UNIVERSITY OF GHANA

COLLEGE OF BASIC AND APPLIED SCIENCES

EVALUATION OF MEDICINES FOR MALARIA VENTURE (MMV) COMPOUND LIBRARY FOR POTENCY AGAINST *P. FALCIPARUM* CLINICAL ISOLATES

By

JERSLEY DIDEWURAH CHIRAWURAH

(102783480)

THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MPHIL MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES DEGREE.

DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY

JULY, 2016

Style Definition: Heading 2: All caps

Style Definition: Heading 4: Font: Bold, Not Italic

Style Definition: TOC 1: Line spacing: Double, Tab stops: 6.26", Right,Leader: …

DECLARATION

The experimental work presented in this thesis was undertaken by me, Jersley D. Chirawurah, at the Department of Biochemistry, Cell and Molecular Biology under the supervision of Prof. Gordon A. Awandare and Dr. Samuel Duodu of the Department of Biochemistry, Cell and Molecular Biology, University of Ghana. I acknowledge all references have been duly cited.

……

Jersley D. Chirawurah (Student)

……

Prof. Gordon A. Awandare (Supervisor)

……..

Dr. Samuel Duodu (Co-Supervisor)

ACKNOWLEDGEMENT

I will, first of all, like to give thanks to the Almighty God for creating the opportunity and strengthening me to undertake this project. I will also like to express my gratitude to my supervisor, Prof. Gordon A. Awandare for the opportunity to work with him and also for his generational leadership that has challenged me to successfully execute this project. I will also like to thank Dr. Samuel Duodu for his indispensable help and commitment that came in handy in the course of this project. I am also very much beholden to Dr. Yaw Aniweh, who provided guidance and direction throughout this project. Many thanks to all members of the Infectious Disease Research Laboratory of the Department of Biochemistry, Cell and Molecular Biology for all their support. I am also very grateful to all the staff of Immunology and Parasitology Departments of the Noguchi Memorial Institute for Medical Research, especially Dr. Michael Ofori and Prof. Niels Quashie for their support. I appreciate all help and support from all the staff of Biochemistry, Cell and Molecular Biology and the West African Center for Infectious Disease Pathogens (WACCBIP). I am also very much beholden to my family for their immense support throughout my studies. Finally, I will like to thank WACCBIP for funding my tuition, stipend and research cost.

DEDICATION

I dedicate this work to God for His guidance and support. I also dedicate this work to my Supervisors and my family for their patience and support.

TABLE OF CONTENTS

DECLARATION ii

ACKNOWLEDGEMENT iii

DEDICATION iv

TABLE OF CONTENTS v

LIST OF TABLES xii

ABSTRACT xiv

CHAPTER ONE 1

1.0 INTRODUCTION 1

1.1.1 Aim 3

1.1.2 Specific Objectives 3

CHAPTER TWO 4

2.0 LITERATURE REVIEW 4

2.1 MALARIA AND THE MALARIA PARASITE 4

2.1.1 Distribution of Malaria Parasites 4

2.1.2 Life Cycle of the Parasite 5

2.1.3 The Burden of Malaria 7

2.2 CLASSES OF CURRENT ANTIMALARIAL DRUGS 8

2.3 COMMON ANTIMALARIAL DRUGS AND THEIR MECHANISMS OF ACTION 9

2.3.1 Quinine 9

2.3.2 Chloroquine 10

2.3.3 Antifolates 10

2.3.4 Mefloquine 11

2.3.5 Atovaquone 11

2.3.6 Artemisinin 12

2.4 ANTIMALARIAL RESISTANCE AND MOLECULAR MARKERS OF DRUG RESISTANCE 13

2.4.1 Antimalarial Drug Resistance 13

2.4.2 Molecular Markers of Drug Resistance 13

2.5 EARLY TREATMENT AS A TOOL FOR MALARIA CONTROL 19

2.6 ARTEMISININ AND ITS COMBINATION THERAPY 19

2.7 VACCINES AS TOOLS FOR MALARIA CONTROL 20

2.8 NEW PARTNERSHIPS FOR MALARIA DRUG DEVELOPMENTS AND THE "MALARIA BOX" COMPOUNDS 21

2.9 SCREENING FOR NEW ANTIMALARIAL COMPOUNDS 22

2.9.1 Microscopy-Based Assays 22

2.9.2 Biochemical-Based Assays 23

2.9. 3 Cell-Based Assays 24

2.10 KEY DRUG TARGETS 25

2.11 DRUG CANDIDATES FROM BLOOD STAGE SCREENS 26

2.12 NEW ANTIMALARIAL DRUG TARGET DISCOVERY 28

CHAPTER THREE 29

3.0 MATERIALS AND METHODS 29

3.1 DRUG ASSAY OPTIMIZATION AND DETERMINATION OF POTENCY OF COMPOUND X 29

3.1.1 *P. falciparum* Culture 29

3.1.2 Drug Assay Culture Preparation 29

3.1.3 Compound X Preparation and Serial Dilutions 30

3.1.4 Growth Inhibition Assay 30

3.1.5 Determination of Mean Parasitemia levels Using Flow Cytometry 32

3.1.6 Determination of Inhibitory Concentrations 32

3.2 SCREENING OF THE MALARIA BOX COMPOUNDS AGAINST CLINICAL ISOLATES 33

3.2.1 *P. falciparum* Clinical Isolates 33

3.2.2 Selection and Preparation of Malaria Box Compounds 33

3.3 SCREENING OF CHLOROQUINE AND ARTESUNATE AGAINST THE CLINICAL ISOLATES 34

3.3.1 Evaluation of the Potency of Chloroquine on the Clinical Isolates 34

3.3.2 Evaluation of the Potency of Artesunate on the Clinical Isolates 35

3.4 GENOTYPING OF CLINICAL ISOLATES USING RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) 36

3.4.1 *P. falciparum* Genomic DNA Extraction 37

3.4.2 Nested PCR for Detecting SNPs in the genomic DNA of the clinical isolates 37

3.4.3 Restriction Digest for SNPs Analysis in the Genomic DNA of the Clinical isolates 38

CHAPTER FOUR 39

4.0 RESULTS 39

4.1 OPTIMIZATION OF THE DRUG ASSAY AND DETERMINATION OF THE POTENCY OF COMPOUND X USING DD2 AND 7G8 39

4.2 SCREENING OF COMPOUNDS FROM THE MALARIA BOX AGAINST CLINICAL ISOLATES 42

4.3 EVALUATION OF THE SENSITIVITY OF THE CLINICAL ISOLATES TO TRADITIONAL ANTIMALARIAL DRUGS 54

4.3.1 Sensitivity of Clinical Isolates to Artesunate 54

4.3. 2 Sensitivity of Clinical Isolates to Chloroquine 55

4.4 GENOTYPING OF CLINICAL ISOLATES USING RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) 56

4.4.1 Polymorphism in the *pfcrt* gene 57

4.4.2 Polymorphisms in the *pfmdr1* gene 58

4.4.3 Polymormisms in the *pfdhps* gene 59

4.4.4 Polymorphisms in the *pfdhfr* gene 60

CHAPTER 5 63

5.0 DISCUSSIONS AND CONCLUSION 63

5.1 SUMMARY 63

5.2 DISCUSSIONS 64

5.3 CONCLUSION 71

5.4 FUTURE STUDIES 71

REFERENCES 72

APPENDIX 96

LIST OF FIGURES

Figure 2. 1: Life cycle of *P. falciparum* 7

Figure 2. 2: Schematic view of the *pfcrt* gene with its 13 exons 15

Figure 2. 3 Schematic view of the *pfmdr1* gene in chromosome 5. 16

Figure 3. 1: Experimental flow chart for the growth inhibition assay and analysis. 31

Figure 3. 2: Experimental flow chart for the restriction fragment length polymorphism (RFLP). 36

Figure 4. 1 Potency of Compound X against Dd2. 40

Figure 4. 2: Potency of Compound X against 7G8. 41

Figure 4. 3 Potency of MMV396797 against four clinical isolates of *P. facliparum*. 43

Figure 4. 4: Potency of MMV006278 against the clinical isolates. 44

Figure 4. 5: Potency of MMV008416 against the clinical isolates. 45

Figure 4. 6: Potency of MMV08956 against the clinical isolates. 46

Figure 4. 7: Potency of MMV0085203 against the clinical isolates. 47

Figure 4. 8: Potency of MMV6787 on clinical isolates. 48

Figure 4. 9: Potency of MMV000753 against the clinical isolates. 49

Figure 4. 10: Potency of MMV007384 against the clinical isolates. 50

Figure 4. 11: Potency of MMV019555 against the clinical isolates. 51

Figure 4. 12: Potency of MMV009015 against the clinical isolates. 52

Figure 4. 13: Potency of artesunate against the clinical isolates. 54

Figure 4. 14: Potency of chloroquine against the clinical isolates. 55

Figure 4. 15: Identification of SNVs in the *pfcrt* gene of the clinical isolates. 57

Figure 4. 16: Identification of SNVs in the pfmdr1 gene of the clinical isolates. 58

Figure 4. 17: Polymorphisms in the *dhps* gene. 59

Figure 4. 18: Identification of SNV in the *pfdhfr* gene of the clinical isolates. 61

Figure A. 1: The *de novo* folate biosynthesis pathway of P. falciparum. 99

LIST OF TABLES

Table 2.1: Summary of the groups of antimalarial drugs based on stage-specific activity..... 9

Table 4. 1: Summary of IC_{50} values for Malaria Box compounds against the four clinical isolates..... 53

Table 4. 2: Summary of polymorphisms in the *pfcrt*, *pfmdr1*, *pfdhps* and *pfdfr* genes in the clinical isolates..... 62

Table A. 1: Nested PCR for the detection of SNPs in the genomic DNA of the clinical isolates 96

Table A. 2: Profile for Restriction Fragment Length Polymorphism. 97

Table A.3: Structures of some conventional antimalarial compounds. 98

LIST OF ABBREVIATIONS

ACT	Artemisinin-based Combination Therapy
CPM	Complete Parasite Media
DHOD	Dihydroorotate Dehydrogenase
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-Linked Immunosorbent Assay
GSK	GlaxoSmithKline
HRP2	Histidine-Rich Protein 2
IC_{50}	50% Inhibitory Concentration
MMV	Medicines for Malaria Venture
MTIP	Myosin Tail Interacting Protein
PCR	Polymerase Chain Reaction
PfCRT	*Plasmodium falciparum* Chloroquine Resistance Transporter
P*f*DHFR	*P. falciparum* Dihydrofolate Reductase
PfDHPS	*P. falciparum* Dihydropteroate Synthase
PfEMP1	*P. falciparum* Erythrocyte Membrane Protein 1
P*f*MDR1	*P. falciparum* Multi-drug Resistance Transporter
PfROM1	*P. falciparum* Rhomboid Proteases 1
P*f*Trx	*P. falciparum* Thioredoxin
P*f*TrxR	*P. falciparum* Thioredoxin Reductase
PLDH	Parasite Lactate Dehydrogenase Enzyme
RFLP	Restriction Fragment Length Polymorphism
SAR	Structural-Activity Relationship
SNV	Single Nucleotide Variation

ABSTRACT

The resistance of *Plasmodium falciparum* to artemisinin and its derivatives underscore the need for new sets of antimalarials with novel mechanisms of action. Towards the development of new, affordable and easily accessible antimalarial drugs for endemic regions, there is the need to screen more compounds for their antimalarial activity. Several studies on different compound classes have been conducted using laboratory strains of *P. falciparum*. However, not much is known of their potency on clinical isolates. In this study, an optimal *in vitro* growth inhibitory assay was established and used to screen ten selected compounds from the Malaria Box against four clinical isolates from three endemic areas in Ghana (Accra-two, Navrongo-one and Kintampo-one). From the assays, MMV085203, MMV006787 and MMV008956 were found to have IC_{50} values below 500 nM with the most efficacious being MMV085203 (IC_{50} values between 55 nM-83 nM). MMV006787 was also found to be more potent against the clinical isolates compared to its activity against laboratory strain of *P. falciparum*, as was reported by previous studies. The Navrongo isolate (N093) was more sensitive to artesunate and most of the Malaria Box compounds but resistant to chloroquine. Genomic studies using restriction fragment length polymorphism (RFLP) identified N093 to harbour the mutant *pfcrt* allele (T76) while the other three had the wild-type *pfcrt* allele (K76). Therefore, the presence of the K76T mutation in N093 seems to confirm data from other studies that suggest that the presence of this mutation increases the sensitivity of malaria parasites to artemisinin-based drugs and resistance to chloroquine. The use of clinical isolates in this study further demonstrates the need to validate potential drug compounds using clinical isolates in addition to the use of laboratory-adapted strains for drug development.

CHAPTER ONE

1.0 INTRODUCTION

Malaria remains a major cause of death in resource-limited settings. Although the global incidence of malaria cases declined by about 30% in the past decade, malaria caused be *P. falciparum* still remains a major problem in sub-Saharan Africa, accounting for about 10% of deaths in children under 5 years (WHO, 2015). In Ghana, malaria accounted for about 33% of all out-patient department (OPD) cases, and 49% of hospital admissions for children below five years in 2014 according to the National Malaria Control Programme of Ghana (Ghana health service, 2015). Despite, the numerous interventions to combat malaria over the decades, issues such as the development of insecticide and drug resistance have militated against the global aim to eliminate of malaria.

In 2006, artemisinin-based combination therapies (ACT) were recommended as the first-line drugs for the treatment of malaria in all endemic regions due to wide-spread resistance to traditional antimalarial drugs like chloroquine, sulfadoxine and pyrimethamine (WHO, 2015). However, the emergence of artemisinin-resistant strains in Western Cambodia (Yeung *et al.*, 2009) underscores the need for new sets of antimalarial drugs with novel mechanisms of action that can counter the emergence of drug-resistant malaria parasites (Ashley *et al.*, 2014, Dondorp *et al.*, 2012).

Vaccines have played a crucial role in the control of infectious diseases like smallpox, polio and measles (Rieckmann *et al.*, 2016, Tomori, Van Riper, 1954). It therefore, seems to suggest that the development of a cost-effective and readily available malaria vaccine would be germane in the eradication of malaria (Rogo *et al.*, 2006). However, all efforts to develop an efficient malaria vaccine remain elusive. There is therefore the need to consider other compounds in the antimalarial drug development pipeline to quickly make available new drugs for controlling drug-resistant malarial parasites.

Due to the poor economic conditions and the limited market returns, there has not been much investment in the development of potential drugs (Veeken *et al.*, 2000). Although new strategies and policies have been developed for eliminating malaria, it will be very challenging to completely wipe off malaria across the globe with the current antimalarials (Alonso *et al.*, 2011). The development of safe and single-dose therapies for mass drug administration to asymptomatic carriers with the ability to block downstream malaria transmission will be an important global malaria elimination strategy (Diagana, 2015). The need to develop new drug therapies for malaria with mechanisms of action that differ from existing antimalarial drugs has led to the identification of new antimalarial agents with novel targets. However, not until recently has there been new antimalarial agents that passed Phase II clinical trials (P *et al.*, 2015, Burrows *et al.*, 2014, Flannery *et al.*, 2013, Burrows *et al.*, 2011, Kato *et al.*, 2016, Fidock, 2016). Towards the development of new, affordable and easily accessible antimalarial drugs for endemic regions, the Medicines for Malaria Venture (MMV) in collaboration with pharmaceutical companies like Novartis, have identified over 20,000 compounds with antiplasmodial activity (Spangenberg *et al.*, 2013). To further accelerate the development of new antimalarial drugs, MMV assembled a total of 400 of these compounds called the Malaria Box which comprises 200 drug-like compounds and 200 probe-like compounds representing the chemical diversity of the 20,000 hit compounds. Although these compounds have been shown to have activity in *in vitro* assays, not all possible targets for these compounds have been explored. This makes the Malaria Box very attractive for further exploration of their targets and mechanisms of action. The Malaria Box compounds are freely available to researchers who want to participate in the drug discovery process but do not have the capacity and resources to resynthesize these compounds (Spangenberg *et al.*, 2013). Several studies have been conducted on these compounds using laboratory strains of *P. falciparum*, however, very little data have been reported on their potency on clinical isolates.

Therefore, in this study ten compounds from the Malaria Box were screened against four clinical isolates from three endemic areas in Ghana (Accra-two, Navrongo-one and Kintampo-one), using *in vitro* growth inhibitory assay. The use of clinical isolates provides a better understanding of the potency of these compounds for antimalarial drug development for endemic regions.

1.1.1 Aim

The aim of this study was to screen and identify potent compounds from Malaria Box against clinical isolates of *P. falciparum.*

1.1.2 Specific Objectives

1. To develop an optimal drug assay for screening Malaria Box compounds and determine the potency of Compound X against laboratory-adapted strains of *P. falciparum* (Dd2 & 7G8).

2. To determine the potency of selected compounds from the Malaria Box against clinical isolates of *P. falciparum.*

3. To evaluate the sensitivity of the clinical isolates to standard antimalarial drugs.

4. To detect mutations in *pfcrt*, *pfmdr*, *dhfr* and *dhps* genes in the clinical isolates and the possible effects of the mutation(s) on the sensitivity of the clinical isolates to the Malaria Box compounds.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 MALARIA AND THE MALARIA PARASITE

2.1.1 Distribution of Malaria Parasites

Human malaria is caused by different species of the apicomplexan *Plasmodium* parasite. Several species of the *Plasmodium* parasite are associated with disease burden, but *Plasmodium falciparum* (*P. falciparum*), *P. malariae*, *P. vivax*, *P. ovale*, *P. knowlesi* and more recently *P. cynomolgi* (Warren *et al.*, 1966, Rahman, 1982, Greenwood, 2002, Ta *et al.*, 2014, Wiwanitkit, 2011, Berry *et al.*, 2011, Cox-Singh *et al.*, 2008b, Baird, 2009) are known to infect humans. As obligate parasites, they live and develop in human hepatocytes and erythrocytes. About 80% of the global cases of malaria-associated deaths occur in sub-Saharan Africa and are predominantly caused by *P. falciparum* (WHO, 2015). The high burden of malaria in sub-Saharan Africa can be partly attributed to the presence of an efficient mosquito vector in addition to excellent ecological conditions that facilitate all year transmission of malaria (Sachs *et al.*, 2002). The high mortality and morbidity rates associated with *P. falciparum*-related infection and the increasing reports of emergence of resistant strains to current chemotherapeutic agents has put *P. falciparum* at the center stage of most research efforts (Ashley *et al.*, 2014, Bloland, 2001, Bowden *et al.*, 1982, Dondorp *et al.*, 2012). *P. vivax* is also widely distributed and is estimated to cause about 40 % of the annual malaria infections worldwide especially in Asia and the Americas (Mendis *et al.*, 2001). However, the global distribution of *P. vivax* is limited by the presence of Duffy negative blood phenotype. The Duffy blood group antigen is a glycoprotein on the host erythrocyte surface that is involved in mopping up of excess toxic chemokines under some disease conditions (Middleton *et al.*, 1997).

The Duffy blood group antigen also serves as an important receptor through which *P. vivax* invade host cells (Miller *et al.*, 1975). *P. vivax* associated-malaria is not common in Africa where most people lack the Duffy coat (Price *et al.*, 2007) .

Previously, *P. vivax* was not associated with severe malaria disease, but recent reports indicate clinically severe disease with its infection and occasionally multiple episodes of relapse (Carlton *et al.*, 2008). *P. ovale* (also known as tertian malaria parasite) is least distributed globally and is associated with benign disease outcomes (Faye *et al.*, 1998). It usually co-infects with *P. falciparum* or *P. malariae* (Dinko *et al.*, 2013, Fancony *et al.*, 2012). *P. malariae* (a quartan malaria parasite) is equally less distributed globally and usually co-infects with other malaria parasites associated with a mild form of the disease. *P. malariae* is unique among the *Plasmodium* spp., due to the fact that its infection can persist in a patient for up 40 days (Hase *et al.*, 2014). Its chronic infection has been linked with nephrotic syndrome (Halleux D Fau - Moerman *et al.*,2014, Hendrickse, 1976). *P. knowlesi* is often misdiagnosed as *P. malariae* and is mostly found in South East Asia (Cox-Singh *et al.*, 2008a). *P. cynomolgi* is a tertian malaria parasite that causes zoonotic infection in humans (Ta *et al.*, 2014). Like *P. vivax*, *P. cynomolgi* also has a dormant liver stage (Krotoski *et al.*, 1982) and a preference for invading immature red blood cells (Krotoski *et al.*, 1982, Warren *et al.*, 1966). Unlike *P. vivax* that cannot be cultured *in vitro*, *P. cynomolgi* has been successfully adapted to short-term *in vitro* culture, which makes *P. cynomolgi* an ideal model parasite for studying *P. vivax* biology and for identifying novel drugs against the dormant liver stage parasite (Nguyen-Dinh *et al.*, 1981).

2.1.2 Life Cycle of the Parasite

P. falciparum is the most widely studied of all the *Plasmodium* species and has been shown to exhibit different invasive forms as per its life cycle.

The parasite is known to have a complex life cycle involving a sexual stage that occurs in a mosquito vector and an asexual stage that occurs in the human host (Fig.2.1). For the malaria parasite to survive, it must have the ability to invade host cells.

The host gets infected with *P. falciparum* through the bite of an infected mosquito (female anopheles) when it is taking a blood meal from the host. During this process, the parasites in the salivary glands of the mosquito get injected into the skin and eventually find their way into the blood vessels and subsequently infect hepatocytes (Yamauchi *et al.*, 2007). After a silent infectious phase in the liver (hepatocytes), exo-erythrocytic merozoites are formed and subsequently find their way into the blood stream as membrane-bound merosomes that rupture, enabling the parasites to access circulating erythrocytes (Prudêncio *et al.*, 2011). The merozoites then rapidly invade erythrocytes within which they grow, multiply and cause a dramatic remodeling of the host red blood cells, conferring it with an ability to adhere to other cells. The parasites in the form of schizonts burst out after 48 hours and invade other healthy erythrocytes. These invasion events are often associated with severe pathology and morbidity in the host organism, culminating in symptoms such as fever, anaemia, lactic acidosis and coma (Miller *et al.*, 2002). The ability of *P. falciparum* to invade erythrocytes of all ages also serves as an important virulence factor and this occurs in a complex, multi-step process characterized by specific molecular interactions (Cowman *et al.*, 2006). After a number of invasion events, the malaria parasite develops into gametocytes and can be found circulating in the blood. A mosquito that comes to feed on the blood of an infected host then takes up these gametocytes in the form of male and female gametes.

With *P. falciparum*, the gametocytes are found in circulation between 7 to 15 days after initial infection of the host organism (Drakeley *et al.*, 2006). After the mosquito picks up the gametocytes, they subsequently develop into gametes and combine to form a zygote. The zygote then differentiates into ookinetes that penetrate the inner gut wall of the mosquito into

the outer gut wall, where they differentiate into the sporozoites. These sporozoites then migrate to the salivary glands of the mosquito, in preparation for another invasion event in a susceptible host.

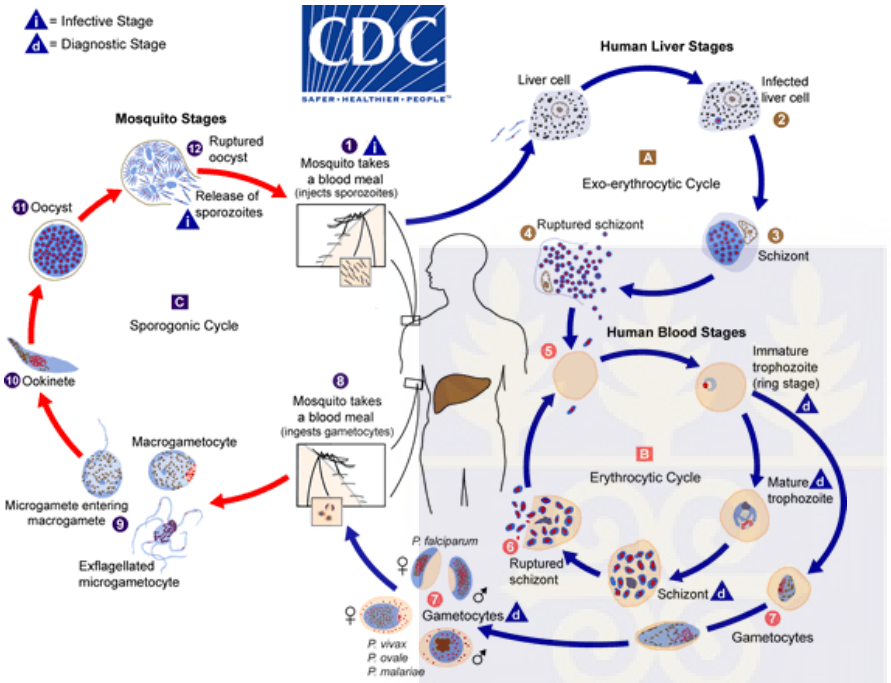


Figure 2. 1: Life cycle of *P. falciparum*
(Courtesy: the Center for Disease Control & Prevention, Division of Parasitic Disease).

2.1.3 The Burden of Malaria

Historically, malaria was found in almost every crevice of human habitats. However, through concerted efforts in malaria control programmes such as mass spraying, proper sanitation and the use of drugs, malaria was eliminated from many developed countries like the United States of America and Russia. Although some of these measures were also implemented in resource-limited settings, poor economic conditions have militated against the sustained implementation of these sound measures in malaria control (Arrow *et al.*, 2004, Sachs *et al.*, 2002).

This was further aggravated by the emergence of drug resistant parasites, insecticide resistance and relaxation of malaria control efforts (Alonso *et al.*, 2011, Feachem *et al.*, 2010, Rahmah *et al.*, 2015).

The incidence of malaria was reported to have decreased by 37% between 2000 and 2015 with a concomitant decline in malaria-associated deaths by 48% (WHO, 2015). However, malaria still remains one of the top four causes of deaths in children in sub-Saharan Africa, accounting for about 10% of child mortality (WHO, 2015). Thus, despite a general decline in malaria cases globally, malaria still remains a major problem in sub-Saharan Africa. In 2015, 3.2 billion people were estimated to be at risk of malaria infection with over 214 million malaria cases and about 438000 malaria-associated deaths globally. About 80% of these malaria-associated deaths were estimated to occur in sub-Saharan Africa and predominantly caused by *P. falciparum* (WHO, 2015). According to the National Malaria Control Programme of Ghana, malaria accounted for about 33 % of all OPD cases, and 49 % of hospital admissions for children below five years in 2014 (Ghana health service, 2015). The global decline in malaria cases is currently threatened by reports of drug-resistant strains to the first-line antimalarials (artemisinin and its combination therapy) (Ashley *et al.*, 2014). Although there are currently no reports of resistance to artemisinin-based drugs in Ghana yet (Kwansa-Bentum *et al.*, 2011), the emergence of artemisinin resistant parasites in other part of the globe (Dondorp *et al.*, 2012), calls for development of new set of antimalarials with novel mechanisms of action against resistant parasites.

2.2 CLASSES OF CURRENT ANTIMALARIAL DRUGS

The major classes of antimalarial drugs include those based on quinine (Chloroquine, mefloquine, amodiaquine and halofantrine) or other aminoquinolines (primaquine and tafenoquine), Antifolates (pyrimethamine, proguanil, cycloguanil, dapsone and sulfadoxine),

Artemisinin and its derivatives (artesunate, artemether/arteether, Coartem) and the hydroxynaphthoquinone atovaquone (Baniecki *et al.*, 2007, Bloland, 2001).

However, based on the stage-specific action, this antimalarials can also be broadly classified into five groups (Bruce-Chwatt, 1962).

Table 2.1: Summary of the groups of antimalarial drugs based on stage-specific activity.

Drug group	Stage-specific action	Drug types/Examples
Casual prophylactic	Pre-erythrocytic forms of *P. falciparum* and *P. vivax*	Proguanil and pyrimethamine
Schizonticidal drugs	Asexual erythrocytic stages of all species of malaria parasites	Quinine and 4-aminoquinolines (chloroquine, amodiaquine)
Gametocytocidal drugs	Active against malaria parasite gametocytes	8-aminoquinolines like primaquine
Sporontocidal drugs	Inhibit the sporogonic phase of the parasite development	Proguanil, chloroproguanil and pyrimethamine
Anti-relapse drugs	Active against exo-erythrocytic phase of *P.vivax* and *P. malariae*	Pamaquine, primaquine and quinocide

2.3 COMMON ANTIMALARIAL DRUGS AND THEIR MECHANISMS OF ACTION

2.3.1 Quinine

Quinine (structure in table A.3) was obtained from the bark of cinchona tree commonly found in South America and became prominent for the treatment of intermittent fevers worldwide. The mechanism of action of quinine is not clearly defined, but studies have shown that it inhibits the polymerization of heme and heme catalase activity (Ribeiro *et al.*, 1997, Slater, 1993). Quinine is still used to treat malaria despite reports of resistance to this compound between 1844 and 1910 (Talisuna *et al.*, 2004).

2.3.2 Chloroquine

Chloroquine (structure in table A.3) is a member of the class of antimalarial compounds known as the 4-amino quinolines. Chloroquine acts by accumulating in the digestive food vacuole of the parasite at high concentrations that inhibit heme detoxification. The digestive food vacuole of the parasite is acidic with a pH of about 4.7. Therefore when chloroquine enters the vacuole, it becomes protonated, which makes it difficult to diffuse out of the digestive food vacuole. Chloroquine then caps hemozoin molecules (a product from the breakdown of hemoglobin), inhibiting further biocrystallization of heme. The accumulation of heme and the formation of heme-chloroquine complex is toxic to the parasite and disrupts membrane function, resulting in cell lysis. Chloroquine also produces a secondary effect by inhibiting DNA and RNA biosynthesis and the degradation of ribosomes (Foote *et al.*, 1994). Chloroquine was first successfully synthesized in 1934 and together with DDT became the two vital tools recommended by the WHO's programme for eradicating malaria globally(Arrow *et al.*, 2004). However, resistant strains of *P. falciparum* to this drug were discovered on the Thai-Cambodian border in 1957 (Harinasuta *et al.*, 1965). Chloroquine-resistant *P. falciparum* strains were also later discovered in non-immune travelers who visited Kenya and Tanzania (Lobel *et al.*, 1985) as well as in places like Sudan, Uganda, Zambia and Malawi, confirming the wide-spread resistance to this drug.

2.3.3 Antifolates

Antifolate drugs (structures in table A.3) act by inhibiting the activity of two crucial enzymes used in folate synthesis by the malaria parasite, dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) (Nzila, 2006). These two enzymes play a crucial role in *de novo* folate biosynthesis (Fig. A.1).

Therefore, drugs that block the activity of enzymes in the folate metabolic pathway result in inhibition of the biosynthesis of pyrimidines (with a reduction in DNA, serine and methionine formation), purines and some essential amino acids. Antifolates can be grouped into two classes, Type-1 antifolates (sulfonamides and sulfones) which closely look like p-aminobenzoic acid (PABA) and Type-2 antifolates which include pyrimethamine, biguanides and triazine metabolites, quinazolines). Type-1 antifolates act by binding to the active site of dihydropteroate synthase and inhibiting the enzyme from catalyzing dihydroxymethyldidydropterin to form dihydropteroate. Type-2 antifolates act by inhibiting dihydrofolate synthase from reducing H_2folate to H_4folate (Saifi *et al.*, 2013).

2.3.4 Mefloquine

Mefloquine (structure in table A.3) is a 4-quinoline methanol that was developed by Walter Reed Army Institute of Research (WRAIR) with the goal of finding a new drug against chloroquine resistant plasmodium parasites. Mefloquine was said to be very effective against malaria caused by *P. falciparum* when it was taken effectively (Rieckmann *et al.*, 1974). Mefloquine interacts less strongly with free heme compared to chloroquine.

However, it has been shown to interfere with the polymerization of heme in *in vitro* assays, lower or similar to the effect seen with chloroquine (Slater, 1993). Resistant parasite strains against this pharmaceutical agent became more pronounced when the drug became very commonly used (Hoffman *et al.*, 1985).

2.3.5 Atovaquone

Atovaquone (structure in table A.3) belongs to the class of antimalarial compounds called hydroxynaphthoquinones. Atovaquone was produced and sold under the trade name Malarone that is composed of atovaquone and proguanil (Nixon *et al.*, 2013).

Atovaquone alone is believed to affect mitochondrial electron transfer chain, however, when it is combined with proguanil, it perturbs mitochondrial membrane potential (Baggish *et al.*, 2002). Atovaquone is a ubiquinone analog and when it binds to cytochrome *bc1* complex in the electron transport chain in the mitochondria of the malaria parasite, it disposes of electrons produced by the enzyme dihydroorotate dehydrogenase during the synthesis of pyrimidines, resulting in the death of the parasite (Hammond *et al.*, 1985).

2.3.6 Artemisinin

Artemisinin (structure in table A.3) is an antimalarial agent isolated from *Artemisia annua* (commonly known as sweet wormwood). The use of the sweet wormwood for medicinal purposes was known to Chinese herbalists over 2000 years ago as *qing-hao* (Klayman, 1985). It was discovered by some Chinese researchers who observed that an ethyl ether extraction of qing-hao was potent in clearing *Plasmodium berghei* in mice, with an efficacy similar to chloroquine and quinine. Shortly afterward, studies were conducted to determine the structure of the compound. It was later synthesized and new derivatives were developed and tested in clinical trials (Greenwood, 2002). Artemisinin and its derivatives are known as sesquiterpene lactones. They are considered prodrugs that quickly hydrolyse to dihydroartemisinin, (biologically active form) to generate reactive oxygen species (ROS) (Shandilya *et al.*, 2013). Although the exact mode of action of artemisinin is not known, this compound has been hypothesized to possess an endoperoxide bond that enables it interact with either iron or heme to produce free radicals that bind to heme to form a heme-artemisinin complex that interferes with the formation of hemozoin (Wang *et al.*, 2017, Lucantoni *et al.*, 2013, Shandilya *et al.*, 2013). Artemisinin and its derivatives are currently widely used to treat malaria globally, but the emergence of drug resistance against this drug in Western Cambodia highlights the need for new antimalarials with a novel mechanism of action (Ashley *et al.*, 2014, Dondorp *et al.*,

2012).

2.4 ANTIMALARIAL RESISTANCE AND MOLECULAR MARKERS OF DRUG RESISTANCE

2.4.1 Antimalarial Drug Resistance

Reports of chloroquine resistance emerged in the 1950s. However, chloroquine-resistant malaria parasites emerged in Africa two decades later and ever since, the malaria parasites have continuously developed resistance to almost all the chemotherapeutic agent including sulphadoxine-pyrimethamine (SP) (Bloland, 2001, Talisuna *et al.*, 2004, Cui *et al.*, 2015). The acquisition of mutations in various genes mediate resistance to different drugs in malaria parasites (Cowman *et al.*, 1994, Ashley *et al.*, 2014, Korsinczky *et al.*, 2004). This is largely caused by selective pressure from the abuse or extensive use of antimalarial drugs (Cui *et al.*, 2015). Antimalarial drug resistance has been associated with the risk of developing anemia, low birth weight, increased malaria transmission and epidemics as well as malarial-associated deaths (Arrow *et al.*, 2004). Drug resistance affects the efficacy of antimalarial drugs for malaria treatment. The levels of drug resistance vary from one geographical area to another, such that a drug that may be less efficacious at one place will be efficacious in other places (Bloland, 2001). Antimalarial drug resistance can be determined by observing the ability of the drug to clear parasitemia within a certain period of time *in vivo*. One other way of detecting drug resistance in the laboratory setting is through the use of *in vitro* assays that enable the assessment of how sensitive the malaria parasite is to the drug (Petersen *et al.*, 2011). The use of molecular techniques can also detect the genetic polymorphisms associated with drug resistance is useful in monitoring the emergence of drug resistance across the globe, and defining molecular mechanisms underpinning the resistance (Burrows *et al.*, 2014).

2.4.2 Molecular Markers of Drug Resistance

Advances made in the field of molecular and cell biology especially with the development of

polymerase chain reaction (PCR) has made it possible to easily probe the genome of an organism to rapidly detect and characterize genetic markers of drug resistance in *P. falciparum* (Alonso *et al.*, 2011).

The most common genes used as markers of antimalarial drug resistance include dihydrofolate reductase gene (*pfdhfr*) and the dihydropteroate synthase gene (*pfdhps*); which mediate resistance to the antifolate drugs, pyrimethamine and sulfadoxine; the *P. falciparum* chloroquine resistance transporter (*pfcrt*) which mediates resistance to chloroquine (Cooper *et al.*, 2005) and the *P. falciparum* multidrug resistance gene (*pfmdr*) that controls the level of resistance in some of the chloroquine-resistant parasites (Duraisingh *et al.*, 2005). Mutations in the *dhfr* gene at positions 108, 59, 51 and 164 have been associated with resistance to pyrimethamine (Wang *et al.*, 1997). Also, experiments conducted using *in vitro* assays has shown that *P. falciparum* parasites with point mutations in the *dhps* gene are less sensitive to sulfadoxine (Wang *et al.*, 1995).

Pfcrt

Chloroquine resistance has been associated with mutations in the *pfcrt* gene that encodes the PFCRT protein found on the digestive food vacuole of the malaria parasite. The *pfcrt* gene is found on chromosome 7 and has 13 exons (Fig. 2.2). This gene encodes the transmembrane protein PFCRT belonging to the drug metabolite transporter family and has 10 putative transmembrane domains found on the digestive food vacuole (Awasthi *et al.*, 2013). The mutation on this gene is a lysine that is non-synonymously substituted with threonine at codon position 76 (K76T) on chromosome 7 which is the main determinant of chloroquine sensitivity and resistance (Wellems *et al.*, 1991). There are mutations in other regions such as C72S, M74I, N75E and A220S that also influence chloroquine resistance in synergy with K76T. The mutations in *pfcrt* gene at codon positions 72-76 have been shown to influence higher levels

of resistance to chloroquine compared to amodiaquine in Southeast Asia and Africa, but a higher level of resistance to amodiaquine in South America (Reed *et al.*, 2000).

This makes the K76T mutation an important molecular marker of chloroquine resistance and is widely used to monitor chloroquine resistance globally.

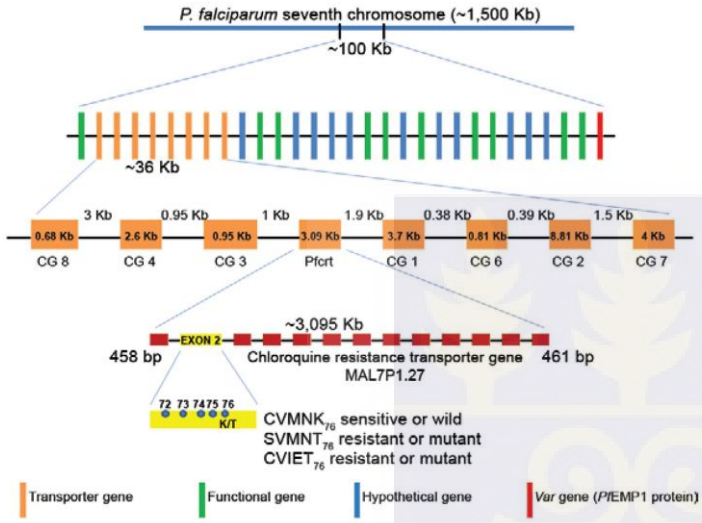


Figure 2. 2: Schematic view of the *pfcrt* gene with its 13 exons. The K76T mutation is found within ~100 Kb segment in chromosome 7 of *Plasmodium falciparum* genome. (Courtesy: (Awasthi *et al.*, 2013)).

Pfmdr1

Studies from *in vitro* assays suggest the level of hloroquine resistance is influenced by mutations in the *P. falciparum* multidrug resistance gene (*pfmdr*) that codes for the transmembrane protein P-glycoprotein homolog 1 (Pgh1) (Chauhan *et al.*, 2014). The *pfmdr1* gene has one exon found on chromosome 5 (Fig. 2.3), which encodes the transmembrane protein, P-glycoprotein homolog 1. P-glycoprotein homolog 1 has two helical transmembrane

domains and a nucleotide-binding fold serving as ATP binding site (Reiling *et al.*, 2015). Mutations at codon positions N86Y, Y184F, S1034C, N1042D and D1246Y as well as copy number variations of *pfmdr1* are associated with the emergence of multi-drug resistance.

The presence of these mutations also influences the levels of resistance to drugs like quinine, halofantrine, lumefantrine and artemisinin (Duraisingh *et al.*, 2005).

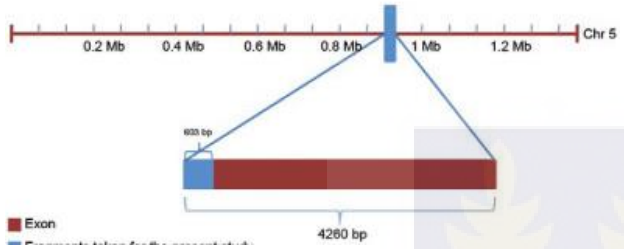


Figure 2. 3 Schematic view of the *pfmdr1* gene in chromosome 5.
(Courtesy: (Sutar *et al.*, 2011))

Pfmrp

P. falciparum multidrug resistance-associated protein (*pfmrp*) gene encodes a protein that aids the transport of organic anionic substrate like oxidized glutathione, glucuronate, sulfate conjugate and drugs (Briolant *et al.*, 2010). *pfmrp* is thought to influence the levels of resistance to chloroquine, quinine, primaquine, piperaquine and artemisinin, but not directly determining drug resistance. The gene encoding this protein is a member of the ABC transporter family and has one exon found on chromosome one. Studies have shown that parasites with mutations at codon position Y191H and A437S in *pfmrp* are less sensitive to chloroquine, quinine, primaquine, piperquine and artemisinin, compared to the wild-type parasite (Mu *et al.*, 2003, Ursing *et al.*, 2006). Raj *et al.* (2009) studied the function of PFMRP protein by knocking out the gene encoding for this protein using parasites that are sensitive and resistant to chloroquine,

quinine, primaquine, piperaquine and artemisinin.

They discovered that the resistant parasites also became highly sensitive to the drugs, but then the already sensitive ones had even higher sensitivities to chloroquine and quinine (Raj *et al.*, 2009). Therefore, mutations in this gene are also used to monitor drug resistance.

Pfdhfr-ts

P. falciparum dihydrofolate reductase (PFDHFR) is a protein that is encoded by the *pfdhfr-ts* gene found on chromosome 4 with one exon (Fowler *et al.*, 2006). This enzyme plays a dual role by aiding the thymidylate synthase to synthesize dTMP and dihydrofolate reductase to reduce dihydrofolate to tetrahydrofolate (Hyde, 2009). Antifolate drugs such as pyrimethamine and cycloguanil act by inhibiting the activity of *pfdhfr* (Fig.A.1) which is crucial for generating pyrimidine that serves as a precursor for replicating DNA (Foote *et al.*, 1994). A point mutation at codon position S108D on the *pfdhfr* protein has been shown to be responsible for pyrimethamine resistance and mutations at N51I, C59N and I164L control the level of resistance to pyrimethamine (Sirawaraporn *et al.*, 1997). Also, mutations at codon positions A16V and S108T in *pfdhr* is associated with *P. falciparum* resistance to cycloguanil (Galindo *et al.*, 2010). Thus, the presence of this mutations in the *pfdhfr* genes is used to monitor drug resistance.

Pfdhps

The *P. falciparum* dihydropteroate synthase (PFDHPS) is an enzyme encoded by three exons in the *pfdhps* gene on chromosome eight. This enzyme catalyzes a reaction involving p-aminobenzoic acid to form dihydrofolate which is a precursor for the synthesis of pyrimidine (Fig.A.1) used for DNA replication (Gregson *et al.*, 2005). Point mutations in the *pfdhps* enzyme at codon positions S436A/F, A437G, L540E, A581G and A613T/S) have been shown

to modulate the level of resistance to sulfadoxine in *P. falciparum* (Galindo *et al.*, 2010).The use of sulfadoxine as a monotherapy resulted in the emergence of resistant parasites against this drug, which led to the development of a combination therapy involving sulfadoxine and pyrimethamine (SP). However, shortly after the introduction of SP combination therapy, *P. falciparum* isolates developed resistance to SP, and this was linked to mutations in the *pfdhfr* and *pfdhps* genes respectively (Galindo *et al.*, 2010, Gresty *et al.*, 2014).

bc1

Atovaquone acts by competitively binding to ubiquinol binding site on cytochrome b (*cytb),* which interferes with the electrochemical potential of the membrane of the mitochondrion and resulting in the death of the parasite (Mather *et al.*, 2005). Point mutations at codon position Y268N/S/C in the ubiquinol binding site (highly conserved region) of *cytb* gene has been shown to be responsible for atovaquone resistance in clinical isolates of *P. falciparum* (Korsinczky *et al.*, 2000, Reed *et al.*, 2000). Therefore, these mutations are used to detect resistance to atovaquone.

Kelch 13

Studies have identified non-synonymous mutations in the kelch repeat region of the *K13* propeller domain at codon positions Y493H, R539T, I543T and C580Y to be associated with less sensitivity of *P. falciparum* to artemisinin in both laboratory and clinical isolates (Ariey *et al.*, 2014). This makes the use of polymorphisms in the *K13* propeller gene a crucial molecular marker for monitoring emergence and spread of resistance of strains of *P. falciparum* to artemisinin.

2.5 EARLY TREATMENT AS A TOOL FOR MALARIA CONTROL

Malaria parasites in infected erythrocytes are able to proliferate at a rate of about 6 to 20 parasites per cycle with an efficiency of about 30 to 90 %. However, in the presence of an efficacious anti-malarial agent, parasitemia can be reduced by 100 to 10000-fold in each cycle at their maximal effect (White, 1999, White, 1997). Therefore, early and effective administration of antimalarial chemotherapy helps control the replication of the parasite and minimizes the effects of clinical complications associated with malaria. This is very crucial in our quest to control malaria transmission and reduce malarial-associated mortality and morbidity.

2.6 ARTEMISININ AND ITS COMBINATION THERAPY

In the past five decades, very few new drugs have been developed for the treatment of malaria including artemisinin and its combination therapies (ACT) (Alonso *et al.*, 2011). ACTs were adopted as the first-line antimalarial therapy against blood stage malaria infections in an effort to curtail the emergence of drug resistance (WHO, 2015). The concept behind the combination therapy is to couple a fast acting drug like artemisinin that has a short half-life with a moderate or slow acting drug with a longer half-life to clear the remaining parasites (Cui *et al.*, 2009). The high efficacy and good tolerance properties of the ACTs have been a tremendous contribution to the fall in malaria cases and malaria-associated deaths globally (Arrow *et al.*, 2004). Irrespective of reports of decreasing sensitivities of *P. falciparum* to ACTs in South-East Asia, artesunate-amodiaquine remains effective in the treatment of malaria in Africa where it is used as the first-line treatment for malaria. The world Malaria Report has reported a treatment failure of less than 10% in African countries where this drug is currently used as first or second-line drug for the treatment of malaria (WHO, 2015).

Some studies have identified malaria parasites from Ghana with mutations mediating artemisinin drug resistance, however, ACTs (artesunate-amodiaquine) are still effective for treating malaria in Ghana (Kamau *et al.*, 2015, Kwansa-Bentum *et al.*, 2011). With reports of the emergence of drug-resistant strains against artemisinin in Western Cambodia (Ashley *et al.*, 2014), there is the need to continue the hunt for new antimalarials that target all stages of the parasite to counter and suppress the emergence of drug-resistant strains.

2.7 VACCINES AS TOOLS FOR MALARIA CONTROL

Vaccines have been described as offering a cost-effective and high-impact means of controlling infectious diseases. For decades now, vaccines have been used to control infectious disease agents like smallpox, measles, diphtheria and poliomyelitis (Rogo *et al.*, 2006). Although several efforts and investments have gone into the development of an efficacious malaria vaccine, the most promising one so far has been the RTS,S/AS01. This vaccine targets the circumsporozoite proteins of invading *P. falciparum* at the pre-erythrocytic stage (Gosling *et al.*, 2016). Although clinical trials show a substantial reduction in clinical malaria cases, the RTS,S/AS01 vaccine is less than 50% efficient and it is not able to offer a whole year protection (Rts, 2014). Studies focused on the development of an erythrocytic stage vaccine have also not yielded fruitful outcomes. This has largely been attributed to the fact that we do not have a complete understanding of the invasion process coupled with a complex life cycle of the parasite. Also, the ability of the malaria parasite to selectively switch invasion pathways using several invasion proteins to distract the host immune system, have all contributed to the challenges associated with the development of effective malaria vaccine.

.

2.8 NEW PARTNERSHIPS FOR MALARIA DRUG DEVELOPMENTS AND THE "MALARIA BOX" COMPOUNDS

Pharmaceutical companies play a crucial role in the research and development of new drugs. Since developing new antimalarials require a lot of investment, the pharmaceutical companies that have the financial clout to pursue new drug discovery programmes for tropical diseases like malaria have demonstrated little interest. This is largely hinged on the fact that areas with high burden of malaria are often found in resource-limited regions with several economic challenges, which make such economies unattractive to merit the risk of making such huge investment with the expectation of little returns from the investments (Veeken *et al.*, 2000). Despite these challenges there is still a need to develop new antimalarial drugs with novel mechanisms of action that can control drug resistant-parasites in endemic regions. A lot of efforts are already being made towards the development of new highly efficacious antimalarial drugs that are affordable, accessible, and safe for treating malaria in endemics areas in the tropics. New collaborations involving some pharmaceutical companies like Novartis and public institutions such as Medicine for Malaria Vaccine (MMV) are currently advancing the search for new antimalarial drugs. To accelerate the research and development of new antimalarial drugs for endemic regions, the Medicines for Malaria Venture (MMV) collaborated with Novartis, St. Jude Children's Research Hospital and GlaxoSmithKline (GSK) to undertake a high-throughput screening of more than 4 million compounds. About 20,000 of these compounds were found to possess blood-stage antimalarial activity in *in vitro* drug assays (Spangenberg *et al.*, 2013). In order to facilitate further exploration on these compounds, MMV assembled a set of 400 unique compounds from the 20,000 hits called the "Malaria Box". The Malaria Box represents the chemical diversity of the 20,000 hit compounds, comprising 200 drug-like and 200-probe-like compounds. The Malaria Box concept allows biologists to participate in the drug discovery process by exploring the targets and mechanisms of action of these compounds without the need to resynthesize them.

Work done so far on these compounds has largely been based on the use of laboratory-adapted strains of *P. falciparum* and not much has been reported on their potency against clinical isolates. To get a better picture of the potency of these compounds for developing new sets of antimalarial drugs for endemic regions, it will be important to investigate the activity of these compounds on field isolates, as well as determine if the data differ significantly from that of laboratory-adapted strains.

2.9 SCREENING FOR NEW ANTIMALARIAL COMPOUNDS

The discovery of new antimalarial compounds with novel mechanisms of action is a very vital step for the development of new antimalarial drugs to counter drug-resistant parasites. Antimalarial drug efficacies can be tested using *in vivo, in vitro*, animal models studies and molecular markers. High throughput screening of a large number of compounds requires initial *in vitro* testing followed by an animal testing *in vivo* studies (Baniecki *et al.*, 2007). *In vitro* assays are initially performed using laboratory strains of *P. falciparum*. These assays qualify potent compounds for further testing and development based on their concentrations required to inhibit 50 % of the parasite growth (IC_{50}). Screening of structurally diverse small molecules libraries against *P. falciparum* has been achieved using various approaches involving microscopy-based assays, cell-based assays or biochemical assays (Hay *et al.*, 2009).

2.9.1 Microscopy-Based Assays

One of the ways of screening for antimalarial activity of a chemical compound against the malaria parasite is to grow the parasite in different concentrations of the drug in a multi-well plate and then compare parasitemia levels in wells with the compound to wells without the compound, using Giemsa-stained slides (Woodrow *et al.*, 2015).

However, this approach is laborious and does not provide the platform for high-throughput analysis with several compounds at a time. Another challenge of using microscopy is that the outcome may vary from person to person.

2.9.2 Biochemical-Based Assays

Hypoxanthine Uptake-Based Assay

Hypoxanthine-uptake based assay is a biochemical assay that was introduced for high-throughput analysis (Abiodun *et al.*, 2010). Plasmodium parasites are unable to synthesize purines *de novo* and therefore salvage these metabolic precursors from the host for their growth and development (Arnold *et al.*, 2016). Therefore, adding hypoxanthine to the parasite culture media makes it possible for the parasites to incorporate this radiolabeled purine into their nucleic acid, which can then be detected by radioactivity (Desjardins *et al.*, 1979). This assay allows for faster analysis with reduced variability of the outcome and is suitable for screening of a large number of compounds at a time. However, the use of radioactive material and the high cost of equipment limits the use of this method (Noedl *et al.*, 2002).

P. falciparum Lactate Dehydrogenase (PfLDH)-Based Assay

Based on the disadvantages of the hypoxanthine uptake assay, new assays that do not involve the use of radioactive material such as the lactate dehydrogenase enzyme-based assay were introduced (Penna-Coutinho *et al.*, 2011). The malaria parasite lactate dehydrogenase (pLDH) is an enzyme involved in the glycolysis pathway and can be used to determine the viability of the parasite. In the pLDH assay, pLDH levels are measured in a double-site enzyme-linked LDH immunodetection assay using monoclonal antibodies that are specific for pLDH (Druilhe *et al.*, 2001).

Histidine-Rich Protein 2-Based Assay

Another non-radioactive assay involves the use of Histidine-Rich Protein 2 (HRP2) to detect parasitemia levels in drug assays. *P. falciparum* produces histidine and alanine-rich proteins in the course of its proliferation and development. The levels of histidine-rich protein 2 (HRP2) correspond with levels of parasitemia and can be measured using ELISA. The advantage of this assay is that it is suitable for testing slow-acting drugs (Noedl *et al.*, 2002, Noedl *et al.*, 2003).

2.9. 3 Cell-Based Assays

Although biochemical assays can be alternatively used for the initial screening of potential drugs, its use is limited by the fact that the compounds may have intricate interactions with the organism at different levels (Flannery *et al.*, 2013). Therefore, biochemical assays may not accurately reflect the complex interactions between the compound and the organism. This problem has prioritized the use of cell-based assays that more accurately represent the response of an organism to the compounds being screened. In cell-based assays, the malaria parasite in culture is incubated with the test compound for a period and the efficacy of the compound is evaluated for its ability to inhibit parasite growth and development. Though this approach might appear simple, the challenge here is to obtain reproducible data and to be able to conduct the assay for a large number of compounds in very a cost-effective manner using very small test volumes (Flannery *et al.*, 2013). Cell-based assays also represent invaluable tools for evaluating the toxicity of a compound on the organism. Technological advances in developing miniaturized cell-based assays and improved signal detection and processing techniques has made it relatively easier and faster to conduct dose-response experiments on several compounds in 384 or 1536 well assay plates during primary screens (Riss, 2005).

High-throughput screens using cell-based assays has aided in the discovery of hundreds of compounds which were not known in the past five years (Mokgethi-Morule *et al.*, 2016). These compounds have the potential of being developed as antimalarial drugs for endemic regions.

SyBr-Green 1-Based Assay

Flow cytometry has facilitated high-throughput screening of several antimalarial compounds using DNA-binding fluorescent dyes such as SyBr Green 1 that stain the parasite's DNA (Bennett *et al.*, 2004). In this assay, the parasites are incubated under different concentrations of drug and the assay is subsequently stopped at either 48 hours or 72 hours. SyBr Green 1 is added to each well in the assay which stains the parasites DNA. Given the fact that the erythrocytes do not contain DNA, the DNA dye stains only the parasite's DNA and the flow cytometer can be used to determine the level of fluorescence from the DNA dyes that corresponds to the parasitemia. Assays that require the use of flow cytometry are fast, require fewer sample volumes and produce more objective data with less background (Woodrow *et al.*, 2015). However, the cost of the flow cytometer and running cost limits its use (Karl *et al.*, 2009).

2.10 KEY DRUG TARGETS

Some of the well-known chemically validated antimalarial drug targets include the dihydrofolate reductase (DHFR) and *bc1* that are inhibited by the antifolates (pyrimethamine and proguanil) and atovaquone respectively. Because inhibition of DHFR is selective for parasite enzyme over the human enzyme, this enzyme has been employed in *in silico* analysis involving the co-crystal structures of DHFR-substrate complexes to identify potential antimalarial compounds that bind wild-type DHFR and the mutated forms (Sirawaraporn *et al.*, 1997, Yuthavong *et al.*, 2012).

In silico analysis was also employed in the design of P218 (a diaminopyrimidine) which is specific for *Plasmodium* DHFR with concentrations in the nanomolar range and binds both wild-type and mutant DHFR at the same active site as the natural substrate (Yuthavong *et al.*, 2012). One other important target is dihydroorotate dehydrogenase (DHOD) which is a mitochondrial enzyme involved in the *de novo* synthesis of pyrimidines and a major mitochondrial electron transport chain for intraerythrocytic malaria parasites. A high-throughput screen conducted in 2005 against recombinant DHOD identified a new chemical class called triazolopyridines which had greater than 5000 fold specificity for *P. falciparum* DHOD over that of the human DHOD (Baldwin *et al*., 2005, Gujjar *et al.*, 2009). Carboxamides is another class of chemical agents that were identified to inhibit DHOD activity in a high-throughput screening involving a recombinant DHOD and *P. falciparum, P. berghei* and *P.vivax* (Booker *et al*., 2010, Patel *et al*., 2008). Although triazolopyridines and carboxamides both target DHOD, structural analysis on their binding pockets suggest that these two chemical classes bind to overlapping and distinct sites on DHOD (Patel *et al.*, 2008). The carboxy terminal of myosin A and the myosin tail interacting protein (MTIP) of the malaria parasite have also been identified as new drug targets (Patel *et al.*, 2008). The interactions between the carboxy-terminal tail of myosin A and the myosin tail interacting protein are important for erythrocyte invasion. Therefore inhibitors targeting these proteins will stalk invasion and proliferation.

2.11 DRUG CANDIDATES FROM BLOOD STAGE SCREENS

Blood stage screens take advantage of the fact that human erythrocytes do not have nuclei, to identify new drug candidates. In blood stage screens, the ability of malaria parasites with nuclei to grow in the presence of the chemical compound can be assessed.

The level of nucleic acid after the incubation period correlates with parasitemia levels and a read-out can be obtained using fluorescent DNA-binding dyes (SYBR Green 1) to evaluate the effects of the chemical agent on the growth of the parasite (Fidock *et al.*, 2004). *P. falciparum* lactate dehydrogenase may also be used to evaluate the effect of the chemical agent on the development of the parasite. Based on these approaches, more than 4 million chemicals have been screened with about 0.4% to 1% of the compounds demonstrating blood-stage activity against *P. falciparum* (Gamo *et al.*, 2010). The Genomics Institute of Novartis Research Foundation (GNF), GlaxoSmithKline (GSK) and the St. Jude Children's Research Hospital (SJCRH) have conducted many high-throughput screens in 384 or 1536-well format plate and have identified more than 20,000 of the hit compounds to have antimalarial activity (Guiguemde *et al.*, 2010, Plouffe *et al.*, 2008). KAE609 (structure in table A.3) is one of the most promising compounds that was discovered in the screening of about 10,000 natural compounds against blood-stage *P. falciparum* (Rottmann *et al.*, 2011). This compound belongs to a class of compounds called spiroindolones, which have been identified to be very potent against blood stage *P. falciparum* and *P. vivax* clinical isolates at low nanomolar concentrations. The mechanism of action of spiroindolones is novel and they target the *P. falciparum* outer membrane transporter, PfATP4. PfATP4 was previously thought to be a calcium pump but has been recently identified to be crucial for maintaining sodium homeostasis in the parasite. KAE609 is one of the first drug candidates to enter Phase II clinical trials in 20 years (Spillman *et al.*, 2013). GSK in their screening efforts has also identified compounds with 47 different scaffolds that were selected for Structural-Activity Relationship (SAR) analysis and lead identification (Calderón *et al.*, 2011). One compound from these screens (TCMDC-139046) (structure in table A.3) with a scaffold containing an indoline core has been found to be active against *P. falciparum*. However, the indoline core is known to inhibit serotonin receptors in humans, which seems to limit the use of this drug agent as a

potential antimalarial, because of the possible side effect. However double-divergent structure-activity relationship analysis is being conducted to modify these compounds and improve their efficacy and selectivity against *P. falciparum* with reduced activity against the human receptor (Calderón *et al.*, 2012).

2.12 NEW ANTIMALARIAL DRUG TARGET DISCOVERY

New antimalarial therapeutics can be designed to target proteins and pathways that are germane to the parasite's development. Developing progeny parasite clones that are resistant to a particular compound and comparing their genome to that of the sensitive parent can be useful in the discovery new drug targets (Dharia *et al.*, 2009, Meister *et al.*, 2012). This approach has been used in the discovery of targets such as PfATP4, *P. falciparum* cyclic amine resistance locus (PfCARL) and lysyl tRNA synthetase that are currently being screened as potential antimalarial agents for malaria control. The protein biosynthetic pathway in malaria parasites also offers a rich source of novel antimalarial drug targets. Three tRNA sythetases have been chemically validated as targets in the protein biosynthetic pathway (Crowther *et al.*, 2011). The metabolism of hemoglobin and release of heme in *P. falciparum* results in the generation of reactive oxygen species such superoxide anions, increasing the oxidative stress and eventually death of the parasite. The glutathione and thioredoxin systems provide a way for the parasite to maintain redox homeostasis and antioxidant defense, given the fact that the *P. falciparum* lacks glutathione peroxidase and catalase (Jortzik *et al.*, 2012). Studies using genetic and chemical tools have shown that thioredoxin reductase and glutathione are crucial for parasite survival (Müller, 2003). Thus *P. falciparum* thioredoxine reductase appears to be a novel antimalarial drug target.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 DRUG ASSAY OPTIMIZATION AND DETERMINATION OF POTENCY OF COMPOUND X

3.1.1 *P. falciparum* Culture

P.falciparum-infected erythrocytes of Dd2 and 7G8 were maintained at 37ºC *in vitro* in a plastic culture flask at pH 7.4 using human group O+ erythrocytes at 2-4 % hematocrit in complete parasite media (CPM). The media contains RPMI 1640 (Sigma Aldrich) with 5 mg/mL Albumax II (Gibco), 10 µg/mL gentamycin, 0.2 µg/mL hypoxanthine, 2 mM L-glutamine, 25 mM HEPES, 23.8 mM NaHCO3 supplemented with 2% inactivated human AB+ serum. A gas mixture of 94% nitrogen, 5% CO_2, 1% oxygen was bubbled through the media for 1-3 minutes.

3.1.2 Drug Assay Culture Preparation

The parasitemia of each malaria parasite was determined by counting a total of 500 infected and uninfected red cells under a light microscope. The percentage parasitemia was then estimated as: (Number of infected cells/ total number of cell count)*100.

P. falciparum Dd2 and 7G8 parasite cultures were then synchronized using 5% D-Sorbitol (Sigma-Aldrich). In the synchronization step, 5 ml of the parasite cultures were first transferred into 15 ml falcon tubes and centrifuged at 1500 rpm for 5 minutes. The supernatant was then discarded and 5 ml of sorbitol was added. The mixture was then incubated for 10 minutes, followed by centrifugation at 1500 rpm. The supernatant was then discarded followed by three washes with RPMI.

After the synchronization step, a parasite culture of 1 % parasitemia and 2 % hematocrit was prepared by diluting the synchronized rings parasites with packed uninfected erythrocytes and complete parasite media (CPM).

Uninfected erythrocytes at 2% hematocrit were also prepared for use as background control. Since Compound X was dissolved in 100% concentration of Dimethyl sulfoxide (DMSO) (Sigma Life Science), a final working concentration of 10 % DMSO in RPMI corresponding to the level of DMSO in the 100 µM dilution was prepared for use as the negative control.

3.1.3 Compound X Preparation and Serial Dilutions

Compound X was an unknown anti-plasmodial drug obtained from Dr. Yaw Aniweh (Nanyang Technological University, Singapore). A stock solution of 10 mM was prepared by dissolving 0.519 mg of the compound in 2 ml of 100% DMSO. For each assay, 20 µl of the stock solution was aliquoted and added to 180 µl of RPMI to obtain a working concentration of 1mM. The rest of the serial dilutions (from 750 µM to 15.3 µM) were conducted using RPMI containing 10% DMSO. These serial dilutions provided final well concentrations from 100 µM to 1.53 µM containing 1% DMSO.

3.1.4 Growth Inhibition Assay

The drug assay was conducted in a 96 well plate, by aliquoting 90 µL of the parasite culture containing 1% parasitemia and 2% hematocrit in triplicate wells. 10 µL of the compound was then added by pipetting up and down several times to ensure a uniform mixture of the compound with the culture. A portion of the parasite media (10 µL) containing RPMI with 10% DMSO was added to the DMSO control wells and 10 µL of RPMI media was added to the NO COMPOUND control wells. The background control (uninfected erythrocytes) wells were filled with 100 µL of uninfected erythrocytes at 2% hematocrit.

In order to control evaporative loss, 200 µL of sterile PBS was added to the surrounding outer wells. The plates were bagged and sealed in an airtight transparent plastic bags with mixed blood gas (94% nitrogen, 5% CO_2, 1% oxygen).

The bagged plates were incubated for 48 hours at 37°C. A summary of the steps in conducting the drug assay is shown in figure 3.1.

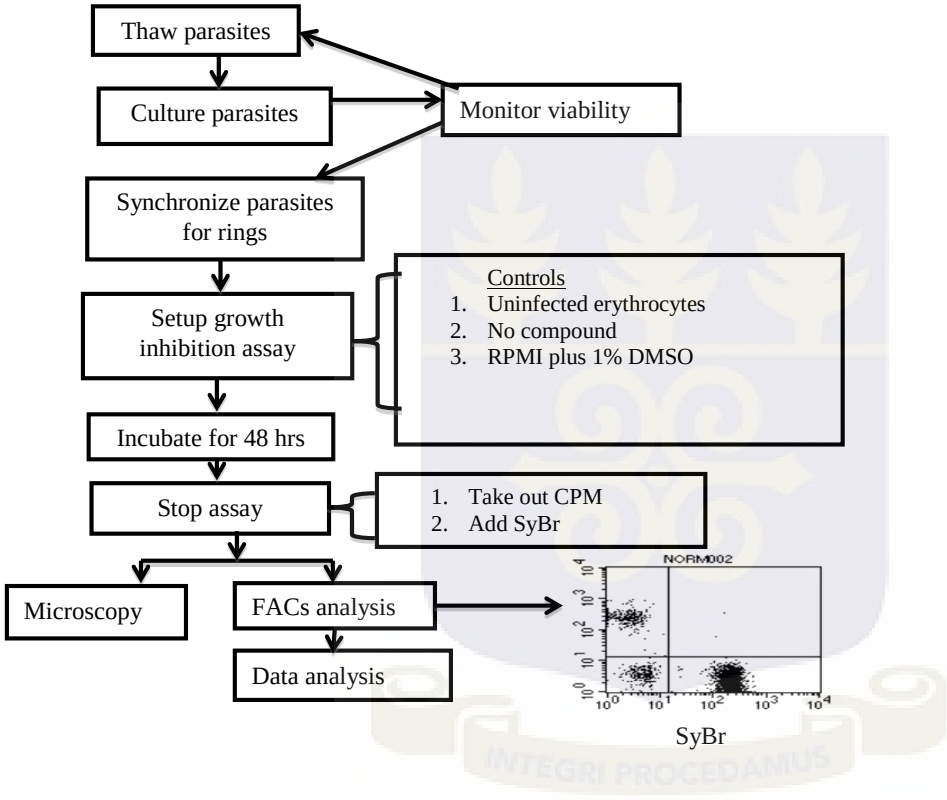


Figure 3. 1:An experimental flow chart for the growth inhibition assay and analysis.

The growth inhibition assay was conducted using sorbitol-synchronized rings and different concentration of the compound. Uninfected erythrocytes were used as a background control, RPMI was used as negative control and RPMI plus 1% DMSO was used as DMSO control. SyBr Green 1 fluorescent dye was used to stain the parasite DNA. Flow cytometry was used to determine the mean parasitemia levels. Data was analysed using GraphPad prism. Microscopy was used to monitor the assay for possible contaminations.

3.1.5 Determination of Mean Parasitemia levels Using Flow Cytometry

After 48 hours of incubation, 80 µL of the CPM was taken out of the assay wells and replaced with an equal volume of SYBR Green I (Invitrogen). The assay plates were then incubated for 30 minutes at 37 ºC. Prior to the flow cytometry analysis, 10 µL of the culture sample from each well was added to 500 µL of FACS sheath solution (BD Biosciences) in FACS tubes. The parasitemia corresponding to each culture well were then determined using the BD FACS LSRFortessa flow cytometer. The blue laser of the flow cytometer excited the SYBR Green I fluorescent dye that stained the parasite DNA and the fluorescence signal was detected by a 530/30 filter. The forward and side scatter parameters were used to select erythrocytes. The BD FACSDiva Software (v8.0.1) then used to determine the mean parasitemia corresponding with infected erythrocytes that are stained with SyBr Green 1 for 50,000 cells per well.

3.1.6 Determination of Inhibitory Concentrations

In order to evaluate the potency of the compounds, sigmoidal dose-response curves were first generated with GraphPad Prism (Version 6.01), using mean parasitemia values from DMSO control wells as the negative control. The 50% inhibitory concentration (IC_{50}) values were then estimated from the dose response curves. The IC_{50} values correspond to the concentration of the compound at which the parasitaemia is reduced by half. Closely reproducible IC_{50} values were used to qualify the assay as optimal. The potency of Compound X against Dd2 and 7G8 was assessed by comparing the IC_{50} values based on criteria used by other studies for classifying the antiplasmodial activity of a compound. The inhibitory concentrations of the compounds against parasites was presented as IC_{50} values with 95% confidence interval.

3.2 SCREENING OF THE MALARIA BOX COMPOUNDS AGAINST CLINICAL ISOLATES

3.2.1 *P. falciparum* Clinical Isolates

Four isolates (EIMK239, EIMN093, EIMA156 and EIMA160) were randomly selected from archived samples stored in liquid nitrogen at the Noguchi Memorial Institute for Medical Research. The samples were collected from Accra, Navrongo and Kintampo as part of an ongoing study on the erythrocyte invasion mechanism. The parasites were thawed and maintained in culture as described for the laboratory-adapted strains.

3.2.2 Selection and Preparation of Malaria Box Compounds

The library of 400 Malaria Box compounds was provided by Medicines for Malaria Venture (MMV) at concentrations of 10 mM in dimethyl sulfoxide (DMSO) in 384-well microtiter plates (Spangenberg *et al.*, 2013). A total of 10 compounds out of the 400 Malaria Box compounds were selected for screening against the clinical isolates (N093, A156, A160 and K239). These included two compounds (MMV000753, MMV007384) which were identified as Beta-hematin inhibitors (Fong *et al.*, 2015), five compounds with activity against thioredoxin reductase (MMV006278, MMV085203, MMV008956, MMV396797, MMV008416) (Tiwari *et al.*, 2016a) and the last three compounds (MMV009015, MMV019555, MMV006787) which were identified to be active against the Dd2 strain of *P. falciparum* (Ullah *et al.*, 2016). Working concentrations of the compounds were prepared from 10 mM to 1 mM using RPMI and subsequently from 50 µM to 0.00064 µM with RPMI containing 2.5% DMSO. The parasite culture (90 µL) was then incubated with 10 µl of the compound (25 µM to 0.064 nM) in 96-well plate at 37^{o}C.

A portion of the parasite media (80 µL) was then taken out and replaced with equal volume of SyBr Green 1 and incubated at 37 ºC for 30 minutes.

The mean parasitemia was determined by flow cytometry using the FACDiva software. The background effect (RBCs control) was subtracted from the mean parasitemia values of both treated and untreated wells. GraphPad Prism was then employed in plotting the mean parasitemia levels expressed as percentages against the concentrations tested. GraphPad Prism was also used to generate sigmoid dose-response curves and to estimate the IC_{50} values for each of the compounds against the clinical isolates. The mean parasitemia values from wells with RPMI containing 0.25% DMSO was used as negative control. The IC_{50} values provided a way to measure the potency of the compounds against the clinical isolates.

3.3 SCREENING OF CHLOROQUINE AND ARTESUNATE AGAINST THE CLINICAL ISOLATES

3.3.1 Evaluation of the Potency of Chloroquine on the Clinical Isolates

A stock concentration of 10 mM solution of chloroquine (WRAIR Inventory Lab) was prepared by dissolving 3.199 mg of chloroquine powder in 1 mL sterile distilled water. This was further diluted serially with sterile distilled water to obtain working concentrations of 500 µM to 0.00064 µM (1:5 dilution).The diluted chloroquine solution was screened against the four clinical isolates at final well concentrations between 25 µM to 0.0064 nM.

The assay was conducted using a similar approach as was described for screening the clinical isolates against the Malaria Box compounds. The negative control was distilled water in place of chloroquine.

Sigmoid dose-response curves were plotted in GraphPad Prism after determining the mean parasitemia levels with wells containing distilled water as the negative control. IC_{50} values were then determined from the dose-response graphs for chloroquine against each of the

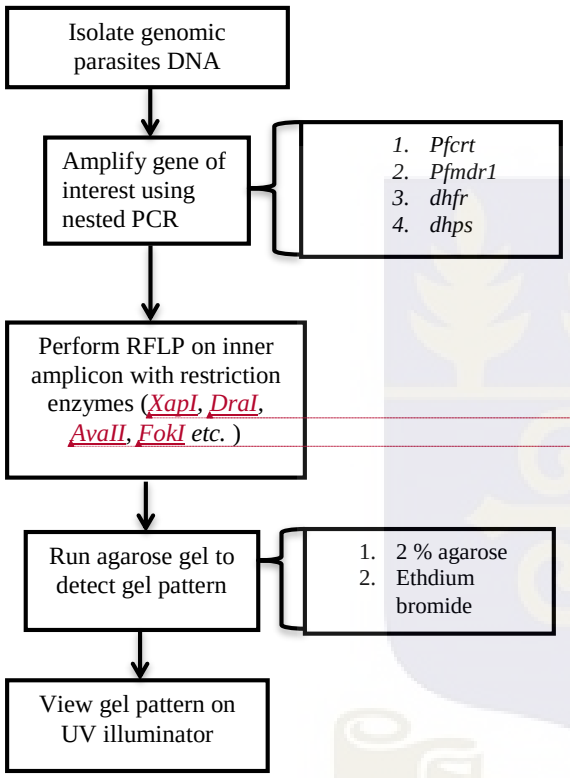
clinical isolates. The potency of chloroquine against the clinical isolates was determined by comparing the IC_{50} values obtained to reference IC_{50} values.

3.3.2 Evaluation of the Potency of Artesunate on the Clinical Isolates

A stock concentration of 1 mM artesunate (Sigma) was prepared by dissolving 0.384 mg of artesunate powder in 1 mL DMSO (100%). A 1:5 dilution was used to obtain working concentrations from 1 mM to 1.6 µM using RPMI and then serially diluted further using RPMI containing 1% DMSO to obtain concentrations from 0.32 µM to 0.02048 nM. The screening of artesunate against the clinical isolates was conducted using a similar approach as was used for screening of the Malaria Box compounds. The final well concentrations of artesunate were from 32 nM to 0.002048 nM and the negative control well had 0.1 % final DMSO concentration.

3.4 GENOTYPING OF CLINICAL ISOLATES USING RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

RFLP was employed in detecting mutations in four genes, *Pfcrt*, *Pfmdr1*, *Pfdhfr* and *Pfdhps* that mediate resistance to chloroquine and antifolate drugs. The procedure is summarized in Fig. 3.2.



Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt
Formatted: Font: 12 pt

Figure 3. 2: Experimental flow chart for the restriction fragment length polymorphism (RFLP). A nested polymerase chain reaction (PCR) was used to amplify genes of interest (*pfcrt, pfmdr1, dhfr* and *dhps*). The restriction enzymes were used to digest the genes of interest in order to detect single nucleotide variations that serve as molecular markers of drug resistance using restriction fragment polymorphism (RFLP) analysis. The digested gene products were run on a 2% agarose gel stained with ethidium bromide and the band patterns were viewed on ultraviolet trans-illuminator.

3.4.1 *P. falciparum* Genomic DNA Extraction

P. falciparum genomic DNA was extracted from the four clinical isolates (N093, A156, A160 and K239) using the QIAamp blood midi kit (QIAGEN).

A volume of 900 µL of the isolate was added to 100 µL QIAGEN protease in a 15 mL centrifuge tube. Also, 1.2 mL of the lysing buffer AL was added to the mixture and then mixed thoroughly by gently inverting the tube 15 times followed by one-minute additional vortex. The mixture was incubated at 70ºC for 10 minutes after which 1 mL 98% ethanol was added followed by another thorough mixing. The resulting mixture was then transferred onto a QIAamp Midi column in a 15 mL tube and then centrifuged at 3000 rpm for 3 minutes. The filtrate was discarded and 2 mL of the washing buffer AW1 was added to the column followed by centrifugation at 3600 rpm for 2 minutes. The resulting filtrate was also discarded and 2 mL of another washing buffer AW2 was added and centrifuged at 3600 rpm for 30 minutes. The QIAamp Midi column was then placed in a new 1.5 mL microcentrifuge tube and 200 µL of elution buffer AE was added and incubated at room temperature for 5 minutes. The mixture was centrifuged at 3600 rpm for 5 minutes and the filtrate which contains the *P. falciparum* genomic DNA was collected and stored at -20ºC.

3.4.2 Nested PCR for Detecting SNPs in the genomic DNA of the clinical isolates

In order to determine the presence or absence of single point mutations in the genomes of isolates, regions flanking the mutations of interest in the target genes were amplified using the Analytik Jena Thermocycler (Table A.1). Briefly, using the first set of primers, outer PCR was carried out in the first reaction at a final reaction volume of 25 µL consisting of 6.5 µL nuclease free water, 12.5 µL 2X Maxima Hot Start Green PCR Master Mix (Thermo Scientific), 0.5 µL of each of the forward and reverse primers and 5 µL of the isolated DNA.

The products obtained from the outer PCR reaction were used in the second PCR run using the second sets of primers.

The reaction volumes were also about 25 µL as was used in the first run, instead, 10.5 µL of nuclease-free water and 1 µL of the PCR amplicon were used. About 5 µL of both the outer and nested PCR products were separated on a 2% ethidium bromide-stained agarose gel for one hour at 110V and then visualized under a benchtop UV trans-illuminator.

3.4.3 Restriction Digest for SNPs Analysis in the Genomic DNA of the Clinical isolates

The resulting nested products from the second PCR containing the mutations of interest were digested with restriction endonucleases (BioLabs). Each of the reactions was set at a total volume of 15 µL which included 8.2 µL nuclease-free water, 1.5 µL 10X CutSmart buffer, 0.3 µL NEB restriction enzymes (*XapI*, *DraI*, *AvaII*, *FokI*, *M1uCl*, *XmnI* and *BsrI*) (See table A. 2 for details) and 5 µL of the nested DNA product. A volume of 10 µL of each of the digested products was separated on a 2% ethidium bromide-stained agarose gel for 1 hour at 110V and then visualized under UV light.

CHAPTER FOUR

4.0 RESULTS

4.1 OPTIMIZATION OF THE DRUG ASSAY AND DETERMINATION OF THE POTENCY OF COMPOUND X USING DD2 AND 7G8

To optimize the drug assay for screening the clinical isolates against the Malaria box compounds, two laboratory strains of *P. falciparum* (Dd2 and 7G8) were initially tested against compound X. Growth of Dd2 was inhibited by Compound X in a dose dependent manner, displaying more than 50% inhibition relative to DMSO control from 100 µM to 12.5 µM (Fig 4.1, A). However, at concentrations of 6.25 µM and below, only about 20% growth inhibition was observed. To estimate the IC_{50} values of Compound X, a dose response curve was plotted and the data was fitted to a non-linear regression curve. The trend of growth inhibition was consistent across three separate experiments performed, with IC_{50} values ranging from 9.02 µM (95% CI: 8.29-9.80) to 11.55 µM (95% CI: 10.65-12.53) (Fig 4.1, A-C).

The close reproducibility of thee IC_{50} values from the three independent experiments, indicated that the assays were optimal for screening the Malaria Box compounds. To further confirm that the assay was optimal, the protocol was also used to screen Compound X against 7G8 (multi-drug resistant strain of *P. falciparum*) at concentrations from 100 µM to 1.56 µM. A dose-dependent response was observed with IC_{50} values of 6.00 µM (95% CI: 4.80 to 7.50) and 6.57 µM (95 % CI: 4.804 to 9.008) in the first and second experiments respectively (Fig. 4.2 A and B). The closely reproducible IC_{50} values in the two assays further qualifies the drug assay as optimal.

Also, the IC_{50} values observed for Compound X against Dd2 and 7G8, were within the expected range (6-10 µM) for Compound X. This further confirmed the drug assay as optimal for screening the Malaria box compounds. Using the criteria proposed by Batista *et al*., (2009),

Compound X can be said to have very good antimalarial activity against Dd2 and 7G8 drug-resistant strains of *P. falciparum* as the IC_{50} values were within 1-20 µM range assigned for drugs with good potency.

Activity of Compound X against Dd2

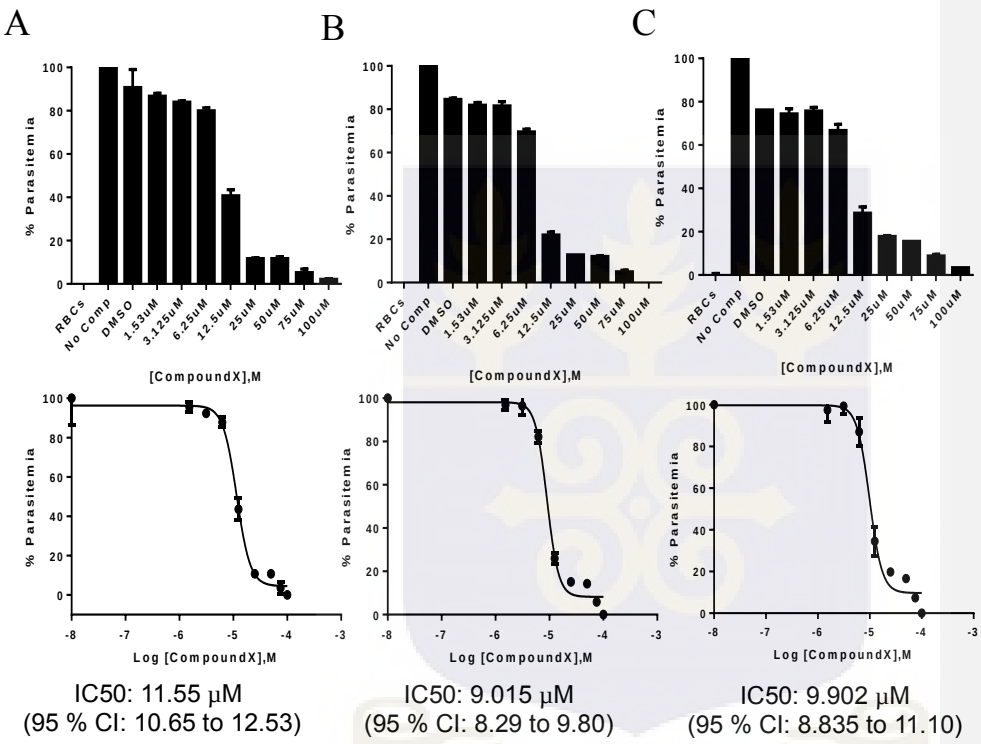


Figure 4. 1 Potency of Compound X against Dd2.
Panel A-C are bar graphs and dose-response curves for three independent experiments (A is experimental 1, B is experiment 2 and C is experiment 3) showing the response of Dd2 to Compound X at concentrations between 100 µM to 1.53 µM. Erythrocytes (RBCs) were used as a background control, No Compound (No comp) control were wells with no compound treatment and DMSO control were wells with 1% final DMSO concentration.

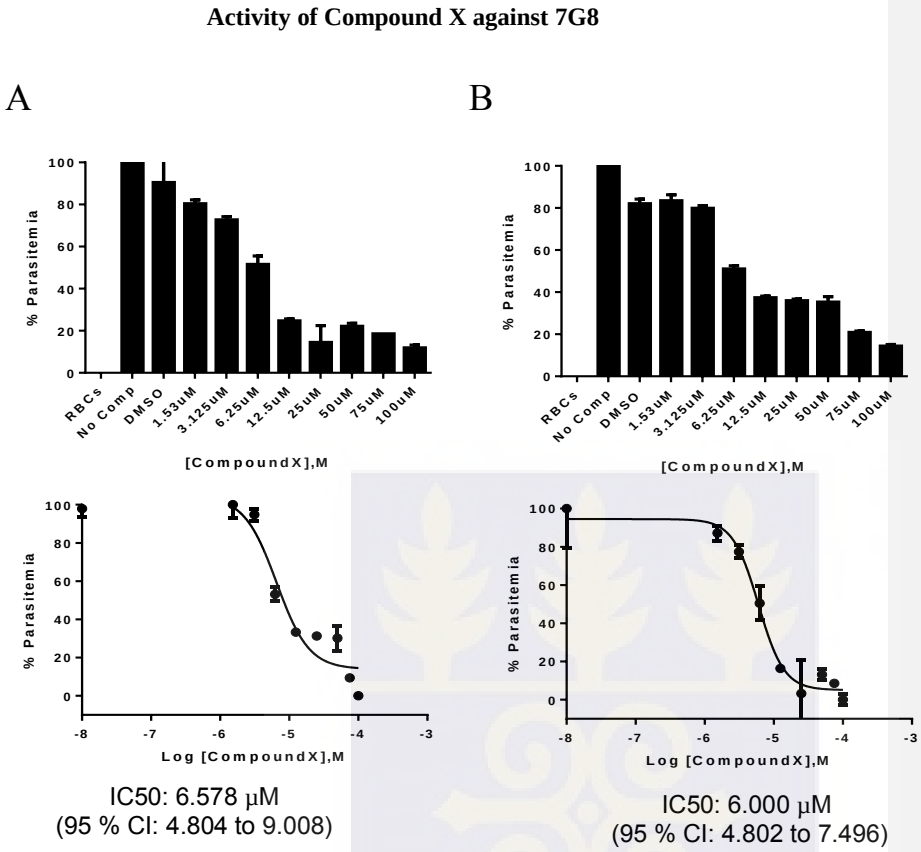


Figure 4. 2: Potency of Compound X against 7G8.
Panel A and B (A is experiment 1 and B is experiment 2) are bar graphs and dose-response curves showing the response of 7G8 to Compound X at concentrations between 100 µM to 1.53 µM. Erythrocytes (RBCs) were used as a background control, No Compound (No comp) control were wells with no treatment and DMSO control were wells with 1% final DMSO concentration.

4.2 SCREENING OF COMPOUNDS FROM THE MALARIA BOX AGAINST CLINICAL ISOLATES

In order to evaluate the potency of the Malaria Box compounds against clinical isolates, ten compounds that have been found to have potency against laboratory strains were screened against four clinical isolates (N093, A160, A156 and K239). Table 4.1 contains a summary of the IC_{50} values of the ten compounds against the four clinical isolates obtained from this study. In order to identify very potent compounds that can be further explored for antimalarial drug development, compounds with IC_{50} values below 500 nM against the clinical isolates were selected. Three of Malaria Box compounds (MMV006787, MMV085203 and MMV008956) with IC_{50} values below 500 nM across the four clinical isolates were identified and selected for further exploration (Fig. 4.6, Fig. 4.7 and Fig. 4.8). MMV006787, MMV085203 and MMV008956 compounds will be further pursued for their stage-specific activity, target identification and molecular mechanisms of action.

MMV396797

MMV396797 generally had an excellent potency against all the clinical isolates with IC_{50} values less than 1µM (Fig.4.3 A-D). About 70% growth inhibition was observed at concentrations between 25 µM to 1µM. Based on the estimated IC_{50} values, MMV396797 was more efficacious against N093 at 659.9 nM, followed by A156 at a concentration of 701.1 nM, K239 at 801.6 nM and A160 at 835.8 nM.

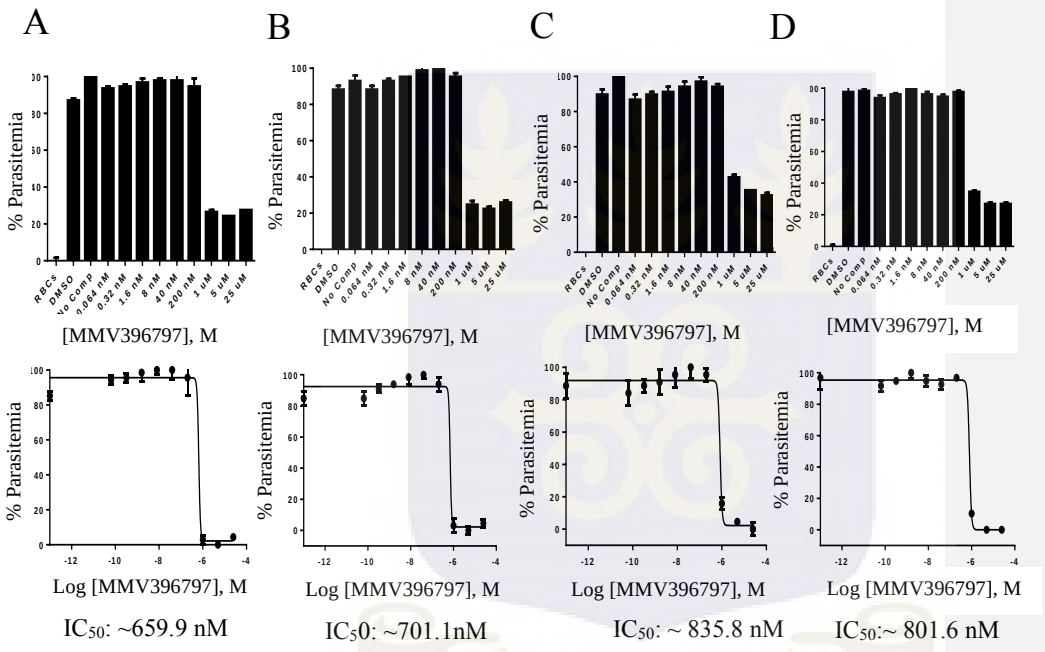


Figure 4. 3 Potency of MMV396797 against four clinical isolates of *P. falciparum*.
Panel A-D are bar graphs and dose-response curves showing the response of clinical isolates (A is N093, B is A156, C is A160 and D is K239) to MMV396797 at concentrations from 25 µM to 0.064 nM. Uninfected erythrocytes (RBCs) were used as a background control, No comp= wells with RPMI media only and DMSO= wells with RPMI plus 0.25% DMSO.

MMV006278

MMV006278 exhibited good antimalarial activity against the clinical isolates with IC_{50} values greater than 1 µM (Fig.4.4 A-D). Generally, a dose-dependent relationship was observed at concentrations between 25 µM and 200 nM, but beyond this range, there was relatively no inhibition compared to the no compound control (DMSO). In this study, MMV006278 was more potent against N093 with IC_{50} of 1.90µM (Fig.4.4 A) followed by K239 and A160 (Fig.4.4, D and C) with IC_{50} of 4.34 µM and 9.46 µM, respectively. MMV006278 was about two-fold less potent against A156 compared to the other three clinical isolates with IC_{50} of 18.41 µM (Fig.4.4 B).

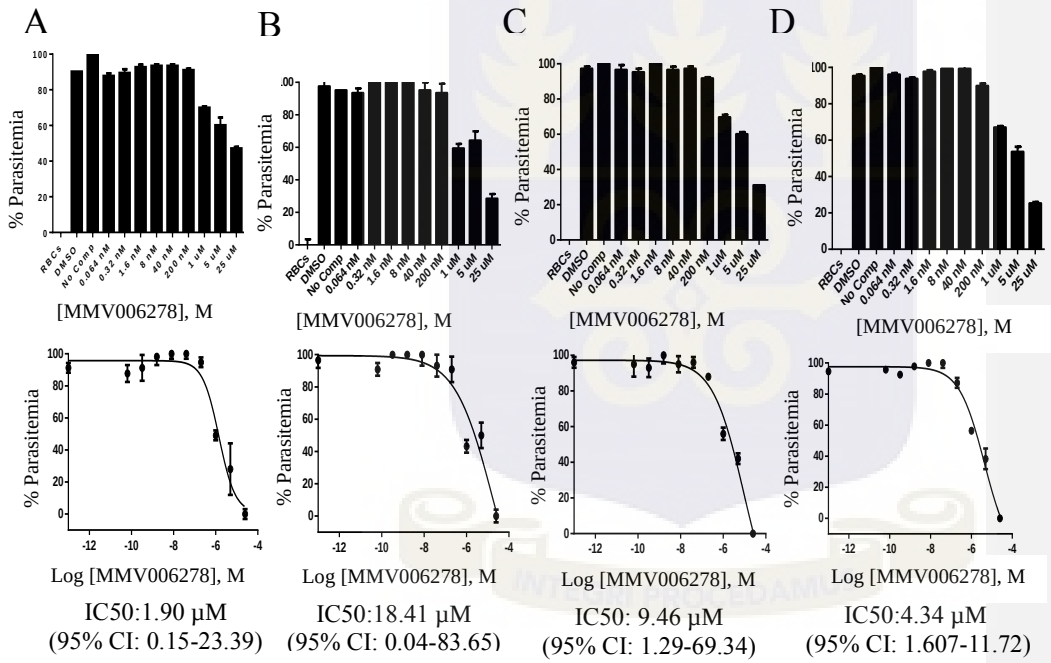


Figure 4. 4: Potency of MMV006278 against the clinical isolates.
Panel A-D are bar graphs and dose-response curves showing the response of clinical isolates (A is N093, B is A156, C is A160 and D is K239) to MMV006278 at concentrations from 25 µM to 0.064 nM. Uninfected erythrocytes (RBCs) were used as a background control, No comp= wells with culture plus RPMI and DMSO= wells with RPMI plus 0.25% DMSO.

MMV008416

MMV008416 was generally not very active against the clinical isolates with IC_{50} values beyond 1 µM (Fig.4.5 A-D). Estimated IC_{50} values from the dose-response curves suggest MMV008416 was 2-fold more active against K239 with IC_{50} of 1.70 µM (Fig.4.5 D) compared to A160 that had an IC_{50} value of 3.51 µM (Fig.4.5 C). MMV008416 was not active against N093 and A156 which had IC_{50} values beyond the micromolar range (Fig.4.5 A and B).

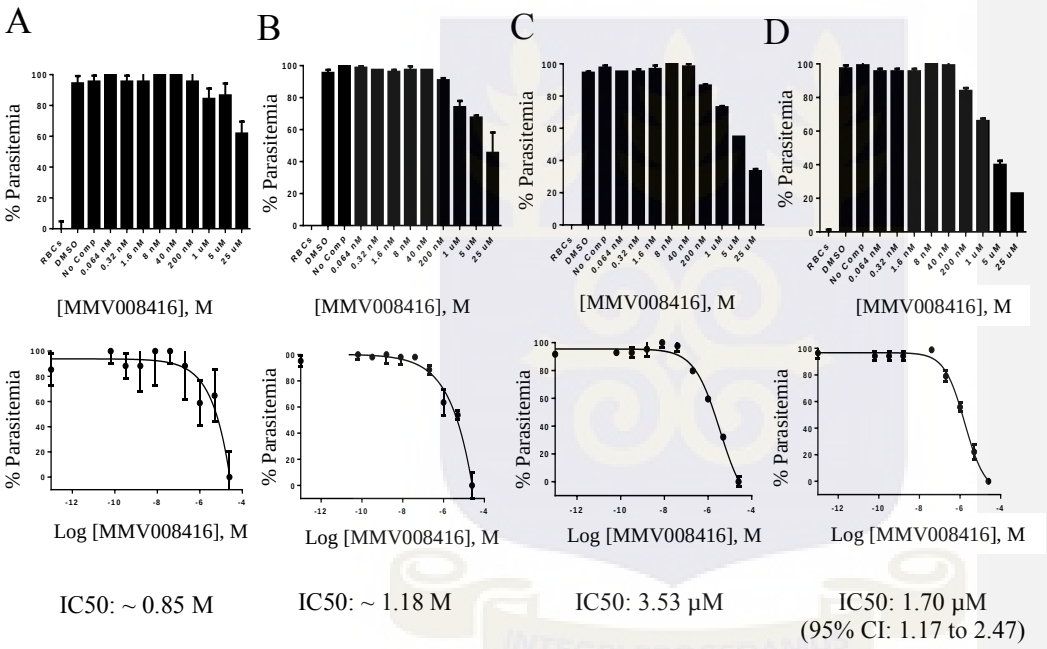


Figure 4. 5: Potency of MMV008416 against the clinical isolates.
Panel A-D are bar graphs and dose-response curves showing the response of clinical isolates (A is N093, B is A156, C is A160 and D is K239) to MMV008416 at concentrations from 25uM to 0.064nM. Uninfected erythrocytes (RBCs) were used as a background control, No comp= wells with culture plus RPMI and DMSO= wells with RPMI plus 0.25% DMSO.

MMV008956

Generally, MMV008956 was very potent against the clinical isolates with IC_{50} values below 250 nM. At concentrations between 25 µM to 1 µM, parasite growth was inhibited by more than 50% in all the clinical isolates (Fig.4.6 A-D). Also, about 40% inhibition of growth was observed at 200 nM in K239, A160 and N093 (Fig.4.6 A-C) which was twice the inhibition observed at 200 nM in A156 (Fig.4.6 D). MMV008956 was more potent against K239 at a 50% inhibitory concentration of 43.89 nM (Fig.4.6 D) which was more than 2-fold the IC_{50} of MMV008956 against A160 (Fig.4.6 C), more than 3-fold the IC50 against N093 (Fig.4.6 A) and almost 5-fold the IC_{50} against observed for this compound against A156 (Fig.4.6 B).

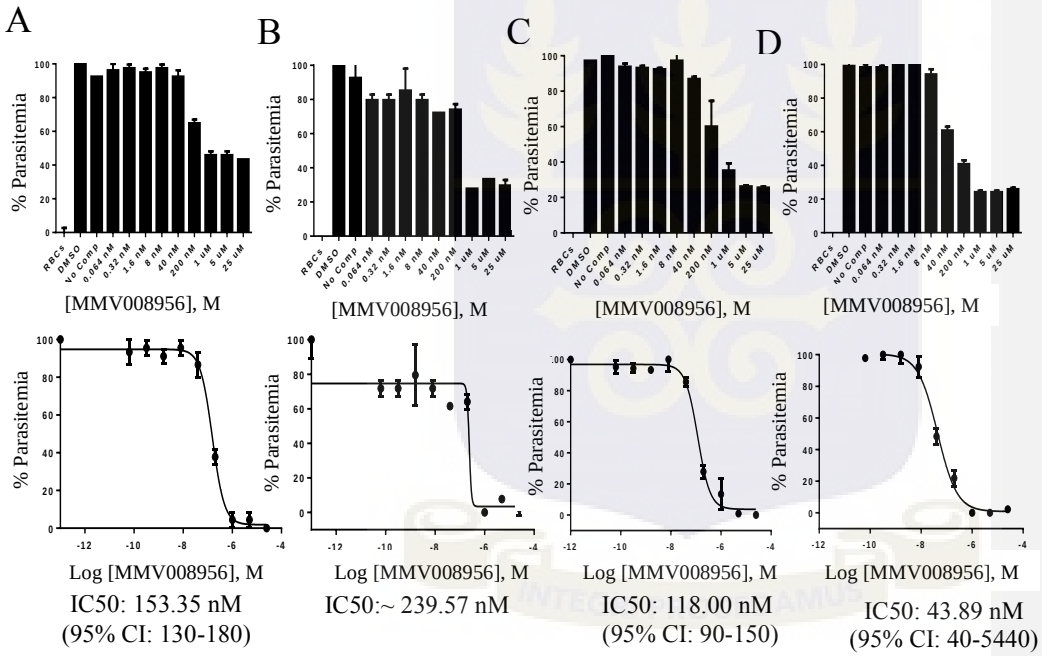


Figure 4. 6: Potency of MMV008956 against the clinical isolates.
Panel A-D are bar graphs and dose-response curves showing the response of clinical isolates (A is N093, B is A156, C is A160 and D is K239) to MMV008956 at concentrations between 25µM to 0.064nM. Uninfected erythrocytes (RBCs) were used as a background control, No comp= wells with culture plus RPMI and DMSO= wells with RPMI plus 0.25% DMSO.

MMV085203

MMV085203 was the most potent compound in this study with IC_{50} values below 100 nM (fig.4.7 A-D). MMV085203 inhibited the growth of the clinical isolates by more than 60% at concentrations of 25 µM to 200 nM and about 20% inhibition at 40 nM in all the clinical isolates. There was relatively no growth inhibition beyond the 40 nM concentrations relative to the DMSO control (Fig.4.7 A-D). MMV085203 was more potent against N093 with an IC_{50} value of 55.83 nM (Fig. 4.7 A). The IC_{50} values of MMV085203 against K239 and A156 were also observed to be 58.02 nM and 59.15 nM (Fig. 4.7 B-D) respectively, which were similar to that of the IC_{50} of MMV085203 against N093. However, MMV085203 had an IC_{50} of 82.32 nM when screened against A160, which is about 20 nM less the potency of MMV085203 against the other three clinical isolates.

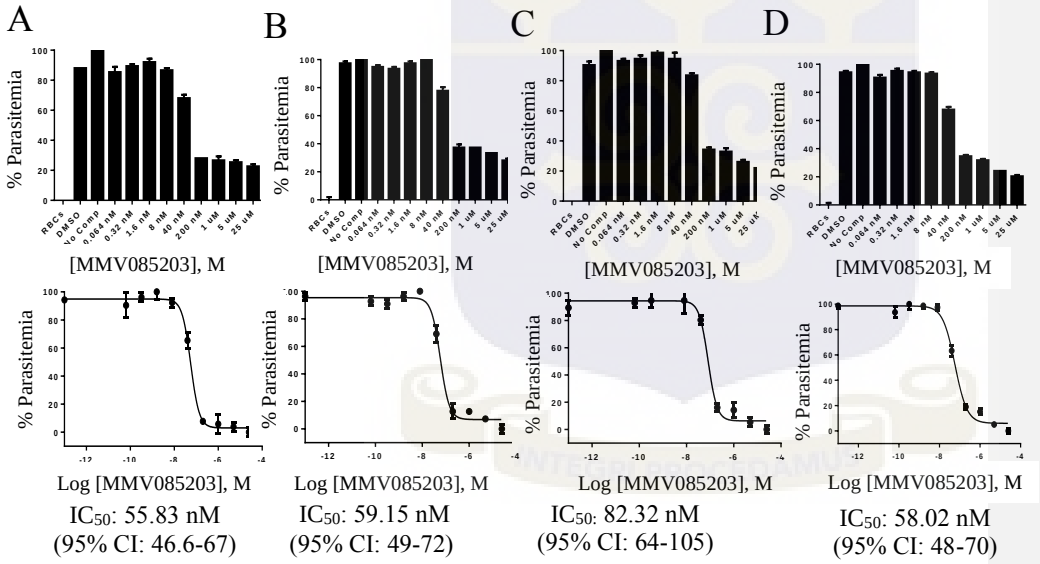


Figure 4. 7: Potency of MMV085203 against the clinical isolates.
Panel A-D are bar graphs and dose-response curves showing the response of clinical isolates (A is N093, B is A156, C is A160 and D is K239) to MMV085203 at concentrations between 25µM to 0.064nM. Uninfected erythrocytes (RBCs) were used as background control, No comp= wells with culture plus RPMI and DMSO= wells with RPMI plus 0.25% DMSO.

MMV006787

MMV006787 was generally very potent against all the clinic isolates with IC_{50} values below 1 µM (Fig.4.8 A-D). N093, A160 and K239 were sensitive to MMV006787 with more than 60% growth inhibition at concentrations between 25 µM to 1 µM compared to A156 that had about 40% inhibition at these same concentrations (Fig. 4.8 A-D). MMV006787 was more potent against N093 compared to the other isolates with an IC_{50} was 190 nM. The potency of MMV006787 against N093 was about 2-fold the potency against K239 (IC_{50} of 320.60 nM), about 3-fold the potency against of A160 (IC_{50} of 418.60 nM) (Fig.4.8 C) and about 4-fold the potency of this compound against A156 (IC_{50} of 939.60 nM) (Fig.4.8 B).

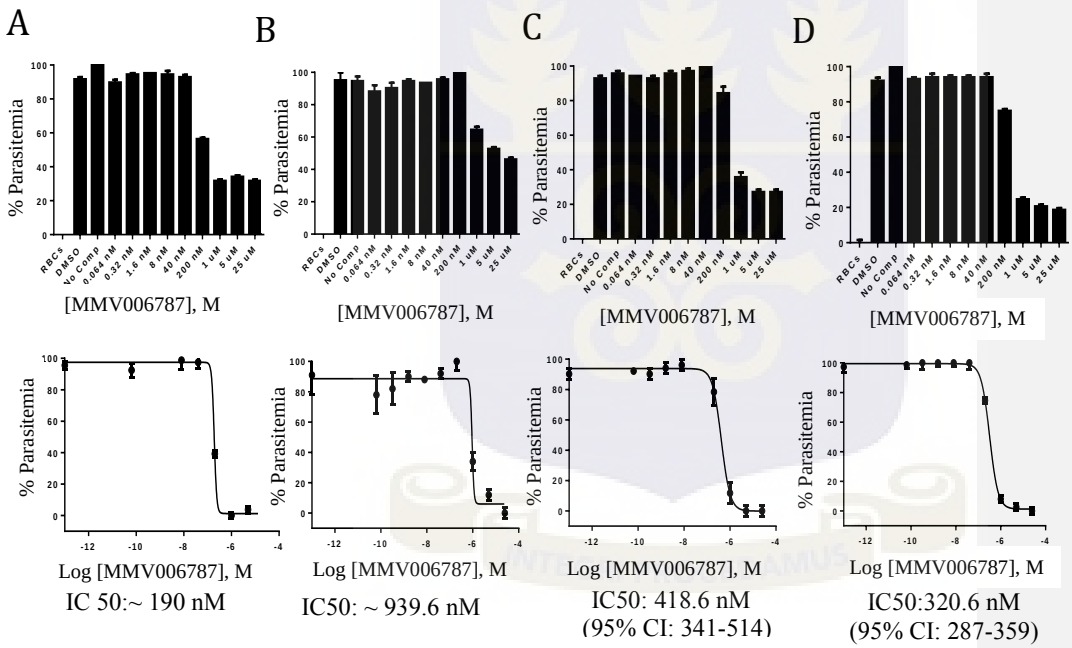


Figure 4. 8: Potency of MMV006787 on clinical isolates.
Panel A-D are bar graphs and dose-response curves showing the response of clinical isolates (A is N093, B is A156, C is A160 and D is K239) to MMV006787 at concentrations between 25 µM to 0.064nM. Uninfected erythrocytes (RBCs) were used as a background control, No comp= wells with culture plus RPMI and DMSO= wells with RPMI plus 0.25% DMSO.

MMV000753

Except for MMV000753 against N093, this compound was generally very potent against all the clinical isolates with IC_{50} values below 1 µM (Fig.4.9 A-D). A dose-dependent inhibition of parasite growth was also observed between 25 µM to 200 nM. Beyond this range, parasitemia levels were similar to that of the DMSO indicating little or no inhibition of growth. This compound was very active against A156 with an IC_{50} value of 769.60 nM followed by A160 with an IC_{50} of 811.10 nM and K239 with an IC_{50} of 940.70 nM. MMV000753 was less active against N093 compared to the other three isolates with an IC_{50} value of greater than 1 µM (Fig. 4.9 A).

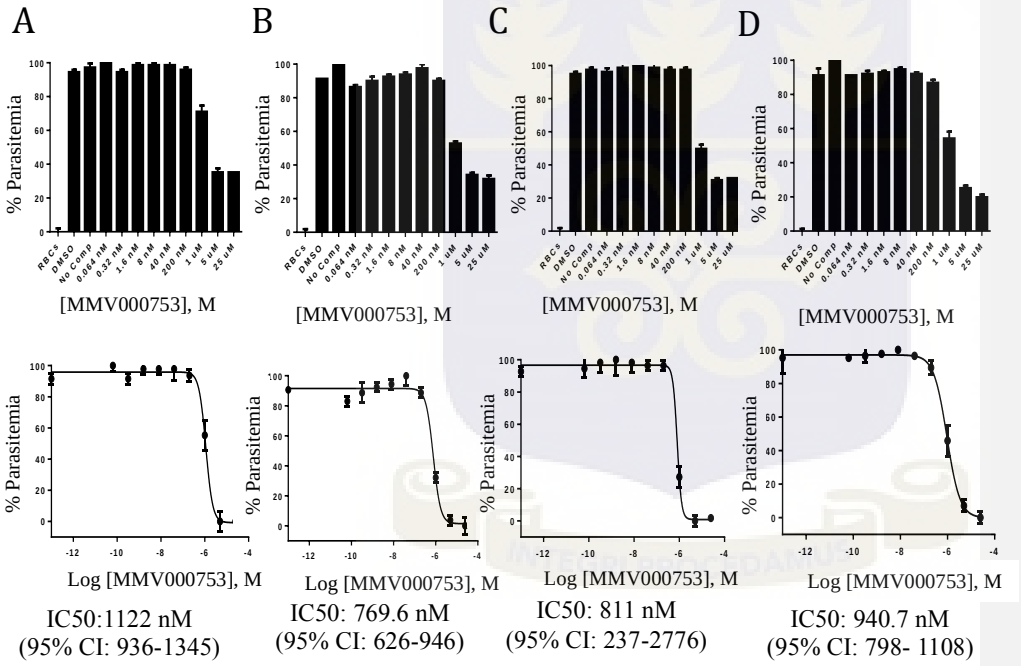


Figure 4. 9: Potency of MMV000753 against the clinical isolates.
Panel A-D are bar graphs and dose-response curves showing the response of clinical isolates (A is N093, B is A156, C is A160 and D is K239) to MMV000753 at concentrations between 25 µM to 0.064nM. Uninfected erythrocytes (RBCs) were used as background control, No comp= wells with culture plus RPMI and DMSO= wells with RPMI plus 0.25% DMSO.

MMV007384

MMV007384 had good antimalarial properties against N093 and A160 but was ineffective against A156 and K239 (Fig.4.10 A-D). From the dose-response curves, N093 was the most sensitive clinical isolate to MMV007348 with an IC_{50} of 7.26 µM (Fig.4.10 A), followed by A160 with an IC_{50} value of 16.16 µM which was more than twice the IC_{50} of this compound against N093. K239 had an IC_{50} value of 100.2 µM which was about nine (9) times the IC_{50} of N093 and about five (5) times the IC_{50} of A160. The IC_{50} value for A156 could not be estimated because there was no dose-response relationship (Fig.4.10 B).

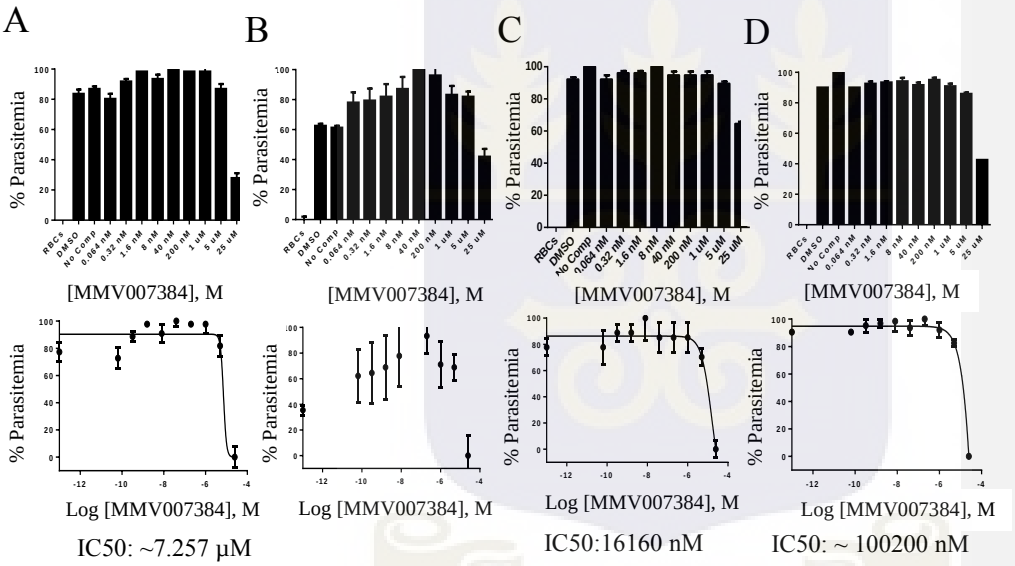


Figure 4. 10: Potency of MMV007384 against the clinical isolates.
Panel A-D are bar graphs and dose-response curves showing the response of clinical isolates (A is N093, B is A156, C is A160 and D is K239) to MMV007384 at concentrations between 25 µM to 0.064 nM. Uninfected erythrocytes (RBCs) were used as a background control, No comp= wells with culture plus RPMI and DMSO= wells with RPMI plus 0.25% DMSO.

MMV019555

MMV019555 had excellent potency against A156, A160 and K239 with IC_{50} values below 1 µM, but good antimalarial activity against N093 with IC_{50} 3.318 (Fig.4.11. A-D). A156 was the most sensitive to MMV019555 compared to the other isolates at a 50 % inhibitory concentration of 280.90 nM (Fig.4.11 B). K239 and A160 were also found to be sensitive to MMV019555 at 399.90 nM and 867.2 nM respectively. N093 was the least sensitive to MMV01955 with an IC50 value of 3.32 uM (about eleven times the IC50 of the MMV019555 against A156).

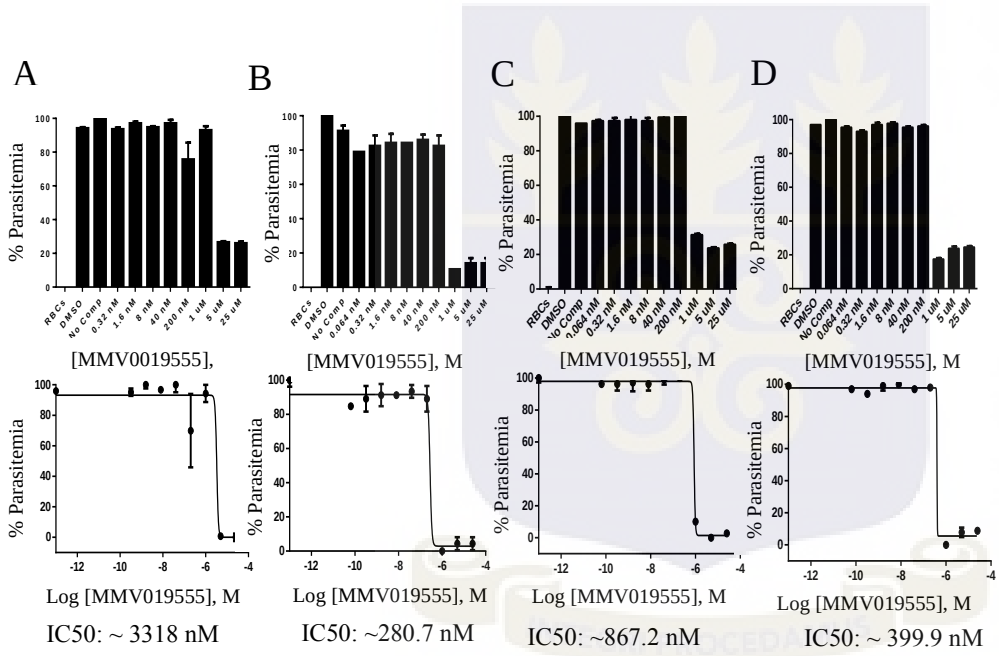


Figure 4. 11: Potency of MMV019555 against the clinical isolates.
Panel A-D are bar graphs and dose-response curves showing the response of clinical isolates (A is N093, B is A156, C is A160 and D is K239) to MMV019555 at concentrations between 25µM to 0.064 nM. Uninfected erythrocytes (RBCs) were used as a background control, No comp= wells with culture plus RPMI and DMSO= wells with RPMI plus 0.25% DMSO.

MMV009015

MMV009015 had excellent activity against N093, A156 and A160 but good antimalarial activity against K239 (Fig.4.12 A-D). At concentrations between 25 µM and 1 µM, MMV009015 reduced the parasitemiae by about 60% across the four clinical isolates. MMV009015 was more potent against N093 with IC_{50} of 275.6 nM and less active against K239 with IC_{50} of 1009 nM.

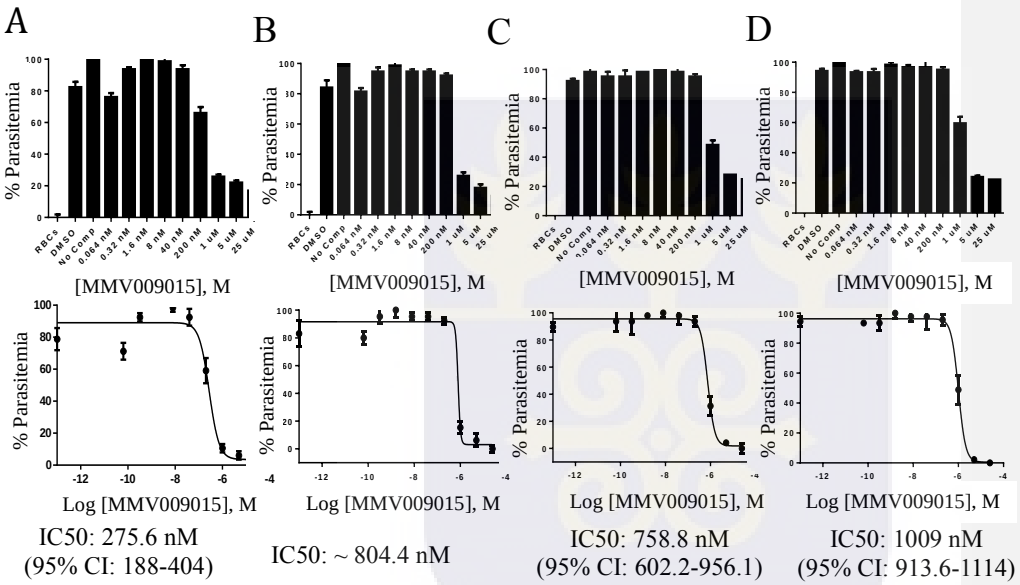


Figure 4. 12: Potency of MMV009015 against the clinical isolates.
Panel A-D are bar graphs and dose-response curves showing the response of clinical isolates (A is N093, B is A156, C is A160 and D is K239) to MMV009015 at concentrations between 25 µM to 0.064 nM. Uninfected erythrocytes (RBCs) were used as a background control, No comp= wells with culture plus RPMI and DMSO= wells with RPMI plus 0.25% DMSO.

Table 4. 1: Summary of IC_{50} values for Malaria Box compounds against the four clinical isolates.

Compound ID	N093 IC_{50} (nM)	A156 IC_{50} (nM)	A160 IC_{50} (nM)	K239 IC_{50} (nM)	Reported IC_{50}
MMV006787	~ 190	~ 939.60	418.60	320.60	Dd2 IC50 (906.6 nM)
MMV006278	1896	18410	9464	4339	PfTrxR assay IC_{50} (3.5 µM)
MMV008956	153.50	~ 239.50	118	43.89	PfTrxR assay IC_{50} (6.6 µM)
MMV008416	~ $0.85*10^6$	~ $1.18*10^6$	3531	1702	PfTrxR assay IC_{50} (7.5 µM)
MMV000753	1122	769.60	811.10	940.70	D6 IC_{50} (1212nM), C235 IC_{50} (1609 nM)
MMV085203	55.83	59.15	82.32	58.02	PfTrxR assay IC_{50} (0.9 µM)
MMV007384	~ 7257	Data did not converge	16160	~ $0.2*10^3$	D6 IC_{50} (2165 nM) C235 IC_{50} (3469 nM)
MMV396797	~ 659.90	~ 701.10	~ 835.80	~ 801.60	PfTrxR assay IC_{50} (4.8 µM)
MMV009015	275.6	~ 804.40	758.80	1009	Dd2 IC_{50} (1093 nM)
MMV019555	~ 3318	~ 280.70	~ 867.20	~ 399.90	Dd2 IC_{50} (545.3 nM)

N093 was found to be more sensitive to the majority of compounds used in this study compared to the other clinical isolates. The last column is IC_{50} values reported from previous studies on these compounds.

4.3 EVALUATION OF THE SENSITIVITY OF THE CLINICAL ISOLATES TO TRADITIONAL ANTIMALARIAL DRUGS

4.3.1 Sensitivity of Clinical Isolates to Artesunate

In order to assess the sensitivity of the clinical isolates to the standard antimalarial drugs currently used, artesunate was used to screen against the clinical isolates at concentrations between 32 nM and 0.00248 nM. This range of concentrations were selected based on the reference IC_{50} values for artesunate. From the dose-response curves, artesunate was more potent against N093 with an IC_{50} of 2.69 nM, followed by A160 with an IC_{50} of 3.09 nM and K239 with an IC_{50} of 4.78 nM (Fig.4.13 A-D). Artesunate was less active against A156 compared to the other three isolates with an IC_{50} value of 12.68 nM which is about 6-fold the IC_{50} of artesunate against N093 (Fig.4.13 B).

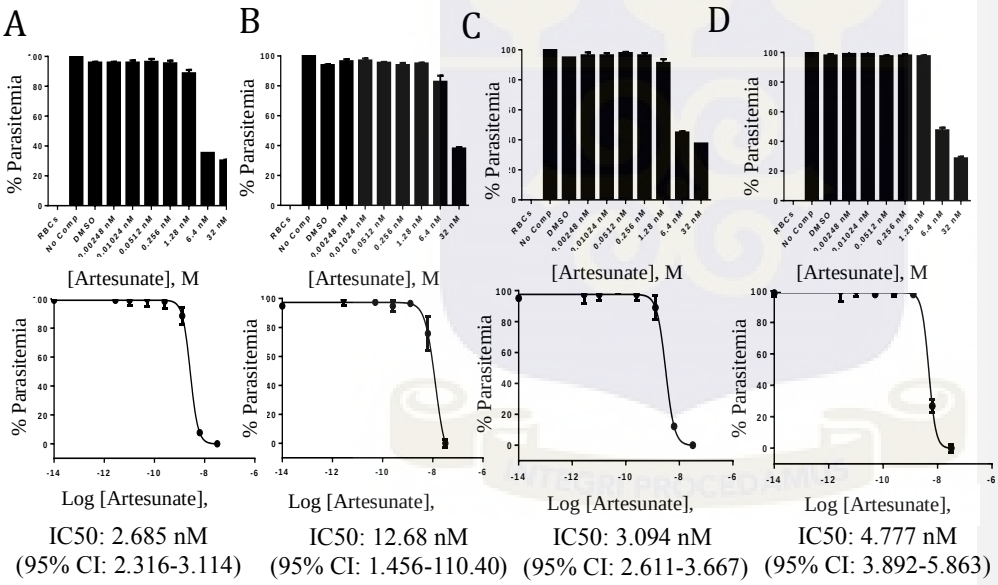


Figure 4. 13: Potency of artesunate against the clinical isolates.
Panel A-D are bar graphs and dose-response curves showing the response of clinical isolates (A is N093, B is A156, C is A160 and D is K239) to artesunate at concentrations between 32 nM to 0.00248 nM. Uninfected erythrocytes (RBCs) were used as background control, No comp= wells with culture plus RPMI and DMSO= wells with RPMI plus 0.1% DMSO.

4.3. 2 Sensitivity of Clinical Isolates to Chloroquine

The potency of chloroquine was also evaluated against the clinical isolates in order to better understand the response of the parasites to the Malaria Box compounds. In this study, 10 mM of chloroquine was serially diluted to yield working concentrations of 250 µM to 0.64 nM (1:5 dilution) using filtered sterile distilled water. From the dose-response curves, chloroquine was more potent against A160 with an IC_{50} of 22.22 nM, followed by K239 with an IC_{50} of 26.82 nM and finally A156 with an IC_{50} of 31.50 nM (Fig.4.14 A-D). Chloroquine was less active against N093 compared to the other isolates with an IC_{50} of 121.20 nM which is about 5-fold the IC_{50} observed for chloroquine against A160 (Fig.4.14 A and C).

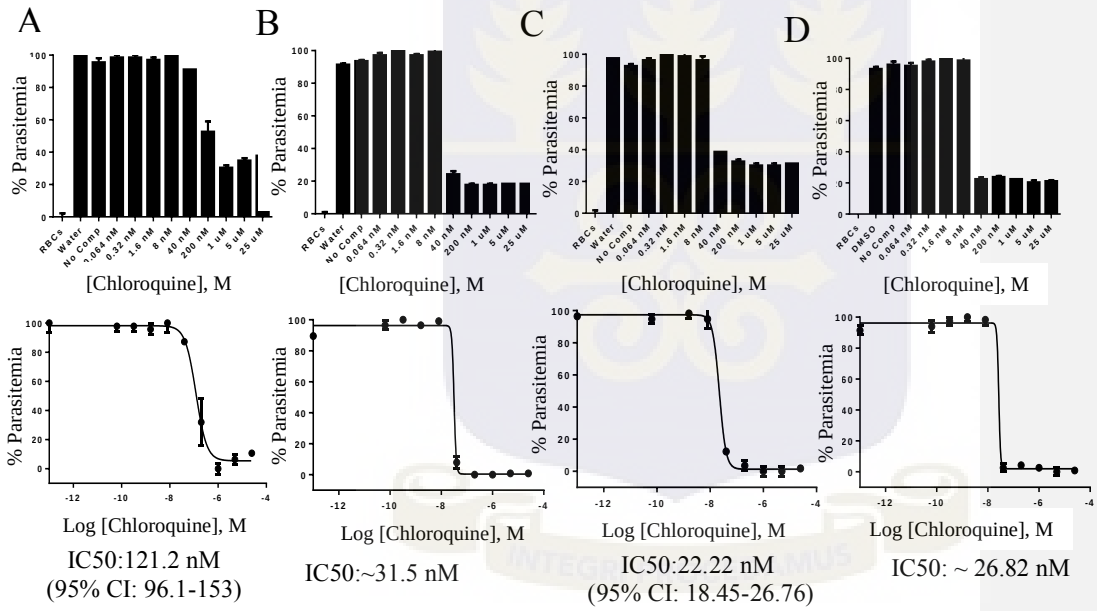


Figure 4. 14: Potency of chloroquine against the clinical isolates.
Panel A-D are bar graphs and dose-response curves showing the response of clinical isolates (A is N093, B is A156, C is A160 and D is K239) to chloroquine at concentrations between 25uM to 0.064nM. Uninfected erythrocytes (RBCs) were used as a background control, No comp= wells with culture plus RPMI and Water= wells with RPMI plus filtered sterile distilled water.

4.4 GENOTYPING OF CLINICAL ISOLATES USING RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

RFLP was used to identify polymorphisms in *pfcrt*, *pfmdr1*, *pfdhps* and *pfdhfr* genes in all the clinical isolates that mediate chloroquine, multidrug, sulfadoxine and pyrimethamine resistance respectively. In this part of the study, N093 showed mutations that mediate chloroquine, multi-drug, sulfadoxine and pyrimethamine resistance respectively. A156 had was sensitive to chloroquine, but had mutations that mediate multidrug resistance as well as sulfadoxine and pyrimethamine resistance. A160 was sensitive to both chloroquine and pyrimethamine but harboured single nucleotide variations mediating multidrug and sulfadoxine resistance. K239, on the other hand was sensitive to chloroquine and sulfadoxine, but multi-drug resistant. K239 showed a mixed response to pyrimethamine. Table 4.2 contain a summary of the results of the RFLP analysis.

4.4.1 Polymorphism in the *pfcrt* gene

After extracting genomic DNA from the four clinical isolates (N093, K239, A160 and A156), two sets of primers were used to successfully amplify the *pfcrt* gene, which is a 134 base pair (bp) amplicon (Fig.4.15 A). Dd2 was used as the positive control that contains the mutant K76T polymorphism and 3D7 was the negative control with wild-type allele. After enzymatic digestion (using *XapI* restriction enzyme), N093 was observed to have band pattern similar to positive control with a 134 bp band size. K239, A160 and A156 all had band patterns similar to the wild type with a band size of 100 bp (Fig.4.15 B). Therefore, N093 was the only isolate that had the K76T mutation and the other three had the wild type allele.

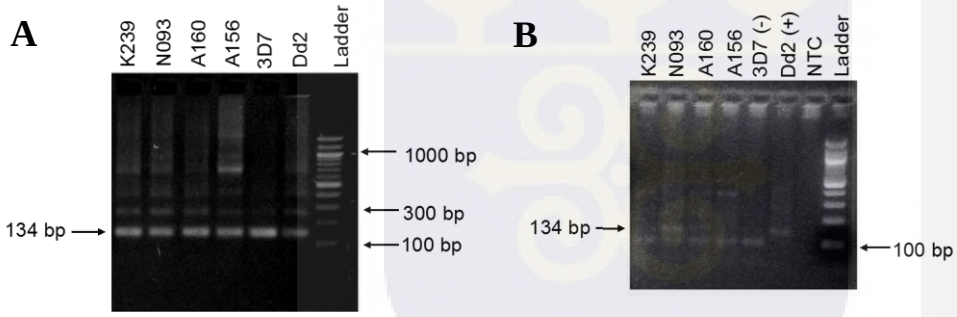


Figure 4. 15: Identification of SNVs in the *pfcrt* gene of the clinical isolates.
(A) Band patterns in the clinical isolates on a 2% agarose gel after nested PCR amplification of the *pfcrt* gene. (B) Band patterns after enzymatic digestion for the detection of K76T mutation in the *pfcrt* gene. 3D7 has the wild type allele and was used as negative control (lane 5). Dd2 has the mutant allele and was used as the positive control (lane 6). Lane 7 is the molecular weight marker (ladder). Lanes 1-4 are the clinical isolates.

4.4.2 Polymorphisms in the *pfmdr1* gene

A 500 bp region of the *pfmdr1* gene was successfully amplified (Fig.4.16 A) and digested with *XapI* and *DraI* restriction enzymes to identify polymorphisms at codon positions N86Y and Y184F on the *pfmdr1* gene (table A. 2). K239 and N093 were found to have N86Y mutation similar to Dd2, which was the positive control, but A160 and A156 had the wild type allele similar to the 7G8, which was the negative control (Fig.4.16 B). Also, all the clinical isolates were found to have the mutant Y184F mutation similar to7G8, which was the positive control (Fig.4.16 C). Therefore, all the clinical isolates were multi-drug resistant parasites.

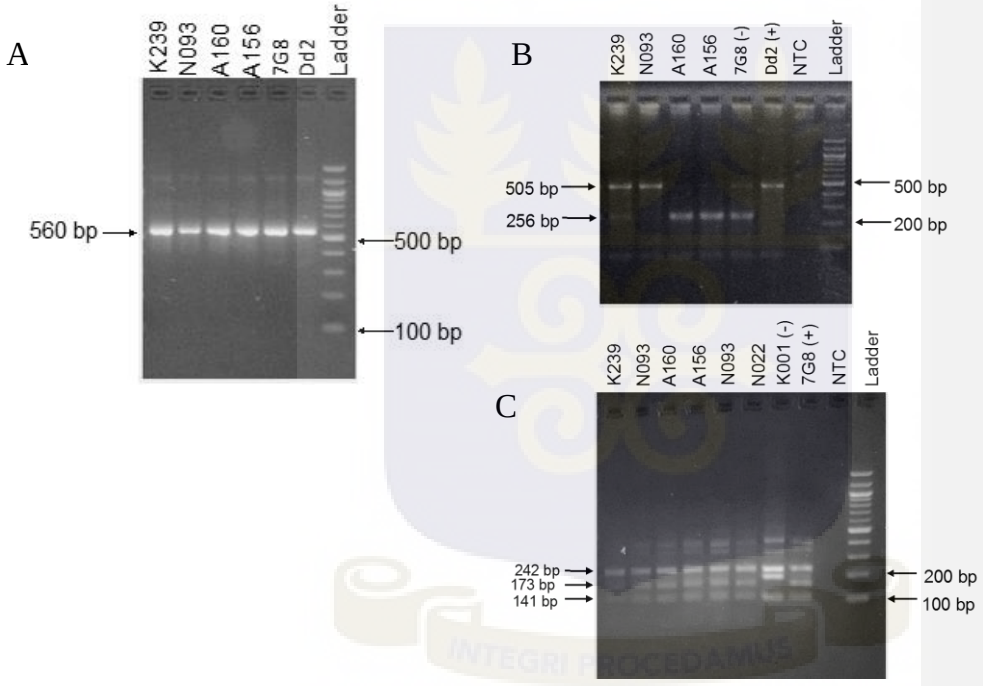


Figure 4. 16: Identification of SNVs in the pfmdr1 gene of the clinical isolates.
(A) Band patterns in the clinical isolates on a 2% agarose gel after nested PCR amplification of the *pfmdr1* gene. (B) Band patterns after enzymatic digestion of *pfmdr1* gene for the detection of N86Y mutation. From left to right are clinical isolates (lanes 1 to 4), negative control (lane 5), positive control (lane 6), Non-template control (NTC) in lane 7 and DNA ladder (lane (8). (C) Band patterns after enzymatic digestion of *pfmdr1* gene for the detection of Y184F mutation. From left to right are clinical isolates (lanes 1 to 5), negative control (lane 7), positive control (lane 6 and 8), NTC lane 9 and molecular weight marker (lane 10).

4.4.3 Polymormisms in the *pfdhps* gene

A 438 bp section of the *dhps* gene that is known to contain polymorphisms that mediate sulfadoxine resistance was successfully amplified using nested PCR (Fig.4.17 A). After digesting the 438 bp amplicon using *AvaII* restriction enzyme that targets polymorphism at codon position A437G (table A. 2). N093, A156 and A160 were found to habour the mutant allele with band patterns similar to K001 which was the positive control and K239 had the wild type allele with band patterns similar to K255 which was the negative control (Fig.4.17 B). None of the isolates had the L540E mutant allele, with band patterns similar to N151 (negative control) that is known to have the wild type allele (Fig.4.17 C).

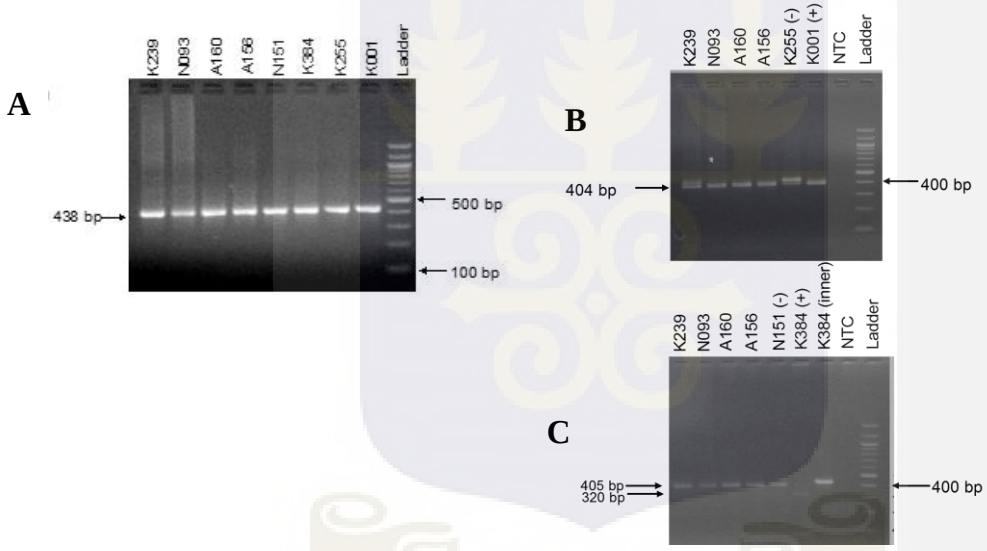


Figure 4. 17: Polymorphisms in the *dhps* gene.

(A) Band patterns in the clinical isolates on a 2% agarose gel after nested PCR amplification of the *pfdhps* gene.

(B) Band patterns after enzymatic digestion of *pfdhps* gene for the detection of A437G mutation. From left to right are clinical isolates (lanes 1 to 4), negative control (lane 5), positive control (lane 6), Non-template control (NTC) in lane 7 and molecular weight marker (lane (8). (C) Band patterns after enzymatic digestion of *pfdhps* gene for the detection of L540E mutation. From left to right are clinical isolates (lanes 1 to 4), negative control (lane 5), positive control (lane 6), DNA template control (lane 7), NTC (lane 8) and molecular weight marker (lane 9).

4.4.4 Polymorphisms in the *pfdhfr* gene

A nested PCR was used to amplify a 326 bp section of the *dhfr* gene that is known to have polymorphisms that mediate resistance to pyrimethamine (Fig.4.18 A). RFLP was then used to examine polymorphisms at codon position 51, 59 and 108 on the 326 bp regions of the *dhfr* gene. N093 and A156 were found to have band patterns similar to N116 (positive control) which has the N51l mutation in the *dhfr* gene (Fig.4.18 B). A160, on the other hand had band patterns similar to the negative control (N151) that is known to have the wild type allele. K239 had band patterns similar to both negative and positive controls, which seems to suggest a mixed infection (Fig.4.18 B). For polymorphism at position 59 that has cysteine to arginine mutation, K239, N093 and A156 were found to have band patterns similar to the positive control (N116) that has the mutant allele, but A160 had a similar band pattern to the negative control that has the wild-type allele (Fig.4.18 C). N093 and A156 also had band patterns similar to positive control, that suggest a polymorphism at codon position 108 with a change from serine to asparagine, but A160 had band pattern similar to the negative control, suggesting the presence of the wild-type allele. K239 had band patterns similar to the wild type and mutant allele that indicate a mixed infection.

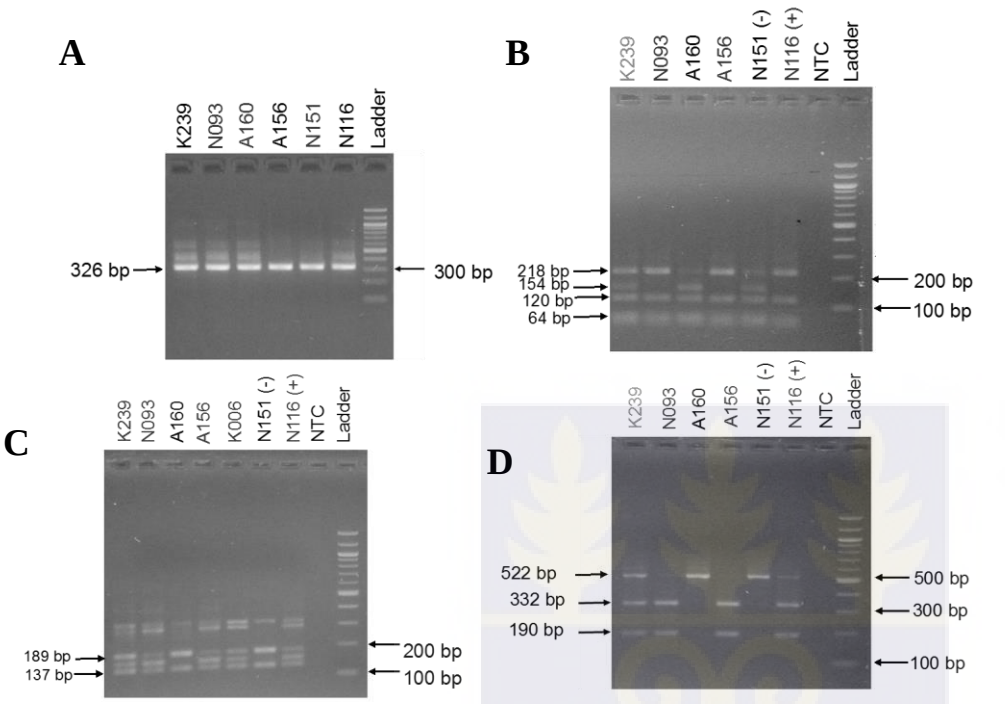


Figure 4. 18: Identification of SNV in the *pfdhfr* gene of the clinical isolates.
(A) Band patterns in the clinical isolates on a 2% agarose gel after nested PCR amplification of the *pfdhfr* gene. (B) Band patterns after enzymatic digestion of *pfdhfr* gene for the detection of N51l mutation. From left to right are clinical isolates (lanes 1 to 4), negative control (lane 5), positive control (lane 6), Non-template control (NTC) in lane 7 and molecular weight marker (lane (8). (C) Band patterns after enzymatic digestion of *pfdfr* gene for the detection of C59R mutation. From left to right are clinical isolates (lanes 1 to 4), negative control (lane 6), positive control (lane 5and 7), NTC (lane 8) and molecular weight marker (lane 9). (D) (C) Band patterns after enzymatic digestion of *pfdhfr* gene for the detection of S108N mutation. From left to right are clinical isolates (lanes 1 to 4), negative control (lane 5), positive control (lane 6), NTC (lane 7) and molecular weight marker (lane 8).

Table 4. 2: Summary of polymorphisms in the *pfcrt*, *pfmdr1*, *pfdhps* and *pfdfr* genes in the clinical isolates.

	PFCRT		PFMDR1				PFDHPS				PFDHFR					
	K76T		**N86Y**		**Y184F**		**G437A**		**L540E**		**N51I**		**C59N**		**S108N**	
Sample ID	**W**	**M**	**W**	**M**	**W**	**M**	**W**	**M**	**W**	**M**	**W**	**M**	**W**	**M**	**W**	**M**
A156	✓		✓			✓		✓	✓			✓		✓		✓
A160	✓		✓			✓		✓	✓		✓		✓		✓	
N093		✓		✓		✓		✓	✓			✓		✓		✓
K239	✓			✓		✓	✓	✓	✓		✓	✓	✓	✓	✓	✓

W= wild type, M= mutant

CHAPTER 5

5.0 DISCUSSIONS AND CONCLUSION

5.1 SUMMARY

The present study sought to screen and identify compounds from the Malaria Box with potency against clinical isolates of *P. falciparum*. In order to achieve this aim, an optimal drug assay was first developed for screening the Malaria Box compounds. This was accomplished by screening Compound X against two drug-resistant laboratory strains of *P. falciparum* (Dd2 and 7G8) using growth inhibition assay. Dose-response graphs were plotted and used to estimate IC_{50} values in GraphPad prism. Closely reproducible IC_{50} values were used to qualify the assay as optimal. After the growth inhibition assay was optimized, ten (10) compounds from the Malaria Box were selected and screened against four (4) clinical isolates N093, A156, A160 and K239. IC_{50} values were estimated from the dose-response curves were and this was used to assess the potency of the selected compounds. The potency of the compounds was assessed based on criteria used by used by Batista *et al.* for classifying the antiplasmodial activity of a compound. Compounds in the range of IC_{50} <1 µM have excellent potency; those in the IC_{50} range of 1-20 µM have good activity, those in IC_{50} of 20-100 µM have moderate activity; those in the IC_{50} range of 100-200 µM have low activity, and compounds in IC_{50} > 200 µM are inactive. The IC_{50} values were also used to select compounds with IC_{50} values below 500 nM that can be prioritized for further exploration. The clinical isolates were also screened against chloroquine and artesunate in order to establish the phenotypes of the clinical isolates based on their response to standard antimalarial drugs. The IC_{50} values were determined and compared with the standard reference values. This helped establish if the clinical isolates were drug resistant or susceptible isolates. To confirm the phenotypes observed from the growth inhibition assay, restriction fragment length polymorphism (RFLP) analysis was used to determine the genotypes of the clinical isolates.

Four genes (*pfdfps, pfmdr1, pfdfr* and *pfcrt*) were assessed for single nucleotide variations (SNV) that confer resistance to various antimalarial drugs. This also provided a way to validate the data obtained from the phenotypic screening.

5.2 DISCUSSIONS

The study aimed to identify potent compounds from the Malaria Box library of 400 compounds that are active against clinical isolates of *P. falciparum.* Potent compounds from this study with new mechanisms of action will be instrumental in accelerating the development of new drugs against drug-resistant parasites. The challenge with the development of the drug screening assay was to obtain reproducible data as well as conduct the assay for a large number of compounds in a very cost-effective manner using very small test volumes (Flannery *et al.*, 2013). The reproducible IC_{50} values (Fig.4.1 and Fig.4.2) observed for Compound X against Dd2 and 7G8 strains of *P. falciparum* indicates the assay was optimal. According to the system of criteria used by Batista *et al.* (2009), for considering the antiplasmodial activity of a given compound, Compound X can be said to have good antiplasmodial activity. Although the IC_{50} values for Compound X are several folds higher than that obtained for conventional drugs like chloroquine and artesunate, structural-activity relationship studies can be conducted to further refine Compound X to improve its potency for antimalarial drug developments.

Once the assay was optimal, it was used to screen the Malaria Box compounds against the clinical isolate. Although several drug-screening studies had identified compounds from Malaria Box with high potency against laboratory-adapted isolates of *P. falciparum*, not much has been reported on the potency of these drugs against clinical isolates. It was observed from this study that the efficacy of MMV007384 against A156 did not follow a dose-response pattern, suggesting low sensitivity or resistance to this compound. On the contrary, N093 appears to be most sensitive to artesunate and each of the malaria box compounds screened

(MMV006787, MMV006278, MMV085203, MMV007384, MMV396797 and MMV009015), but showed high resistance to chloroquine. Gresty *et al.* (2014), have observed that the presence of K76 T single nucleotide variation (SNV) in the *pfcrt* gene of *P. falciparum* clinical isolates that mediates resistance to chloroquine also decrease the susceptibility of the parasites to quinine and halofantrine, but increases their sensitivity to artemisinin and lumefantrine. It was therefore thought that N093 might harbour the K76T mutation which is modulating its sensitivity to most of the Malaria Box compounds. This hypothesis was confirmed in the genotyping analysis, where N093 was identified to be the only clinical isolate, out of the four isolates tested, shown to possess K76T mutation conferring resistance to chloroquine and sensitivity to artesunate. Nevertheless, all the clinical isolates were found to have polymorphisms in the *pfmdr1* gene that is known to modulate the level of chloroquine resistance (Duraisingh *et al.*, 2005, Sutar *et al.*, 2011). Three of the ten Malaria Box compounds (MMV006787, MMV008956 and MMV085203) were identified as inhibitors of the enzyme *P. falciparum* thioredoxin reductase (P*f*TrxR) enzyme. The thioredoxin reductase (P*f*Trx) is part of the thioredoxin system that is crucial for the maintenance of redox homeostasis and antioxidant defense in *P. falciparum* (Jortzik *et al.*, 2012). The breakdown of hemoglobin in *P. falciparum* results in the production of heme and superoxide anions which increases the oxidative stress in the parasite, leading to its death (Müller, 2003). Superoxide dismutase catalyses the dismutation of superoxide anions to hydrogen peroxide in order to eliminate the superoxide anions. This is followed by *P. falciparum* peroxiredoxins converting the hydrogen peroxide formed to water to complete the antioxidant defense. After playing this crucial role, peroxiredoxins becomes inactive and thus require P*f*Trx to convert it back to its active form. P*f*Trx also becomes inactive after converting peroredoxins to their active form and also requires P*f*TrxR to convert P*f*Trx back to its active form (Jortzik *et al.*, 2012, Müller, 2003). Therefore,

processes that inhibit the activity of the P*f*TrxR will result in an increase in oxidative stress and eventually the death of the parasite.

Studies have shown P*f*TrxR is a novel target for the design of new antimalarial drugs (Tiwari *et al.*, 2016b). Therefore identifying compounds that inhibit this target means these compounds can be useful in controlling drug-resistant parasites. This will be important for the global elimination of malaria. Although MMV008956 and MMV085203 both target P*f*TrxR, MMV085203 was more efficacious at inhibiting this target than MMV008956. This could mean MMV085203 might be binding more strongly to the active site of this enzyme compared to MMV008956. The IC_{50} values reported for the inhibitory activity of MMV008956 and MMV085203 against P*f*TrxR from other studies were 6.6 µM and 0.9 µM respectively. These IC_{50} values are more than ten-fold higher than IC50 values observed for these compounds in the present study (average IC_{50} of 152.22 nM and 63.83 nM, respectively). These observations might be because P*f*TrxR is probably acting as a secondary target for killing the parasite as was recently proposed by Tiwari *et al*., 2016. Generally, MMV006787 was very potent against the clinical isolates compared to the Dd2 laboratory strain of *P. falciparum*. MMV000753 which has been identified as an inhibitor of hemozoin formation, was found in this study to be more efficacious against the clinical isolates than was reported in D6 (a chloroquine sensitive strain of *P. falciparum*) and C235 (multi-drug resistant strain of *P. falciparum*) by previous studies (Fong *et al.*, 2015).This observation further confirms the need to use clinical isolates in screening for new drug compounds in addition to the use of laboratory strain. Transcriptional profile studies conducted using of MMV006787 against *P. falciparum*, identified MMV006787 to down-regulate DNA replication, histone chaperones and initiation of translation. However, proteins involved in the pyruvate metabolism, methionine and polyamine metabolism were up-regulated (Wah *et al.*, 2015). Pyruvate metabolic pathway is important for the conversion of pyruvate acetyl-CoA, which forms an important precursor for the

tricarboxylic acid cycle and type II fatty acid synthesis. Inhibition of proteins in the pyruvate metabolic pathway has been shown to prevent plasmodium parasites from forming exo-erythrocytic merozoites during the late liver stage development and thus preventing initiation of blood stage infection (Pei *et al.*, 2010). Also, MMV006787 has been found to cause the up-regulation of parasite exported proteins such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) as well as transporters of the mitochondrial and apicoplast membranes (Wah *et al.*, 2015). The apicoplast has been identified to play a key role in the synthesis of type II fatty acids as well as isoprenoid, iron-sulfur cluster and heme biosynthesis, respectively (Lim *et al.*, 2010, Van Dooren *et al.*, 2006). In *P. falciparum*, Isoprenoid biosynthesis takes place in the apicoplast and are essential for cellular processes such as tRNA isopentenylation and protein prenylation as well as the synthesis of Vitamin E, carotenoids, ubiquinone and dolichols (Guggisberg *et al.*, 2014). Therefore isoprenoids synthesis through the methylerythritol phosphate pathway appears to be an attractive antimalarial drug target for the development of new antimalarials. Also, the mitochondrion of *Plasmodium* species has also been identified as a viable target for antimalarial drug discovery. The *Plasmodium* mitochondrion play key roles in pyrimidine metabolism, active electron transport chain, coenzyme Q and iron-sulphur cluster biosynthesis (Van Dooren *et al.*, 2006). In *Plasmodium* parasites, the mitochondrion and apicoplast have been shown to be crucial for heme biosynthesis (Lim *et al.*, 2010). Some studies have identified the shuttling of heme intermediates between the mitochondrion and apicoplast during heme biosynthesis (Van Dooren *et al.*, 2006). Heme biosynthetic pathway thus appears to be an important pathway, since heme serves as an important prosthetic group for many proteins. It therefore, seems to suggest that MMV006787 might be targeting an essential proteins in the transporters of mitochondrial and apicoplast membranes that could be interfering with critical processes in these organelles and hence affecting the survival of the parasite. So in an attempt to survive, the parasite engages structures and processes that will

provide more energy for the synthesis of essential proteins and also the generation of new structures and new cells to replace dead ones.

Therefore considering the fact that, MMV006787 has targets which are novel and was identified as one of the compounds with IC_{50} below 500 nM range against the clinical isolates, means that this compound can be further explored for new antimalarial drugs development. This compound can be either used as a monotherapy or in combination with existing drugs to counter drug-resistant isolates.

Wah *et al.*, (2015) also found that cultured *P. falciparum* parasites in the presence of MMV008416, MMV085203 and MMV008956 had invasion-related proteins, parasite exported proteins (PfEMP1) and "the acidocalcisome" down-regulated. However, proteins involved in transcription, translation and inositol phosphate metabolism were up-regulated (Wah *et al.,* 2015). Inositol phosphate (3)–dependent Ca(2+) release in *Plasmodium* parasites, has been associated with growth as well as regulation and synchronization of the parasite cell cycle(Alves *et al.*, 2011). Some studies have also shown that when phosphatidylinostitol (5,5)-biphosphate hydrolysis is blocked, *P. falciparum* merozoites are not able to egress from infected human erythrocytes (Brochet *et al.,* 2014). Therefore the primary target of MMV008416, MMV085203 and MMV008956 could be essential proteins involved in inositol metabolism that affect the proliferation of parasite and threatens its survival in addition to their secondary target which has been identified to be P*f*TrxR. It is possible that in a bid to survive, the parasites up-regulated proteins involved in inositol phosphate metabolism as well as transcription and translation of new proteins to replace damaged ones that were probably affected by these compounds. MMV006278 was identified as one of five Malaria Box compounds with activity against P*f*TrxR with an IC_{50} of 3.5 µM (Tiwari *et al.*, 2016a). However, in this study, an average IC_{50} of 8.527 µM was observed which is about 2-fold the IC_{50} reported for the activity of this compound against P*f*TrxR. This could mean MMV006278

interacts less strongly with P*f*TrxR or was less bioavailable at the target site and therefore was not effective against this target enzyme.

Studies have also shown that MMV006278 up-regulates acidocalcisome proteins in malaria parasites (*Wah et al.,* 2015). Acidocalcisomes are important storage sites for cations and phosphorus that play key roles in pyrophosphate and polyphosphate metabolism and the maintenance of calcium homeostasis, maintenance of intracellular pH and osmoregulatory functions (Miranda *et al.,* 2008). Ca (2+) signaling following the release of Ca (2+) from acidocalcisome has been shown to be crucial for the invasion of new uninfected erythrocytes and proliferation (Docampo *et al.*, 2011, Glushakova *et al.*, 2013). Therefore, this compound was found to be less efficacious against the clinical isolates, it may be further explored for a possible combination therapy with existing antimalarial drugs, considering the fact that this compound has a novel target compared to existing antimalarials.

MMV000753 which has been identified as an inhibitor of hemozoin formation, was found in this study to be more efficacious against the clinical isolates than was reported in D6 (a chloroquine sensitive strain of *P. falciparum*) and C235 (multi-drug resistant strain of *P. falciparum*) by previous studies (Fong *et al.*, 2015).This observation further confirms the need to use clinical isolates in screening for new drug compounds in addition to the use of laboratory strain. MMV000753 has also been identified to affect proteins in the chaperone system of *P. falciparum* (Wah *et al.* 2015). The chaperone system in *Plasmodium* is important for the proper folding of proteins, host cell remodeling and response to stress in the environment of the cell (Acharya *et al.*, 2007). Some studies have also shown that inhibition of PfHsp90 prevents *P falciparum* parasites from progressing to the trophozoite stage (Banumathy *et al.*, 2003). It therefore, appears that MMV000753 with novel targets that can be explored further for antimalarial drug development either as a monotherapy or in combination with existing drugs.

MMV019555 was found to be less effective against the N093 which was identified to be the only chloroquine resistant strain in this study.

However, its potency against the multi-drug resistant and chloroquine-sensitive clinical isolates was comparable to the potency reported from other studies of this compound against Dd2 (Ullah, *et al.*, 2016).

MMV019555 has been identified to affect the functions of several proteases involved in invasion and egress of *P. falciparum* into erythrocytes (Wah *et al*. 2015). One of the implicated proteases includes subtilisin-like serine proteases (PfSUB1 and PfSUB2) that are germane for the processing of merozoites surface proteins used by the parasite for invasion and egress out of host erythrocytes (Li *et al.*, 2012). *P. falciparum* rhomboid proteases (PfROM1 and PfROM2) have also been shown to mediate the cleavage of a variety of substrates with transmembrane domains which are most likely engaged in the invasive stages of *P. falciparum*. Studies have also shown that when cysteine proteases are blocked with broad-spectrum cysteine protease inhibitors, *Plasmodium* parasites are not able to effectively invade host erythrocytes (Francis *et al.*, 1997). Proteases in *P. falciparum* also play a major role in the degradation of hemoglobin for the synthesis of essential amino acids required for growth and proliferation. Protein transport, protein homeostasis and host cell remodeling are all essential functions within the parasite that require proteases (Francis *et al.*, 1997). This has made proteases an attractive target for the design of new antimalarial drugs. Therefore, identifying MMV019555 with inhibitory activity against *P. falciparum* proteases suggest this compound can be further explored for antimalarial drug developments especially in combination with existing drugs.

5.3 CONCLUSION

In this study an optimal growth inhibition drug assay was developed for screening and identifying active compounds with antiplasmodial activity. This assay was used to determine the potency of Compound X that was found to have good antimalarial properties against Dd2 and 7G8. The assay was subsequently used to screen ten compounds from the Malaria Box against four clinical isolates. MMV006787, MMV0085203 and MMV008956 were identified to have excellent antimalarial activity against all the clinical isolates with IC_{50} values below 500 nM. MMV0085203 was the most potent of the three compounds with IC_{50} values below 100 nM. The drug assay was also used to screen the clinical isolates against chloroquine and artesunate. N093 was found to be resistant to chloroquine but sensitive to artesunate. RFLP analysis was also conducted to confirm the phenotypes of the isolates. N093 was also identified in this study as the only clinical isolate with the K76T mutation that conferred resistance to chloroquine but sensitivity to artesunate and the Malaria Box compounds.

Therefore, the use of clinical isolates in this study further shows the need to validate potential drug compounds using clinical isolates for their further development.

5.4 FUTURE STUDIES

Three compounds from the Malaria Box were identified to have excellent potency with IC_{50} values below 500 nM. The next stage of this study will be to conduct stage-specific assays to determine whether these compounds act at the ring, trophozoite or schizont stages of the parasite. After the stage-specific analysis, target validations will be conducted to identify the possible targets for these compounds. This will be followed by the elucidation of the mechanisms of action of these compounds.

REFERENCES

Abiodun, O. O., Gbotosho, G. O., Ajaiyeoba, E. O., Happi, C. T., Hofer, S., Wittlin, S., Sowunmi, A., Brun, R. & Oduola, A. M. J. (2010). Comparison of SyBr green I-, pico green-, and [3h]-hypoxanthine-based assays for *in vitro* antimalarial screening of plants from Nigerian ethnomedicine. *Parasitology Research,* 106**,** 933-939.

Acharya, P., Kumar, R. & Tatu, U. (2007). Chaperoning a cellular upheaval in malaria: heat shock proteins in plasmodium falciparum. *Molecular And Biochemical Parasitology,* 153**,** 85-94.

Alonso, P. L., Brown, G., Arevalo-Herrera, M., Binka, F., Chitnis, C., Collins, F., Doumbo, O. K., Greenwood, B., Hall, B. F., Levine, M. M., Mendis, K., Newman, R. D., Plowe, C. V., Rodríguez, M. H., Sinden, R., Slutsker, L. & Tanner, M. (2011). A research agenda to underpin malaria eradication. *PloS Medicine,* 8**,** E1000406.

Alves, E., Bartlett, P. J., Garcia, C. R. S. & Thomas, A. P. (2011). Melatonin and ip(3)-induced ca(2+) release from intracellular stores in the malaria parasite plasmodium falciparum within infected red blood cells. *The Journal Of Biological Chemistry,* 286**,** 5905-5912.

Ariey, F., Witkowski, B., Amaratunga, C., Beghain, J., Langlois, A.-C., Khim, N., Kim, S., Duru, V., Bouchier, C., Ma, L., Lim, P., Leang, R., Duong, S., Sreng, S., Suon, S., Chuor, C. M., Bout, D. M., Menard, S., Rogers, W. O., Genton, B., Fandeur, T., Miotto, O., Ringwald, P., Le Bras, J., Berry, A., Barale, J.-C., Fairhurst, R. M., Benoit-Vical, F., Mercereau-Puijalon, O. & Menard, D. (2014). a molecular marker of artemisinin-resistant plasmodium falciparum malaria. *Nature,* 505**,** 50-55.

Arnold, M. S., Engel, J. A., Chua, M. J., Fisher, G. M., Skinner-Adams, T. S. & Andrews, K. T. (2016). Adaptation of the [3H]Hypoxanthine Uptake Assay for In Vitro-Cultured Plasmodium knowlesi Malaria Parasites. *Antimicrob Agents Chemother,* 60**,** 4361-3.

Arrow, K. J., Panosian, C. & Gelband, H. (2004). Saving lives, buying time: economics of malaria drugs in an age of resistance. *Drugs***,** 125.

Ashley, E. A., Dhorda, M., Fairhurst, R. M., Amaratunga, C., Lim, P., Suon, S., Sreng, S., Anderson, J. M., Mao, S., Sam, B., Sopha, C., Chuor, C. M., Nguon, C., Sovannaroth, S., Pukrittayakamee, S., Jittamala, P., Chotivanich, K., Chutasmit, K., Suchatsoonthorn, C., Runcharoen, R., Hien, T. T., Thuy-Nhien, N. T., Thanh, N. V., Phu, N. H., Htut, Y., Han, K.-T., Aye, K. H., Mokuolu, O. A., Olaosebikan, R. R., Folaranmi, O. O., Mayxay, M., Khanthavong, M., Hongvanthong, B., Newton, P. N., Onyamboko, M. A., Fanello, C. I., Tshefu, A. K., Mishra, N., Valecha, N., Phyo, A. P., Nosten, F., Yi, P., Tripura, R., Borrmann, S., Bashraheil, M., Peshu, J., Faiz, M. A., Ghose, A., Hossain, M. A., Samad, R., Rahman, M. R., Hasan, M. M., Islam, A., Miotto, O., Amato, R., Macinnis, B., Stalker, J., Kwiatkowski, D. P., Bozdech, Z., Jeeyapant, A., Cheah, P. Y., Sakulthaew, T., Chalk, J., Intharabut, B., Silamut, K., Lee, S. J., Vihokhern, B., Kunasol, C., Imwong, M., Tarning, J., Taylor, W. J., Yeung, S., Woodrow, C. J., Flegg, J. A., Das, D., Smith, J., Venkatesan, M., Plowe, C. V., Stepniewska, K., Guerin, P. J., Dondorp, A. M., Day, N. P. & White, N. J. (2014). spread of artemisinin resistance in plasmodium falciparum malaria. *The New England Journal Of Medicine,* 371**,** 411-23.

Awasthi, G. & Das, A. (2013). Genetics of chloroquine-resistant malaria: A haplotypic view. *Memorias do Instituto Oswaldo Cruz,* 108**,** 947-961.

Baggish, A. L. & Hill, D. R. (2002). Antiparasitic agent atovaquone. *Antimicrobial Agents And Chemotherapy,* 46**,** 1163-1173.

Baird, J. K. (2009). Malaria zoonoses. *Travel Med Infect Dis,* 7.

Baldwin, J., Michnoff, C. H., Malmquist, N. A., White, J., Roth, M. G., Rathod, P. K. & Phillips, M. A. (2005). High-throughput screening for potent and selective inhibitors of plasmodium falciparum dihydroorotate dehydrogenase. *Journal Of Biological Chemistry,* 280**,** 21847-21853.

Baniecki, M. L., Wirth, D. F. & Clardy, J. (2007). High-throughput plasmodium falciparum growth assay for malaria drug discovery. *Antimicrobial Agents And Chemotherapy,* 51**,** 716-723.

Banumathy, G., Singh, V., Pavithra, S. R. & Tatu, U. (2003). Heat shock protein 90 function is essential for plasmodium falciparum growth in human erythrocytes. *The Journal Of Biological Chemistry,* 278**,** 18336-45.

Bennett, T. N., Paguio, M., Gligorijevic, B., Seudieu, C., Kosar, A. D., Davidson, E. & Roepe, P. D. (2004). Novel, rapid, and inexpensive cell-based quantification of antimalarial drug efficacy. *Antimicrobial Agents And Chemotherapy,* 48**,** 1807-1810.

Berry, A., Iriart, X., Wilhelm, N., Valentin, A., Cassaing, S., Witkowski, B., Benoit-Vical, F., Menard, S., Olagnier, D., Fillaux, J., Sire, S., Le Coustumier, A. & Magnaval, J. F. (2011). Imported Plasmodium knowlesi malaria in a French tourist returning from Thailand. *Am J Trop Med Hyg,* 84.

Bloland, P. B. (2001). Drug resistance in malaria. *Geneva: World Health Organization.*

Booker, M. L., Bastos, C. M., Kramer, M. L., Barker, R. H., Skerlj, R., Sidhu, A. B., Deng, X., Celatka, C., Cortese, J. F., Guerrero Bravo, J. E., Crespo Llado, K. N., Serrano, A. E., Angulo-Barturen, I. I., Jim??Nez-D??Az, M. A. B. N., Viera, S., Garuti, H., Wittlin, S., Papastogiannidis, P., Lin, J. W., Janse, C. J., Khan, S. M., Duraisingh, M., Coleman, B., Goldsmith, E. J., Phillips, M. A., Munoz, B., Wirth, D. F., Klinger, J. D., Wiegand, R. & Sybertza, E. (2010). Novel Inhibitors Of Plasmodium Falciparum Dihydroorotate Dehydrogenase With anti-malarial activity in the mouse model. *Journal Of Biological Chemistry*, 285**,** 33054-33064.

Bowden, D. K., Bastien, P., Douglas, F. P., Muir, J. W. & Tambisari, E. (1982). Chloroquine-resistant plasmodium falciparum malaria in vanuatu. *Med J Aust*, 2.

Briolant, S., Henry, M., Oeuvray, C., Amalvict, R., Baret, E., Didillon, E., Rogier, C. & Pradines, B. (2010). Absence of association between piperaquine in vitro responses and polymorphisms in the pfcrt, pfmdr1, pfmrp, and pfnhe genes in plasmodium falciparum. *Antimicrob Agents Chemother*, 54**,** 3537-44.

Brochet, M., Collins, M. O., Smith, T. K., Thompson, E., Sebastian, S., Volkmann, K., Schwach, F., Chappell, L., Gomes, A. R., Berriman, M., Rayner, J. C., Baker, D. A., Choudhary, J. & Billker, O. (2014). Phosphoinositide metabolism links cgmp-dependent protein kinase g to essential ca2+ signals at key decision points in the life cycle of malaria parasites. *Plos Biology*, 12.

Bruce-Chwatt, L. J. (1962). Classification of antimalarial drugs in relation to different stages in the life-cycle of the parasite: commentary on a diagram. *Bulletin Of The World Health Organization*, 27**,** 287-290.

Burrows, J. N., Burlot, E., Campo, B., Cherbuin, S., Jeanneret, S., Leroy, D., Spangenberg, T., Waterson, D., Wells, T. N. & Willis, P.(2014). Antimalarial drug discovery - the path towards eradication. *Parasitology*, 141**,** 128-39.

Calderón, F., Barros, D., Bueno, J. M., Coterón, J. M., Fernández, E., Gamo, F. J., Lavandera, J. L., León, M. L., Macdonald, S. J. F., Mallo, A., Manzano, P., Porras, E., Fiandor, J. M. & Castro, J. (2011). An invitation to open innovation in malaria drug discovery: 47 quality starting points from the tcams. *Acs Medicinal Chemistry Letters*, 2**,** 741-746.

Calderón, F., Vidal-Mas, J., Burrows, J., De La Rosa, J. C., Jiménez-Díaz, M. B., Mulet, T., Prats, S., Solana, J., Witty, M., Gamo, F. J. & Fernández, E. (2012). A divergent sar study allows optimization of a potent 5-ht2c inhibitor to a promising antimalarial scaffold. *Acs Medicinal Chemistry Letters*, 3**,** 373-377.

Carlton, J. M., Adams, J. H., Silva, J. C., Bidwell, S. L., Lorenzi, H., Caler, E., Crabtree, J., Angiuoli, S. V., Merino, E. F., Amedeo, P., Cheng, Q., Coulson, R. M. R., Fernandez-Becerra, C., Gilson, P. R., Gueye, A. H., Guo, X., Kang, S., Kooij, T. W. A., Korsinczky, M., Meyer, E. V., Nene, V., Paulsen, I., White, O., Ralph, S. A., Ren, Q., Sargeant, T. J., Salzberg, S. L., Stoeckert, C. J., Sullivan, S. A., Yamamoto, M. M., Hoffman, S. L., Wortman, J. R., Gardner, M. J., Galinski, M. R., Barnwell, J. W. & Fraser-Liggett, C. M. (2008). Comparative genomics of the neglected human malaria parasite plasmodium vivax. 455.

Chauhan, K., Pande, V. & Das, A. (2014). DNA sequence polymorphisms of the pfmdr1 gene and association of mutations with the pfcrt gene in indian plasmodium falciparum isolates. *Infect Genet Evol,* 26**,** 213-22.

Cooper, R. A., Hartwig, C. L. & Ferdig, M. T. (2005). Pfcrt is more than the plasmodium falciparum chloroquine resistance gene: a functional and evolutionary perspective. *Acta Trop,* 94**,** 170-80.

Cowman, A. F. & Crabb, B. S. (2006). Invasion of red blood cells by malaria parasites. *Cell,* 124**,** 755-766.

Cowman, A. F., Galatis, D. & Thompson, J. K. (1994). Selection for mefloquine resistance in Plasmodium falciparum is linked to amplification of the pfmdr1 gene and cross-resistance to halofantrine and quinine. *Proceedings of the National Academy of Sciences of the United States of America,* 91**,** 1143-1147.

Cox-Singh, J., Davis, T. M. E., Lee, K.-S., Shamsul, S. S. G., Matusop, A., Ratnam, S., Rahman, H. A., Conway, D. J. & Singh, B. (2008a). Plasmodium knowlesi malaria in humans is widely distributed and potentially life-threatening. *Clinical Infectious Diseases : An Official Publication Of The Infectious Diseases Society Of America,* 46**,** 165-171.

Cox-Singh, J. & Singh, B. (2008b) . Knowlesi malaria: newly emergent and of public health importance? *Trends Parasitol,* 24.

Crowther, G. J., Napuli, A. J., Gilligan, J. H., Gagaring, K., Borboa, R., Francek, C., Chen, Z., Dagostino, E. F., Stockmyer, J. B., Wang, Y., Rodenbough, P. P., Castaneda, L. J., Leibly, D. J., Bhandari, J., Gelb, M. H., Brinker, A., Engels, I., Taylor, J., Chatterjee, A. K., Fantauzzi, P., Glynne, R. J., Van Voorhis, W. C. & Kuhen, K. L. (2011). Identification of inhibitors for

putative malaria drug targets amongst novel antimalarial compounds. *Molecular And Biochemical Parasitology,* 175**,** 21-29.

Cui, L., Mharakurwa, S., Ndiaye, D., Rathod, P. K. & Rosenthal, P. J. (2015). Antimalarial Drug Resistance: Literature review and activities and findings of the icemr network. *The American Journal Of Tropical Medicine And Hygiene,* 93**,** 57-68.

Cui, L. & Su, X. Z. (2009). Discovery, mechanisms of action and combination therapy of artemisinin. *Expert Rev Anti Infect Ther,* 7**,** 999-1013.

Desjardins, R. E., Canfield, C. J., Haynes, J. D. & Chulay, J. D. (1979). Quantitative activity semiautomated technique. *Antimicrobial Agents And Chemotherapy,* 16**,** 710-718.

Dharia, N. V., Sidhu, A. B. S., Cassera, M. B., Westenberger, S. J., Bopp, S. E., Eastman, R. T., Plouffe, D., Batalov, S., Park, D. J., Volkman, S. K., Wirth, D. F., Zhou, Y., Fidock, D. A. & Winzeler, E. A. (2009). Use of high-density tiling microarrays to identify mutations globally and elucidate mechanisms of drug resistance in plasmodium falciparum. *Genome Biology,* 10**,** R21.

Diagana, T. T. (2015). Supporting malaria elimination with 21st century antimalarial agent drug discovery. *Drug Discov Today,* 20**,** 1265-70.

Dinko, B., Oguike, M. C., Larbi, J. A., Bousema, T. & Sutherland, C. J. (2013). Persistent detection of plasmodium falciparum, p. malariae, p. ovale curtisi and p. ovale wallikeri after act treatment of asymptomatic ghanaian school-children. *Int J Parasitol Drugs Drug Resist,* 3**,** 45-50.

Docampo, R. & Moreno, S. N. J.(2011). Acidocalcisomes. *Cell Calcium,* 50**,** 113-119.

Dondorp, A. M., Nosten, F., Yi, P., Das, D., Hanpithakpong, W., Lee, S. J., Ringwald, P., Imwong, M., Chotivanich, K. & Lim, P. (2012). Artemisinin resistance in plasmodium falciparum malaria. *N Engl J Med,* 361**,** 455-467.

Drakeley, C., Sutherland, C., Bousema, J. T., Sauerwein, R. W. & Targett, G. A. T. (2006). The epidemiology of plasmodium falciparum gametocytes : weapons of mass dispersion. 22.

Druilhe, P., Moreno, A., Blanc, C., Brasseur, P. H. & Jacquier, P. (2001). A Colorimetric in vitro drug sensitivity assay for plasmodium falciparum based on a highly sensitive double-site lactate dehydrogenase antigen-capture enzyme-linked immunosorbent assay. *American Journal Of Tropical Medicine And Hygiene,* 64**,** 233-241.

Duraisingh, M. T. & Cowman, A. F. (2005). Contribution of the pfmdr1 gene to antimalarial drug-resistance. *Acta Tropica,* 94**,** 181-90.

Fancony, C., Gamboa, D., Sebastiao, Y., Hallett, R., Sutherland, C., Sousa-Figueiredo, J. C. & Nery, S. V. (2012). Various pfcrt and pfmdr1 genotypes of plasmodium falciparum cocirculate with p. malariae, p. ovale spp., and p. vivax in Northern Angola. *Antimicrob Agents Chemother,* 56**,** 5271-7.

Faye, F. B. K., Konaté, L., Rogier, C. & Trape, J.-F. (1998). Plasmodium ovale in a highly malaria endemic area of Senegal. *Transactions Of The Royal Society Of Tropical Medicine And Hygiene,* 92**,** 522-525.

Feachem, R. G., Phillips, A. A., Targett, G. A. & Snow, R. W.(2010). Call to action: priorities for malaria elimination. *The Lancet,* 376**,** 1517-1521.

Fidock, D. A. 2016. Drug discovery: Chemical diversity targets malaria. *Nature,* 538**,** 323-325.

Fidock, D. A., Rosenthal, P. J., Croft, S. L., Brun, R. & Nwaka, S. (2004). Antimalarial drug discovery: efficacy models for compound screening. *Nat Rev Drug Discov,* 3**,** 509-520.

Flannery, E. L., Chatterjee, A. K. & Winzeler, E. A. (2013). Antimalarial drug discovery: approaches and progress towards new medicines. *Nature Reviews. Microbiology,* 11**,** 849-862.

Fong, K. Y., Sandlin, R. D. & Wright, D. W. (2015). Identification Of B-Hematin inhibitors in the MMV Malaria Box. *International Journal For Parasitology: Drugs And Drug Resistance,* 5**,** 84-91.

Foote, S. J. & Cowman, A. F. (1994). The mode of action and the mechanism of resistance to antimalarial drugs. *Acta Tropica,* 56**,** 157-171.

Fowler, E. V., Chavchich, M., Chen, N., Peters, J. M., Kyle, D. E., Gatton, M. L. & Cheng, Q. (2006). Physical linkage to drug resistance genes results in conservation of var genes among West Pacific Plasmodium falciparum isolates. *Journal Of Infectious Diseases,* 194**,** 939-948.

Francis, S. E., Sullivan, D. J. & Goldberg, D. E. (1997). Hemoglobin metabolism in the malaria parasite Plasmodium falciparum. *Annual Review Of Microbiology,* 51**,** 97-123.

Francis, S. E., Sullivan, D. J. & Goldberg, D. E. (1997). Hemoglobin metabolism in the malaria parasite Plasmodium falciparum. *Annual review of microbiology,* 51**,** 97-123.

Galindo, J. A., Cristiano, F. A., Knudson, A., Nicholls, R. S. & Guerra, A. P. (2010). Point mutations in dihydrofolate reductase and dihydropteroate synthase genes of Plasmodium falciparum from three endemic malaria regions in Colombia. *American Journal Of Tropical Medicine And Hygiene,* 30**,** 56-64.

Gamo, F.-J., Sanz, L. M., Vidal, J., De Cozar, C., Alvarez, E., Lavandera, J.-L., Vanderwall, D. E., Green, D. V. S., Kumar, V., Hasan, S., Brown, J. R., Peishoff, C. E., Cardon, L. R. & Garcia-Bustos, J. F. (2010). Thousands of chemical starting points for antimalarial lead identification. *Nature,* 465**,** 305-310.

Glushakova, S., Lizunov, V., Blank, P. S., Melikov, K., Humphrey, G. & Zimmerberg, J. (2013). Cytoplasmic free Ca2+ is essential for multiple steps in malaria parasite egress from infected erythrocytes. *Malaria Journal,* 12**,** 41.

Gosling, R. & Von Seidlein, L. (2016). The Future of the RTS,S/AS01 malaria vaccine: an alternative development plan. *Plos Med,* 13**,** E1001994.

Greenwood, B. (2002). The fever trail: in search of the cure for malaria. *Nat Med,* 8**,** 1346.

Gregson, A. & Plowe, C. V. (2005). Mechanisms of resistance of malaria parasites to antifolates. *Pharmacol Rev,* 57**,** 117-45.

Gresty, K. J., Gray, K.-A., Bobogare, A., Wini, L., Taleo, G., Hii, J., Cheng, Q. & Waters, N. C. (2014). Genetic mutations in Plasmodium falciparum and Plasmodium vivax dihydrofolate reductase (Dhfr) And dihydropteroate synthase (Dhps) in Vanuatu And Solomon Islands Prior to the introduction of artemisinin combination therapy. *Malaria Journal,* 13**,** 1-7.

Guggisberg, A. M., Amthor, R. E. & Odom, A. R. (2014). Isoprenoid biosynthesis in Plasmodium falciparum. *Eukaryotic Cell,* 13**,** 1348-1359.

Guiguemde, W. A., Shelat, A. A., Bouck, D., Duffy, S., Crowther, G. J., Davis, P. H., Smithson, D. C., Connelly, M., Clark, J., Zhu, F., Jiménez-Díaz, M. B., Martinez, M. S., Wilson, E. B., Tripathi, A. K., Gut, J., Sharlow, E. R., Bathurst, I., Mazouni, F. E., Fowble, J. W., Forquer, I., Mcginley, P. L., Castro, S., Angulo-Barturen, I., Ferrer, S., Rosenthal, P. J., Derisi, J. L., Sullivan, D. J., Lazo, J. S., Roos, D. S., Riscoe, M. K., Phillips, M. A., Rathod, P. K., Van Voorhis, W. C., Avery, V. M. & Guy, R. K. (2010). Chemical genetics of Plasmodium falciparum. *Nature,* 465**,** 311-315.

Gujjar, R., Marwaha, A., El Mazouni, F., White, J., White, K. L., Creason, S., Shackleford, D. M., Baldwin, J., Charman, W. N., Buckner, F. S., Charman, S., Rathod, P. K. & Phillips, M. A. (2009). Identification of a metabolically stable triazolopyrimidine-based dihydroorotate dehydrogenase inhibitor with antimalarial activity in mice. *Journal Of Medicinal Chemistry,* 52**,** 1864-1872.

Hammond, D. J., Burchell, J. R. & Pudney, M. (1985). Inhibition of pyrimidine biosynthesis de novo in Plasmodium falciparum by 2-(4-T-Butylcyclohexyl)-3-Hydroxy-1,4-naphthoquinone in vitro. *Mol Biochem Parasitol,* 14**,** 97-109.

Harinasuta, T., Suntharasamai, P. & Viravan, C. (1965). Chloroquine-resistant falciparum malaria in Thailand. *The Lancet,* 286**,** 657-660.

Hase, R., Uwamino Y Fau - Muranaka, K., Muranaka K Fau - Tochitani, K., Tochitani K Fau - Sogi, M., Sogi M Fau - Kitazono, H., Kitazono H Fau - Hosokawa, N. & Hosokawa, N. [Plasmodium malariae malaria with more than a 4-month incubation period: difficult to distinguish from a relapse of Plasmodium vivax malaria].

Hay, S. I., Guerra, C. A., Gething, P. W., Patil, A. P., Tatem, A. J., Noor, A. M., Kabaria, C. W., Manh, B. H., Elyazar, I. R. F., Brooker, S., Smith, D. L., Moyeed, R. A. & Snow, R. W. 2009. A world malaria map: Plasmodium falciparum endemicity in (2007). *Plos Medicine,* 6**,** 0286-0302.

Heinberg, A., Siu, E., Stern, C., LawrencE, E. A., Ferdig, M. T., deitsch, K. W. & Kirkman, L. A. (2013). Direct evidence for the adaptive role of copy number variation on antifolate susceptibility in Plasmodium falciparum. *Molecular microbiology,* 88**,** 702-712.

Hoffman, S., Dimpudus, A., Campbell, J., Marwoto, H., Sukri, N., Rustama, D., Punjabi, N., Oetomo, H. S., Harun, S., Heizmann, P. & Laughlin, L. (1985). RII And RIII type resistance of Plasmodium Falciparum to combination of mefloquine and sulfadoxine/pyrimethamine in Indonesia. *The Lancet,* 326**,** 1039-1040.

Hyde, J. E. (2009). Europe PMC funders group exploring the folate pathway in Plasmodium falciparum. 94**,** 191-206.

Jortzik, E. & Becker, K. (2012). Thioredoxin and glutathione systems in Plasmodium falciparum. *Int J Med Microbiol,* 302**,** 187-94.

Kamau, E., Campino, S., Amenga-Etego, L., Drury, E., Ishengoma, D., Johnson, K., Mumba, D., Kekre, M., Yavo, W., Mead, D., Bouyou-Akotet, M., Apinjoh, T., Golassa, L., Randrianarivelojosia, M., Andagalu, B., Maiga-Ascofare, O., Amambua-Ngwa, A., Tindana,

P., Ghansah, A., Macinnis, B., Kwiatkowski, D. & Djimde, A. A. (2015). K13-Propeller Polymorphisms in Plasmodium falciparum Parasites From Sub-Saharan Africa. *The Journal of Infectious Diseases*, 211**,** 1352-1355.

Karl, S., Wong, R. P., St Pierre, T. G. & Davis, T. M. (2009). A comparative study of a flow-cytometry-based assessment of in vitro Plasmodium falciparum drug sensitivity. *Malar J*, 8**,** 294.

Kato, N., Comer, E., Sakata-Kato, T., Sharma, A., Sharma, M., Maetani, M., Bastien, J., Brancucci, N. M., Bittker, J. A., Corey, V., Clarke, D., Derbyshire, E. R., Dornan, G. L., Duffy, S., Eckley, S., Itoe, M. A., Koolen, K. M., Lewis, T. A., Lui, P. S., Lukens, A. K., Lund, E., March, S., Meibalan, E., Meier, B. C., Mcphail, J. A., Mitasev, B., Moss, E. L., Sayes, M., Van Gessel, Y., Wawer, M. J., Yoshinaga, T., Zeeman, A. M., Avery, V. M., Bhatia, S. N., Burke, J. E., Catteruccia, F., Clardy, J. C., Clemons, P. A., Dechering, K. J., Duvall, J. R., Foley, M. A., Gusovsky, F., Kocken, C. H., Marti, M., Morningstar, M. L., Munoz, B., Neafsey, D. E., Sharma, A., Winzeler, E. A., Wirth, D. F., Scherer, C. A. & Schreiber, S. L. (2016). Diversity-oriented synthesis yields novel multistage antimalarial inhibitors. *Nature*, 538**,** 344-349.

Klayman, D. L. (1985). Qinghaosu (Artemisinin): an antimalarial drug from china. *Science*, 228**,** 1049-1055.

Korsinczky, M., Chen, N., Kotecka, B., Saul, A., Rieckmann, K. & Cheng, Q. (2000). Mutations in Plasmodium falciparum cytochrome b that are associated with atovaquone resistance are located at a putative drug-binding site. *Antimicrobial Agents and Chemotherapy*, 44**,** 2100-2108.

Korsinczky, M., Chen, N., Kotecka, B., Saul, A., Rieckmann, K. & Cheng, Q. (2000). Mutations in Plasmodium falciparum cytochrome b that are associated with atovaquone resistance are located at a putative drug-binding site. *Antimicrobial Agents And Chemotherapy*, 44**,** 2100-2108.

Krotoski, W. A., Bray, R. S., Garnham, P. C. C., Gwadz, R. W., Killick-Kendrick, R., Draper, C. C., Targett, G. A., Guy, M. W., Koontz, L. C. & Cogswell, F. B. (1982). Observations on early and late post-sporozoite tissue stages in primate malaria. II. the hypnozoite of Plasmodium cynomolgi bastianellii. from 3 to 105 days after infection, and detection of 36- to 40-hour pre-erythrocytic forms. *American Journal Of Tropical Medicine And Hygiene*, 31**,** 211-225.

Kwansa-Bentum, B., Ayi, I., Suzuki, T., Otchere, J., Kumagai, T., Anyan, W. K., Osei, J. H., Asahi, H., Ofori, M. F., Akao, N., Wilson, M. D., Boakye, D. A. & Ohta, N. (2011). Plasmodium falciparum isolates from southern ghana exhibit polymorphisms in the SERCA-Type PFATPASE6 though sensitive to artesunate in vitro. *Malar J*, 10**,** 187.

Li, H., Child, M. A. & Bogyo, M. (2012). Proteases as regulators of pathogenesis: examples from the apicomplexa. *Biochimica Et Biophysica Acta - Proteins And Proteomics*, 1824**,** 177-185.

Lim, L. & Mcfadden, G. I. (2010). The evolution, metabolism and functions of the apicoplast. *Philosophical Transactions Of The Royal Society Of London. Series B, Biological Sciences*, 365**,** 749-763.

Lobel, H. O., Campbell, C. C. & Roberts, J. M. (1985). Originally published as volume 1, issue 8433 fatal malaria in us civilians. *The Lancet,* 325**,** 873.

Lucantoni, L., Duffy, S., Adjalley, S. H., Fidock, D. A. & Avery, V. M. (2013). Identification of MMV Malaria Box Inhibitors of Plasmodium falciparum Early-Stage Gametocytes Using a Luciferase-Based High-. 57**,** 6050-6062.

Mather, M. W., Darrouzet, E., Valkova-Valchanova, M., Cooley, J. W., Mcintosh, M. T., Daldal, F. & Vaidya, A. B. (2005). Uncovering the molecular mode of action of the antimalarial drug atovaquone using a bacterial system. *J Biol Chem,* 280**,** 27458-65.

Meister, S., Plouffe, D. M., Kuhen, K. L., Bonamy, G. M. C., Wu, T., Barnes, W., Bopp, S. E., Borboa, R., Bright, A. T., Che, J., Dharia, N. V., Gagaring, K., Gettayacamin, M., Gordon, P., Groessl, T., Kato, N., Lee, M. C. S., Mcnamara, C. W., Fidock, D. A., Nagle, A., Nam, T.-G., Richmond, W., Roland, J., Rottmann, M., Zhou, B., Froissard, P., Glynne, R. J. & Mazier, D. (2012). NIH public access. 334**,** 1372-1377.

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

Meshnick, S. R. (1998). Artemisinin antimalarials: mechanisms of action and resistance. *Medecine Tropicale : Revue Du Corps De Sante Colonial,* 58**,** 13-17.

Middleton, J., Neil, S., Wintle, J., Clark-Lewis, I., Moore, H., Lam, C., Auer, M., Hub, E. & Rot, A. (1997). Transcytosis and surface presentation Of IL-8 By venular endothelial cells. *Cell,* 91**,** 385-395.

Miller, L. H., Mason, S. J., Dvorak, J. A., Mcginniss, M. H. & Rothman, I. K. (1975). Erythrocyte receptors for (Plasmodium knowlesi) malaria: Duffy Blood group determinants. *Science,* 189**,** 561-563.

Miranda, K., Souza, W. D., Plattner, H., Hentschel, J., Kawazoe, U., Fang, J. & Moreno, S. N. J. (2008). Acidocalcisomes in Apicomplexan parasites. *Experimental Parasitology,* 118**,** 2-9.

Mokgethi-Morule, T. & N'da, D. D. (2016). Cell based assays for anti-Plasmodium activity evaluation. *Eur J Pharm Sci,* 84**,** 26-36.

Mu, J., Ferdig, M. T., Feng, X., Joy, D. A., Duan, J., Furuya, T., Subramanian, G., Aravind, L., Cooper, R. A., Wootton, J. C., Xiong, M. & Su, X. Z. (2003). Multiple transporters associated with malaria parasite responses to chloroquine and quinine. *Molecular Microbiology,* 49**,** 977-989.

Müller, S. (2003). Thioredoxin reductase and glutathione synthesis in Plasmodium falciparum. *Redox Report : Communications In Free Radical Research,* 8**,** 251-5.

Nguyen-Dinh, P., Gardner, A. L., Campbell, C. C., Skinner, J. C. & Collins, W. E. 1981. Cultivation In Vitro Of The Vivax-Type Malaria Parasite Plasmodium Cynomolgi. *Science,* 212**,** 1146-1148.

Nixon, G. L., Moss, D. M., Shone, A. E., Lalloo, D. G., Fisher, N., O'neill, P. M., Ward, S. A. & Biagini, G. A. (2013). Antimalarial pharmacology and therapeutics of atovaquone. *J Antimicrob Chemother,* 68**,** 977-85.

Noedl, H., Wernsdorfer, W. H., Miller, R. S. & Wongsrichanalai, C. (2002). Histidine-rich protein II : a novel approach to malaria drug sensitivity testing histidine-rich protein II. *Antimicrobial Agents And Chemotherapy,* 46**,** 1658-1664.

Noedl, H., Wongsrichanalai, C. & Wernsdorfer, W. H. (2003). Malaria drug-sensitivity testing: new assays, new perspectives. *Trends In Parasitology.*

Nzila, A. (2006). The past, present and future of antifolates in the treatment of Plasmodium falciparum infection. *J Antimicrob Chemother,* 57**,** 1043-54.

Patel, V., Booker, M., Kramer, M., Ross, L., Celatka, C. A., Kennedy, L. M., Dvorin, J. D., Duraisingh, M. T., Sliz, P., Wirth, D. F. & Clardy, J. (2008). Identification and characterization of small molecule inhibitors of Plasmodium falciparum dihydroorotate dehydrogenase. *Journal Of Biological Chemistry,* 283**,** 35078-35085.

Pei, Y., Tarun, A. S., Vaughan, A. M., Herman, R. W., Soliman, J. M. B., Erickson-Wayman, A. & Kappe, S. H. I. (2010). Plasmodium pyruvate dehydrogenase activity is only essential for the parasite's progression from liver infection to blood infection. *Molecular Microbiology,* 75**,** 957-971.

Penna-Coutinho, J., Cortopassi, W. A., Oliveira, A. A., França, T. C. C. & Krettli, A. U. (2011). Antimalarial activity of potential inhibitors of *Plasmodium falciparum* lactate dehydrogenase enzyme selected by docking studies. *Plos One,* 6**,** E21237.

Petersen, I., Eastman, R. & Lanzer, M. (2011). Drug-resistant malaria: molecular mechanisms and implications for public health. *Febs Lett,* 585**,** 1551-62.

Plouffe, D., Brinker, A., Mcnamara, C., Henson, K., Kato, N., Kuhen, K., Nagle, A., Adrián, F., Matzen, J. T., Anderson, P., Nam, T.-G., Gray, N. S., Chatterjee, A., Janes, J., Yan, S. F., Trager, R., Caldwell, J. S., Schultz, P. G., Zhou, Y. & Winzeler, E. A. (2008). In silico activity profiling reveals the mechanism of action of antimalarials discovered in a high-throughput screen. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 105**,** 9059-64.

Price, R. N., Tjitra, e., guerra, c. a., yeung, s., white, n. j. & anstey, n. m. (2007). vivax malaria: neglected and not benign. *American Journal Of Tropical Medicine And Hygiene*, 77**,** 79-87.

Rahmah, Z., Sasmito, S. D., Siswanto, B., Sardjono, T. W. & Fitri, L. E. 2015. Parasitemia Induces high plasma levels of interleukin-17 (IL-17) And low levels of interleukin-10 (IL-10) and transforming growth factor in pregnant mice infected with malaria. *Malaysian Journal Of Medical Sciences*, 22**,** 25-32.

Rahman, K. M. (1982). Epidemiology of malaria in Malaysia. *Rev Infect Dis*, 4.

Raj, D. K., Mu, J., Jiang, H., Kabat, J., Singh, S., Sullivan, M., Fay, M. P., Mccutchan, T. F. & Su, X.-Z. (2009). Disruption of a Plasmodium falciparum multidrug resistance-associated protein (Pfmrp) alters its fitness and transport of antimalarial drugs and glutathione. *The Journal Of Biological Chemistry*, 284**,** 7687-7696.

Reed, M. B., Saliba, K. J., Caruana, S. R., Kirk, K. & Cowman, A. F. (2000). Pgh1 modulates sensitivity and resistance to multiple antimalarials in Plasmodium falciparum. *Nature*, 403**,** 906-909.

Reiling, S. J. & Rohrbach, P. (2015). Monitoring Pfmdr1 transport in Plasmodium falciparum. *Malar J,* 14**,** 270.

Ribeiro, M. C. D. A., Augusto, O. & Ferreira, A. M. D. C. (1997). Influence of quinoline-containing antimalarials in the catalase activity of ferriprotoporphyrin IX. *Journal Of Inorganic Biochemistry,* 65**,** 15-23.

Rieckmann, A., Villumsen, M., Sorup, S., Haugaard, L. K., Ravn, H., Roth, A., Baker, J. L., Benn, C. S. & Aaby, P. (2016). Vaccinations against smallpox and tuberculosis are associated with better long-term survival: A Danish case-cohort study 1971-2010. *Int J Epidemiol.*

Rieckmann, K. H., Trenholme, G. M., Williams, R. L., Carson, P. E., Frischer, H. & Desjardins, R. E. (1974). Prophylactic activity of mefloquine hydrochloride (WR 142490) in drug resistant malaria. *Bulletin Of The World Health Organization,* 51**,** 375-377.

Riss, T. (2005). Selecting cell - based assays for drug discovery screening. *Cell Note Issue***,** 16-21.

Rogo, K., Oucho, P. & Mwalali, P. (2006). Maternal mortality. *Disease and mortality in sub-Saharan Africa 2nd Edition***,** 223-236.

Rottmann, M., Mcnamara, C., Yeung, B. K. S., Lee, M. C. S., Russell, B., Seitz, P., Plouffe, D. M., Dharia, N. V., Tan, J., Cohen, S. B., Spencer, K. R., González-Páez, G. E., Suresh, B., Goh, A., Suwanarusk, R., Jegla, T., Schmitt, E. K., Beck, H.-P., Brun, R., Nosten, F. & Renia, L. (2011). Spiroindolones, a new and potent chemotype for the treatment of malaria. *Science,* 329**,** 1175-1180.

Rts, S. C. T. P. (2014). Efficacy and safety of the RTS,S/AS01 malaria vaccine during 18 months after vaccination: A Phase 3 randomized, controlled trial in children and young infants at 11 African Sites. *Plos Med*, 11**,** E1001685.

Sachs, J. & Malaney, P. (2002). The economic and social burden of malaria. *Nature*, 415**,** 680-685.

Saifi, A., Saifi, M. A., Beg, T., Harrath, A. H., Suleman, F., Altayalan, H. & Quraishy, S. A. (2013). Antimalarial drugs: mode of action and status of resistance. *African Journal Of Pharmacy And Pharmacology*, 7**,** 148-156.

Shandilya, A., Chacko, S., Jayaram, B. & Ghosh, I. (2013). A plausible mechanism for the antimalarial activity of artemisinin: A computational approach. *Scientific Reports*, 3**,** 2513.

Sirawaraporn, W., Sathitkul, T., Sirawaraporn, R., Yuthavong, Y. & Santi, D. V. (1997). Antifolate-resistant mutants of Plasmodium falciparum dihydrofolate reductase. *Proceedings Of The National Academy Of Sciences*, 94**,** 1124-1129.

Slater, A. F. G. (1993). Chloroquine: Mechanism of drug action and resistance in Plasmodium falciparum. *Pharmacology & Therapeutics*, 57**,** 203-235.

Spangenberg, T., Burrows, J. N., Kowalczyk, P., Mcdonald, S., Wells, T. N. C. & Willis, P. (2013). The open access Malaria Box: A drug discovery catalyst for neglected diseases. *Plos One*, 8.

Spillman, N. J., Allen, R. J. W., Mcnamara, C. W., Yeung, B. K. S., Winzeler, E. A., Diagana, T. T. & Kirk, K. (2013). Na(+) regulation in the malaria parasite plasmodium falciparum involves the cation ATPASE PFATP4 and is a target of the spiroindolone antimalarials. *Cell Host & Microbe,* 13**,** 227-37.

Sutar, S. K. D., Gupta, B., Ranjit, M., Kar, S. K. & Das, A. (2011). Sequence analysis of coding DNA fragments of Pfcrt and Pfmdr-1 genes in Plasmodium falciparum isolates from Odisha, India. *Memorias Do Instituto Oswaldo Cruz,* 106**,** 78-84.

Ta, T. H., Hisam, S., Lanza, M., Jiram, A. I., Ismail, N. & Rubio, J. M. (2014). First case of a naturally acquired human infection with Plasmodium cynomolgi. *Malaria Journal,* 13**,** 1-7.

Talisuna, A. O., Bloland, P. & D'alessandro, U. (2004). History, dynamics, and public health importance of malaria parasite resistance. *Clin.Microbiol.Rev.,* 17**,** 235-254.

Tiwari, N., Reynolds, P. & Calderón, A. (2016). Preliminary Lc-Ms based screening for inhibitors of Plasmodium falciparum thioredoxin reductase (PfTrxR) among a set of antimalarials from the Malaria Box. *Molecules,* 21**,** 424.

Tomori, O. From Smallpox eradication to the future of global health: innovations, application and lessons for future eradication and control initiatives.

Ursing, J., Zakeri, S., Gil, J. P. & Bjorkman, A. (2006). Quinoline resistance associated polymorphisms in the Pfcrt, Pfmdr1 and Pfmrp Genes Of Plasmodium falciparum in Iran. *Acta Trop,* 97**,** 352-6.

Van Dooren, G. G., Stimmler, L. M. & Mcfadden, G. I. (2006). Metabolic maps and functions of the Plasmodium mitochondrion. *Fems Microbiology Reviews*, 30**,** 596-630.

Van Riper, H. E. (1954). Immunization in poliomyelitis. *The Journal Of Bone & Joint Surgery*, 36**,** 893-1019.

Veeken, H. & Pécoul, B. (2000). Editorial : Drugs for ' neglected diseases ': A bitter pill. 5**,** 309-311.

Wah Wah, N. (2015). System biology approach for functional annotation of the genome of human malaria parasite Plasmodium falciparum and antimalarial drug discovery (Ph.D). *Nanyang technological University*.

Wang, K. S., Li, J., wang, Z., Mi, C., Ma, J., piao, L. X., Xu, G. H., Li, X. & Jin, X. (2017). Artemisinin inhibits inflammatory response via regulating NF-κB and MAPK signaling pathways. *Immunopharmacology and Immunotoxicology*, 39**,** 28-36.

Wang, P., Brooks, D. R., Sims, P. F. G. & Hyde, J. E. (1995). A mutation-specific PCR system to detect sequence variation in the dihydropteroate synthetase gene of Plasmodium falciparum. *Molecular And Biochemical Parasitology*, 71**,** 115-125.

Wang, P., Lee, C.-S., Bayoumi, R., Djimde, A., Doumbo, O., Swedberg, G., Dao, L. D., Mshinda, H., Tanner, M., Watkins, W. M., Sims, P. F. G. & Hyde, J. E. (1997). Resistance to antifolates in Plasmodium falciparum monitored by sequence analysis of dihydropteroate synthetase and dihydrofolate reductase alleles in a large number of field samples of diverse origins. *Molecular And Biochemical Parasitology*, 89**,** 161-177.

Warren, M., Skinner, J. C. & Guinn, E. (1966). Biology of the simian malarias of Southeast Asia. I. Host cell preferences of young trophozoites of four species of Plasmodium. *Journal Of Parasitology*, 52**,** 14-16.

Wellems, T. E., Walkerijonah, A. & Panton, L. J. (1991). Genetic mapping of the chloroquine-resistance locus on Plasmodium falciparum chromosome 7. 88**,** 3382-3386.

White, N. (1999). Antimalarial drug resistance and combination chemotherapy. *Philosophical Transactions Of The Royal Society Of London. Series B, Biological Sciences*, 354**,** 739-749.

White, N. J. (1997). Assessment of the pharmacodynamic properties of antimalarial drugs in vivo. *Antimicrobial Agents And Chemotherapy*, 41**,** 1413-1422.

WHO (2015). World Malaria Report 2015.

Woodrow, C. J (2015). Comparison between flow cytometry, microscopy, and lactate dehydrogenase-based enzyme-linked immunosorbent assay for Plasmodium falciparum drug susceptibility testing under field conditions. *J Clin Microbiol*, 53**,** 3296-303.

Wiwanitkit, V. (2011). Human knowlesi malaria and neurological complication: a new thing to be discussed in tropical neurology. *Acta Neurol Taiwan*, 20.

Yamauchi, L. M., Coppi, A., Snounou, G. & Sinnis, P. (2007). Plasmodium sporozoites trickle out of the injection site. 9**,** 1215-1222.

Yeung, S., Socheat, D., Moorthy, V. S. & Mills, A. J. (2009). Artemisinin resistance on the Thai-Cambodian border. *Lancet*, 374.

Yuthavong, Y., Tarnchompoo, B., Vilaivan, T., Chitnumsub, P., Kamchonwongpaisan, S., Charman, S. A., Mclennan, D. N., White, K. L., Vivas, L., Bongard, E., Thongphanchang, C., Taweechai, S., Vanichtanankul, J., Rattanajak, R., Arwon, U., Fantauzzi, P., Yuvaniyama, J., Charman, W. N. & Matthews, D. (2012). Malarial dihydrofolate reductase as a paradigm for drug development against a resistance-compromised target. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 109**,** 16823-8.

APPENDIX

Table A. 1: Nested PCR for the detection of SNPs in the genomic DNA of the clinical isolates

Gene	SNP	PCR	Primers Sequence	PCR Conditions	Amplicon Sizes (bp)
Pfcrt	K76T	Outer	F: 5'CCGTTAATAATAAATACACGCAG3' R: 5'CGGATGTTACAAAACTATAGTTACC 3'	94ºC (3min) followed by 30 cycles of 94ºC (30s), 56ºC (30s), 68ºC (1min) and 68ºC (3min)	537
		Nested	F: 5' TGTGCTCATGTGTTTAAACTT 3' R: 5' CAAAACTATAGTTACCAATTTTG 3'	94ºC (5min) followed by 25 cycles of 94ºC (30s), 48ºC (30s), 68ºC (1min) and 68ºC (3min)	134
Pfmdr1	N86Y Y184F	Outer	F: 5' TGT TGA AAG ATG GGT AAA GAG CAG AAA GAG 3' R: 5' TAC TTT CTT ATT ACA TAT GAC ACC ACA AAC A 3'	94ºC (120s) followed by 40 cycles of 94ºC (60s), 45ºC (30s), 68ºC (60s) and 68ºC (300s)	639
		Nested	F: 5' GTC AAA CGT GCA TTT TTT ATT AAT GAC CA**T TT**A 3' R:: 5' AAA GAT GGT AAC CTC AGT ATC AAA GAA GAG 3'	94ºC (120s) followed by 40 cycles of 94ºC (60s), 45ºC (30s), 68ºC (60s) and 68ºC (300s)	560
Pfdhps	A437G L540E	Outer	F: 5' AAC CTA AAC GTG CTG TTC AA 3' R: 5' AAT TGT GTG AAT TGT CCA CAA 3'	94ºC (180s) followed by 40 cycles of 94ºC (60s), 50 ^{0}C (60s), 68ºC (120s) and 68ºC (300s)	438
		Nested	F: 5' TGC TAG TGT TAT AGA TAT AGG ATG AGC ATC 3' R: 5' CTA TAA CGA GGT ATT GCA TTT AAT GCA AGA A 3'	94ºC (120s) followed by 40 cycles of 94ºC (30s), 45ºC (30s), 68ºC (60s) and 68ºC (300s)	711
Pfdhfr	N51I C59R 108N	Outer	F: 5' TTT ATG ATG GAA CAA GTC TGC 3' R: 5' AGT ATA TAC ATC GCT AAC AGA 3'	94ºC (120s) followed by 40 cycles of 94ºC (30s), 50ºC (30s), 68ºC (60s) and 68ºC (300s)	642
		Nest 1	F: 5' TTT ATG ATG GAA CAA GTC TGC GAC GTT 3' R: 5' AAA TTC TTG ATA AAC AAC GGA ACC TTT TA 3'	94ºC (120s) followed by 35 cycles of 94ºC (30s), 45ºC (30s), 68ºC (60s) and 68ºC (300s)	522
		Nest 2	F: 5' GAA ATG TAA TTC CCT AGA TAT GGA ATA TT 3' R: 5' TTA ATT TCC CAA GTA AAA CTA TTA GAG CTT C 3'	94ºC (120s) followed by 35 cycles of 94ºC (30s), 45ºC (30s), 68ºC (60s) and 68ºC (300s)	326

*F = Forward primer and R = Reverse primer

Table A. 2: Profile for Restriction Fragment Length Polymorphism.

Gene	SNP Position	Restriction Enzyme	Incubation °C/(min)	Expected band size (bp)	
				Wildtype	Mutant
Pfcrt	K76T	*XapI*	37 (15)	34, 100	134
Pfmdr	N86Y	*XapI*	37 (15)	249, 256	505, 45
	Y184F	*DraI*	37 (15)	242, 204, 114	242, 173, 114
Pfdhps	A437G	*AvaII*	37 (15)	438	404, 34
	K540E	*FokI*	37 (60)	405, 33	320, 85, 33
Pfdhfr	N51I	*M1uCl*	37 (15)	190, 154, 64	218, 120, 64
	C59R	*XmnI*	37 (15)	189, 137	163, 137, 26
	S108N	*BsrI*	65 (15)	326	180, 146

Formatted: Font color: Auto

Formatted: Font: 12 pt

Formatted: Font: 12 pt

Formatted: Font: 12 pt

Formatted: Font: 12 pt

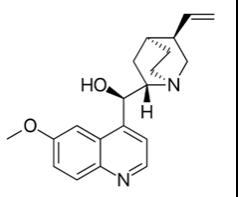
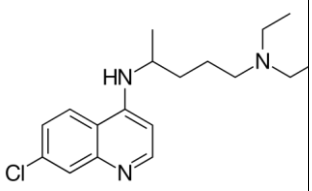
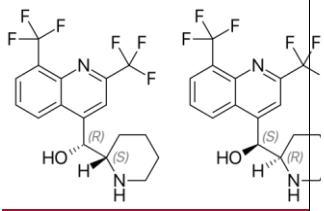
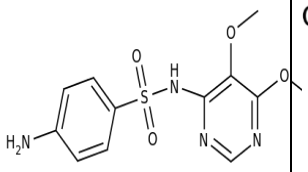
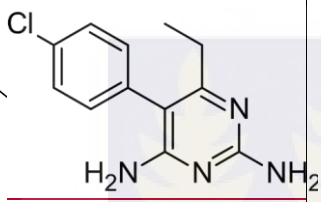
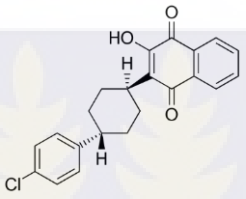
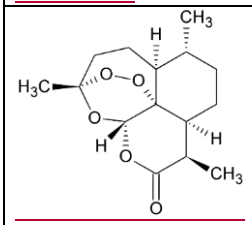
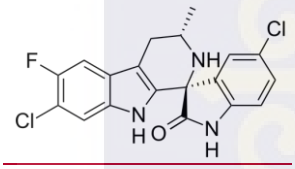
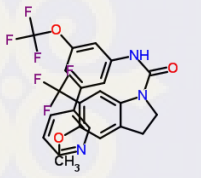
Formatted: Font: 12 pt

Formatted: Font: 12 pt

Formatted: Font: 12 pt

Formatted: Left, Indent: First line: 0.5", Line spacing single, Tab stops: Not at 2.4"

Table A.3: Structures of some antimalarial drugs and compounds (in clinical trials).

 Quinine	 Chloroquine	 Mefloquine
 Sulfadoxine	 Pyrimethamine	 Atovaquone
 Artemisinin	 KAE 609	 TCMDC-139046

Formatted: Font: (Default) Times New Roman, Font color: Auto

Formatted: Font: (Default) Times New Roman, Font color: Auto

Formatted: Heading 5, Left, Line spacing: single, Ta stops: Not at 2.4"

Formatted: Font: (Default) Times New Roman, Font color: Auto

Formatted: Font: (Default) Times New Roman

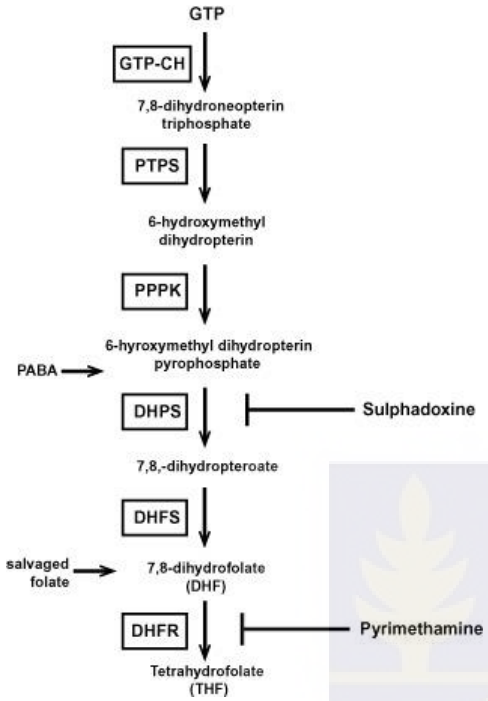


Figure A. 1: **The *de novo* folate biosynthesis pathway of P. falciparum**. Enzymes in the folate pathway are boxed and substrates are in plain text. Abbreviations: Guanosine triphosphate-cyclohydrolase (GTP-CH), pyruvolytetrahydropterin synthase (PTPS), hydroxymethyldihydropterin pyrophosphokinase (PPPK), dihydropteroate synthase (DHPS), dihydrofolate synthase (DHFS), dihydrofolate reductase (DHFR). GTP-CH is the first enzyme in the folate biosynthesis pathway. Inhibitors of folate biosynthesis are shown at the right of the pathway: sulphadoxine (SDX), pyrimethamine (PYR). Para-aminobenzoic acid (PABA) enters as a substrate for DHPS. Salvaged folate can also enter the pathway upstream of DHFR. (Adapted and modified from (Heinberg *et al.*, 2013))

Formatted: Heading 4 Char, Font: (Default) +Body (Cambria), Font color: Auto

Formatted: Heading 4 Char, Font: (Default) +Body (Cambria), Not Bold, Font color: Auto

Formatted: Heading 4 Char, Font: (Default) +Body (Cambria), Font color: Auto