EVALUATION OF ANTIPLASMODIAL ACTIVITIES OF CHLOROQUINE ENCAPSULATED HEPARIN-FUNCTIONALIZED SOLID LIPID NANOPARTICLES

MUGA JOSEPH OWINO

PG/MSC/00028/2012

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR AWARD OF THE DEGREE OF MASTER OF SCIENCE IN CELL AND MOLECULAR BIOLOGY

DEPARTMENT OF ZOOLOGY

SCHOOL OF PHYSICAL AND BIOLOGICAL SCIENCES

MASENO UNIVERSITY

DECLARATION

I declare that this thesis is my own original work and has not been presented in any institution of higher learning for the award of a degree.

Muga Joseph Owino

Reg. No.:PG/MSC/00028/2012

Signature..... Date.....

This thesis has been submitted for examination with the approval of the University and the following student supervisors:

Prof. Walter G.Z.O. Jura, Ph.D.

Department of Zoology, School of Physical and Biological Sciences,

Maseno University

Sign..... Date.....

Dr. Jeremiah W. Gathirwa, Ph.D.

Kenya Medical Research Institute,

Center for Traditional Medicine and Drug Research

Nairobi, Kenya.

Sign…..…….. Date…….....

Dr. Matshawandile Tukulula, Ph.D.

Council for Scientific and Industrial Research (CSR), Polymers and Composites (P&C)

Material Sciences and Manufacturing

South Africa.

Sign…….……..... Date….....

ACKNOWLEDGEMENTS

I am grateful to my university supervisor, Professor Walter G.Z.O. Jura for his guidance from the development of the study proposal to the completion of the thesis writing. My gratitude also goes to Dr. Jeremiah Gathirwa, my project supervisor, for his support and guidance in the development of the proposal and more specifically with the bioassay studies at KEMRI. Appreciation also goes to Dr. Matshawandile Tukulula for his timely advice, help, support and supervision throughout my study, especially during the nanoformulation phase of the study in Pretoria, South Africa. Special thanks also go out to the staff of the Polymers and Composites division at the Council of Science and Industrial Research (CSIR), specifically Ms Debra Marima, for their assistance with the characterization of the nanoformulations. I am much indebted to the staff at the Center for Traditional Medicine Development Research (CTMDR) at KEMRI in Nairobi, for their assistance with both the *In vitro* and *In vivo* bioassays. Special thanks to Mr. Njokah Muturi and Mr. Japheth Ngetich for their assistance at the centre without which this work will not have been possible. I also appreciate Mr. Lucas Ogutu of animal house at KEMRI for preparing the mice used in the *in vivo* study. I wish to express my heartfelt appreciation to my wife Angeline and our sons, Michael and Patrick, for their love and understanding when I was away undertaking my study. Thank you all.

DEDICATION

To my parents, Francis Muga and Florah Muga, who used chloroquine to treat me and my siblings for malaria when we were young and for their prayers.

ABSTRACT

Chloroquine was the mainstay of antimalarial chemotherapy because of its safety profile, effectiveness and the relatively cheap cost. However, the emergence of chloroquine-resistant *Plasmodium falciparum* parasites has rendered this drug ineffective in most regions where malaria is endemic. Most antimalarial drugs that act through blood - stage - specific mechanism are no longer effective due to the rapid emergence of drug resistance while the costs of development of new drugs continue to rise. Ligand-mediated nanoparticulate drug delivery system can mitigate chloroquine resistance mechanisms and provide a potential cure for malaria. The objective of this study was to evaluate antiplasmodial activity of chloroquine-encapsulated heparin functionalized, solid lipid nanoparticles (SLNs). Specifically, the study determined the physicochemical properties, antiplasmodial activity of the nanoformulated SLNs against *P. falciparum in vitro* and *P.berghei* in mice. The modified double-emulsion solvent evaporation technique was used to prepare the nanoparticles. The semi-automated micro-dilution technique was adapted in assessing the *in vitro* antiplasmodial activity by use of tritium labeled hypoxanthine. The uptake of tritium labeled hypoxanthine was measured by Beta counter and recorded inform of counts per minute (CPM). The CPMs were then computed to give drug concentration capable of inhibiting 50% of the *P. falciparum* (IC_{50}), as a function of antiplasmodial efficacy. The 4-day suppressive test with modification was used to evaluate antiplasmodial activity against *P. berghei* in mice. The mean percentage parasitaemia between treatments were compared with respect to the negative control. Kruskal Wallis test was used to analyze the statistical significance at $p< 0.05$ among the treatment groups. The mean particle size, zeta potential, drug loading, and encapsulation efficiency of the SLN-CQ were 444.5 ± 6.9 nm, 9.41 ± 0.376 mV, 25%, and 90%, while for the SLN- HEP-CQ they were 374.6 ± 7.6,-4.06 ± 0.091, 21 and 78% respectively. SLN-CQ, SLN-HEP and SLN-HEP-CQ showed moderate antiplasmodial activities against chloroquine sensitive (D6) strain of *P. falciparum in vitro.* The nanoformulated drugs, SLN- HEP, SLN-CQ and SLN- HEP-CQ, showed significant antiplasmodial activity against CQ sensitive *P. berghei in vivo* in comparison to the negative control group. The *in vivo* results showed that this nanoformulated drugs worked as effective as the standard chloroquine drug and hence can be developed further to improve their efficacy.

TABLE OF CONTENTS

OPERATIONAL TERMS

Diploid: Cells containing a full set of chromosomes.

Gametes: Reproductive elements, male and female.

Gametocytes: Precursors of the sexual forms of the malaria parasite, which release either male or female gametes within the stomach of the mosquito.

Bioavailability: The rate and extent to which an active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action.

(ACT): Is an antimalarial combination therapy with an Artemisinin derivative as one component of the combination given for at least 3 days.

Haploid: Cells containing a half set of chromosomes.

Merozoite: The form of the malaria parasite that invades red blood cells

Resistance: Ability of a parasite strain to survive and or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject.

Treatment failure: Treatment failure is defined as an inability to clear malarial parasitaemia or resolve clinical symptoms despite administration of an antimalarial medicine. Treatment failure is not, however, always due to drug resistance, and many factors can contribute, mainly by reducing drug concentrations. These factors include incorrect dosage, poor patient

compliance in respect of either dose or duration of treatment, poor drug quality and drug interactions.

Therapeutic index: The margin between the doses resulting in a therapeutic efficacy and toxicity to other organ systems.

***In vitro* assays**: *In vitro* assays are used to monitor drug resistance by measuring the intrinsic sensitivity of *Plasmodium falciparum* to antimalarial drugs. Parasites are exposed to a precise concentration of drug and observed for inhibition of maturation into schizonts. They offer a more objective approach to determining parasite resistance, as they are based on direct contact between parasites and incremental drug concentrations.

Ad libitum: The "free-feeding" weight of an animal, as opposed, for example, to the weight after a restricted diet or pair feeding.

Malaria control: Reducing the malaria disease burden to a level at which it is no longer a public health problem.

Malaria elimination: The interruption of local mosquito-borne malaria transmission; reduction to zero of the incidence of infection caused by human malaria parasites in a defined geographical area as a result of deliberate efforts; continued measures to prevent re-establishment of transmission are required.

Malaria eradication: Permanent reduction to zero of the worldwide incidence of infection caused by a particular malaria parasite species. Intervention measures are no longer needed once eradication has been achieved.

Morbidity: The number of cases of a given disease in relation to the overall population (quoted e.g. as cases per 100000 or per million per annum).

Mortality rate: The number of deaths expressed as a proportion of the number of individuals who have contracted the disease in a particular outbreak, or over a specified period of time.

Nanoencapsulation: Entrapment of active principle in the matrix of a nanoparticulate drug carrier system.

Nanotechnology: Nanotechnology is the study, design, creation, synthesis, manipulation, and application of functional materials, devices, and systems through control of matter at the nanometer scale (1–1000 (nanometers), and the exploitation of novel phenomena and properties of matter at that scale.

Nanomedicine: It is the application of nanotechnology in medicine and includes the delivery and targeting of pharmaceutical, therapeutic, and diagnostic agents using nanoparticles.

Liposomes: They are microscopic vesicles consisting of one or more concentric spheres of lipid bilayers separated by aqueous or buffer compartments.

Solid Lipid Nanoparticles: They are solid, submicronic particulate carriers with a size ranging from 1 to 1000 nm and consisting of highly purified triglycerides, complex glyceride mixtures or even waxes as matrix.

Drug delivery: Mechanism of directing the active principle to the required site.

Free drug:	Drug not incorporated in a drug carrier system e.g. a tablet.
Formulation:	Combination of active ingredients with the appropriate excipients
Excipients:	Inactive ingredients employed for the purpose of dilution, protection, stability, controlled release, taste, fillers, coloring, disintegration, etc

ABBREVIATIONS /ACRONYMS

ACT: Artemisinin-based combination therapy

CQ: Chloroquine diphosphate

CQS: Chloroquine sensitive

CQR: Chloroquine Resistant

DHFR: Dihydrofolate reductase

DHPS: Dihydropteroate synthase

FP: Fe (II) - protoporphyrin IX

GDP: Gross Domestic Product

HEP: Heparin

***p*RBC**: Red blood cell infected with malaria parasite

PfCRT: Gene encoding *P. falciparum* chloroquine resistance transporter

Pfdhfr: Gene encoding *P. falciparum* dihydrofolate reductase

Pfdhps: Gene encoding *P. falciparum* dihydropteroate synthase

Pfmdr1: Gene encoding *P. falciparum* multidrug resistance 1 protein

PfEMP: *Plasmodium falciparum* Erythrocyte Membrane Protein 1

PVA: Polyvinyl alcohol (surfactant)

SLN: Solid Lipid Nanoparticle

MOH: Kenya Ministry of Health

UNICEF: United Nations Children's Fund

***u*RBCs**: Red blood cell not infected with malaria parasite

WHO: World Health Organization

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

CHAPTER ONE: BACKGROUND

1.0 Introduction

Malaria represents a significant global burden and poses a great challenge to drug discovery and delivery efforts due to its intracellular nature and disseminated locations (WHO, 2016). Worldwide, prevalence of the disease is estimated to be 212 million clinical cases in 2015. Mortality due to malaria stood at 429,000 deaths worldwide in 2015 with 90 % of these occurring in sub-Saharan Africa. Children under the age of 5 years accounted for 303,000 (70%) of the deaths (WHO, 2016). It is the leading cause of morbidity and mortality in Kenya where it affects more than 70% of the population (NMCP, 2016; PMI, 2016; KHIS, 2016). Five *Plasmodium* species known to infect humans and cause malaria include *P. falciparum, P. vivax*, *P. malariae*, *P. ovale and P. knowlesi*, with *P. falciparum* being the most virulent form of human malaria (Kobayashi *et al.,* 2013).The high morbidity and mortality (99% of deaths) in sub-Saharan Africa were caused by *P. falciparum*, the most virulent of the five malarial parasites that infect humans and the widespread presence of *Anopheles gambiae* in this region. *Anopheles gambiae* is the most effective malaria vector and also the most difficult to control (Santos-Magalhaes and Mosqueira, 2009). Much of this morbidity and mortality could be avoided if drugs available to patients were efficacious, of high quality, and used correctly (Gaurvika *et al.*, 2012).

Chloroquine (CQ), a 4-aminoquinoline drug was discovered in 1934 by Hans Andersag and co-workers at the Bayer laboratories and introduced into clinical practice in 1947 for the prophylactic treatment of malaria (Salituna *et al.,* 2004). CQ acts by accumulating inside the digestive food vacuole of the malaria parasite by a process that involves ion trapping following protonation, specific transport, and/or binding to a receptor like heme (Shujatullah *et al.,* 2012). CQ was used for several decades as the antimalarial drug of choice due to its safety, high efficacy and low cost (Hyde, 2007). Due to the widespread prevalence of CQ resistant (CQR)

parasites, CQ was removed from the front-line antimalarial chemotherapy in the late 1990s (WHO, 2001). Other drugs for treatment of malaria include amodiaquine, lumefantrine, mefloquine and sulfadoxine – pyrimethamine, artemisinin and artemisinin based combination therapies (Wilson *et al.,* 2013). Most of these drugs are no longer effective due to the emergence of chloroquine resistant *P.falciparum* and the multi-drug resistant *P.falciparum.* More recent drugs have emerged to treat CQR malaria, but there remain concerns about costs and safety (Burgess *et al.,* 2007). A report of resistance to the artemisinin-based combination therapy (ACT) in South East Asia has further complicated malaria control efforts. Artemisinins are the most potent compounds in the antimalarial drug arsenal and no suitable replacements are expected any time soon (Gaurvika *et al.,* 2012).

Current drugs suffer from non-specific targeting and resistance leading to non-target toxicity (Ekambaram *et al*., 2012). There is therefore an urgent need to reformulate the available antimalarials in an innovative way that will circumvent the resistance mechanisms and deliver the drug to the target site. The use of nanotechnology, specifically drug delivery, has emerged as the best way of delivering the drugs in the site of target while mitigating resistance (Burgess *et al*., 2006). Studies have shown that encapsulation of CQ in liposomes coated with anti-pRBC increased drug efficacy against both CQS and CQR strains of *P.berghei* in mice (Urban and Fernandez-Bisquets 2014~~)~~ hence the choice of solid lipid nanoparticles that is less toxic (Puri *et al.,* 2009) is a more viable option for delivering CQ to the site of action. Using this nanotechnology, solid lipid particles of sizes less than 500nm can be prepared in which CQ is entrapped and heparin salt added for targeting plasmodium falciparum erythrocyte membrane protein on the surface of malaria parasite infected red blood cells (Omwoyo *et al*., 2014). These nanosized particles acquire new physicochemical properties due to their small size, surface structure and high surface area. These properties include size, shape, zeta potential and

polydispersity index and allow nanoparticulate systems to overcome current limitations of conventional formulation (Martinho *et al.*, 2011).

Problem Statement

Malarial transmission control is one of the main goals of global efforts focusing on increased access to insecticide nets, diagnostic tests, vaccines to prevent disease, and novel therapies. ACTs currently constitute the last effective and most tolerable treatment for multi-drug resistant *P. falciparum* in uncomplicated malaria due to its rapid action. However, the efforts to eliminate malaria have been frustrated due to resistance of mosquito vectors to insecticides, resistance of the parasites to drugs, socioeconomic problems, and the lack of effective vaccines. Malaria parasites have developed resistance to cheaper drugs, including chloroquine, while current drugs used to treat CQR malaria are expensive and have adverse side-effects. The main problems in malaria treatment are the non-specific targeting of antimalarials to intracellular parasites and multiple drug resistance. Antimalarial drug delivery currently rely on the administration of compounds with little or no specificity for the main target cell, the *Plasmodium*-infected red blood cell (*pRBC*), and thus require high doses which in most cases lead to off-target toxicity (Ekambaram *et al.*,2012). This study therefore sought to reformulate CQ using solid lipid nanoparticles and functionalize the nanoparticles with heparin for targeting parasitisized red blood cells (*p*RBCs). Urban and Fernandez-Bisquets (2014) recently showed that heparin nanoparticles specifically target *p*RBC over noninfected red blood cells (*u*RBCs). Studies have shown that encapsulation of CQ in liposomes coated with anti-*p*RBC increased drug efficacy against both CQS and CQR strains of *P.berghei* in mice (Urban and Fernandez-Bisquets 2014). Thus, in this study, solid lipid nanoparticles that were less toxic, more stable, had controlled drug release and could be functionalized for targeting was preferred because of its superior properties. The aim was to attempt to design more stable and robust heparin functionalized SLNs, so to circumvent the short comings of Urban and Fernandez-Bisquets (2014) nanoparticles.

1.1 Justification and significance of the Study

Increased efforts in antimalarial drug discovery are urgently needed to develop safe and affordable new drugs to counter the spread of malaria parasites that are resistant to existing agents (Omwoyo *et al*., 2014). However, in the absence of new drugs, drug reformulation, more specifically drug delivery, seems more attractive (Burgess *et al*., 2006). Thus, properly designed drug delivery systems such as heparin-functionalized solid lipid nanoparticle can deliver the drug cargo, in the site of interest, that are low and safe to non-target tissues while at the same time are locally high and lethal enough to the malaria parasite (Abhijit *et al.,* 2007). Since a study by showed that Santos-Magalhaes and Mosqueira, 2010, CQ-encapsulated liposomes completely cured mice infected with CQ-resistant *P.berghei,* the nanoformulated drugs were tested against CQR (W2) and CQS (D6) strains of *P. falciparum* as well. The cost of producing the nanoformulated CQ will be affordable since chloroquine is readily available while the other excipients used in the formulation are also readily available at relatively cheap prices (Omwoyo *et al.,* 2014). The results obtained will inform future research in use of nanotechnology in the design of cost effective target specific novel drug delivery systems for CQ and other antimalarials. The synergistic antiplasmodial effect of SLN and heparin was an indication that further optimization might give rise to a potential drug candidate for treatment of malaria. These findings will also be used by researchers as baseline for studies on targeted nanomedicines for malaria and other parasitic diseases afflicting majority of the world's poor countries. It will also inform policy makers, governments, donors and pharmaceutical firms who allocate resources for research and development in the control and eradication of malaria. This study therefore has the potential to restore the use of old and toxic drugs by modifying their bio-distribution and reducing toxicity. These findings could lead to a formulation that is cheap, efficacious, non-toxic, target specific and patient friendly antimalarial treatment. This will benefit malaria endemic regions of

sub-Saharan Africa, south East Asia and the Mediterranean where malaria exhorts a huge socioeconomic burden.

1.2 Objectives

1.2.1 Broad objective

To synthesize and evaluate antiplasmodial activity of CQ encapsulated in heparin-functionalized solid lipid nanoparticles (SLNs) *in vitro* and *in vivo*.

1.2.2 Specific objectives

1. To prepare and determine physicochemical properties of the CQ encapsulated heparin functionalized SLNs comprising of particle size, zeta potential, polydispersity index, drug loading and encapsulation efficiency.
2. To determine antiplasmodial activity of the CQ encapsulated heparin functionalized SLNs against *P. falciparum in vitro*.
3. To determine *in vivo* antiplasmodial activity of the CQ encapsulated heparin functionalized SLNs against *P. berghei* in mice.

1.3 Research Questions

1. What are the physicochemical properties of CQ encapsulated in heparin functionalized solid lipid nanoparticles that are essential for antimalarial drug delivery?
2. What effects do CQ encapsulated in heparin functionalized solid lipid nanoparticles have against *P. falciparum in vitro*?

3. What effects do CQ encapsulated in heparin functionalized solid lipid nanoparticles have against *P.berghei* in mice?

CHAPTER TWO: LITERATURE REVIEW

2.0 Malaria

Malaria is an acute febrile illness and is the world's most important tropical parasitic disease, and kills more people than any other communicable disease except tuberculosis (WHO, 2016). In many developing countries and in Africa especially, malaria exacts an enormous toll on lives and medical costs of patients, and in days of labour lost (WHO, 2014). Malaria is a vector-borne infection caused by a parasite of the genus *Plasmodium* from the phylum Apicomplexa. Plasmodia are obligate intracellular parasites that are able to infect and replicate within the erythrocytes after a clinically silent replication phase in the liver. Five species (*P. falciparum, P. malariae, P. ovale, P. vivax and P. knowlesi*) are responsible for natural infection in human beings (Spinello *et al.,* 2012).

2.1 Malaria Transmission

Malaria infection results from injection of sporozoites by an infected female anopheles mosquito during a blood meal (Baer *et al.,* 2007). These sporozoites migrate through the skin into the circulation and then to the liver where they invade hepatocytes and develop into merozoites within a few minutes (Urban and Fernandez-Bisquets, 2014). Merozoites exit liver cells and re-enter the bloodstream where they invade red blood cells, undergo asexual replication and release new merozoites from the infected red blood cells causing fever and illness. The process by which parasitic merozoites invade erythrocytes involves the following steps: attachment, apical reorientation, junction formation, and formation of a protective parasitophorous vacuole where the parasite proliferates (Kirk, 2001). The parasite does not elicit immune response during the intraerythrocytic stage because the RBC lacks the mechanism to process and present antigens to the immune system (Kobayashi *et al.,* 2013). During maturation in the RBCs, the parasite modifies the plasma membrane of the host RBC to

meet its needs for growth and multiplication and also cause adhesion of the parasitized RBC (*p*RBC) to the vascular endothelium. These modifications are essential for membrane transport and evasion of the host immune system. As the intraerythrocytic cycle proceeds from the ring to trophozoite and schizonts phases, different proteins of plasmodium origin are expressed on the surface of the RBCs giving rise to formation of positively charged knobs. The knobs appear at trophozoite stage and increase to maximum number at the schizonts stage. *P.falciparum* erythrocyte membrane protein 1(*PfEMP1*) is located in these knobs (Urban and Fernandez-Bisquets, 2014). The asexual blood stages are responsible for all the symptoms and pathologies of malaria hence resident parasites inside *p*RBCs are the main target for chemotherapy. During the initial burst, some merozoites develop into gametocytes that circulate in the bloodstream where they are ingested by a mosquito during a blood meal and mature into gametes (sexual stage of life cycle). Upon fertilization, female gametes develop into ookinetes that burrow through the mosquito's midgut wall and form oocysts on the exterior surface. Thousands of active sporozoites develop inside the oocysts and eventually bursts, releasing sporozoites into the body cavity that travel to the mosquito's salivary glands (National Institute of Health, 2014). Both the human- and mosquito-based life cycle of the malaria parasite are summarized in Figure 1.

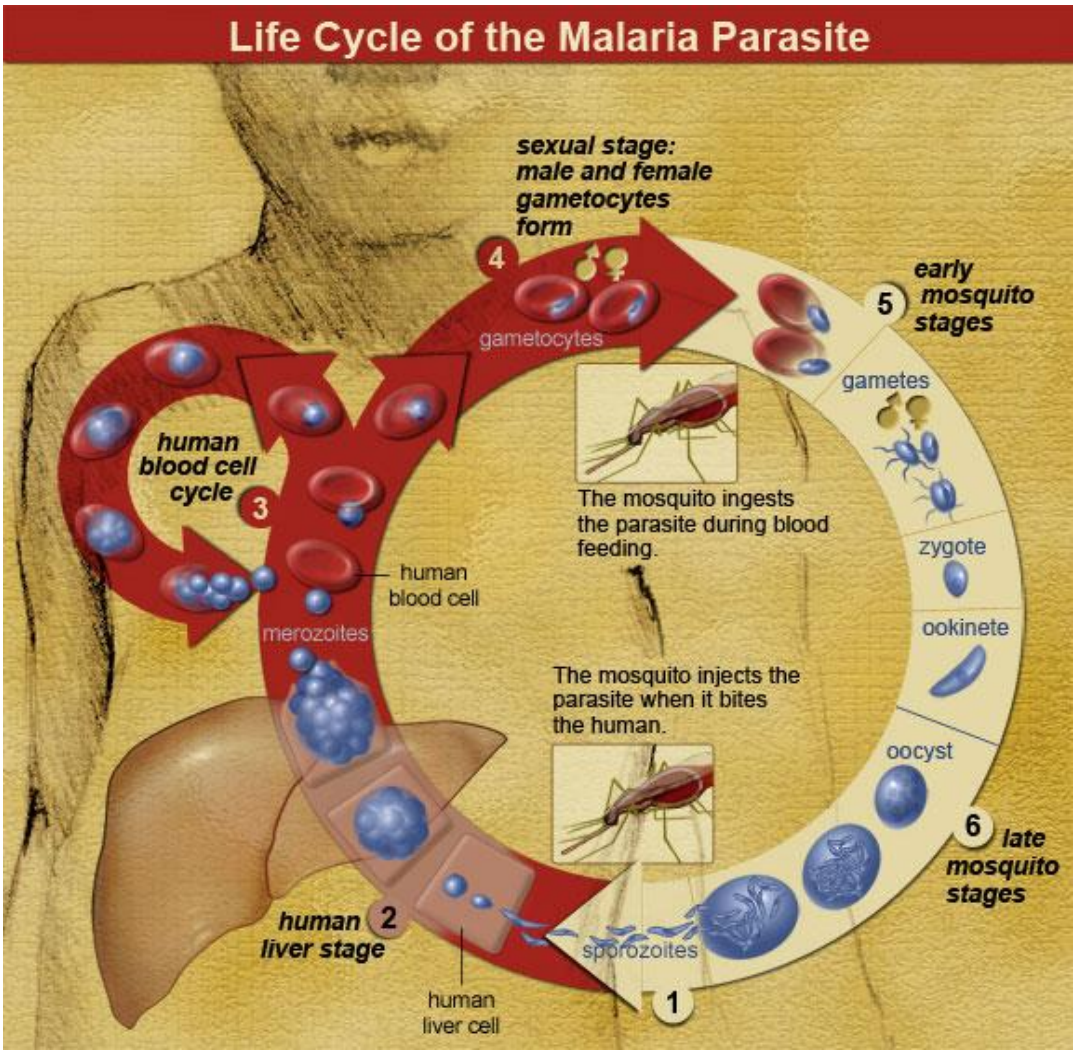


Figure 1 Life cycle of malaria parasites (Adapted from http:www.niaid.nih.gov/topics/malaria/pages/lifecycle/aspx, 2014 accessed on 01/04/2014)

2.2 Epidemiology

It is estimated that 3.2 billion people are at risk of malaria worldwide with sub Saharan Africa accounting for 88% of all malaria cases and 90% of the deaths of in 2015. (KHIS, 2015) *P.falciparum* is responsible for most malaria deaths, especially in Africa. *P.vivax* is the most geographically widespread species but produces less severe symptoms. *P.vivax* is now found mostly in the tropics, especially throughout Asia. In Central and

Western Africa, *P. vivax* infections are rare because of the high prevalence of the RBCs Duffy negative phenotype in the population, which interferes with merozoites entry into the RBCs. *P.ovale* infections are also rare but may be encountered in sub-Saharan Africa and in Asia. *P. malariae infections are* spread in sub-Saharan Africa, in Southeast Asia, in Indonesia, in many islands in Western Pacific and in areas of the Amazon Basin of South America. Its distribution overlaps with that of *P. falciparum* (Autino *et al.*, 2012).

2.3 Drugs for Treatment of Malaria

The most effective treatment for *P.falciparum* infection is the use of artemisinins in combination with other antimalarials which decreases resistance to any single drug component. The peroxide bridge of artemisinin reacts with iron from heme to form reactive carbon centered radicals which kill the parasite by attacking either the sarco/endoplasmic reticulum Ca^{2+-}ATPase or the parasite's DNA (Butler *et al.*, 2010). Other antimalarials used in combination with artemisinin include: amodiaquine, lumefantrine, mefloquine and sulfadoxine-pyrimethamine (WHO, 2010). Table 1 summarizes the different classes of antimalarials used for the treatment and prophylaxis of malaria. Quinine is an alkaloid; it accumulates in the food vacuoles of *Plasmodium* species, especially *P.falciparum*. Quinine acts by inhibiting the hemozoin biocrystallization, thus facilitating an aggregation of cytotoxic heme. Amodiaquine is a 4-aminoquinolone anti-malarial drug similar in structure and mechanism of action to CQ. Amodiaquine is now available in a combined formulation with Artesunate and is among the ACTs recommended by the World Health Organization (WHO, 2010). The principal available antimalarial drugs and their chemical families are shown in Table 1.

Table 1 Principal available Antimalarial Drugs (WHO Global report on Antimalarial drug efficacy and drug resistance: 2000 - 2010).

Chemical family	Drugs
4-Aminoquinolines	Chloroquine, amodiaquine, piperaquine
Amino-alcohols	Quinine, quinidine, mefloquine, halofantrine, Lumefantrine
Sulfonamides and sulfones	Sulfadoxine, sulfalene, dapsone
Biguanides	Proguanil, chlorproguanil
Diaminopyrimidine	Pyrimethamine
8-Aminoquinoline	Primaquine
Sesquiterpene lactones	Artemisinin, arteether, artemether, artesunate, Dihydroartemisinin
Naphthoquinone	Atovaquone
Antibiotics	Azythromycin, clindamycin, doxycycline, tetracycline

Pyrimethamine is used in the treatment of uncomplicated malaria and acts by inhibiting dihydrofolate reductase in the parasite thus preventing the biosynthesis of purines and pyrimidines, and thereby halting the processes of DNA replication, cell division and reproduction. Proguanil is a synthetic derivative of pyrimidine and acts in a similar manner to pyrimidine. Sulfadoxine and sulfamethoxypyridazine are specific inhibitors of the enzyme dihydropteroate synthetase in the tetrahydrofolate synthesis pathway of malaria parasites. Primaquine is a highly active 8-aminoquinolone that is used in treating all types of malaria infection. It is the only known drug to cure both relapsing malaria infections and acute cases.

Artemisinin has a very rapid action and the vast majority of acute patients treated show significant improvement within 1–3 days of receiving treatment. Its derivatives include Artemether, Artesunate and Dihydroartemisinin. Doxycycline is one of the more prevalent antimalarial drugs prescribed, due to its relative effectiveness and cheapness (Whitty *et al.*, 2008). The steady decline in effectiveness of existing mono-therapies with no clear

alternative, most malaria-endemic countries in Africa and Asia have adopted ACTs as antimalarial drug policy (Whitty *et al.,* 2008). According to WHO guidelines 2010, ACTs should be used in preference to amodiaquine plus sulfadoxine-pyrimethamine for the treatment of uncomplicated *P.falciparum* malaria (WHO, 2010). Emergence of artemisinin resistance has been confirmed in Cambodia, Myanmar, Thailand, and Viet Nam, the well-known hotspots of multidrug resistance to *P.falciparum* (Dondorp *et al.,* 2011; Fairhurst *et al.,* 2012; WHO, 2014). Similar to the spread of resistance to chloroquine and other antimalarial medicines in the past, there is a possibility that artemisinin resistance will spread or develop independently around the world. This raises a serious concern on the long-term efficacy of artemisinin-based combination therapies, as these combination therapies currently constitute the last effective and most tolerable treatment for multidrug-resistant *P.falciparum* (Na-Bangchang and Karbwang, 2013). Resistance has occurred as a consequence of poor treatment practices, inadequate patient adherence to prescribed antimalarial regimens, and the widespread availability of oral artemisinin-based monotherapies and substandard forms of the drug (WHO, 2013).

2.4 Use of Chloroquine for Treatment of Malaria

Chloroquine is one of the longest-serving of the synthetic antimalarials that appeared over the course of the last century (Talisuna *et al*., 2004). The main advantages of CQ therapy are the fast action in blood parasite stages, low toxicity, good bioavailability from oral dosage form and high volume of distribution in the body and its low cost (Santos-Magalhaes and Mosqueira, 2009). CQ was introduced in 1934 and became the drug of choice for malaria treatment in 1947 (Salituna *et al*., 2004). CQ is a white, bitter, crystalline powder that exists in two polymorphic forms, one melting between 193°C and 195°C, and the other between 210°C and 215°C. CQ is soluble in water at acidic pH, insoluble in most organic solvents (e.g. alcohol, methanol, ether and chloroform (http://www.inchem.org, accessed on 03/04/2014). Each tablet contains 500 mg of Chloroquine diphosphate

USP, equivalent to 300 mg Chloroquine base. Chemically, CQ is known as 7-chloro-4-[[4-(diethyl amino)-1-methylbutyl] amino] quinoline phosphate (1:2) and has the following structural formula (Figure 2):

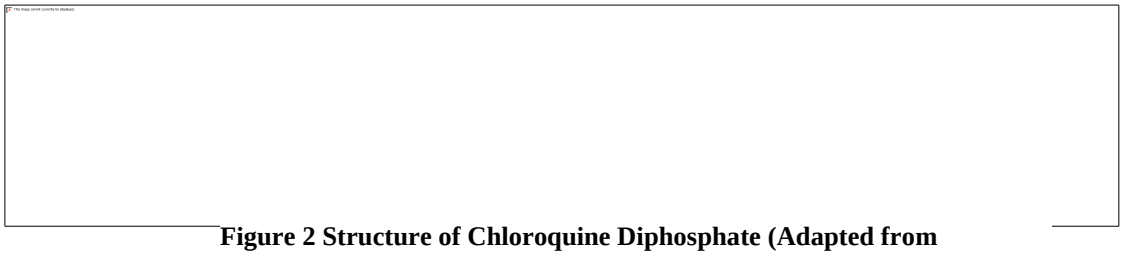


Figure 2 Structure of Chloroquine Diphosphate (Adapted from en.wikipedia/wiki/Chloroquine, accessed on 03/04/2014).

2.5 Mode of Action of Chloroquine

Effective treatment of malaria takes advantage of the differences in metabolism between the malaria parasite and its host cells (Urban and Fernandez-Busquets, 2014). In its erythrocyte stage, *P.falciparum* invades the red blood cells forming the acidic digestive vacuole. Hemoglobin endocytosed from the host erythrocyte cytosol is digested in the acidic digestive vacuole (DV) of the intraerythrocytic malaria parasite to liberate toxic monomeric heme (Chinappi *et al.*, 2010). The parasite detoxifies heme by converting it to hemozoin (malaria pigments) (Chinappi *et al.*, 2010).In the presence of CQ, heme is not converted to hemozoin leading to its accumulation that kills the parasite (Lehane and Kirk, 2008). CQ is a diprotic weak base and, at physiological pH (~7.4), can be found in its un-protonated (CQ), mono-protonated (CQ^{+}) and di-protonated (CQ^{++}) forms (Chinappi *et al.*, 2010). The unprotonated CQ is the only membrane permeable form of the molecule and it freely diffuses into the erythrocyte up to the digestive vacuole (Lehane and Kirk, 2008). In this compartment, chloroquine molecules become protonated and, since membranes are not permeable to charged species, the drug accumulates into the acidic digestive vacuole where it binds to haematin, a toxic byproduct of the hemoglobin proteolysis preventing its incorporation into the hemozoin crystal as illustrated in Figure 3 (Chinappi *et al.*, 2010).

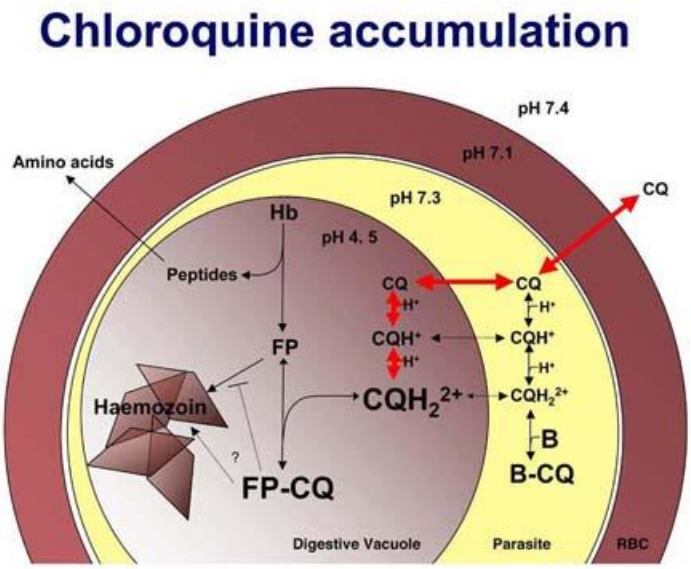


Figure 3 Chloroquine accumulation in digestive vacuole of malaria parasite (Adapted from http://www.phar.cam.ac.uk/research/hladky/images, accessed on 03/04/2014)

2.6 Chloroquine resistance

Drug resistance in malaria is the ability of the parasite strains to survive and/or multiply despite administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject (Erah *et al.,* 2003).Resistance of malaria parasites can be attributed to overuse of antimalarial drugs for prophylaxis, inadequate or incomplete therapeutic treatments of active infections, a high level of parasite adaptability at the genetic and metabolic levels, and a massive proliferation rate that permits selected populations to emerge relatively rapidly (Hyde, 2007). CQ resistance first emerged in Southeast Asia and South America in the late 1950s, and by the late 1970s, it had made its way to the African continent, where it contributed to increased transmission of malaria and deaths (Laufer *et al.,* 2006). Figure 4 shows distribution of Malaria Endemic Areas and Drug Resistance worldwide**.**

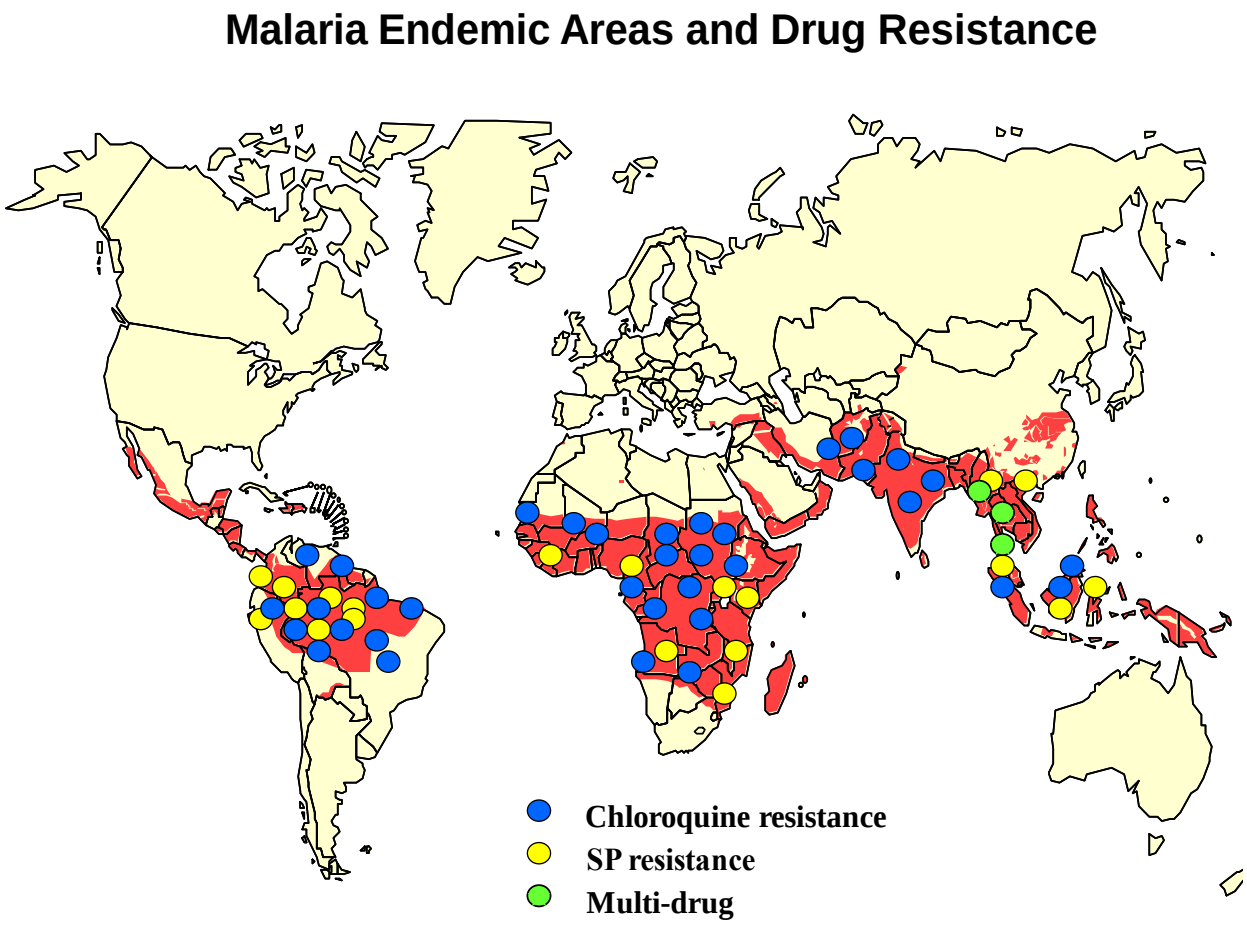


Figure 4 Malaria Endemic Areas and Drug Resistance (adapted from http://www.who.int/pmnch/topics/child/CountdownReportOnly.pdf accessed on 5/04/2014).

Resistance, primarily found in *P. falciparum*, is attributed to mutations on the gene (*PfCRT*) and the gene (*PfMDR1)*, (Wellems *et al.*, 2001; Lewis *et al.*, 2014). The products of the gene expression are two putative protein transporters exhibited by *P. falciparum*; the CQ resistance transporter (CRT) and the *P-glycoprotein* homologue 1 (Pgh1). Mutations in *PfCRT* are believed to confer CQ resistance by reducing the amount of CQ accumulated by the parasite. The bulk of the intra-parasitic CQ is believed to be concentrated within the DV where *PfCRT* is located (Bray *et al.*, 2005). Both CRT and Pgh1 are present on the digestive vacuole membrane of plasmodium (Santos-Magalhaes and Mosqueira, 2009). Resistant isolates have reduced affinity of

Ferriprotoporphyrin (FP)–CQ, (FP–CQ) binding in the digestive vacuole; therefore CQ-resistant isolates have evolved a mechanism whereby the access of CQ to FP is reduced**.** CQ resistance is associated with a decrease in the amount of CQ that accumulates in the digestive vacuole, the site of action for CQ. CQR cells efflux CQ at about 40 times the rate of CQ-sensitive cells (O'Neill *et al.,* 2012). Use of heparin-functionalized solid lipid nanoparticle can bypass the chloroquine transporters and reach the digestive vacuole (Nzekwe *et al.*, 2015).

2.7 Nanotechnology

The application of nanometer (10 to 1000 nm) scale materials (Figure 5) in an innovative way to develop new approaches in drug delivery is called Nanomedicine. Nanosized solid colloidal particles in which the active principles are dissolved, entrapped, and/or to which the active principle is adsorbed or attached are referred to as nanoparticles (Basu *et al.,* 2012). At this scale, materials display different physicochemical properties due to their small size, surface structure and high surface area (Santos-Magalhaes and Mosqueira). These properties allow nanoparticulate systems to overcome current limitations of conventional formulation (free drugs) as they facilitate the intracellular uptake to specific cellular targets (Martinho *et al.,* 2011).

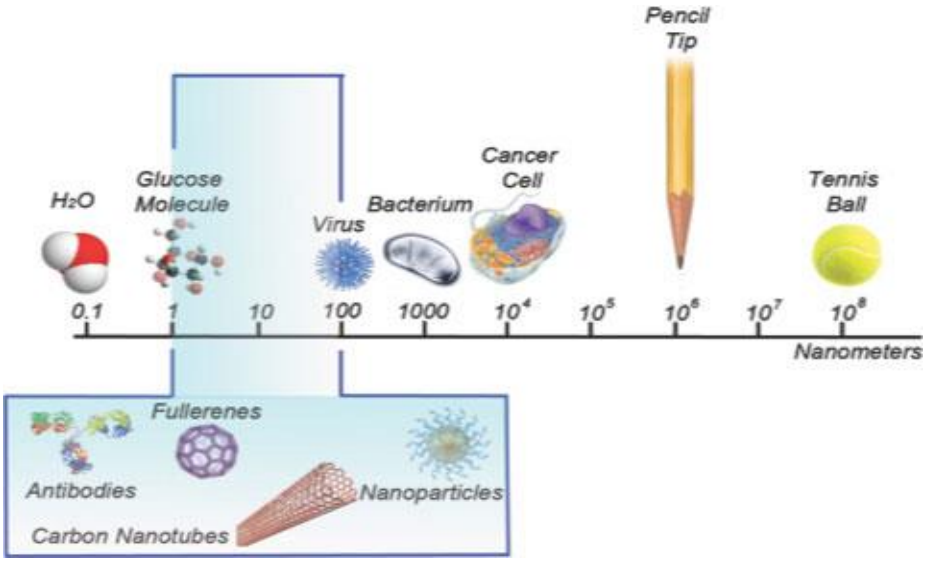


Figure 5 Showing Sizes of antibodies, fullerenes, carbon nanotubes and nanoparticles relative to some common nanoparticles like bacterium, cancer cell, pencil tip and tennis ball (Salamanca - Buentello et al., 2005), source: http://inl.int/what is nanotechnology?

Nanoformulation is the entrapment of chemotherapeutics in nanosized formulations like liposomes and solid lipid nanoparticles among others. A study by Santos-Magalhaes and Mosqueira, 2010, showed that CQ-encapsulated liposomes tested in CQ-resistant *P.berghei* infection in mice cleared all the parasites (Santos-Magalhaes and Mosqueira, 2010). *P.berghei* is the rodent plasmodium parasite that is used in mice model while *P.falciparum* causes infection in humans and is used for in vitro studies. The mode of action of CQ in *P.berghei* and *P. falciparum* is based on inhibition of biocrystallization of hemozoin leading to accumulation toxic heme that kills the parasite (O'Neill *et al.,* 2012).The two differ in the drug delivery system because the carrier system was modeled for *in vivo* system . Thus the CQ-encapsulated liposomes were efficacious to CQ-resistant P.berghei. Also, the mode of action of CQ is the same in liposome and solid lipid nanoparticle but solid lipid nanoparticles provide the possibility of controlled release of drug and targeting, increased drug stability and biocompatibility (Chen *et al.,* 2013). The results indicate that selective homing of CQ to malaria-infected erythrocytes may help to cure the chloroquine resistant malarial infections with reduced ~~dose~~ (Abhijit *et al.,*

2007). Figure 6 shows the pharmaceutical carriers available in the pharmaceutical industry. There are four classes of nanoparticles: Lipid based nanoparticles, polymeric nanoparticles, metal based nanoparticles and biological nanoparticles. Polymeric nanoparticles are structures obtained from synthetic polymers like Polyacrylamide, polyacrylate or natural polymers like albumin, chitosan or gelatin and range in size from 10 t0 100 nm. Metal based nanoparticles are easy to handle with the aid of an external magnetic field. They are of pure metals like cobalt, nickel, manganese and iron or their alloys and oxides (Wilczewska *et al*., 2012). Biological nanoparticles include biological molecules like aptamers, antibodies DNA and RNA that can be used as a nanovector to deliver drugs to site of infection. Lipid-based nanoparticles are the least toxic and are known to increase the solubility of and absorption of the poorly soluble drugs hence suitable for development of drug delivery systems (Puri *et al.*, 2009). Solid lipid, one of the physical forms of lipid, is used to formulate nanoparticles as solid lipid nanoparticles (SLNs). Figure 6 shows common nanocarrier systems in use.

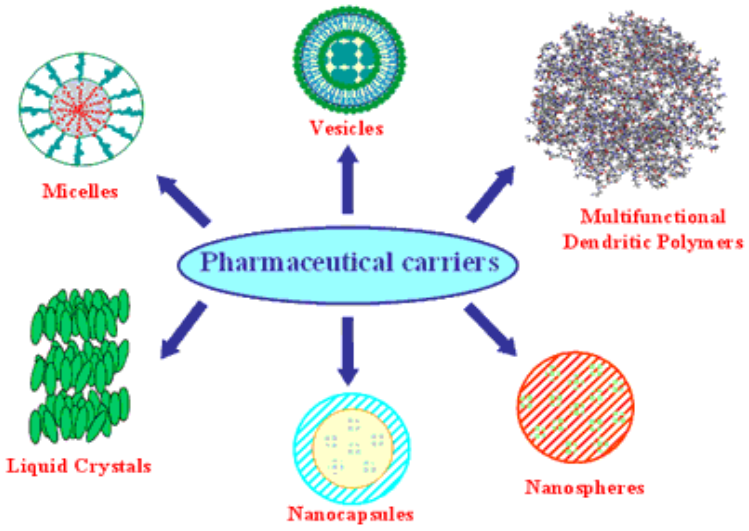


Figure 6 Pharmaceutical carriers (adapted from http://www.azonano.com/oars.asp, accessed on 08/04/ 2014).

Solid lipid nanoparticles (Figure 7) are preferred because they provide the possibility of controlled release of drug and drug targeting, an increase in drug stability, the ability of incorporating both lipophilic and hydrophilic

drugs, and their biocompatibility (Chen *et al.,* 2013). Solid lipid nanoparticles are made up of solid lipids, emulsifier and/or co emulsifier and water (Parhi and Suresh, 2012). The lipids used as a matrix for solid lipid nanoparticle are highly purified triglycerides, fatty acids, steroids or waxes (Abhijit *et al.,* 2007). These lipids melt at temperatures exceeding body temperature (37°C) to retain their solid state at physiological temperatures (Kairemo *et al.*, 2008).

The main drawbacks of conventional malaria chemotherapy are the development of multiple drug resistance and the nonspecific targeting to intracellular parasites, resulting in high dose requirements and subsequent intolerable toxicity (Ekambaram *et al.,* 2012). Nanoformulated drugs show enhanced delivery to or uptake by target cells and reduction in the toxicity of the free drug to non-target organs and hence lead to increase of therapeutic index (De Jong and Paul, 2008). A safe and targeted drug delivery could improve the performance of some classic medicines already on the market including CQ for treatment of malaria (Parveen *et al.,* 2012*).*

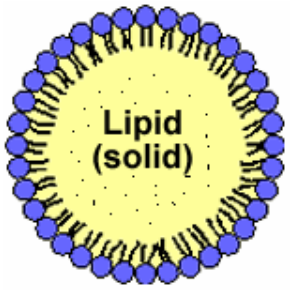


Figure 7 Structure of solid lipid nanoparticle (Ekambaram *et al*., 2012)

SLNs have been used for pulmonary delivery of antimicrobials to treat tuberculosis caused by *Mycobacterium tuberculosis* where drugs such as rifampicin, isoniazide, pyrazinamide were loaded in SLNs systems, and this drug loaded SLNs were then able to decrease the dosing frequency and improve patient compliance (Pandey *et al.,* 2005). Curcumin, an anticancer drug has poor photodegradation and low availability as free drug but showed increased photostability and enhanced anticancer activity against MCF 7 breast cancer cells when

encapsulated in SLNs (Chen *et al.,* 2013). Mitoxantrone-loaded solid lipid nanoparticle local injections were formulated to reduce the toxicity and improve the safety and bioavailability of drug (Lu *et al.,* 2006). Doxorubicin was complexed with soybean-oil-based anionic polymer and dispersed collectively with a lipid in water to form doxorubicin loaded SLNs.This system was able to improve doxorubicin's efficacy and reduce the number of breast cancer cells (Rahul *et al.,* 2011). Therefore, anticancer drugs carried by SLNs, including Mitoxantrone, methotrexate and paclitaxel, may be more effective than free anticancer drugs for breast cancer treatment (Zhuang *et al.,* 2012). Artemether-loaded SLNs offered significant improvement in the antimalarial activity and duration of action compared to conventional injectable formulation. Oral absorption and bioavailability of several drugs were improved after oral administration of the drug-loaded solid lipid nanoparticles (Das and Chaudhury, 2010). A study by Omwoyo *et al*., (2014), demonstrated that nanoformulated primaquine was 20% more effective than conventional primaquine against *P.berghei* in mice. In this formulation, the drug offered protection from enzymatic and chemical hydrolysis during passage through the gastrointestinal tract (Raina *et al.,* 2012).

2.8 Heparin functionalized nanocarriers

Targeting of nanoparticles to the site of *p*RBC can be achieved by conjugating them heparin which interacts selectively with receptors present on the surface of the target cells (Alukda et al., 2011). During the intraerythrocytic cycle, *P.falciparum* erythrocyte membrane protein 1(*PfEMP1*) is expressed at the surface of the *p*RBC and has a binding site for heparin. The circumsporozoite protein in the sporozoites attachment to hepatocytes during the primary stage of liver infection are also targets for heparin, thus heparin can be used to target both liver and erythrocytic stages of the malaria parasites (Fernandez-Bisquets, 2013). A study by Marques *et al,* in 2014 showed that heparin had antimalarial activity by inhibiting invasion of merozoites. Heparin was therefore used both as treatment for malaria and as a targeting ligand but was discontinued due to

its strong anticoagulation action (Urban and Fernandez-Bisquets, 2014). The high specificity of HP binding to *p*RBCs infected with late forms of *P.falciparum* therefore provides the basis for the development of heparin-based SLNs for target delivery of CQ. Marques *et al*. 2014 established that heparin-functionalized liposomes loaded with primaquine increased three-fold the activity of encapsulated drug in *P. falciparum* cultures. The current shortcomings in the treatment and control of malaria, including resistance and side effects to previously efficacious regimens, slow pace of research and development for new drugs, lack of potent vaccine, mosquito resistance to insecticides and high cost of newer antimalarial drugs, nanoformulation remains a viable option. The studies above demonstrate that the potency of previously efficacious drugs can be revived by packaging the drugs in properly designed nanoparticulate system that is targeted for delivery to the site of infection. This antiplasmodial activity could further increase since SLNs have shown better physicochemical properties than liposomal systems hence the choice of SLN over liposomal systems.

CHAPTER THREE: MATERIALS AND METHODS

3.0 Study Location

Encapsulation of CQ and characterization for size, zeta potential, polydispersity index, drug loading and encapsulation efficiency was performed at the Council for Scientific and Industrial Research (CSIR)'s Polymers and Composite Laboratories in Pretoria, South Africa. *In vitro* and *in vivo* studies were performed at the Center for Traditional Drug Development Research, Kenya Medical and Research Institute in Nairobi, Kenya.

3.1 Study Design

Experimental design (post-test only design) with experimental group and two control groups (positive and negative).Treatment consisted of four set ups; drug with heparin, drug with no heparin, no drug and standardised CQ drug. In the in vitro assay, the drugs were tested against CQS (D6) and CQR (W2) strains of P.falciparum with standardized CQ as positive control and no drug as negative control. For the in vivo assay, the drugs were tested against CQS *P.berghei* where standardized CQ was used as positive control and water (placebo) used as negative control.

3.2 Encapsulation of Chloroquine

3.2.1 Method of preparation of CQ-loaded SLNs

Stearic acid was the matrix; polyvinyl alcohol (PVA) and pluronic were used as surfactants to stabilize the emulsion, while chitosan (CS) increased circulation time in the intestines. CS also introduced positive charges on SLNs that enabled functionalization with the negatively charged heparin for targeting of *p*RBCs. Particle size reduction was enhanced with D-lactose monohydrate while sulfanoyl was the antifoaming agent. All materials and reagents were purchased from commercial sources and used as received without any

modifications; CQ analytical grade, stearic acid, low viscous chitosan, polyvinyl alcohol (PVA) of molecular weight 13,000–23,000 partially hydrolyzed (87%–89%), D-lactose monohydrate, sulfanoyl, ethyl acetate (EtOAc) and low molecular weight heparin sodium salt (> 180 USP units/mg) were purchased from Sigma Aldrich and Merck (both in South Africa and Kenya). Purification of the solutions of CS, PVA and D-lactose monohydrate was undertaken via membrane filtration. Magnetic stirrer and hot plate (with max rpm of 1000), high speed homogeniser (max rpm of 8000,Silverson L4R; Silverson Machines Limited, Buckinghamshire, UK), bench top Bucchi mini spray dryer (model B-290; BUCHI Labortechnik AG, Flawil, Switzerland), and UV-VIS instrument (Perkin Elma).

The nanoformulated drugs were prepared in three set ups comprising; drug with heparin, no drug and no heparin. The modified double-emulsion solvent evaporation technique was adapted, with slight modification, from the method previously reported by Omwoyo *et al*.,(2014). Briefly, 100 mg of the CQ salt dissolved in 2 mL aqueous PVA (2%, w/v) solution and 10 mL EtOAC containing 100 mg of Stearic acid were homogenised at 6000 rpm, in an ice-bath, for a period of 5minutes to form the first emulsion (w1/o). This emulsion was then transferred into a second aqueous solution (containing 10 mL PVA (2%), 5 mL of 0.3% Chitosan solution (w/v), 5 mL of 5% D-lactose monohydrate solution and 5 mL of 1% heparin solution) and stirred on the hot plate stirrer for 2 minutes after adding 200 µl of sulfanoyl. Thereafter, the mixture was homogenised at 8000 rpm for 5 minutes and the resulting water-in-oil-in water (w1/o/w2) double emulsion directly fed into the Buchi mini spray dryer set to the following parameter: Outlet temperatures (90 °C, Aspirator =100%, pump= 2mL/min and atomizing pressure set at 7 bars. In the case of no drug or no heparin, the same method was followed without the addition of the drug or heparin.

3.2.2 Characterization of the SLNs

Particle size, polydispersity index (PDI) and zeta potential were measured in triplicates by dynamic laser scattering or photon correlation spectroscopy using a Malvern Zetasizer Nano ZS (Malvern instruments, Malvern, UK). Drug-loading (%DL) and encapsulation efficiency (%EE) were measured using a UV-VIS spectroscopy *via* the indirect method.

The %EE and %DL was calculated using the formulas below:

$$\%EE = (\text{drug in precipitate/total added drug}) \times 100 \quad (1)$$

$$\%DL = (\text{drug in precipitate/drug in precipitate + added excipients}) \times 100 \quad (2)$$

Where, "drug in precipitate" = total drug added -free drug after ultra-centrifugation (indirect method) and "added excipients" = lipids + surfactant mixtures + other ingredients used.

3.3 Bioassays

The nanoformulated drug was evaluated *in vitro* and *in vivo* against malarial parasites. For comparison, free drugs counterparts were similarly evaluated. *P. berghei* strain of malaria parasites was used in the *in vivo* experiment. This was the most appropriate rodent malaria parasite that was able to propagate in mice. *P. falciparum* clones namely: the Sierra Leonean (CQ-sensitive) D6; and the Indochinese (CQ-resistant) W2, were used for the *in vitro* study. The parasite clones were obtained from the Malaria Research and Reference Reagent Resource Center (MR4) and cultured at the Kenya Medical Research Institute at the Centre for Traditional Medicine Development Research from where part of this project was implemented.

3.3.1 *In vitro* anti-Plasmodial bioassay

Parasite cultivation was carried out using previously described procedures (Trager & Jensen 1976; Schlichtherle *et al.*, 2000). The culture medium consisted of RPMI 1640 (10.4 g/l) powdered medium (without PABA) and

lactic acid (LA) dissolved in 960 ml of distilled-autoclaved water (DAW) supplemented with 10% human serum, 25 mM (5.94 g/l) HEPES and 25 mM $NaHCO_3$. Human O positive red blood cells served as the parasites host cells. Test samples were prepared by dissolving in 100% DMSO (Sigma Chemical Co, St Louis, MO, USA) and diluted to lower the concentration of DMSO to ≤ 1% to avoid solvent carry over effects. Stock solution (1 µg/ml) of chloroquine base was prepared for use as a reference drug. The semi-automated micro-dilution technique was adapted in assessing *in vitro* anti-plasmodial activity (Desjardins *et al.,* 1979; Le Bras &Deloron 1983).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Drug at the highest concentration											
B												
C												
D												
E												
F												
G												
	Parasitized no drug								Not parasitized no drug			

Figure 8 Layout of 96-well plate pre-coated with test solutions.

96-well flat-bottom micro-culture plates were pre-coated with test solutions in duplicate (Figure 9). The first row A, contained test solutions of the drug at the highest concentration. Serial dilution was carried out under sterile conditions in a laminar flow hood (Bellco Glass Inc., U.S.A) using a Titertek motorized hand diluter (Flow Laboratories, Uxbridge, UK) from the second (B) to the second last well (G) achieving a 64-fold dilution. The last row of wells (H), served as controls. Thus wells 1-8 of row H served as negative controls (parasitized

and no drug) while wells 9-12 of row H served as a background (non parasitized and no drug). Parasite cultures at ≥80% ring-stage, ≥ 4% percentage parasitaemia (%P), and ≥ 3 % growth rate and at a 6% haematocrit were used in the anti-plasmodial assays. Method described by Trager and Jensen, 1976 was used to ensure correct parasitaemia in culture. Growth media was used to adjust this culture prior to introducing into the wells so as to achieve a parasitaemia of 0.4 % and a haematocrit of 1.5% from which 200 µl were dispensed into each well of the drug pre-coated micro-culture plate. Plates containing parasitized and non-parasitized erythrocytes were incubated at 37 °C in a gas mixture (3%CO_2, 5%O_2, and 92%N_2) for 48 hours after which 25 ul of 0.5 mCi (G-3H) hypoxanthine (Amersham International, Burkinghamshire, UK) in culture medium was added to each well followed by further 18 h incubation. At the end of the incubation the assay plates were frozen to lyse the cultures. The parasite DNA was recovered by harvesting the lysate onto glass-fiber filter plates using cell harvester (Figure 10) and the radioactivity (in counts per minute) was counted using a beta counter (Wallac Micro Beta TriLux). The mean values for uptake of 3H-hypoxanthine in parasitized control and non parasitized control erythrocytes were calculated.

Figure 9 Cell Harvester

The drug concentration capable of inhibiting 50% of the *P .falciparum* (IC_{50}) was determined by logarithmic transformation of drug concentration and radioactive counts per minute (cpm) using the formula:

$IC_{50} = antilog\ (log\ X_1 + [(logY_{50} - log\ Y_1) \times (log\ X_2 - log\ X_1)] / (logY_2 - log\ Y_1))$, where Y_{50} is the cpm value midway between parasitisized and non-parasitisized control cultures and X_1, Y_1, X_2, and Y_2 are the concentrations and cpm values for the data points above and below the cpm midpoints (Sixsmith *et al.*,1984).

3.3.2 In vivo antimalarial bioassay

In vivo bioassay protocol was based on a 4-day suppressive test as described by Peters *et al* (1975) with modification where twenty one (21) mice were divided into seven (7) groups of three (3). The parasites were maintained in a donor mouse by inoculation with 250 µl of a 1:1 (v/v) suspension of erythrocytes infected with *P. berghei* in phosphate buffered saline (PBS). On the day of the experiment the host mouse was anaesthetized by chloroform soaked in cotton wool in a desicator.

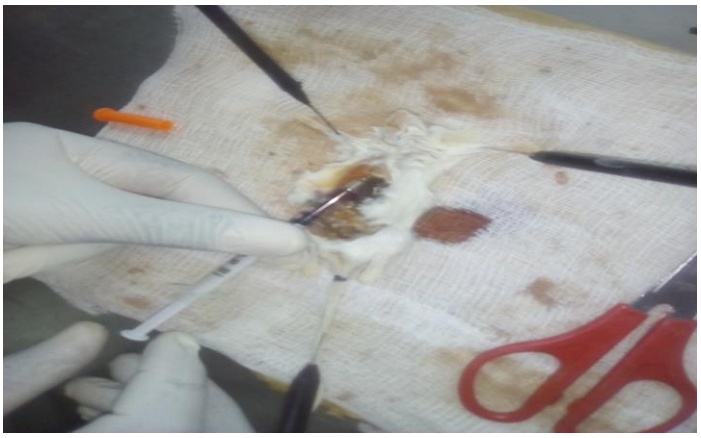


Figure 10 Blood drawn from sacrificed donor mouse

Experimental mice were weighed using the animal scale (Shihana) at the animal house in KEMRI. Whole blood from the donor mouse was drawn by cardiac puncture (Figure 10) into heparinized vacutainer tube containing 0.5 % trisodium citrate and a suspension of *P. berghei* parasitized erythrocytes in PBS was prepared. The test mice were randomly divided into seven groups of three and kept in standard cages at air-conditioned animal room with 22-23 °C. Two groups were negative and positive controls while the rest were treatment groups. Each mouse was injected intraperitoneally (Figure 11) with infected blood containing $2x10^7$ parasitized red blood cells of *P. berghei* ANKA contained in 0.2 ml inoculums on day zero.

The Negative group received Placebo (water), the Positive control group received the control drug (chloroquine) while the Test groups received the test drugs (nanoformulated drugs). The mice receiving test drugs were treated daily from day zero (immediately after infection) up to day 3, with an oral equimolar dose of 20 mg/kg day. Those in the positive control group received chloroquine diphosphate dissolved in PBS to give a volume of 0.2 ml at 10mg/kg day. On day 4 (96 hours post-infection), blood smears were taken by making a thin film from a tail snip of each mouse, fixed in methanol and stained with 10% Giemsa for 20 minutes.

Figure 11 Test mouse injected with P. berghei

The percentage parasitaemia of each mouse in the group was recorded and the mean parasitaemia for the group calculated. Percentage suppression (reductions) was calculated with mean parasitaemia of each group at day 4 using the formula:

$$\%P = 100\ x\ \left(\frac{\text{mean parasitaemia in negative control} - \text{mean parasitaemia in test gp}}{\text{mean parasitaemia in negative control}}\right)$$

3.4 Statistical Analysis and Data Evaluation

The results were recorded as counts per minute (CPMs) per well at each drug concentration and expressed as percentage of the untreated controls. The parasitaemia of each mouse in the group was recorded and the mean parasitaemia for the group calculated at day 4. Kruskal Wallis test was used to analyze the statistical significance at $p< 0.05$ among the treatment groups.

3.5 Ethical Considerations

Prior to commencement, ethical approvals to carry out the study were obtained from the Kenya Medical Research Institute (KEMRI) Ethical Review Committee. Copies of letters of approval are annexed in appendices II and III.

CHAPTER FOUR: RESULTS AND ANALYSIS

4.0 Physicochemical Characterization of Nanoformulated Drugs

The particles sizes of the prepared SLNs ranged from 374.6 to 482.2 nm, while the PDI's and Zeta potential ranged from 0.175 ± 0.021 to 0.346 ± 0.028 nm and -5.73 ± 0.267 to 24.1 ± 0.321 mV, respectively (Table 2). Empty SLN nanoparticles exhibited the largest size whilst SLN loaded with CQ showed a 40 nm reduction in size and those loaded with both CQ and HEP showed a size reduction of 110.6 nm compared to the empty SLN. The empty SLN exhibited a zeta potential of 24.0 ± 0.321mV while the CQ loaded nanoparticles (SLN-CQ) exhibited a reduced positive zeta potential of 9.41 ± 0.376 mV. The drug loading was found to be 25% and 21% for SLN-CQ and SLN-CQ-Hep respectively with encapsulation efficiency of 90% and 78% for SLN-CQ and SLN-CQ-HEP respectively.

Table 2 Summary of physicochemical characteristics of the nanoformulated drugs for size, PDI, zeta potential and encapsulation efficiency

DRUG	Size(nm) (Mean ± std dev.)	PDI (Mean ± std dev.)	Zeta (mV) (Mean± std dev.)	Drug-loading (%)	EE (%)
SLN	482.2 ± 12.0	0.245 ± 0.023	24.0 ± 0.321	-	-
SLN-CQ	444.5 ± 6.9	0.175 ± 0.021	9.41 ± 0.376	25	90
SLN-HEP	379.2 ± 1.2	0.346 ± 0.028	-5.73 ± 0.267	-	-
SLN-HEP-CQ	374.6 ± 7.6	0.272 ± 0.053	-4.06 ± 0.091	21	78

Std dev: standard deviation, PDI: poly dispersity index, Drug: Chloroquine sulphate. SLN and SLN-HEP did not have entrapped CQ thus cell for drug-loading and encapsulation efficiency are blank.

4.1 *In vitro* anti-Plasmodial Bioassay

The anti-plasmodium activity criteria in the *in vitro* assay were defined as high when IC_{50} value was below 10µg/ml, moderate when between 10-50µg/ml and low when between 50-100µg/ml. Drug samples with IC_{50} > 100µg/ml were considered to be inactive (Mbatchi *et al*., 2006).

The *in vitro* antiplasmodial study showed that SLN-CQ, SLN-HEP and SLN-HEP-CQ, possessed moderate antiplasmodial activity against D6 (CQS) strain of *P. falciparum* as shown by IC_{50} values in Table 3. Heparin salt solution and empty SLN displayed IC_{50} values above 100µg/ml, hence no antiplasmodial activity against D6 (CQS) strain of *P.falciparum in vitro.* Against the W2 (CQ-resistant strain of *P.falciparum*), none of the formulations exhibited tested any antiplasmodial activity except CQ which showed low activity of 58.719±2.67ng/ml.

Table 3 Summary of Antiplasmodial Activities of the different nanoformulated drugs

S.No.	Name	W2 IC_{50}s (Mean ± std dev.)	D6 IC_{50}s (Mean ± std dev.)
1	Heparin solution	> 100µg/ml	> 100µg/ml
2	SLN-Nanoparticle	> 100µg/ml	> 100µg/ml
3	SLN-CQ	> 400ng/ml	23.75±3.24ng/ml
4	SLN-HEP	> 100µg/ml	12.98±1.62µg/ml
5	SLN-HEP-CQ	> 400ng/ml	22.97±2.58ng/ml
	CQ	58.719±2.67 ng/ml	5.81±0.18ng/ml

Abbreviations: CQ, chloroquine; SLN, empty solid lipid nanoparticle; SLN-CQ, solid lipid nanoparticle with entrapped CQ; SLN-HEP, solid lipid nanoparticle functionalized with heparin; SLN-HEP-CQ, CQ entrapped in solid lipid nanoparticle functionalized with heparin; D6, Sierra Leonean CQ sensitive *P.falciparum;* W2, Indochinese CQ-resistant *P.falciparum.*

4.2 *In vivo* anti-Plasmodial Bioassay

In this study, the standard 4-day suppressive test was used to evaluate the antimalarial activity of the nanoformulated drugs on CQS *P. berghei* infected mice. Equimolar amounts of the test drugs and the standard

drugs were administered through the oral route. The mean parasitaemia for each treatment group and percent suppression of the drugs are shown in Table 4.

Table 4 Mean parasitaemia and percent suppression of mice in the treatment groups

Test Group	Mean pRBC/1000 RBC	% Suppression
HEPARIN	79.33±8.50	18.21
SLN	40.33±2.89	58.42
SLN-HEP	54.33±5.51	44.00
SLN-CQ	0	100
SLN-HEP-CQ	0	100
Negative Control	97.00±2.65	0
CQ	0	100

Values for parasitaemia are expressed as mean ± standard deviation (PD ± SD) for three mice per group and the % suppression (activity) when compared with the untreated (negative) control.

Table 4 shows the results of mean parasitaemia of mice in the treatment groups ranging from 0 to 80. The mean parasitaemia in SLN-CQ, SLN-HEP-CQ and Chloroquine group was 0 while the mean parasitaemia of untreated control group was 97.00±2.65.

Table 5 Mean rank of Parasitaemia for the nanoformulated drugs

Group Test	Number of observations	Mean rank	χ^2	p-value
HEPARIN	3	17.00		
SLN	3	11.00		
SLN-HEP	3	14.00		
SLN-CQ	3	5.00	19.79	0.003
SLN-HEP-CQ	3	5.00		
Negative Control	3	20.00		
CQ	3	5.00		

A Kruskal-Wallis test showed that there was a significant statistical difference in parasitaemia density across the drug treatments, $\chi^2_{(6)} = 19.79$, p=0.003 , with mean rank parasitaemia density of 17.00 for HEPARIN, 11.00 for SLN, 14.00 for SLN-HEP, 5.00 for each of SLN-CQ, SLN-HEP-CQ and CQ and 20.00 for Negative Control

as shown in Table 5. These results showed that there were statistically significant differences in antiplasmodial activity across the treatment groups compared to the negative control.

SLN-CQ and SLN-HEP-CQ exhibited maximum suppression (100%) while Heparin exhibited the minimum suppression (18.21%) parasitaemia. None of the treatments exhibited higher suppression than the standard drug, CQ. The post hoc test (Table 6) showed that there was statistically significant difference between SLN (p=0.046) and SLN-CQ (p=0.037) and SLN-HEP-CQ to Negative-Control. Similar results were seen when HEPARIN (p=0.037), SLN (p=0.034) and SLN-HEP (p=0.037) were compared to the Standardized drug-CQ. From the findings above, the nanoformulated drugs SLN-CQ and SLN-HEP-CQ were as effective as the standardized drug, CQ.

Table 6 Comparison of parasitemias for the different treatments with Controls

Control Groups	Treatment Group	p-value
Negative-Control	HEPARIN	0.050
	SLN	0.046*
	SLN-HEP	0.050
	SLN-CQ	0.037*
	SLN-HEP-CQ	0.037*
Standardized Drug-CQ	HEPARIN	0.037*
	SLN	0.034*
	SLN-HEP	0.037*
	SLN-CQ	1.000
	SLN-HEP-CQ	1.000

*Significant at $p<0.05$, P-values indicate levels of significance when treatments are compared to controls. SLN, SLN-CQ and SLN-HEP-CQ showed statistically significant difference in activity compared to the negative control. HEPARIN, SLN and SLN-HEP showed statistically significant difference in activity compared to the positive control. SLN-CQ and SLN-HEP-CQ did not show any difference with the positive control.

CHAPTER FIVE: DISCUSSION

Characterization results indicated reduction in size of nanoparticles; this was explained by the fact that the CQ, in the form of the diphosphate salt had an inherent negative charge and exerted a strong electrostatic interaction with the positive outer chitosan layer of the SLNs causing a slight drop in size. Moreover, the addition of the Heparin solution to SLN resulted in a further drop in size and this reduction was also attributed to the electrostatic interaction between the highly negative heparin (Chung *et al*, 2010) and the positively charged chitosan. The combined effect of CQ and heparin therefore explained the smallest size of the SLN-HEP-CQ nanoparticles. Empty SLN exhibited a highly positive Zeta potential (24.0 ± 0.321mV) and this was attributed to the positively charged chitosan. However, the addition of CQ diphosphate resulted in reduction of the positive charge strength of SLN-CQ (9.41 ± 0.376 mV) and was explained by the fact that CQ diphosphate is negatively charged and therefore neutralized some positive charge shown by empty SLNs. A further drop of the surface charge recorded on addition of heparin was attributed to the presence of highly negatively charged heparin. The Zeta potentials were generally low and were indicative of the stability of the nanoemulsion prepared (Honary and Zahir, 2013); It is generally accepted that Zeta potentials from 0 to ±30 mV (low) indicate instability of the nanoemulsion because forces of attraction exceed those of repulsion breaking the dispersion and forming flocculation while those and with values above ±30 mV indicate increased electrostatic repulsive forces between the nanoemulsion droplets and hence resist aggregation (Silva *et al*., 2011; James, 2011). Thus, higher Zeta potential values were therefore associated with stable nanoemulsion (Honary and Zahir, 2013). The Zeta potential values obtained were all negative except for SLN and SLN-CQ resulting to poor uptake by the negatively charged surface of the *p*RBCs. Positively charged particles are taken up better than negative particles due to electrostatic binding to the cell surface (Eleonore Frohlich, 2012). The drug-

loading and encapsulation efficiency were measured via the indirect method on the UV-Vis instrument. Drug loading was found to be 25% and 21% for SLN-CQ and SLN-CQ-HEP respectively. The Encapsulation efficiency was found to be 90% and 78% for SLN-CQ and SLN-CQ-HEP respectively.

The *in vitro* antiplasmodial study showed that SLN-CQ, SLN-HEP and SLN-HEP-CQ, possessed moderate antiplasmodial activity against chloroquine sensitive D6 strain of *P. falciparum* as shown by IC_{50} values between 12ug/ml and 24ng/ml . Interestingly, SLNs containing heparin (SLN-HEP) indicated enhanced antimalarial activity compared to free heparin salt. The observed enhanced activity of heparin in the SLNs could be attributed to slow release profile that is achievable with nanoformulation. Previous studies demonstrated that SLNs formulated through the double emulsion technique used in this study achieved sustained release of the nanoformulated drug over a period of 72 hrs (Omwoyo *et al.,* 2014). This phenomenon is likely to have produced continuous availability of heparin in the cells surroundings thus inhibiting RBC invasion by merozoites (Adams *et al.,* 2006; Carlson *et al.,* 1992). The antiplasmodial activity of the test drugs were however low when compared with that of the standard drug, CQ (IC_{50} 5.81±0.18ng/ml) and this could partly be attributed to the mode of uptake of the nanoformulated drugs. The drugs were designed for uptake in biological environment provided by the *in vivo* assay where the acquired physicochemical characteristics of size, zeta potential and polydispersity index were considered (Martinho *et al.,* 2011, Omwoyo *et al.,* 2014). Against W2 (CQ-resistant strain of *P.falciparum*), none of the formulations exhibited tested any antiplasmodial activity while CQ showed low activity (58.719±2.67ng/ml).

Results for *in vivo* antiplasmodial studies indicate that SLN, SLN-CQ and SLN-HEP-CQ displayed mean percentage suppression of greater than 50 %. Heparin salt alone showed mild antimalarial activity with suppression of 18.21 % and is in agreement with the study by Marques and colleagues in 2014 where they demonstrated that heparin inhibited invasion of RBCs by merozoites. However, nanoformulated heparin (SLN-

HEP), displayed enhanced antimalarial activity compared to free heparin salt. This is due to the fact that nanoformulated heparin had reduced size that was responsible for enhanced uptake (Santos-Magalhaes and Mosqueira, 2009). Free CQ and the nanoformulated drugs (SLN-CQ and SLN-HEP-CQ) completely cleared *P.berghei in vivo* indicating that they are as effective as standard CQ drug. These results showed that there were statistically significant differences in antiplasmodial activity across the treatment groups ($\chi^2_{(6)} = 19.79$) compared to the negative control. The nanoformulations therefore displayed antiplasmodial activity compared to the negative control group. The percentage suppression analysis of the different treatments compared to the negative control and the standard drug showed that SLN-CQ and SLN-HEP-CQ were highly active against *P. berghei* parasites while SLN and SLN-HEP showed percentage suppression of 58.42% and 44% respectively. Heparin had the lowest activity at 18.21%. From the findings above, there was no statistically significant difference in activity between SLN-CQ and SLN-HEP-CQ and the standardized drug, CQ. The nanoformulated CQ was therefore as effective as the standard CQ.

CHAPTER SIX: SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.0 Summary of Study Findings

Prepared CQ loaded nanoparticles were of desired size (below 500 nm), PDI (less than 0.7), zeta potential (moderately positive), % drug loading above 10%, (*Shen et al*., 2017). CQ loaded nanoparticles displayed antiplasmodial activities against CQS but no activity against CQR strains of *P.falciparum.* SLN-HEP displayed moderate antiplasmodial activity against CQS strains of *P.falciparum in vitro*. CQ loaded nanoparticles displayed percentage suppression parasitaemia ranging from 18.21-100% in mice and therefore was as effective as standard CQ drug.

6.1 Conclusions

1. CQ loaded solid lipid nanoparticle were successfully prepared and characterised for size, PDI, zeta potential, drug-loading and encapsulation efficiency.
2. *In vitro* study showed that heparin-functionalized solid lipid nanoparticle, SLN-HEP, SLN-CQ and SLN-HEP-CQ displayed moderate antiplasmodial activity against CQS strains of *P.falciparum in vitro* but no activity against CQR strains of *P.falciparum.* The synergistic effect of heparin and SLN was responsible for the enhanced antiplasmodial activity when heparin is coated onto SLN demonstrated by IC_{50} value of 12.98±1.62µg/ml.
3. From *in vivo* studies, SLN-CQ and SLN-HEP-CQ showed high activity against CQS strains of *P.berghei* contrary to *in vitro* assay results which showed them to have moderate activity.

6.2 Recommendations for application of the study

1. Optimization of previously prepared nanoformulations to achieve and maintain the desired characteristics to improve on their efficacy in the treatment of malaria that will also mitigate on the development of resistant strains of malaria parasites.
2. Nanoformulated drugs should be tested against Chloroquine resistant *P. berghei* in mice and survival times monitored for each treatment group.

6.3 Recommendations for Future Research

1. Physical stability studies of the nanoformulated drug during long storage should be carried out routinely to monitor changes in physicochemical properties with time (i.e. stability studies should be undertaken over a period of time).
2. Further studies should be carried out to exploit the potential of the synergistic antiplasmodial effect of heparin and SLN as drug candidate for treatment of malaria.
3. *In vitro* drug release studies at physiological pH ranges should be carried out to determine drug release rate from the encapsulated nanoparticle.
4. *In vivo* cytotoxicity studies should be carried out to determine safety levels of the nanoformulated drug
5. Further optimization by varying the excipients to achieve nanoformulation with desired characteristics.

REFERENCES

Adams, Y., Freeman, C., Schwartz-Albiez, R., Ferro, V., Parish, C. R., & Andrews, K. T. (2006). Inhibition of *Plasmodium falciparum* Growth In Vitro and Adhesion to Chondroitin-4-Sulfate by the Heparin Sulfate Mimetic PI-88 and Other Sulfated Oligosaccharides. *Antimicrob. Agents Chemother. 50*(8), 2850–2852. http://doi.org/10.1128/AAC.00313-06

Alukda, D., Sturgis, T, and Youan, B.C. (2011). Formulation of Tenofovir-Loaded Functionalized Solid Lipid Nanoparticles intended for HIV Prevention. *J. Pharm. Sci., **100**(8): 3345–3356.*

Antinori, S., Galimberti, L., Milazzo, L, and Corbellino, M. (2012). Biology of human malaria *Plasmodia* including *P.knowlesi*. *Mediterr. J. Hematol. Infect. Dis, **4**(1): e2012013*

Autino, B., Noris, A., Russo, R, and Castelli, F. (2012). Epidemiology of Malaria in Endemic Areas. *Mediterr. J. Hematol. Infect. Dis, **4** (1):e2012060.*
Baer, K., Klotz, C., Kappe, S.H.I., Schnieder, T and Frevert, U. (2007). Release of Hepatic *Plasmodium yoelii* merozoites into the pulmonary microvasculature. *PLoS Pathog., **3**(11):E171*

Barrand, M. A, and Hlardky, S. B. (2014). Ion transport at the blood brain barrier, pH regulation, multidrug-resistance transporters in red blood cells and chloroquine accumulation by malaria parasites (*Plasmodium falciparum*) accessed at www.phar.cam.ac.uk/research/hladky/images,2014.

Barrette, A, and Ringwald, P. (2010).*WHO Global report on Antimalarial drug efficacy and drug resistance pg.15*

Basu, B., Garala K., Bhalodia, R., Joshi, B, and Mehta, K. (2010). Solid lipid nanoparticles: A promising tool for drug delivery system. *J. Pharm. Res., **3**(1): p. 84-9*

Bickii, J., Basco, L.K, and Ringwald, P. (2011).Assessment of Three In Vitro Tests and an In Vivo Test for Chloroquine Resistance in *Plasmodium falciparum* Clinical Isolates. *Antimicrob. Agents Chemother. **55**(7): 3115–3124.*

Birhan, Y.S., Bekhit, A.A. and Hymete, A. *BMC Res Notes (2015) 8:589*

Boyle, M. J., Richards, J. S., Gilson, P. R., Chai, W. & Beeson, J. G. *Interactions with heparin-like molecules during erythrocyte invasion by Plasmodium falciparum merozoites*. *Blood, **115**: 4559–4568 (2010).*

Bray, P. G., Martin, R. E., Tilley, L., Ward, S.A., Kirk, K. and Fidock, D.A. (2005). Defining the role of PfCRT in *Plasmodium falciparum* chloroquine resistance. *Mol. Micro., (2005) **56** (2): 323–333*

Burgess, S.J., Andrews, S.S., Gunsaru, B., Hodson, C.A., Kelly, J.X., Liebman, K., Selzer, A, and Peyton, D.H.(2006). Reversed Chloroquine: Remodeling Chloroquine to Combat Chloroquine-Resistant *Plasmodium falciparum* Malaria. *J. Med. Chem.*,***49****: 5623*

Butler, A.R., Khan, S, and Ferguson, E. (2010). A brief history of malaria chemotherapy. *J. R. Coll. Physicians, Edinb* ***40****:172–7*

Carlson J., Treutiger C.J, Hedlund I., Helmby H., Jepson A., Twumasi P., Kwiatkowski D., Greenwood B.M. and Wahlgren M.(1992). Disruption of Plasmodium falciparum erythrocyte rosettes by standard heparin and heparin devoid of anticoagulant activity. *Am. J. Trop. Med. Hyg.* 46,595–602 (1992).

Chan, C.W., Spathis, R., Reiff, D.M., McGrath, S.E., Garruto, R.M., Lum, J.K. (2012). Diversity of Plasmodium falciparum Chloroquine Resistance Transporter (PfCRT) Exon 2 Haplotypes in the Pacific from 1959 to 1979, *PLoS ONE,* ***7****(1): e30213*

Chen, J., Dai, W.T., He, Z.M., Gao, L., Huang, X., Gong, J.M., Xing, H.Y, and Chen, W.D.
(2013). Fabrication and evaluation of curcumin-loaded nanoparticles based on solid lipid as a new type of colloidal drug delivery system. *Indian J. Pharm. Sci.,* ***75*** *(2): 178–184.*

Chinappi, M., Via, A., Marcatili, P, and Tramontano, A. (2010). Mechanism of Chloroquine resistance in *Plasmodium falciparum*. *PLoS One,* ***5****(11):e14064.*

Centre for Disease Control, 2012,http://www.cdc.gov/malaria/ accessed on12/08/2013

Date, A.A., Joshi, M.D, and Patravale, V.B., (2007). Parasitic diseases: Liposomes and polymeric nanoparticles versus lipid nanoparticles. *Adv. Drug Deliv. Rev.*, **59**: *505–521*

Das, S., and Chaudhury, A. (2011). Recent advances in lipid nanoparticle formulations with solid matrix for oral drug delivery. *AAPS Pharm. Sci. Tech.,* ***12*** *(1):62-76.*

De Jong, W.H, and Borm, P. J.A.(2008). Drug delivery and nanoparticles: Applications and hazards, *Int. J. Nanomedicine,* ***3****(2): 133–149.*

Delves, M., Plouffe, D., Scheurer, C., Meister, S., Wittlin, S., Winzeler, E.A., Sinden, R.E, and Leroy, D.(2012) The Activities of Current Antimalarial Drugs on the Life Cycle Stages of Plasmodium. A Comparative study with human and rodent parasites. *PLoS Med.,* ***9****(2):e1001169.*

Desjardins, R.E., Canfield, C.J., Haynes, J.D., and Chulay, J.D. (1979).Quantitative assessment of anti-malarial activity *in vitro* by an automated dilution technique. *Antimicrob. Agents Chemother.* ***16****: 379*

Dondorp A.M., Fairhurst R.M, Slutsker L., John R. MacArthur J.R., Breman J.L., Guerin P.J, Wellems T.E., Ringwald P., Newman R.D. and Plowe C.V.(2011). The threat of artemisinin-resistant malaria, *N. Engl. J. Med. 365; 12*

Ekambaram, P., Sathali, A, and Priyanka, K.(2012). Solid Lipid Nanoparticles: A Review, *Sci. Revs. Chem. Commun., **2**(1): 80-102*

Frohlich E. (2012). The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. *Int. J. Nanomedicine*

Erah, P.O., Arienmughare, G., and Okhamafe, A, A. O. (2003). *P. falciparum* malaria resistance to chloroquine in five communities in Southern Nigeria. *Afr. J. Biotechnol., **2** (10), pp. 384-389.*

Fairhurst R.M., Nayyar G.M.L., Breman J.G., Hallett R.,Vennerstrom J.L., Duong S., Ringwald P., Wellems T.E., Plowe C.V. and Dondorp A.M. (2012). Artemisinin-Resistant Malaria: Research Challenges, Opportunities, and Public Health Implications. *Am. J. Trop. Med. Hyg., **87**(2):231–241*

Gaurvika, M. L. N., Breman, J.G., Newton, P.N. and Herrington, J. (2012). Poor-quality antimalarial drugs in Southeast Asia and sub-Saharan Africa. *Lancet Infect. Dis., **12**: 488–96*

Honary S and Zahir H (2013). Effect of Zeta Potential on the Properties of Nano-Drug Delivery Systems - A Review (Part 2). *Trop J Pharm Res, **12** (2):*265

Hyde J.E. (2007). Drug-resistant malaria - an insight *FEBS J., **274**(18): 4688–4698.*

National Institute of Health, accessed at http//www.niad.nih.gov/topics/malaria, 2012.

Kairemo, K., Erba, P., Bergström, K., and Pauwels, E. K. J., (2008). Nanoparticles in cancer. *Curr. Radiopharms, **1**, 30-36.*

Kenya Malaria Fact Sheet, Kemri, 2013.

Kobayashi, K., Takemae, T.K., Sugi, T., Ishiwa, A., Gong, H., Recuenco, F.C., Iwanaga, T., Horimoto, T., Akashi, H, and Kato, K. (2013). Analyses of Interactions between Heparin and the Apical Surface Proteins of *Plasmodium falciparum*, *SCIENTIFIC REPORTS | **3**: 3178 |*

KIRK K., (2001). Membrane Transport in the Malaria-Infected Erythrocyte. Physiological Reviews, ***81**(2).*

Laufer, M. K , Thesing, P. C., Eddington, N. D., Masonga, R., Dzinjalamala, F. K., Takala, S. L., Taylor, T. E. and Plowe, C. V (2006). Return of Chloroquine antimalarial Efficacy in Malawi. *N Engl. J. Med., **355**:1959-66.*

Leary, J. F. (2011). "The Importance of Zeta Potential for Drug/Gene Delivery in Nanomedicine" accessed at https://nanohub.org/resources on 27/04/2016

Lewis, A., Wacker, M., Olszewski, K. L., Cobbold, S.A., Baska, K.S., Tan, A., Ferdig, M.T, and Llina, M. (2014). Metabolic QTL analysis links chloroquine resistance in *Plasmodium falciparum* to Impaired Hemoglobin Catabolism. *PLoS Genetics, **10**(1): e1004085*

Le Bras and Durand (2003),.The mechanisms of resistance to antimalarial drugs in *Plasmodium falciparum*. *Fundam. Clin. Pharmacol. **17**(2):147-53.*

Lehane A. M., and Kirk, K. (2008).Chloroquine-Associated H^+ Leak from the Mutations in PfCRT Give Rise to a Chloroquine Resistance-Conferring. *Antimicrob. Agents Chemother. **52**(12):4374.*

Lu B., Xiong, S.B., Yan, H., Yin X.D, and Chao, R.B., (2006). Solid lipid nanoparticles of mitoxantrone for local injection against breast cancer and its lymph node metastases. *Eur. J. Pharm. Sci., 2006, **28**, 86-95.*

Martinho, N., Damgé, C, and Reis, C.P. (2011). Recent Advances in Drug Delivery Systems. *J. Biom/ Nanobiotechnol., 2011, **2**, 510-526.*

Mbatchi, S.F., Mbatchi, B., Banzouzi, J.T., Bansimba, T., Nsonde Ntandou, G.F., Ouamba, J.M., Berry, A. and Benoit-Vical, F. (2006). *In vitro* antiplasmodial activity of 18 plants used in Congo Brazzaville traditional medicine. *J. Ethnopharmacol., **104:** 168-174.*

Marques, J., Moles, E., Urbán, P., Prohens, R., Busquets, M.A., Sevrin, C., Grandfils, C, and Fernàndez-Busquets, X.(2014). Application of heparin as a dual agent with antimalarial and liposome targeting activities toward *Plasmodium*-infected red blood cells. *Nanomedicine, **S1549-9634**(14)00314-1*

Melariri, P., Campbell, W., Etusim, P, and Smith, P. (2012). *In vitro* and *in vivo* antimalarial activity of Linolenic and Linoleic acids and their methyl esters. *Adv. Studies Bio., **4(7):** 333 – 349.*

National Malaria Control Programme accessed at http://www.nmcp.or.ke/index.php/malaria-in-kenya on 2/3/2017

Na-Bangchang, K, and Karbwang, J. (2013). Emerging artemisinin resistance in the border areas of Thailand. *Expert Rev.Clin.Pharmacol.,**6**(3):307-22.*

Nair. R. Kumar, K.S.A., Priya, K.V, and Sevukarajan, M. (2011). Recent Advances in Solid Lipid Nanoparticle Based Drug Delivery Systems. *J. Biomed. Sci. and Res., **3** (2): 368-384).*

Nzekwe I. T., Azodoa V. I.,Agubatab C.O., Naickerc B.,Okored V. and Esimonee C.O. 2015 Preliminary formulation and characterization of solid lipid nanoparticles containing chloroquine and a P-glycoprotein inhibitor: Influences of lipid-surfactant ratios. *J. Chem. Pharm. Res., 7(2): 932-939*

O'Neill, P.M., Barton, V.E., Stephen A. Ward, S.A, and Chadwick, J.(2012). 4-Aminoquinolines: Chloroquine, Amodiaquine and Next-Generation Analogues. *Milestones in Drug Therapy, pp 19-44.*

Omwoyo, W.N., Ogutu,B, B., Oloo, F., Swai, H., Lonji Kalombo, L., Melariri, P, and Mahanga, M.O.(2014). Preparation, characterization and optimization of primaquine-loaded solid lipid nanoparticles, *Int. J.Nanomedicine,**9**: 3865–3874.*

PATH Malaria Vaccine Initiative accessed at www.malariavaccine.org.on 06/04/2017

Pandey, R., Sharma, S, and Khuller G.K.(2005). Oral solid lipid nanoparticle-based antitubercular chemotherapy, *Tuberculosis (Edinb), **85**(5-6):415-20.*

Parhi, R, and Suresh, P.(2012).Preparation and Characterization of Solid Lipid Nanoparticles-*A Review, Curr. Drug Discov. Technol., **9**, 2-16.*

Parveen, S., Misra, R, and Sahoo, S.K. (2012). Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging. *Nanomedicine: NBN **8**(2):147-166*

Pereira, M.R., Henrich, P.P., Sidhu, A.B., Johnson, D., Hardink, J., Van Deusen, J., Lin, J., Gore, K., O'Brien, C., Wele, M., Djimde, A., Chandra, R, and Fidock, D.A.(2009).*In vivo* and *in vitro* antimalarial properties of azithromycin-chloroquine combinations that include the resistance reversal agent amlodipine. *Afr. J.Tradit. Complement. Altern. Med., **6**(2): 163–167.*

Peters, W., Portus, J.H., and Robinson, B.L., 1975. The chemotherapy of rodent malaria, XXII. The value of drug-resistant strains of *P. berghei* in screening for blood schizonticidal activity. *Ann. Trop. Med. Parasitol. 69:155–171.*

President's Malaria Initiative report accessed at https://www.pmi.gov/docs/default-source/default-document-library/country-profiles/kenya_profile.pdf on 2/3/2017

Puri, A., Loomis, K., Smith, B., Lee, J., Yavlovich, A., Heldman, E., and Blumenthal, R. (2009), Lipid-Based Nanoparticles as Pharmaceutical Drug Carriers: From Concepts to Clinic. *Crit. Rev. Ther. Drug Carrier Syst., **26** (6): 523–580*

Raina, N., Goyal, A.K., Pillai C.R, and Rath, G. (2013).Development and characterization of Artemether loaded solid lipid nanoparticles. *Ind.J.Pharm.Edu. Res., **47**(2):*123-128

Salamanca-Buentello, Persad, D, and Singer, P.(2005).Nanotechnology and the Developing World, *PLos Med.,**2**(5): 383-386.*

Santos-Magalhaes, N. S, and Mosqueira, V.C. (2009). Nanotechnology applied to the treatment of malaria, *Adv. Drug Deliv. Rev., **62**(4–5):560–575*

Schlichtherle, M., Wahlgren, M., Perlmann, H, and Scherf, A. (2000). Methods in malaria research, 3rd edn. *MR4/ATCC, Manassas, pages1-74.*

Shahinas, D., Folefoc, A, and Pillai D.R. (2013). Targeting *Plasmodium falciparum* Hsp90.Towards Reversing Antimalarial Resistance, *Pathogens, **2**: 33-54*

Shen S., Wu Y., Liu Y, and Wu D. (2017). High, drug-loading nanomedicines: progress, current status and prospects. *Int J Nanomedicine. **12**:4085-4109.*
Shujatullah, F., Khan, H.M., Khatoon, A., Khan, P.A, and Ashfaq, M. (2012).In Vitro Chloroquine Resistance in *Plasmodium falciparum* isolates from Tertiary Care Hospital. *Malar. Res. Treat., 1- 4*

Silva H.D., Cerqueira M.A. and Vicente A.A. (2012) Nanoemulsion for Food Applications: Development and Characterization. *Food Bioprocess Technol,* 5:854–867

Sixsmith, D.G., Watkins, W. M., Chulay, J.D, and Spencer, H.C. (1984). *In vitro* antimalarial activity of tetrahydrofolate dehydrogenase inhibitors. *Am. J. Trop. Med. Hyg., **33**(5):772–776*

Soppimath, K.S., Aminabhavi, T.M., Kulkarni, A.R, and Rudzinski, W.E. (2001).Biodegradable polymeric nanoparticles as drug delivery devices. *J. Control Release, **70:1**-20*

Talisuna, A.O., Bloland, P., and D'Alessandro U., (2004). History of Antimalarial Drug Resistance. *Clin. Microbiol. Rev., **17**(1):235.*

Tiwari, G., Tiwari, R., Sriwastawa, B., Bhati, L., Pandey, S., Pandey, P, and Saurabh, K. B. (2012). Drug delivery systems: *An updated review, Int. J. Pharm. Investig., **2**(1): 2–11.*

Trager, W, and Jensen, J.B.(1976). Human malaria parasites in continuous culture. *Science, **193** (4254):673-5.*
Wilczewska A.Z., Niemirowicz K., Markiewicz K.H and Car H. (2012). Pharmacological Reports, *64, 1020-1037*

Wellems, T.E, and Plowe, C.V. (2001).Chloroquine-Resistant Malaria. *J. Infect .Dis., **184:**770–6.*

WHO, World Malaria Report, 2014, http://www.who.int/malaria/media/
World_malaria_report_2014/en/.

WHO, World malaria report (2012), www.who.int/malaria/world malaria report-2012

WHO, World malaria report (2010), www.who.int/malaria/world malaria report-2010 pp. 75–86

Whitty, C. J. M., Chandler, C., Ansah, E., Leslie, T, and Staedke, S.G. (2008). Deployment of ACT antimalarials for treatment of malaria: challenges and opportunities. *Malar J, **7** Suppl 1*.

Wilson, D.W., Langer, C., Goodman, C.D., McFadden, G.I, and James G. Beeson, J.G. (2013). Defining the timing of action of antimalarial drugs against *Plasmodium falciparum*. *Antimicrob. Agents Chemother. **57**(3):1455–1467*

UNICEF, 2004, http://www.unicef.org/media/files accessed on 12/08/2013

Urban, P. and Fernandez-Bisquets, X. (2014). Nanomedicine against malaria, *Curr Med Chem, **21:**605-629*

Zhuang, Y.G., Xu, B., Huang, F., Wu, J.J, and Chen, S. (2012). Solid lipid nanoparticles of anticancer drugs against MCF-7 cell line and a murine breast cancer model, *Pharmazie, **67**(11):925-9.*

APPENDICES

Appendix I: Tests for screening for antimalarials

The standard test for screening potential drugs for antiplasmodial activity is a radioactivity-based method that relies upon the incorporation of 3H-hypoxanthine into the DNA of the parasite to measure parasitic replication in red blood cells. This method is very sensitive and it can be used to screen a large number of compounds, but requires hazardous radioactive materials that require special facilities and procedures. Alternatives to the 3 H-hypoxanthine-based methodologies include a labor-intensive and time-consuming microscopic method and several colorimetric assays. 4–6 Colorimetric methods, however, are based on enzymatic activity rather than parasite replication, and in addition, may be subject to artifacts caused by pigments present in crude plant extracts that are frequently used in drug screening programs.

Appendix II: KEMRI ACUC approval

KENYA MEDICAL RESEARCH INSTITUTE

Centre for Traditional Medicine and Drug Research, P.O. Box 54840-00200 NAIROBI, Kenya
Tel. 254 (020) 2722541, 2718349, 0722 205101, 0733 400603
E-mail: ctmdr@kemri.org; Website: www.kemri.org

KEMRI/ACUC/ 03.11.16 **30th November 2016**

Joseph Muga,
CTMDR, KEMRI

Mr. Muga,

RE: Animal use approval for SERU 3325 - "Evaluation of Antiplasmodial Activities of Chloroquine Encapsulated in Heparin – Functionalized Solid Lipid Nanoparticles" protocol

The KEMRI ACUC committee acknowledges the resubmission of the above mentioned protocol. It has been confirmed that all the use of laboratory mice is necessary in achieving the study objectives and issues raised earlier have been adequately addressed.

Approval is granted for a period of one year starting from when the SERU approval will be obtained. If you still intend to handle laboratory animals after the period covered by this 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. In addition, the committee expects the study to provide an annual report on the progress of animal use simultaneously with the annual continuing review report to SERU.

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

The committee wishes you all the best in your work.

Yours sincerely,

Dr. Kononoi Limbaso
Chairperson KEMRI ACUC

KENYA MEDICAL RESEARCH INSTITUTE
★ 30 NOV 2016 ★
ANIMAL CARE AND USE COMMITTEE
Signature: .....

In Search of Better Health

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 09, 2016**

TO: **JOSEPH OWINO MUGA,**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. PETER MWITARI,**
THE DIRECTOR, CTMDR,
NAIROBI

Forwarded 9/12/2016

Dear Sir,

RE: KEMRI/SERU/CTMDR/020/3325 (*RESUBMISSION 2 OF INITIAL*): EVALUATION OF ANTIPLASMODIAL ACTIVITIES OF CHLOROQUINE ENCAPSULATED IN HEPARIN – FUNCTIONALIZED SOLID LIPID NANOPARTICLES.

Reference is made to your letter dated 5th December 2016. The KEMRI Scientific and Ethics Review Unit (SERU) acknowledge receipt of the revised study documents on 6th December 2016.

This is to inform you that the Committee noted that the issues raised at the 254th meeting of the KEMRI Ethics Review Committee (ERC) held on **16th August 2016** have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **9th December, 2016** for a period of one year. Please note that authorization to conduct this study will automatically expire on **December 08, 2017.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **27th October, 2017**.

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

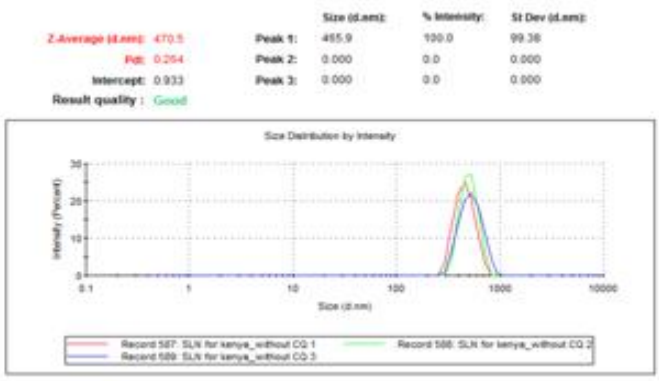
You may now embark on your study.

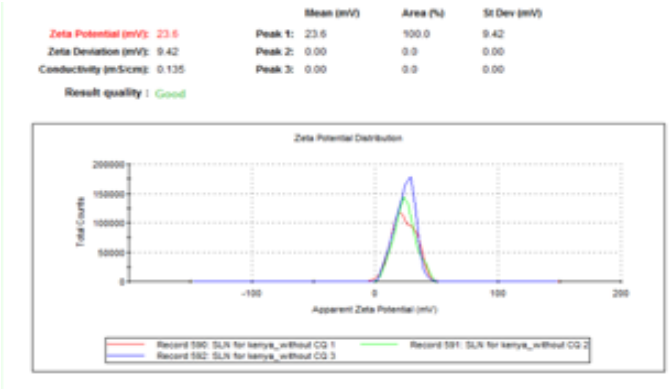
Yours faithfully,

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

Appendix IV: UV-VIS spectrometry curves for nanoformulated CQ

SLN without CQ

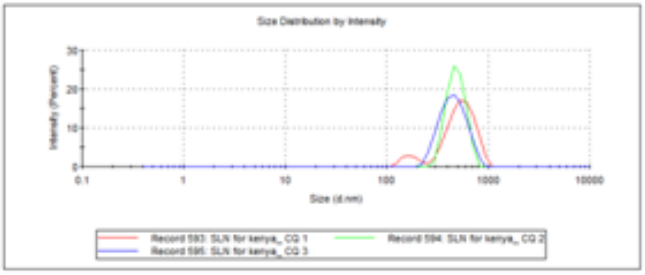




SLN with CQ

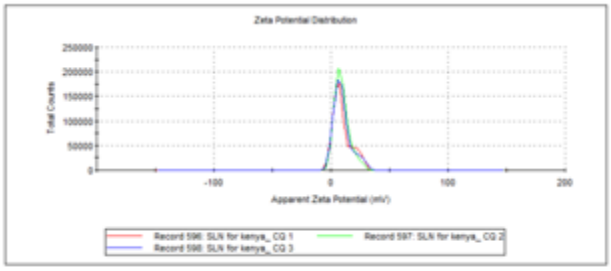
		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 436.8	Peak 1:	563.6	89.9	157.0
PdI: 0.193	Peak 2:	173.6	10.1	34.62
Intercept: 0.937	Peak 3:	0.000	0.0	0.000

Result quality : Good



		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): 9.65	Peak 1:	9.65	100.0	7.68
Zeta Deviation (mV): 7.68	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.229	Peak 3:	0.00	0.0	0.00

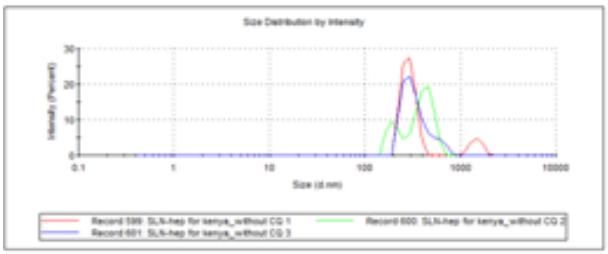
Result quality : Good



SLN – HEP without CQ

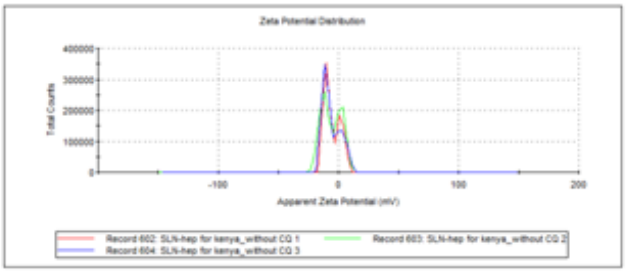
		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 379.8	Peak 1:	286.5	87.2	46.32
PdI: 0.374	Peak 2:	1467	12.8	209.0
Intercept: 0.831	Peak 3:	0.000	0.0	0.000

Result quality : Good



		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -5.43	Peak 1:	-9.30	63.8	3.38
Zeta Deviation (mV): 6.54	Peak 2:	1.91	36.2	3.25
Conductivity (mS/cm): 0.102	Peak 3:	0.00	0.0	0.00

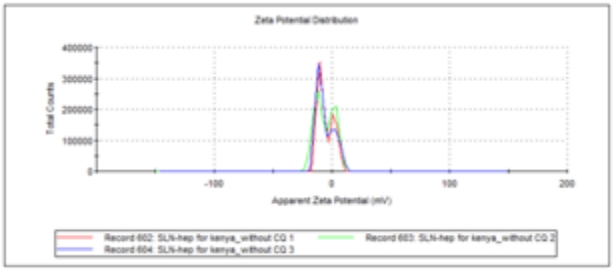
Result quality : Good



SLN – HEP with CQ

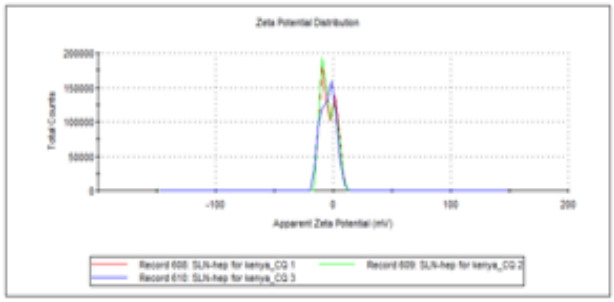
		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -5.43	Peak 1:	-9.30	63.8	3.38
Zeta Deviation (mV): 6.54	Peak 2:	1.91	36.2	3.25
Conductivity (mS/cm): 0.102	Peak 3:	0.00	0.0	0.00

Result quality : Good



		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -3.95	Peak 1:	-7.66	58.5	3.65
Zeta Deviation (mV): 6.16	Peak 2:	1.77	41.5	3.23
Conductivity (mS/cm): 0.156	Peak 3:	0.00	0.0	0.00

Result quality : Good



Appendix V: uptake of tritium labeled hypoxanthine by pRBCs

CPMs for CQ sensitive P. falciparum (D6) isolates

	1 Heparin		**2 SLN**		**3 SLN-CQ**		**4 SLN-HEP**		**5 SLN-HEP-CQ**		**CQ**	
D6	100µg/ml	100µg/ml	100µg/ml	100µg/ml	80ng/ml	80ng/ml	100µg/ml	100µg/ml	80ng/ml	80ng/ml	40ng/ml	40ng/ml
A	8455	8576	10786	8770	7205	6209	9521	9024	104	110	86	94
B	8718	9883	12013	10312	98	84	122	132	80	74	100	82
C	9577	8815	9681	9519	210	194	98	281	76	98	66	80
D	10745	12431	11593	10261	12671	11159	104	106	12264	10729	96	140
E	10369	9927	11446	11683	10298	10555	4150	6615	10192	11270	11762	12012
F	10285	9691	10166	9635	11037	10224	9434	9172	13059	12230	12050	11664
G	10723	9154	9939	10813	9584	10346	10172	11566	11940	12422	11662	12501
H	9368	9992	10251	10527	8381	9035	9958	11436	10772	10475	11537	12035

CPMs for CQ resistant P. falciparum (W6) isolates

	1 Heparin		**2 SLN**		**3 SLN-CQ**		**4 SLN-Hep**		**5 SLN-Hep-CQ**		**CQ**	
W2	100µg/ml	100µg/ml	100µg/ml	100µg/ml	400ng/ml	400ng/ml	100µg/ml	100µg/ml	400ng/ml	400ng/ml	200ng/ml	200ng/ml
A	9309	9446	11814	9144	9140	8283	9801	8484	130	160	106	98
B	9910	8971	7705	10229	8220	7689	8821	7228	7850	9285	136	134
C	8768	9287	10228	11051	10079	9482	10046	9899	8911	10543	652	678
D	13486	10083	10106	11017	9905	10428	10535	11748	11883	10009	8406	8914
E	10861	9975	11144	14018	12496	13031	14934	13916	8725	9162	9239	10795
F	10356	10165	11568	11529	12492	14033	12071	9935	10778	10394	9512	10760
G	10643	8784	10287	11133	10478	10390	12461	11203	10624	9602	9990	10275
H	9078	8563	9386	11105	10170	10763	11269	12090	11009	9137	10188	10212