform you that the Committee notes that the issues raised during the 240th of the KEMRI/Ethics Review Committee (ERC) held on 19th January, 2016 have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **5th May, 2016** for a period of one year. Please note that authorization to conduct this study will automatically expire on **May 04, 2017.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **March 23, 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.

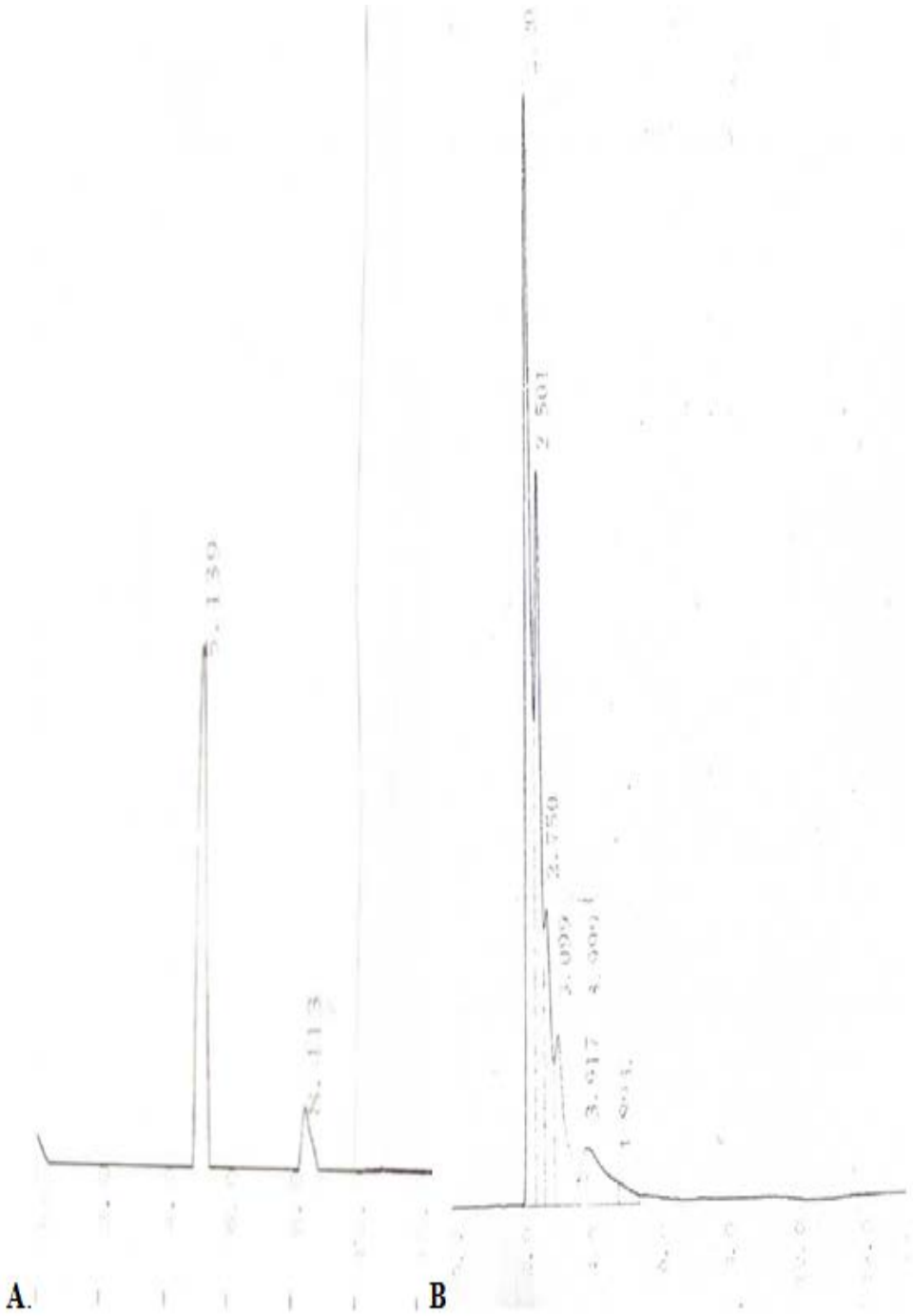
You may embark on the study.

Yours faithfully,

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

## APPENDIX 3 HPLC (Shimadzu) Instrumentation

**APPENDIX 4 HPLC Spectrum for the drugs in HPLC analysis**




**A:** HPLC spectrum for the parent trioxaquine spiked in mice plasma**.** **B:** HPLC spectrum for the trioxaquine in mice plasma.


**C:** HPLC spectrum for the standard Dihydroartemisinin. **D:** HPLC spectrum for 4,7-dichloroquinoline in mice plasma