Studies to elucidate the molecular targets of two potent antimalarial benzoxaborole compounds in *Plasmodium falciparum*

By

Ebere Irene Sonoiki

A dissertation submitted in partial satisfaction of the requirements of the degree of

Doctor of Philosophy

In

Infectious Diseases and Immunity

Committee in charge:

Co-chair: Dr. Eva Harris
Co-chair: Dr. Philip Rosenthal
Inside: Dr. Sangwei Lu
Outside: Dr. Jamie Cate

Spring 2014

ABSTRACT

Studies to elucidate the molecular targets of two potent antimalarial benzoxaborole compounds in *Plasmodium falciparum*

By

Ebere Irene Sonoiki

Doctor of Philosophy in Infectious diseases and immunity

University of California, Berkeley

Dr. Eva Harris, Co-chair
Dr. Philip Rosenthal, Co-chair

With increasing resistance of malaria parasites to available drugs, there is a great need for new antimalarials, ideally with novel mechanisms of action. We are investigating the antimalarial activity and mechanisms of action of benzoxaboroles, a novel class of boron-containing compounds. Compounds 3661, 1467 and related compounds demonstrated good activity *in vitro* against chloroquine-resistant *Plasmodium falciparum* and *in vivo* against murine *Plasmodium berghei* infection (compound 3661: IC_{50} 37 nM against W2-strain *P. falciparum*, ED_{90} 0.3 mg/kg against murine *P.berghei*; compound 1467: IC_{50} 196 nM, ED_{90} 7.4 mg/kg). In an attempt to characterize mechanisms of action, we selected for parasites with decreased sensitivity to 3661 and 1467 by culturing with step-wise increases in concentration of the compounds followed by whole genome sequencing. Sequencing of parasites selected for resistance to 1467 showed several SNPs in the editing domain of a predicted leucyl tRNA synthetase (LeuRS) gene (PF3D7_0622800) and in another gene of unknown function (PF3D7_1218100). Additionally, both compounds were tested for stage-specificity by incubation with test compounds for 8 hour intervals across the parasite erythrocytic life cycle. Both compounds were most active against trophozoite stage parasites. To further understand the mechanism of action of 1467 and the related compound 1474, the incorporation of ^{14}[C] leucine in parasite cultures or parasite extracts including exogenous tRNA was assessed in the presence or

absence of the compounds. Dose-dependent inhibition of both protein synthesis and LeuRS activity was observed for 1467 and 1474, but not 3661 or the control artemisinin, supporting different mechanisms for the different benzoxaboroles. For 3661, *in vitro* resistance selection was also achieved by culturing parasites in step-wise increasing concentration and in a single high concentration of the compound. Cross-resistance was not seen between parasites selected with 3661 and those selected with 1467. Whole genome sequencing of multiple clones selected for resistance to 3661 revealed several SNPs in a gene that codes for a homolog of mammalian cleavage and polyadenylation specificity factor (CPSF; PF14_0364). In summary, we offer strong evidence for unique antimalarial mechanisms of action for two benzoxaboroles, identifying two potential novel antimalarial drug targets. Further investigation of these novel benzoxaborole mechanisms is underway.

LIST OF ABBREVIATIONS

aaRS	Amino acyl tRNA synthetase
ACTs	Artemisinin combination therapies
AL	Artemether-lumefantrine
AlaRS	Alanyl tRNA synthetase
ArgRS	Arginyl tRNA synthetase
AS/AQ	Artesunate-Amodiaquine
AspRS	Asparagyl tRNA synthetase
AsnRS	Asparaginyl tRNA synthetase
β-CASP	Metallo-beta-lactamase, CPSF, Artemis, Snm1, Pso2
CNV	Copy number variation
CPSF	Cleavage and polyadenylation specificity factor
CQ	Chloroquine
CysRS	Cysteinyl tRNA synthetase
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
DP	Dihydroartemisinin-Piperaquine
ED_{90}	Ninety percent effective dose
Fig	Figure
G6PD	Glucose-6-phosphate dehydrogenase
gDNA	Genomic DNA
GlnRS	Glutaminyl tRNA synthetase
GluRS	Glutamyl tRNA synthetase
GlyRS	Glycyl tRNA synthetase
HisRS	Histidinyl tRNA synthetaste
IC_{50}	Fifty percent inhibitory concentration
ILeuRS	Isoleucyl tRNA synthetase
Kb	Kilobase
kDa	Kilodalton
LeuRS	Leucyl tRNA synthetase
LysRS	Lysyl tRNA synthetase
M	Molar
mM	Millimolar

µM	Micromolar
MetRS	Methionyl tRNA synthetase
MMV	Medicines for Malaria Venture
Pfemp1	Plasmodium falciparum erythrocyte membrane protein 1
Pfmdr1	Plasmodium falciparum multidrug resistance gene 1
Pfcrt	Plasmodium falciparum chloroquine resistance transporter
PheRS	Phenylalanyl tRNA synthetase
Pre-mRNA	Messenger RNA precursor
ProRS	Prolyl tRNA synthetase
QPCR	Quantitative polymerase chain reaction
RDTs	Rapid Diagnostic Tests
RBCs	Red blood cells
Rifin	Repetitive interdispersed family
SerRS	Seryl tRNA synthetase
SNPs	Single nucleotide polymorphisms
SP	Sulfadoxine/Pyrimethamine
Stevor	Subtelomeric variable open reading frames
ThrRS	Threonyl tRNA synthetase
tRNA	Transfer ribonucleic acid
TrpRS	Tryptophanyl tRNA synthetase
TryRS	Tyrosyl tRNA synthetase
UTR	Untranslated region
ValRS	Valyl tRNA synthetase
WHO	World Health Organization
WGS	Whole genome sequencing

TABLE OF CONTENTS

Abstract 1
List of Abreviations i
List of Figures v
List of Tables vii
CHAPTER 1: INTRODUCTION 1
1.1: Epidemiology of malaria 1
1.2: Biology of *P. falciparum* 2
1.3: Current malaria drugs and corresponding mechanism of action and resistance 5
1.4: Mechanism of antimicrobial drug resistance 10
1.5: In vitro drug resistance selection as a tool for studying antimicrobial mechanism of action and resistance 10
1.6: Aminoacyl tRNA synthetases 11
1.7: The role of cleavage and polyadenylation specificity factor in 3' end pre-mRNA processing 15
1.8: Requirements for a good antimalarial drug 17
1.9: Benzoxaboroles as novel and promising antimalarial drug candidates 17
1.10: Non-falciparum malaria 20
CHAPTER 2: MATERIALS AND METHODS 21
CHAPTER 3: STUDY OF THE MECHANISM OF ACTION OF THE BENZOX-ABOROLE COMPOUND 1467 34
3.1: 1467 exhibits potent in vitro and in vivo antimalarial action 34
3.2: In vitro morphology and stage specificity of 1467 on RBC stage *P. falciparum* 37
3.3: Selection and analysis of parasites with decreased 1467 sensitivity 39
3.4: Compound 1467 appears to inhibit LeuRS editing activity 50
3.5: Impaired editing in 1467 resistant *P. falciparum* 54
3.6: Discussion 56
CHAPTER 4: STUDY OF THE MECHANISM OF ACTION OF THE BENZOX-ABOROLE COMPOUND 3661 60

4.1: Compound 3661 has excellent in vitro and in vivo antimalarial activity ... 60
4.2: In vitro morphological and stage specific effects of 3661 on RBC stage *P. falciparum* ... 62
4.3: Selection and analysis of parasites with decreased 3661 sensitivity ... 64
4.4: Discussion ... 70
CHAPTER 5: CONCLUDING REMARKS AND FUTURE DIRECTIONS ... 73
CHAPTER 6: REFERENCES ... 75

LIST OF FIGURES

CHAPTER 1

Figure 1.1	Life cycle of the malaria parasite - *P. falciparum*
Figure 1.2	The 48-hour RBC cycle of *P. falciparum*.
Figure 1.3	Two-step aminoacylation reaction catalyzed by aaRS enzymes
Figure 1.4	A diagram depicted mammalian pre-mRNA 3' end processing complex.
Figure 1.5	General structure of benzoxaboroles

CHAPTER 2

Figure 2.1	1467 *in vitro* resistance selection schematic
Figure 2.2	3661 *in vitro* resistance selection schematic

CHAPTER 3

Figure 3.1	Chemical structure of compounds 1467 and 1474
Figure 3.2	Survival curves of *P. bergei*- infected mice treated with 1467
Figure 3.3	Morphological effects of 1467 treatment on *P. falciparum*
Figure 3.4	Stage specificity of 1467 on RBC stage *P. falciparum*
Figure 3.5	Schematic of 1467-resistance selection.
Figure 3.6	Histogram showing copy number variation on chromosome 12 of 1467 resistant clones compared to parental Dd2
Figure 3.7	LeuRS multiple sequence alignments
Figure 3.8	Effect of 1467 on [^{14}C]leucine incorporation in *P. falciparum* parasites

Figure 3.9	Effect of benzoxaboroles on *P. falciparum* LeuRS activity
Figure 3.10	Effect of benzoxaboroles on *P. falciparum* IleuRS and ValRS activity
Figure 3.11	Sensitivity to norvaline of parasites selected for resistance to 1467

CHAPTER 4

Figure 4.1	Structures of compounds 3661 and 7334
Figure 4.2	Survival curves of *P. bergei*- infected mice treated with 3661
Figure 4.3	Morphological effects of 3661 treatment on *P. falciparum*
Figure 4.4	Stage specificity of 3661 on RBC stage *P. falciparum*
Figure 4.5	Schematic of 3661 resistance selection

LIST OF TABLES

CHAPTER 1

Table 1.1	List of standard antimalarial drugs and corresponding class, mechanism of action and resistance status
Table 1.2	Classification of aminoacyl tRNA synthetases

CHAPTER 2

Table 2.1A	List of illumina indices used for gDNA library preparation of 1467 resistant clones
Table 2.1B	List of illumina indices used for gDNA library preparation of 3661 resistant clones
Table 2.2A	List of amplification and sequencing primers for PF3D7_0622800 and PF3D7_1218100
Table 2.2B	List of amplification and sequencing primers for PF3D7_1438500
Table 2.2C	List of amplification and sequencing primers for PF3D7_052300
Table 2.3	Primer sequences used for *pfmdr1* qPCR

CHAPTER 3

Table 3.1	*In vitro* and *in vivo* antimalarial activities of compounds 1467 and 1474
Table 3.2	SNP filtration flow chart showing the criteria and corresponding number of SNPs excluded from further analysis
Table 3.3	Detailed list of genes amplified on chromosome 12 of 1467 resistant clones

Table 3.4	Activity of antimalarials against 2nd and 3rd generation 1467 resistant Dd2 *P.falciparum*
Table 3.5	Relevant SNPs identified in compound1467-resistant *P. falciparum* by whole genome sequencing
Table 3.6	Detailed listing of the SNPs observed in PF3D7_0622800 and PF3D7_1218100 of 1467 resistant Dd2 clones

CHAPTER 4

Table 4.1	*In vitro* and *in vivo* antimalarial activities of compounds 3661 and 7334
Table 4.2	Activity of antimalarials against 2nd and 4th generation 3661 resistant W2 *P. falciparum*
Table 4.3	Relevant SNPs identified in step-wise selected 3661 resistant *P. falciparum* clones
Table 4.4	Relevant SNPs identified in one-time selected 3661 resistant *P. falciparum* clones
Table 4.5	Detailed listing of the SNPs observed in PF3D7_1438500 of 3661 resistant clones.

CHAPTER 1

INTRODUCTION

1.1 Epidemiology of malaria

Malaria is one of the biggest global health challenges in our world today. According to the 2013 World Health Organization (WHO) world malaria report, about 3.4 billion people are at risk of malaria and 1.2 billion are at high risk. In 2012, malaria accounted for about 200 million cases and 627,000 deaths. Most of the malaria deaths occurred in sub-Saharan Africa (90%) and in children under the age of five years (77%). Other regions plagued by malaria include Asia and South America.

Malaria is a protozoan disease caused by apicomplexan parasites of the *Plasmodium* genus and transmitted by the female anopheles mosquito. Plasmodial parasites that infect humans are *P. ovale, P. malariae, P. knowlesi, P. vivax* and *P. falciparum*. Of these, *P. falciparum*, the focus of this dissertation, is the most widely prevalent, with highest endemicity in sub-Saharan Africa (Dondorp et al. 2008, von Seidlein et al. 2012). It also occurs in Asia, South and Central America, and Oceania, albeit with lower incidence (Gething et al. 2011). Due to characteristics such as high blood parasitemia, sophisticated immune evasion techniques, and ability to cause sequestration of infected and uninfected red blood cells (RBCs) in blood vessels within vital organs such as the brain, *P. falciparum* accounts for nearly all severe and fatal cases of malaria (White et al. 2013).

Due to the global public health burden associated with malaria, robust steps are being taken towards control and elimination. Beginning with the launch of the Roll Back Malaria Initiative by WHO in 1998, malaria control activities have increased, as funding from international agencies, governments, philanthropies, and private corporations has increased. Such activities include distribution of long-lasting insecticide-impregnated bed

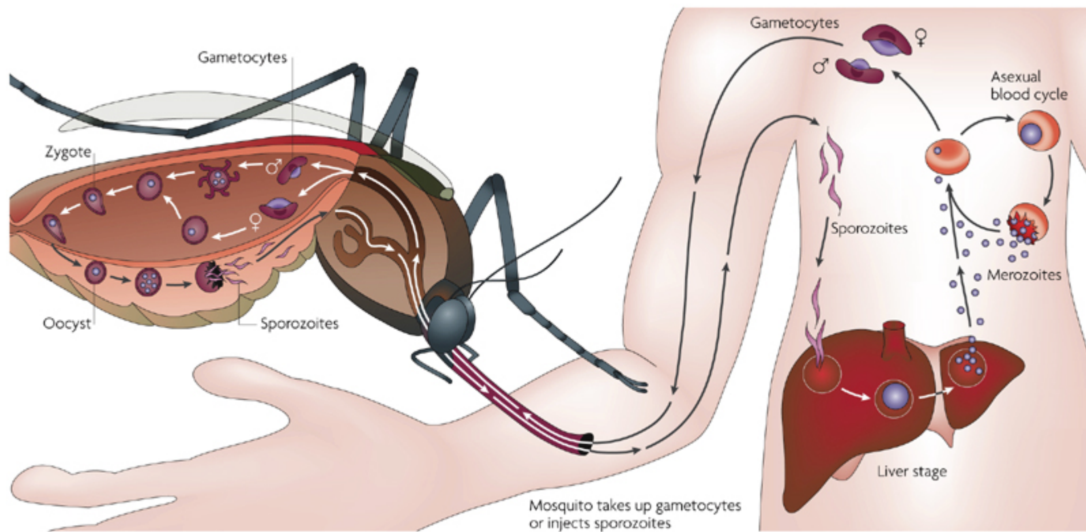
nets, indoor residual spraying of insecticides, intermittent preventive therapy with antimalarials, and expansion of access to artemisinin-based combination therapies (ACTs) and rapid diagnostic tests (RDTs). All together, these interventions have led to decreases in malaria incidence and mortality in several endemic regions. Notwithstanding, this observed progress is far from uniform, as some endemic areas have experienced increases in malaria incidence over the past decade (O'meara et al. 2008, Trape et al. 2011, Jagannathan et al. 2012, Rocker-Feltrer et al. 2012). Clearly, more work is needed, as malaria remains one of the most important infectious diseases in the world.

1.2 Biology of *P. falciparum*

The life cycle of plasmodia is complex, requiring survival in a mosquito vector and in different cell types within the human host (Fig 1.1). In the anopheline mosquito vector, male and female gametocytes, picked up during a blood meal from the human host, join to form a diploid zygote, which progresses to an ookinete in the midgut. The ookinete penetrates the gut membrane where it forms oocysts, leading to release of sporozoites, which then migrate to the salivary gland. When a parasitized mosquito bites a human host, mostly between dusk and dawn, it deposits sporozoites into the bloodstream, which travel to the liver and infect hepatocytes. The parasites then undergo asexual reproduction, releasing merozoites into the blood stream after about 8-10 days. When released into the blood stream, the merozoites invade red blood cells (RBCs) and undergo a 48 hour erythrocytic cycle (Miller et al. 2013). For this dissertation, most of the work is focused on this stage, as is the case for most drug development studies, because it is the stage associated with disease manifestations in humans. In the RBC *P. falciparum* develops from rings (~ 0 – 20 hour after invasion), to trophozoites (~ 20 – 36 hour), to schizonts (~ 36 – 48 hour), followed by release of a new population of merozoites into the blood stream to continue the cycle (Fig 1.2). Alternatively, a fraction of the parasite population develops into male and female gametocytes which, when picked up by the mosquito during a blood meal, starts the sexual reproductive stage in the mosquito. As the parasite develops inside the RBC, it changes the membrane architecture and

composition of the cell by inserting parasite-derived hypervariable antigens, notably erythrocyte membrane protein 1 (pfemp1), into the RBC membrane in order to import nutrients and to enable adherence to endothelial cells. Additionally, the parasites hijack and utilize host proteins, most notably hemoglobin, which the parasites break down to acquire amino acids and iron while crystalizing the resulting toxic haem into non-toxic hemozoin (Rosenthal 1988, Rosenthal and Meschnick 1996, Rosenthal 2003).

In order to efficiently handle the complexities associated with living in such biologically diverse and complex environments, P. *falciparum* expresses different sets of genes during each stage [Bozdech et al. 2003, Le Roch et al. 2003, Llinas et al. 2006]. For the RBC cycle, the ring stage is characterized by expression of transcription and translation enzymes in preparation for the high metabolic activity of the trophozoite stage, during which numerous metabolic proteins are up regulated, including proteases and glycolytic proteins. In the early schizont stage, the parasite prepares for the subsequent invasion of new sets of RBCs by up regulating DNA replication enzymes, cytoskeletal proteins and erythrocytic binding proteins [Bozdech et al. 2003, Le Roch et al. 2003, Llinas et al. 2006].



Nature Reviews | Genetics

Fig 1.1. Life cycle of the malaria parasite - *P. falciparum*. Reproduced from Xinzhuan et al 2007. A parasitized mosquito bites a human host, depositing sporozoites into the bloodstream, which travel to the liver and infect hepatocytes. The sporozoites then undergo asexual reproduction in the hepatocytes, releasing merozoites into the blood stream. The merozoites invade red blood cells (RBCs) and undergo a 48-hour erythrocytic cycle

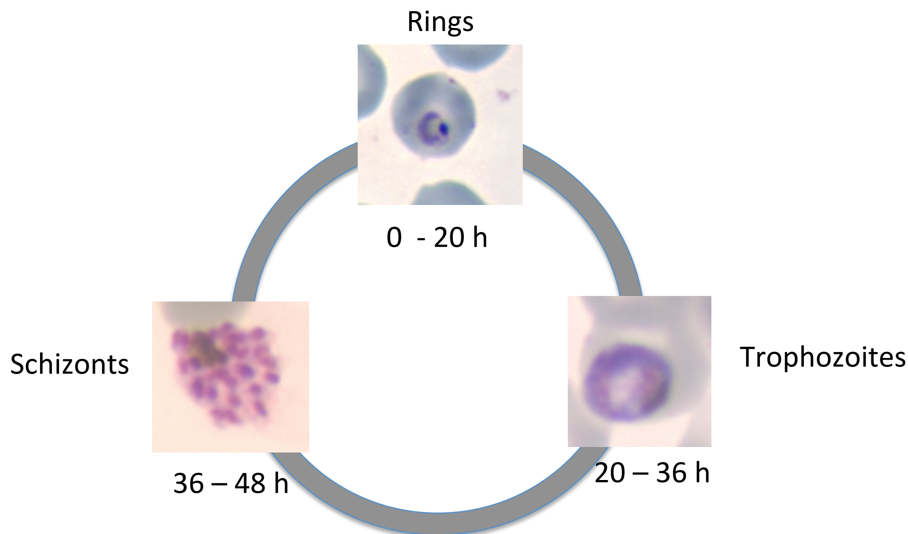


Fig 1.2. The 48-hour RBC cycle of *P. falciparum*. The cycle begins with rings (0 – 20 hours), followed by trophozoites (20 – 36 hours) and schizonts (36 – 48 hours)

1.3 Current malaria drugs and corresponding mechanisms of action and resistance

Much of malaria control has been highly dependent on the development of successful chemotherapy. However drug resistance continues to pose a big challenge, especially for *P. falciparum*, thereby warranting the need for the discovery and development of novel antimalarial drugs. The main classes of antimalarials currently in use include aminoquinolines, antifolates, antibiotics and artemisinin derivatives (Table 1.1).

1.3.1 Aminoquinolines

Aminoquinolines can be sub-divided into 4-aminoquinolines (chloroquine, amodiaquine and piperaquine), 8-aminoquinolines (primaquine), and methanol aminoquinolines (quinine, mefloquine). Chloroquine (CQ) was successfully used as a monotherapy until the emergence of drug resistance in *P. falciparum* in the late 1950s in South America and late 1970s in Africa. Although it remains the drug of choice for other human plasmodia species, it is no longer recommended for treatment for *P. falciparum* in nearly all areas due to widespread resistance. Chloroquine acts by inhibiting formation of hemozoin, a non-toxic polymer made from toxic heme monomers derived from hemoglobin breakdown in the food vacuole [Rosenthal et al. 1988, Chou et al. 1993, Sullivan et al. 1996]. Consequently, as a mechanism of chloroquine resistance, *P. falciparum* mutates the food chloroquine resistance transporter (*pfcrt*), to decrease chloroquine concentration in the food vacuole (Fidock et al. 2000). The most reliable chloroquine resistance marker is the K76T single nucleotide polymorphism (SNP) [Fidock et al. 2000, Sidhu et al. 2002]. Field isolates typically contain multiple additional SNPs in *pfcrt* in addition to mutations in *P. falciparum* multi drug resistance gene 1 (*pfmdr1),* a P-glycoprotein homologue also located on the membrane of the parasite food vacuole, but these are not essential for chloroquine resistance [Foote et al. 1990, Djimde et al. 2001, Dorsey et al. 2001].

Amodiaquine is another 4-aminoquinolone with similar structure to chloroquine. It was introduced as an alternative to chloroquine in regions

with high prevalence of chloroquine resistance because it remains quite active against chloroquine-resistant *P. falciparum*. (Ashley et al. 2006). Although the mechanism of action of amodiaquine remains uncharacterized, polymorphisms in *pfcrt* and *pfmdr1* are highly associated with treatment failure (Happi et al. 2006, Beshir et al. 2010).

Piperaquine, a bisquinoline, was initially used as a monotherapy in parts of Asia in the 1960s until resistance started emerging 10 – 20 years later (Ashley et al. 2006). The drug was resurrected recently in combination with dihydroartemisinin, and this combination has shown excellent efficacy for the treatment (Kamya et al 2007) and prevention (Nankabirwa *et al*. 2014) of malaria. Piperaquine resistance is associated with *pfmdr1* amplification (Eastman et al. 2011, Veiga et al. 2012) and its mechanism of action remains unknown.

Primaquine, an 8-aminoquinoline, is an FDA approved antimalarial drug that has demonstrated activity against gametocytes, thereby preventing transmission from humans to mosquitoes (Shekalaghe et al. 2007, Smithuis et al. 2010, White 2013). It is also the only approved antimalarial drug that acts against hypnozoites, the dormant liver stages of *P. vivax* and *P. ovale*. Hence, it is used in combination with a blood stage-killing drug such as chloroquine as a first line regimen for *P. vivax* and *P. ovale* (Fernando *et al*., 2011). Widespread primaquine use has been hampered by its ability to cause hemolysis in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency (Fernando *et al*., 2011). The mechanism of action of primaquine is unknown.

Quinine is derived from the bark of the cinchona tree and was the first antimalarial drug. However, due to adverse side effects it is mostly recommended only for the treatment of severe malaria. Mefloquine, another methanol quinoline, has been widely used for the treatment and prevention of malaria. However, its use has been on the decline for chemoprevention due to increasing reports of toxicity (Cook 1995). Resistance to both quinine and mefloquine is associated with *pfmdr1* amplification and their mechanism of action is not understood (Pickard et al. 2003, Woodrow and Krishna 2006).

1.3.2 Antifolates

Standard antifolates include sulfadoxine and pyrimethamine (SP). Both act against the folate biosynthetic pathway. Sulfadoxine inhibits dihydropteroate synthase (DHPS) while pyrimethamine inhibits dihydrofolate reductase (DHFR). The drugs have synergistic activity and were previously

used in a fixed formulation to treat uncomplicated malaria. Although widespread use of SP has been discontinued due to high prevalence of resistance, it is still recommended for intermittent preventive therapy during pregnancy in sub-Saharan Africa (Peters et al. 2007). Resistance to pyrimethamine and sulfadoxine is mediated by accumulation of SNPs in the *dhfr* and *dhps* genes respectively (Gregson and Plowe 2005).

Proguanil is another antifolate and is currently paired with atovaquone, a hydroxynapthaquinone, for treating uncomplicated malaria (Osei-Akoto et al. 2005, Cordel et al. 2013), although this regimen is little used in malaria-endemic areas due to high cost. Atovaquone/proguanil (Malarone) is widely used for short-term prophylaxisfor travelers visiting malaria endemic regions (Leshem et al. 2014).

1.3.3 Antibiotics

Some antibiotics, including the tetracyclines and clindamycin, have antimalarial activity, although they are slow acting (Dahl and Rosenthal 2007). Antibiotics act against the apicoplast, an organelle acquired through endosymbiosis from an ancestral prokaryote (Dahl and Rosenthal 2008). Like the mitochondrion, the apicoplast has an independent prokaryote-like protein synthesis apparatus. This apparatus is a target for bacterial protein synthesis inhibitors such as tetracyclines that act against the 30S ribosome in prokaryotes (Chopra and Roberts, 2001, Dahl et al. 2006, McFadden 2011). The antimalarial activity of antibiotics leads to a "delayed-death" phenotype in which parasite death is not observed until after the first RBC cycle (Dahl and Rosenthal, 2007).

Currently, doxycycline monotherapy is effective for prophylaxis, but the drug must be paired with a fast acting drug (quinine) for effective malaria treatment (Tan et al. 2011). Likewise, clindamycin is recommended in combination with quinine for malaria treatment, especially in children (Obonyo and Juma 2012).

1.3.4 Artemisinins.

The artemisinins are the latest class of antimalarial drugs to be widely used. These compounds originate from the *Artemisia annua* plant, extracts of which were used in China for different treatment purposes. The extraordinarily fast antimalarial activity of artemisinins was reported in the

1970s (Miller and Su 2011). Initially, the supply of artemisinin was very limited due to constraints of its natural source. However, with increasing funding and breakthrough engineering of artemisinin-precursor producing genetically modified microbes, arteminisnin availability is expected to significantly increase (Ro et al. 2006, Tsuruta et al. 2009, Paddon et al. 2013).

Arteminisin was initially used as a monotherapy until formulations of artemisinin-based combination therapies (ACTs) were introduced in order to improve antimalarial efficacy and help avert drug resistance. ACTs are composed of an artemisinin derivative - dihydroartemisinin, artesunate or artemether paired with a longer-acting partner drug such as amodiaquine, lumefantrine, piperaquine, mefloquine, or SP. Current regimens include artemether-lumefantrine (AL), dihydroartemisinin-piperaquine (DP), artesunate-SP (AS/SP), artesunate-mefloquine, and artesunate-amodiaquine (AS/AQ).

Given the prevalence of resistance of *P. falciparum* to the aforementioned antimalarial drugs, ACTs have played a pivotal role in the successes that have been achieved towards the elimination of malaria to date. However, reports of resistance of *P. falciparum*, in the form of delayed parasite clearance, to artemisinin monotherapy and some ACTs have emerged in Southeast Asia (Noedl et al. 2008, Dondorp et al. 2009, Amaratunga et al. 2012, Phyo et al. 2012, Ariey et al. 2013). Since then, several research groups have worked actively to discover the mechanism of artemisinin resistance in *P. falciparum,* which remained elusive until recently (Mungthin et al. 2010, Cheeseman et al. 2012, Cui et al. 2012). In Jan 2014, a research group identified mutations in the K-13 propeller domain of the protein encoded by *PF3D7*_1343700 gene in *P. falciparum* as an important and reliable resistance marker by comparing the genome of artemisinin resistant parasites selected *in vitro* with field isolates from Cambodia [Ariey et al. 2014]. Although it is generally hypothesized that artemisinins act via free radical damage (Meshnick et al. 1993), the specific mechanism of action of the compounds remains unconfirmed.

Table 1.1: List of standard antimalarial drugs and corresponding class, mechanism of action and resistance status

Class	Drug	Drug target/Mechanism of action	Resistance status
4-aminoaquinolines	Chloroquine (CQ)	Inhibits hemozoin formation	High
	Amodiaquine	Similar to CQ, but not confirmed	High
	Piperaquine	Unknown	High
8-aminoquinolines	Primaquine	Unknown	None documented
Methanol-aminoquinolines	Quinine	Unknown	Low to mid-level
	mefloquine	Unknown	High
Antifolates	Sulfadoxine	Dihydro pteroate synthetase	High
	Pyrimethamine	Dihydro folate reductase	High
ACTs	Dihydroartemisinin-Piperaquine	Unknown	Emerging in Asia
	Arthemether-lumefantrine	Unknown	Emerging in Asia
	Artesunate-SP	Unknown	Common
	Artesunate-amodiaquine	Unknown	Common
Hydroxynapthaquinone	Atovaquone	Mitochondrial, cytochrom bc1 complex	Rare
Antibiotics	Doxycycline	Apicoplast, specific target unknown	Emerging
	Clindamycin	Apicoplast, specific target unknown	Emergine

1.4 Mechanisms of antimicrobial drug resistance

Antimalarial drug resistance is often accompanied by genetic changes in the form of SNPs, copy number variations (CNV) and insertion/deletion events. In some cases, resistance can be due to mutations in gene(s) that code for transmembrane transport proteins. Alternatively, resistance can be caused by mutations in genes that code for proteins involved in the molecular pathway targeted by the inhibitor in question, also indicative of the mechanism of action. SNPs have been implicated in drug resistance to several antimicrobials including antimalarials, [Ramaswamy et al*.* 2003, Prichard 2007, Gniadkowski 2008, Strasfield and Chou 2010, Georghiou et al. 2012, Espedido et al. 2012, Bondaryk et al. 2013, Rosenthal 2013]. CNVs are also common mediators of drug resistance [Henry et al. 2000, Singh N. et al*.* 2003, Singh & Rosenthal 2004, Brochet et al. 2008, Price et al. 2004, Guler et al. 2013]. While insertion/deletion events are the least common in antimalarial resistance, they have been implicated in resistance to some antiviral [Menendez-Arias et al. 2006] and antibacterial [Brody et al. 2008] agents.

1.5 *In vitro* drug resistance selection as a tool for studying antimicrobial mechanisms of action and resistance

In vitro resistance selection is a valuable technique that has been used to identify mechanism(s) of action of antimicrobials. Examples include antibacterials, [Ramaswamy et al*.* 2003, Brody et al. 2008, Gniadkowski 2008, Georghiou et al. 2012, Espedido et al. 2012], antifungals [Henry et al. 2000, Bondaryk et al. 2013], antivirals [Menendez-Arias et al. 2006, Strasfield and Chou 2010], and antiprotozoans [Price et al*.* 2004, Singh & Rosenthal 2004, Prichard 2007, Rosenthal 2013, Guler et al. 2013]. For studies of *P. falciparum*, there are two approaches to *in vitro* drug resistance selection; one entails stepwise selection in dose increments over time while the other involves treatment of a large number of parasites with one high concentration of drug (Rathod et al. 1997). The stepwise method requires more time, but offers the advantage of offering insights into genetic changes associated with different levels of resistance (Guler et al. 2013).

Since the publication of the plasmodial genome sequence in 2002 (Gardner et al. 2002), *in vitro* resistance selection has been central to studies

of antimalarial drug resistance and action. As with clinical isolates, the two major genetic changes associated with *in vitro* drug pressure are SNPs and CNVs in transporters or target genes. A notable breakthrough aided by *in vitro* drug selection in *P. falciparum* is the recent discovery of the highly sought-after artemisinin resistance molecular marker detailed above [Ariey et al. 2014]. Other notable findings aided by this technique include the following:

- Confirmation of falcipain-2 and falcipain-3 as the targets of antimalarial cysteine protease inhibitors (Singh and Rosenthal 2004)
- Identification of ATPase4 as the target of spiroindolones, a promising class of antimalarial drug candidates (Rottmann et al. 2010)
- Identification of phosphatidylinositol-4-OH kinase as the target of imidazopyrazines, another promising class of antimalarial drug candidates (McNamara et al. 2013)
- Identification of dihydroorotate dehydrogenase (DHODH) as the target of yet another promising class of antimalarial inhibitors (Guler et al. 2013)

1.6 Aminoacyl tRNA synthetases.

Aminoacyl tRNA synthetases (aaRS) are essential in the protein synthesis process in all kingdoms of life. Hence aaRS enzymes have structural homology between species of both prokaryotic and eukaryotic origins, although significant differences exist (Eriani et al. 1990). The main function of aaRS enzymes is to catalyze the attachment of amino acids to their cognate tRNAs. This process occurs in two steps involving activation of the amino acid by adenylation followed by transfer of the adenylated amino acid to its cognate tRNA [Fig 1.3; Carter 1993, Ling et al. 2009]. AaRS enzymes are divided into two classes based on structural differences that determine which hydroxyl group on the 3' terminal adenosine of the tRNA is attached to the amino acid (2' hydroxyl for Class I or 3' hydroxyl for Class II) [Eriani et al. 1990, Schmidt and Schimmel 1995, Lee et al. 2004, Ling et al. 2009]. Class I and II aaRS enzymes are further differentiated by the structure of their active sites. Class I aaRS enzymes are mostly monomeric and have active sites made

up of six parallel beta strands linked to four alpha helices (Rossmann fold) and two conserved motifs (Eriani et al., 1990). These include CysRS, MetRS, ValRS, LeuRS, IleuRS, ArgRS, GluRS, GlnRS, TyrRS and TrpRS (Sugiura et al. 2000, Ribas et al. 2001). On the contrary, class II aaRS enzymes are often multimeric and have three conserved motifs in their active site including AlaRS, GlyRS, SerRS, ThrRS, ProRS, HisRS, AsnRS, AspRS, LysRS and PheRS (Cusack et al. 1991, Leberman et al. 1991, Ruff et al. 1991). Each class is further subdivided into sub-groups based on the chemical properties of the associated amino acid (Table 1.2) [Cusack 1997]. In order to increase the fidelity of the protein synthesis process, some aaRS enzymes have an editing domain that hydrolyzes mis-aminoacylated tRNAs (Silvian et al. 1999, Beebe et al. 2003, Wolfe et al. 2005). Protein synthesis has an average error rate of 1 in 3000 amino acids (Jakubowski and Goldman 1992). Studies have shown that amino acyl tRNA synthesis with less than average ability to distinguish between cognate and non-cognate amino acids have an editing function [Eldred and Schimmel 1972, Leatherbarrow et al. 1985). Unlike class II, class I editing domains (known as connective peptide 1) are highly conserved in different species [Beebe et al. 2003]. Current understanding of class I editing is based on studies of ILeuRS, ValRS and LeuRS, through which we've learned that class I editing can occur pre or post transfer of amino acid to its cognate tRNA [Silvian et al. 1999, Lincecum et al. 2003, Seiradake et al. 2009, Cvetestic et al. 2012, Li et al. 2013]. Pre-transfer editing takes place at the aminoacylation active site, where a charged amino acid (amino acid attached to adeonosine monophosphate) is hydrolyzed prior to attachment to a non-cognate tRNA (Ling et al. 2012). Post-transfer editing is the better understood process, and takes place in the editing domain, where an amino acid is hydrolyzed after it has been attached to a non-cognate tRNA [Silvian et al. 1999, Lincecum et al. 2003, Seiradake et al. 2009, Cvetestic et al. 2012, Li et al. 2013].

There is increasing interest in the exploration of aaRS enzymes as potential antimicrobial targets, especially for prokaryotic targets [Kim et al. 2003, Vondenhoff et al. 2011, Pham et al., 2013]. Several natural products from prokaryotic organisms inhibit aaRS activity in other prokaryotes. An example is mupirocin (pseudomonic acid), which is produced by *Pseudomonas fluorescens*, a gram-negative bacterium. Mupirocin inhibits the activity of ILeuRS in gram-positive and gram-negative bacteria (Class and Deshong 1995, Kim et al. 2003, Gurney and Thomas 2011). It is used topically for the treatment and elimination of drug-resistant *Staphylococcus aureus*

colonization (Thomas et al. 2010, Seah et al. 2012, Bathoorn et al. 2012). Other natural aaRS inhibitors include borrelidin (ThrRS), indolmycin (TrpRS), furanomycin (IleRS), granaticin (LeuRS), ochratoxin A (pheRS) and cispentacin (ProRS) (Nass et al. 1969, Tanaka et al. 1969, Ogilvie et al. 1975, Werner et al. 1976, Konrad and Roschenthaler, 1977, Konishi et al. 1989). Although these natural products are successful at inhibiting microbial aaRS activity *in vitro*, their *in vivo* activity have been limited by poor bioavailability and lack of selectivity, disqualifying them as viable drugs (Vondenhoff and Van 2011). As a result, great efforts have been made to design and synthesize novel and selective aaRS inhibitors. Despite the high structural conservation between aaRS enzymes between kingdoms, some successes have been attained in bacteria and yeast (Barker 2006, Beyer et al. 2004, Rock et al. 2007, Gaston et al. 2011, Baker et al. 2011, Hu et al. 2013).

For apicomplexans such as malaria parasites, the idea of targeting aaRS enzymes has been entertained due to the presence of the apicoplast – an organelle of prokaryotic origin recently validated as the sole cellular compartment responsible for isoprenoid precursor biosynthesis in *P. falciparum* (Dahl and Rosenthal, 2008, Yeh and DeRisi 2011). In *P. falciparum*, the apicoplast has its own prokaryotic-like protein synthesis apparatus, including aaRS enzymes. Hence, there are two copies of most of the 20 aaRS enzymes in *P. falciparum*, with some exceptions (McFadden 2011, Pham et al. 2014). Mupirocin is an example of a drug that has been shown to successfully target the apicoplast IleRS in *P. falciparum* (Istvan et al. 2011).

Inhibitors of the cytoplasmic eukaryotic aaRS enzymes in protozoans have also been successfully synthesized including *P. falciparum*, *B. malayi* and *T. brucei* (Ding et al. 2011, Hoepfner et al. 2012, Zhang et al. 2013, Azcarate et al. 2013, Pham et al. 2014, Kalidas 2014, Pham et al. 2014). Specifically for *P. falciparum*, several synthetic aaRS inhibitors have been reported including A5 (AlaRS), mupirocin (IleRS), 4-thiaisoleucine (IleRS), cladosporin (LysRS), Lysyl-adenylate analogues (LysRS), halofuginone (ProRS) and borrelidin (ThrRS) [Otoguro et al. 2003, Istvan et al. 2011, Keller et al. 2012, Hoen et al. 2013, Hoepfner et al. 2013, Sugawara et al. 2013].

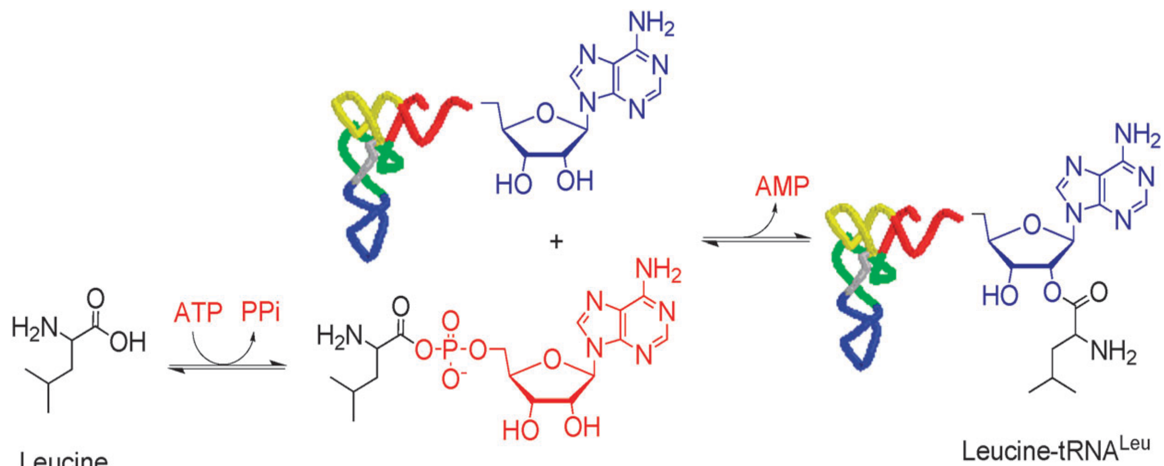


Fig 1.3: Two-step aminoacylation reaction catalyzed by aaRS enzymes. Image adapted from Baker et al. 2010. AMP = adenosine monophosphate, PPi = pyrophospate

Table 1.2: Classification of aminoacyl tRNA synthetases

Class I	**Subclass**	**Class II**	**Subclass**
MetRS	1a	HisRS	IIa
IleRS		ProRS	
LeuRS		SerRS	
ValRS		ThrRS	
CysRS		AspRS	IIb
ArgRS		AsnRS	
GlnRS	1b	LysRS	
GluRS		GlyRS	IIc
TyrRS	1c	AlaRS	
TrpRS		PheRS	

1.7 The role of cleavage and polyadenylation specificity factor (CPSF) in 3' end pre-mRNA processing

The co-transcriptional processing of messenger RNA precursor (pre-mRNA) is an essential activity in eukaryotic organisms. This entails capping of the 5' end of the mRNA, splicing, cleavage and polyadenylation at the 3' end (Coulgan and Manley 1997, Proudfoot 2004, Mandel et al. 2008), all of which are required for the production of mature mRNA. The 3' end processing of pre-mRNA is important because it promotes the transport of mRNA from the nucleus to the cytoplasm (Huang and Carmichael 1996, Vinciguerra and Stutz 2004). Pre-mRNA 3' end processing also enhances translation and the stability of the mRNA while in the cytoplasm, protecting it from degradation by nucleases (Sachs et al. 1997, Wickens et al. 1997, Ford et al. 1997, Preiss and Hentze 1998). Our current understanding of pre-mRNA 3' end processing is based upon extensive research in mammalian cells and yeast. There are conserved sequence elements in the 3' untranslated region (UTR) of pre-mRNAs that are required for successful 3' end processing (Dominski et al. 2005). In mammals, the primary sequence elements include the hexamer polyadenylation signal (PAS: AAUAAA), the cleavage site (CA) and the downstream element (DSE) (Mandel et al. 2008). Studies have also reported the involvement of 14 and 20 proteins divided into sub-complexes in mammals and yeast respectively (Mandel et al. 2008). In mammalian cells, the sub-complexes include poly(A) polymerase (PAP), poly(A) binding protein (PABP), symplekin, the C-terminus of the RNA polymerase II largest subunit, cleavage stimulation factor (CSF), cleavage factor 1 (CF I_m), cleavage factor II (CF II_m) and cleavage and polyadenylation specificity factor (CPSF) (Fig 1.4; Moore and Sharp 1984, Gilmartin et al. 1988, Murthy and Manley 1995, Dantonel et al. 1997, McCracken et al. 1997, Mandel et al. 2006). Of relevance to this dissertation is CPSF.

In humans CPSF has five subunits (CPSF-30, 73, 100, 160 and hFip1), all of which are required for successful cleavage and polyadenylation. For CPSF-30, the function is not well characterized. Reported data on hFip1 (human polyadenylation factor 1) shows that it interacts with PAP and other components in the machinery, suggestive of a role in bringing PAP close to the polyadenylation site (Preker et al. 1995, Helming et al. 2001, Kaufmann et al. 2004). CPSF-160 is the largest of the subunits, and it has been shown to initiate 3' end processing by binding to the PAS sequence element, resulting in recruitment of other proteins (Moore et al. 1988, Murthy and Manley 1995).

Although, CPSF-73 and CPSF-100 both have a β-CASP (named for metallo-beta-lactamase, CPSF, Artemis, Snm1, Pso2) domain, recent evidence suggests that both proteins have different functions. A study of CPSF-73 in mammalian cells showed that it is responsible for the endonuclease activity at the cleavage site (Mandel et al. 2006, Dominski et al. 2007). On the contrary, study of the CPSF-100 homolog in yeast showed no endonuclease activity, allegedly due to its lack of a zinc-binding motif required for cleavage activity (Mandel et al. 2006).

So far, the 3' end processing of pre-mRNA has not been characterized in apicomplexans. A search of the *P. falciparum* genome on plasmodb.org, a genomic database for plasmodial parasites, identified three genes designated as putative CPSF according to sequence and structural homology to mammalian proteins (two on chromosome 3, one on chromosome 14). A thorough evaluation of the role of CPSF in *P. falciparum* and other apicomplexan parasites is needed to further explore their potential as drug targets.

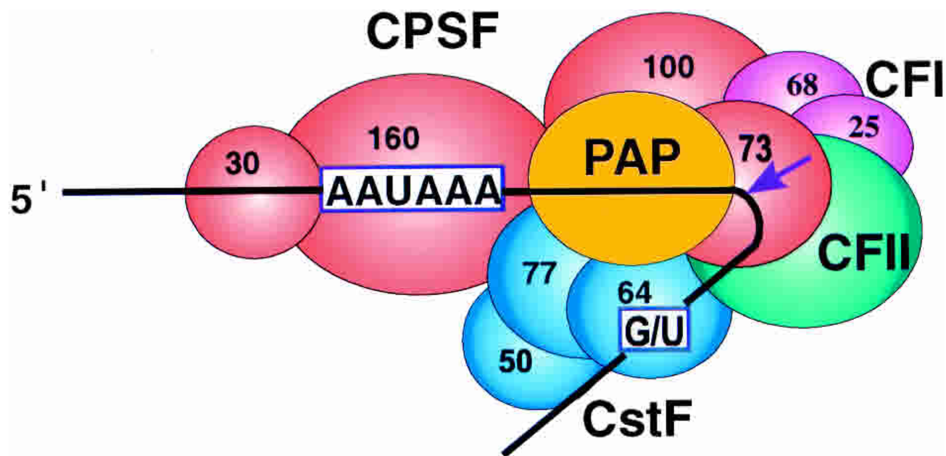


Fig 1.4: A diagram depicting mammalian pre-mRNA 3' end processing complex. Adapted from Ryan et al. 2004.

1.8 Requirements for a good antimalarial drug

The discovery of new drugs to treat malaria is especially challenging. In addition to general drug requirements for efficacy and safety, antimalarial drug candidates are expected to meet several challenging criteria, as elaborated by the Medicines for Malaria Ventures (MMV). Based on the MMV product profile, new antimalarials should a) be safe in children and in pregnant women, b) have low cost of manufacturing, c) lead to rapid resolution of symptoms within 3 days, d) require no longer than 3 days of dosing, and ideally only a single dose, e) have low tendency to select for resistance , f) be orally bioavailable, and g) have stable formulation and packaging.

In addition to these criteria, which involve action against erythrocytic stages, activity against non-erythrocytic stages are desired, in particular action against liver and mosquito stages.

1.9 Benzoxaboroles as novel and promising antimalarial drug candidates

Benzoxaboroles are a novel class of boron-containing compounds. They are characterized by the presence of a five-member boron-containing ring (oxaborole ring) attached to a benzene ring with at least one side chain (Fig 1.5). Until recently, boron-containing compounds were not seriously considered as drug candidates due to misconceptions regarding toxicity of boron, over-reactivity and instability associated with boronic acid and earlier synthetic chemistry and manufacturing difficulties (Del Rosso and Plattner 2014). Over time, some of these reservations have been dispelled with advances in boron synthetic chemistry and emerging evidence in favor of boron as a drug component (Baker et al. 2009, Ciaravino et al. 2013). There are several qualities that make boron a good drug component. Most importantly, its empty p-orbital makes it more electrophilic than carbon or nitrogen, thereby increasing its propensity to interact with a wide variety of targets via reversible covalent bonds (Baker et al. 2011, Jacobs et al. 2011, Del Rosso and Plattner 2014). Furthermore, several studies have shown the essentiality of boron in the oxaborole ring for activity, as replacement of

boron with carbon leads to loss of activity (Rock et al. 2007, Zhang et al. 2011]. Currently, the only FDA approved boron-containing drug is bortezomib (trade name: Valcade), a proteasome inhibitor that induces apoptosis, for treating multiple myeloma (Ling et al. 2002, Das et al. 2013). However, several boron-containing compounds are in drug development, including benzoxaboroles that have demonstrated potent activity against infectious pathogens, including bacteria (Hernandez et al. 2013, Hu et al. 2013), fungi (Rock et al. 2007), trypanosomes and malaria parasites (Zhang et al. 2011, Jacobs et al. 2011). Tavaborole, an anti-fungal LeuRS benzoxaborole, is currently being reviewed by the FDA for treatment of onychomycosis, a fungal nail infection (Alley et al. 2007).

Several benzoxaboroles are currently in drug development pipelines for treatment of various diseases. These include gram-negative bacterial infection, pneumococcal infection, tuberculosis, onychomycosis, hepatitis C virus infection, African trypanosomiasis and inflammatory disease. Characterization of the mechanisms of action of some of these antimicrobial benzoxaboroles have identified novel targets, including bacterial LeuRS (*M. tuberculosis*, *S. pneumonia, S. aeruginosa, E. coli, Enterobacteriaceae*) (Hernandez et al. 2013, Hu et al. 2013), bacterial β-lactamase (Xia et al. 2011), fungal LeuRS (*S. cerevisiae*) (Rock et al. 2007), *Trypanosoma brucei* LeuRS (Ding et al. 2011, Jacobs et al. 2011), mammalian rho-activated kinase (Akama et al. 2009), phosphodiesterase-4 (Akama et al. 2013), and NS3 protease in hepatitis C virus (Li et al. 2010). Considering the successful design of benzoxaboroles to treat other infectious diseases, we set out to identify compounds of this class with antimalarial activity.

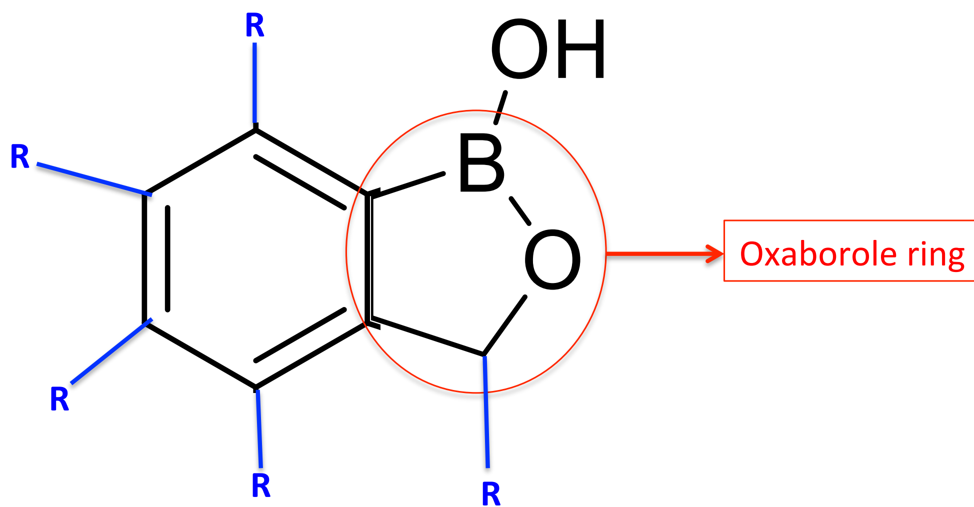


Fig 1.5: General structure of benzoxaboroles

1.10 Non-*falciparum* malaria

P. vivax is another widely distributed species, with high prevalence in South America, Asia and the horn of Africa. Unlike other human plasmodial species, it along with *P. ovale* can persist in the liver in a dormant form known as hypnozoites, resulting in relapsing disease after successful eradication of erythrocyte stages (Gething et al. 2012). Although vivax malaria has been largely associated with benign disease, recent reports have challenged this notion by showing it to be associated with severe malaria in some countries (Price et al. 2009).

P. ovale is predominantly found in sub-Saharan Africa and the Islands of the western Pacific, with more recent reports in parts of Southeast Asia, the Middle East and India (Collins and Jeffery 2005). It is usually associated with benign disease. (Mueller et al. 2007).

P. malariae causes mild disease with quartan (every 4th day) fever manifestatios , unlike the other species that exhibits tertian (every 3rd day) fever symptoms, excluding *P. knowlesi* (Collins and Jeffery 2007). Its distribution coincides with *P. falciparum,* appearing in malaria endemic areas in sub-Saharan Africa, Southeast Asia, the western Pacific and parts of South America (Collins and Jeffery 2007).

P. knowlesi was characterized as a strictly zoonotic malaria parasite until recent reports associated it with human malaria in Malaysia (Singh B. et al. 2004). Since then, it has been reported in other regions including Thailand, Vietnam, Indonesia, Singapore and the Philippines (Lee et al. 2011).

CHAPTER 2

MATERIALS AND METHODS

Culture of malaria parasites
Erythrocytic stages of *P. falciparum* (strains W2, Dd2 or 3D7) were cultured using standard methods at 2% hematocrit in RPMI-1640 (Invitrogen) medium supplemented with 0.5% Albumax, 2 mM l-glutamine, 100 mM hypoxanthine, 5 µg/ml gentamicin, 28 mM $NaHCO_3$ and 25 mM HEPES at 37°C in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2.

Activity of test compounds against cultured *P. falciparum*
P. falciparum strains were synchronized by treatment with 5% D-sorbitol and cultured in duplicate 96 well culture plates (200 µL per well) in the presence of 3-fold serially diluted test compounds ranging from 0.056-1000 nM or, for controls, in solvent (≤0.2% DMSO) alone. After 48 hours, the cultures were fixed with 2% formaldehyde for 24 hours at 37°C or 48 hours at room temperature, the fixed cells were stained with 4 nM YOYO-1 dye (Molecular Probes), and counts of treated and control cultures were determined using a fluorescence-activated cell sorter. IC_{50} values were calculated by nonlinear regression using Prism Graphpad software.

***In vivo* activity of test compounds against murine malaria**
Groups of five Swiss Webster mice were infected intraperitoneally with $6x10^6$ *P.berghei* infected erythrocytes collected from a previously infected mouse and then treated, beginning 1 hour after inoculation, with varying concentrations of test compounds or chloroquine as a positive control, by daily oral gavage for four days. Test compounds were solubilized in 55% polyethylene glycol 300, 25% propylene glycol and 20% water (55/25/20/PEG300/PG/H_20). Negative controls were treated with vehicle only. Infections were monitored by daily microscopic evaluation of Giemsa-

stained blood smears. ED_{90} values, based on comparisons of parasitemias between treated and control animals on the fourth day after the initiation of treatment, were calculated using Prism Graphpad software. Mice were followed until blood parasitemia reached 50%, with frequent blood smears. Mice with >50% parasitemia were euthanized.

Stage Specificity Assay

Stage specific activity of test compounds was analyzed according to Shenai et al. 2002. Synchronous W2 strain *P. falciparum* were cultured in triplicate wells in 96-well culture plates with test compound (2 µM 1467, 0.37 µM 3661 or 1.3 µM chloroquine) for 8 hour intervals, beginning at the ring stage. At the end of each interval, the cultures were washed three times, resuspended in culture media without drug, and cultures were continued. After 48 hours, when control parasites were at the ring stage, the cultures were fixed with 2% formaldehyde and processed using FACs as detailed above. Parasitemias were compared to those of controls as described above.

Morphology of treated parasites

W2-strain *P.falciparum* were cultured in the presence of test compound (4 µM 1467, 4 µM 1474 or 0.37 µM 3661) and monitored by light microscopy at the indicated times (12, 24, 36, 48 and 60 hours post treatment).

Selection of parasites with decreased sensitivity to compound 1467

Dd2-strain *P. falciparum* were cultured in stepwise increasing concentrations of compound1467 (Fig 2.1). Media was changed and fresh 1467 was added to cultures daily. Initially, triplicate 10 ml cultures, each containing a clonal population of 6×10^7 asynchronous parasites, were subjected to 0.4 µM compound1467. Once the treated parasites grew at rates comparable to those of untreated controls, the concentration of compound1467 was increased, following the scheme described in Fig 2.1 until three generations of resistance were achieved. Subsequently resistant clones from each generation were generated by limiting-dilution. This entails diluting parasite cultures to achieve ≤ 1parasite per well in a 96-well plate and cultured until growth can be detected by light microscopy (~ 2 to 3 weeks). Cloning is presumed successful if parasite growth is detected in only a small fraction of the 96 wells (example 20%). Detection of growth in most of the 96 wells is an indication of a failed cloning process, in which case, the process was repeated. The most

resistant clones were also cultured without drug pressure to evaluate the stability of the resistant phenotype.

Selection of parasites with decreased sensitivity to compound 3661
W2 and Dd2-strain *P. falciparum* were cultured separately in stepwise increasing concentrations of compound 3661. Initially, triplicate 10 ml cultures, each containing 6×10^7 asynchronous parasites, were subjected to 0.037μM compound 3661. Once the treated parasites grew at rates comparable to those of untreated controls, the concentration of compound3661 was increased, following the scheme described in Fig 2.2A & B. Following completion of selection, resistant clones were generated by limiting-dilution, as detailed above, before analysis.

Additionally, a one-time drug pressure approach was used to select for 3661 resistance in Dd2-strain *P. falciparum* by the laboratory of our collaborator Dr. David Fidock at Columbia University. Here, a larger population (2×10^9) of clonal Dd2-strain asynchronous parasites was subjected to a single high concentration (170 nM; 5 fold IC_{50}) of 3661 in three independent culture flasks. Media was changed and fresh 3661 was added to each culture daily. After the number of days 29 to 45 days, parasite growth, comparable to untreated parasite population, was observed, marking the end of selection (Fig 2.2C). The resulting parasites with decreased 3661 sensitivity were cloned by limiting dilution and sent to our laboratory, where whole genome libraries were prepared and sequenced.

Dd2: 6×10^7 parasites/flask

0.4µM (31 days)

1st generation IC_{50} : 0.87 – 1.7µM

1µM (73 days)

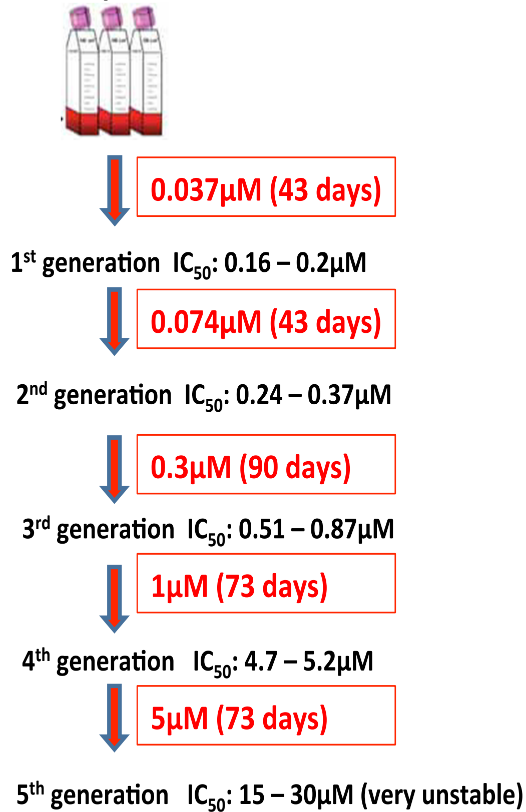
2nd generation IC_{50} : 3.4 – 9uM

10µM (103 days)

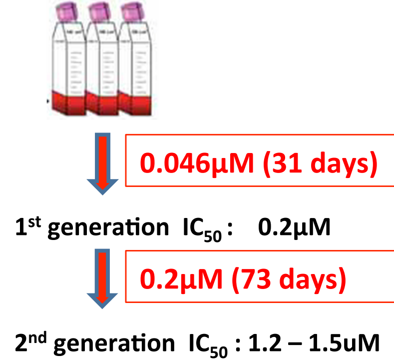
3rd generation IC_{50}: 25 – 40µM

Figure 2.1: 1467 *in vitro* resistance selection schematic

A



B



C

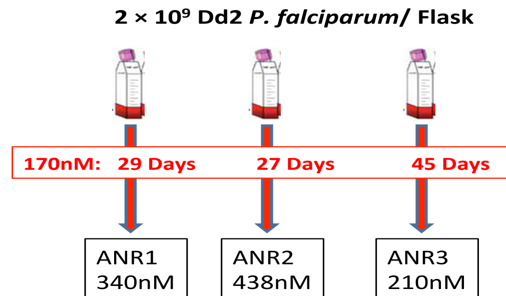


Figure 2.2. Step-wise 3661 *in vitro* resistance selection schematic using A) W2-strain and B) Dd2-strain *P. falciparum*. C) Schematic of one-concentration 3661 selection in Dd2-strain *P. falciparum*.

Genomic DNA Isolation

For whole genome sequencing, genomic DNA (gDNA) from parental parasites and those selected for resistance to 1467 or 3661 was extracted as follows. Synchronized *P. falciparum*-infected erythrocytes (100mL, 2% hematocrit, 10% parasitemia) were treated with 0.15% saponin for 5 minutes on ice to lyse erythrocytes followed by three washes in PBS. Parasite pellets were then lysed in 150 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl pH 7.5, 0.1% L-loril sarkosil (Sigma Aldrich) and 200 mg/ml proteinase K (Qiagen) overnight at 37°C. The samples were then subjected to one extraction with phenol/chloroform/isoamyl alcohol (25:24:1) pH 7.9 (Ambion), treatment with 0.05mg/mL RNAse A (1 hour at 37°C), two additional phenol/chloroform extractions, one chloroform extraction, and then ethanol precipitation. All phenol/chloroform extractions were done using light phase lock tubes (5 Prime). For dideoxy sequencing, gDNA was extracted using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions.

Whole Genome Sequencing

gDNA libraries were prepared from 100 ng DNA using the Nextera DNA Sample Prep Kit (Illumina) according to the manufacturer's instructions, with the exception that the number of cycles and extension temperature in the bridge amplification step was reduced to 6 and 65°C for 6 minutes respectively (Guler et al 2013). Each library was barcoded with unique sets of two indices from the Nextera Index Kit (Illumina) to allow multiple samples to be run on one flow cell (Table 2.1A & B). Next, fragments of 360-560 bp were extracted and collected using Lab Chip XT (Caliper Life Sciences) according to the manufacturer's instructions. The selected fragments were amplified by limited-cycle PCR using Kapa HiFi DNA polymerase (Kapa Biosystems) with dNTPs with an 80% AT coding bias (6 cycles of 95°C for 10 sec, 58°C for 30 sec, 65°C for 6 min using Bio-Rad S1000 thermal cycler). The primers used for both PCR steps were AATGATACGGCGACCACCGA and CAAGCAGAAGACGGCATACG. The concentrations of the libraries were determined with a DNA Bioanalyzer (Agilent), and libraries were pooled at concentrations of 2 nM per library. Library samples were then sequenced at the Center for Advanced Technology at UCSF, where the remainder of library preparation steps, as detailed in Guler et al 2013, were completed, followed by sequencing on a HiSeq 2000 system (Illumina). Analysis of the whole genome sequencing data was carried out as detailed in Guler et al 2013. The generated sequence data for each library was aligned to the 3D7 reference genome using

Bowtie (Langmead 2009), discarding reads with >1 nucleotide mismatch and multiple alignments across the genome. For SNP identification, reads from compound1467 resistant clones were matched to reads from the parental strain and the top 200 SNPs per chromosome were chosen and subsequently filtered based on standard parameters. SNPs were characterized as real if the number of reads covering the nucleotide position was >10 and if the frequency of the SNP was at least 80% across the reads. Searches for novel SNPs included only non-synonymous SNPs in exonic regions excluding hypervariable genes (pfemp1, rifin, and stevor) as these unique *P. falciparum* genes are inherently polymorphic. Copy number variation was analyzed using the UCSC Genome Browser (Kent et al. 2002).

Table 2.1A: List of illumina indices used for gDNA library preparation of 1467 resistant clones

	index 1 (i7)	Index 2 (i5)
Dd2_1467_1st generation clone 1	TAAGGCGA	TATCCTCT
Dd2_1467_2nd generation clone 1	CGTACTAG	AGAGTAGA
Dd2_1467_3rd generation clone 1	TCCTGAGC	TATCCTCT

Table 2.1B: List of illumina indices used for gDNA library preparation of 3661 resistant clones

	index 1 (i7)	Index 2 (i5)
Dd2_3661_1st generation clone 1	GGACTCCT	AGAGTAGA
Dd2_3661_2nd generation clone 1	TAGGCATG	AGAGTAGA
W2_3661_1st generation clone 1	AGGCAGAA	TAGATCGC
W2_3661_2nd generation clone 1	TCCTGAGC	TAGATCGC
W2_3661_3rd generation clone 1	GGACTCCT	CTCTCTAT
W2_3661_4th generation clone 1	TAGGCATG	CTCTCTAT
W2_3661_5th generation clone 1	TCCTGAGC	TATCCTCT
W2_parent	TAAGGCGA	TAGATCGC
Dd2_parent	GGACTCCT	TATCCTCT

Dideoxy Sequencing

The *P. falciparum* LeuRS gene PF3D7_0622800 was amplified in four fragments using the Phusion Hot Start II High-Fidelity DNA Polymerase kit (Thermo Scientific) with 80% AT dNTPs (95°C for 3 min, 30 cycles of 95°C for 10 sec, 52°C for 30 sec, 65°C for 1 min and a final extension at 68°C for 10 min). The amplified fragments were cleaned using ExoSAP-IT (Affymetrix), mixed with sequencing primers and sequenced at the UCSF Genome Core Facility. For the *P. falciparum* PF3D7_1218100 gene, the same approach was followed, but only one 800 bp fragment, including the M416T position, was amplified and sequenced. For the *P. falciparum* CPSF gene PF3D7_1438500 and *pfmdr1* gene (PF3D7_052300), the same approach was used as for the LeuRS gene detailed above. For a list of PCR and sequencing primers used, see Tables 2.2A, 2.2B and 2.2C.

Table 2.2A: List of amplification and sequencing primers for PF3D7_0622800 and PF3D7_1218100

PCR amplification Primers	Sequencing primers
PF3D7_0622800	
Forward: 5'-CTTTTGTTAGAATTTGTTACTATAAAA Reverse: 5'-AAAGTCTTCATTCCATATTTTATTTT	5'-AGGGTAAGGGAAATTGCCTGT 5'-TCCGTTTTCGTTTCATTGTACAGG 5'-TGATCCTTAGCCTTTGATGA 5'-TGGTAGTAGAGTTACTATTTTTAGT
Forward: 5'-AATGTCAAAGATTTTTATTCTATATTT Reverse: 5'-CATTATATGTTTGGAAATTATTCTTT	5'-GCTTTAAGGTGCTACCAAGGAA 5'-ACTTTCATCAACCCTAATGA 5'-CCTGTGTGTCTAGTGATAGTACGG 5'-AGCTGCAATACATTTGACGT
Forward: 5'-TATGAAAACATTTAATTGTAGAAAAT Reverse: 5'-AAAATGTATCTTTCTTAAGAATATAAG	5'-TGGTTAGATGATTGGTCATGT 5'-ATTTCCATAATTTATATACCATTGATT 5'-TGATGTACGTATATCTGGTAAAGATT
Forward: 5'-TATAGAAGATTCTAATTTTAATACTGATA Reverse: 5'-GTGGTTTTATTTTACAAAATTTATTAAC	5'-ACAAATAAATGTAAAGAATCCTATGA 5'-ATCAAATTATATAACTTCATAATAGCA 5'-TCTGAAGAAGAACATACAAC 5'-AAGAAGTCTTAATTTGGGAGA
PF3D7_1218100	
Forward: 5'-CGTATGCAATAATAATTTTTCTTATA Reverse: 5'-ATACGTAATATTCTTCATCATTATAA	5'-GATCAAAATTTTCATATGAATTTTTC 5'-TTTTTACTATTTATGATCCACATAA 5'-GTAATATTCTTCATCATTATAAAAAC

Table 2.2B: List of amplification and sequencing primers for PF3D7_1438500

PCR amplification Primers	Sequencing primers
Forward: 5'-TAAATAGTTTCCTTTGACAAATATTAA	5'-TCCATGTCAACAAAAACTTGTACA
Reverse: 5'-TTAATAGAAGAAAAACAATATTTATCT	5'-TGCTGATGTGCTTACTGATCA
Forward: 5'-TATAATTCCTTCAATTTGATTATTTAC	5'-TCCCAACGAAATAAGAGAATCA
Reverse: 5'-ATATATGAACGTTGTTAATAAGAATAA	5'-GGCTTCCCCTGGTATGCTAC
Forward: 5'-TAAATGTTTCATAAATACATAATGATT	5'-TGTTTCTATTAAATCCATAGTTT
Reverse: 5'-AAAAAGAAATAGGAAAAAGGATTTA	5'-TCACTAGCACCCCCTAGACA

Table 2.2C: List of amplification and sequencing primers for PF3D7_052300

PCR amplification Primers	Sequencing primers
Forward: 5'-TTTATATATTGATATATGTGTACATAGCTTATTTC Reverse: 5'-TTTATATATTGATATATGTGTACATAGCTTATTTC	5'-GATGTAATTACATCCATACAATAACTTGATATCATTG 5'-GAGGTTGAAAAAGAGTTGAACAAAAAGAGTACCGC 5'-GTACGAAATTTATAACAATTTTTACATATG 5'-GGTGCCTCAGTTATATCCATTTTATTAGGTGTAC
Forward: 5'-AAGTTATTGTGGAGAAAAGACTATATTAAAC Reverse: 5'-AATTGTATTGGCATATCTTATAGTACTTAATC	5'-GGAGAAAAGACTATATTAAACAAATTTAATTTGTCCG 5'-CCATTAGTTGAAAATAATGATGATGGAG 5'-GTACTAAATACTCAGATTTATTATCTAAAGAAG 5'-GGTGCCTCAGTTATATCCATTTTATTAGGTGTAC
Forward: 5'-GTATCATCATTACCAGATAAATATGATAC Reverse: 5'-TATGAAATGAGAGAAAATAACAATATTATTTAC	5'-CATCATTACCAGATAAATATGATACCTTAGTAGG 5'-GAGGGTAGCTATATTATTGAACAAGGTAC 5'-GAAAGCACCAAACAATTTACGTATC
Forward: 5'-TATAGAAGATTCTAATTTTAATACTGATA Reverse: 5'-CCATTATTATTAAATACAGTATAATCTTCTGC	5'-CAACAAAATAGGAGAAAAAGTCG 5'-GCGGAGTTTTTGCATTTAGTTCAGATGATG 5'-CCTTATTTACTTTTATATTTACTGGTAG 5'-GCCTATTGGTTTGGATCCTTCTTAATTAAAAGAGG
Forward: 5'-TTTACATGTGATAGTAAAAAAACTACAGC Reverse: 5'-TTAAAATAATAAATTGAGAATATAATTTCTGG	5'-TTTACATGTGATAGTAAAAAAACTACAGC 5'-GAAAATATCAAATTTGGAAGAGAAGATGC 5'-CTATTGCCCACAGAATTGCATCTATAAAAC

Quantification of *pfmdr1* copy number by real-time PCR (QPCR)

Amplification reactions were done as multiplex PCR in MicroAmp 96 well plates (Applied Biosystems) in 25 µL, containing TaqMan mastermix buffer (8% glycerol, 0·625 U DNA polymerase, 5·5 mM $MgCl_2$, 300 µM dNTP, 600 nM), reaction reference dye ROX (5-carboxy-X-rhodamine), pH 8·3), 300 nM of each forward and reverse primer, 100 nM of each probe, and 2.5 µL DNA template. The amplification protocol used was 95°C for 10 min, 50 cycles of 95°C for 15 s, and 58°C for 1 min using Applied Biosystems 7500 Real Time PCR machine. β-tubulin was used as the internal reference. See table 2.3 for primer and probe sequences.

The resulting fluorescence data were analyzed by a comparative C_t method as detailed in Price et al. 2004, comparing changes in fluorescence signal of the target (*pfmdr1*) relative to the internal reference (β-tubulin). All signals were normalized to the passive reference signal (ROX). The detection threshold was set above the mean baseline value for fluorescence of the first fifteen cycles.

Table 2.3: Primer sequences used for *pfmdr1* qPCR

Primer	Sequence
pfmdr1-1F	5'-TGCATCTATAAAACGATCAGACAAA
pfmdr1-1R	5'-TCGTGTGTTCCATGTGACTGT
β-tubulin -1F	5'-TGATGTGCGCAAGTGATCC
β-tubulin -1R	5'-TCCTTTGTGGACATTCTTCCTC
pfmdr1-probe	5'- 6FAM-TTTAATAACCCTGATCGAAATGGAACCTTTG-TAMRA
β-tubulin -probe	5'- VIC-TAGCACATGCCGTTAAATATCTTCCATGTCT-TAMRA

Protein Synthesis Assay

Synchronous trophozoites of Dd2-strain *P. falciparum* at 10 – 15% parasitemia were washed three times with leucine-free RPMI media (Invitrogen) and resuspended to 5% hematocrit in the same medium. Next, 100 µL of each culture was mixed with 0X, 1X, 10X or 100X the IC_{50} of test compound in triplicate, followed by incubation for 15 minutes at 37°C. Then, 0.5 µCi of ^{14}[C]leucine (324mCi/mmol, Perkin Elmer) was added to each aliquot and incubated for two additional hours at 37°C. Subsequently, infected erythrocytes were treated with 0.15% saponin (as described above) and resulting parasite pellets were resuspended in 100 µL 0.02% sodium

deoxycholate (Sigma Aldrich) to lyse the parasites, followed by addition of 100 µL ice cold 10% (w/v) TCA to precipitate proteins. Precipitates were transferred to 0.45 µm nitrocellulose membrane filter plates (Millipore Multiscreen HTS, MSHAN4B50), washed four times with 400µL ice cold 5% TCA and air-dried. ^{14}[C] leucine incorporation was determined after adding 60µL OptiPhase 'Supermix' scintillation cocktail (PerkinElmer) to the filter plates, followed by counting in a Wallac MicroBeta Trilux model 1450 liquid scintillation counter.

Aminoacyl tRNA synthetase Activity Assay

W2 strain *P. falciparum* trophozoites (1 L culture at 10 – 15% parasitemia, 2% hematocrit) were collected and treated with 0.15% saponin as described above, washed three times with PBS, and resuspended in 5 mL extraction buffer (20 mM bisTris-Cl, 1 mM EDTA, 0.1mM phenylmethylsulfonyl fluoride and one complete protease inhibitor tablet [Roche]). The resuspended pellets were lysed by two freeze-thaw cycles and centrifuged at 2000xg for 10 minutes. The supernatant was then centrifuged at 147,000×g for 2 hours and the supernatant used as the source of LeuRS. The LeuRS activity assay was carried out as described previously (Rock et al. 2007). A 500 µL reaction mixture containing 100 uL of parasite extract, 50 mM HEPES-KOH (pH 8.0), 30 mM $MgCl_2$, 30 mM KCl, 2.5uCi [^{14}C]leucine (324 mCi/mmol, Perkin-Elmer) or [^{14}C]isoleucine (220 mCi/mmol, Perkin-Elmer) or [^{14}C]valine (0.05 mCi/mL, Perkin-Elmer), 0.2 mg/mL *S. cerevisiae* or *E. coli* tRNA, 0.02% (w/v) BSA and 1 mM DTT was pre-incubated with specified concentrations of test compound for 20 minutes at room temperature. Reactions were initiated by addition of 4 mM ATP and incubated at 30°C. After 10 minutes, three 75 µL aliquots were transferred to separate wells in a 96-well 0.45 µm multiScreen nitrocellulose membrane filter plate (Millipore Multiscreen HTS, MSHAN4B50) followed by precipitation using 150 µL ice cold 10% TCA, washing four times using 400 µL ice cold 5% TCA and drying. Incorporated [^{14}C]amino acid (leucine, isoleucine or valine) was quantified as described above.

Norvaline Sensitivity Assay

Synchronized parasites were cultured at 1% parasitemia in either leucine-free or complete RPMI-1640 medium (Invitrogen) with varying concentrations of L-norvaline (Sigma Aldrich). After 48 hours, the parasites were fixed with 200 uL 2% formaldehyde and parasitemias were determined as described above.

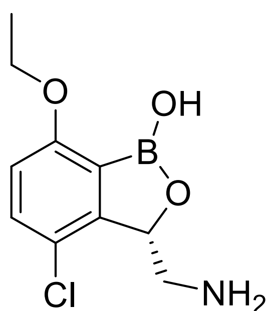
CHAPTER 3

STUDY OF THE MECHANISM OF ACTION OF THE BENZOXABOROLE COMPOUND 1467

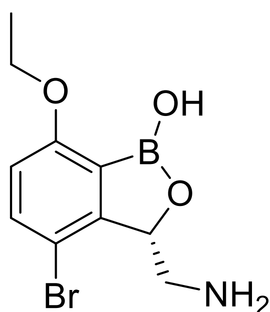
3.1 **1467 exhibits potent *in vitro* and *in vivo* antimalarial activity.**

To evaluate the potential for benzoxaboroles as antimalarial drugs, we screened 8112 compounds from the Anacor library for activity against cultured W2 strain *P. falciparum*. This screen identified 326 compounds with IC_{50} < 1 μM from which 29 compounds with less than mid nanomolar IC_{50} have been tested in a murine malaria model.

In this section we discuss results for compounds 1467 and 1474 (Figure 3.1), related benzoxaboroles that were identified in the initial screen. Compounds 1467 and 1474 demonstrated nanomolar activity against three strains of cultured *P. falciparum* (Table 3.1). Activities were similar against laboratory strains known to be sensitive (3D7) and resistant (W2, Dd2) to chloroquine and other established antimalarials. The compounds did not exert apparent toxicity against cultured Jurkat cells at concentrations up to 100 μM (Table 3.1). When administered orally once daily to *P. berghei*-infected mice for four days, the compounds were highly efficacious, with day four ED_{90} of 7.4 mg/kg/day for 1467 and 16.2 mg/kg/day for 1474 (Table 3.1). At dosages of 100-200 mg/kg/day both compounds afforded long-term (34 day) cures in 40-60% of infected mice (Figure 3.2). Thus, compounds 1467 and 1474 were deemed promising new antimalarial agents worthy of additional study.



Compound1467



Compound1474

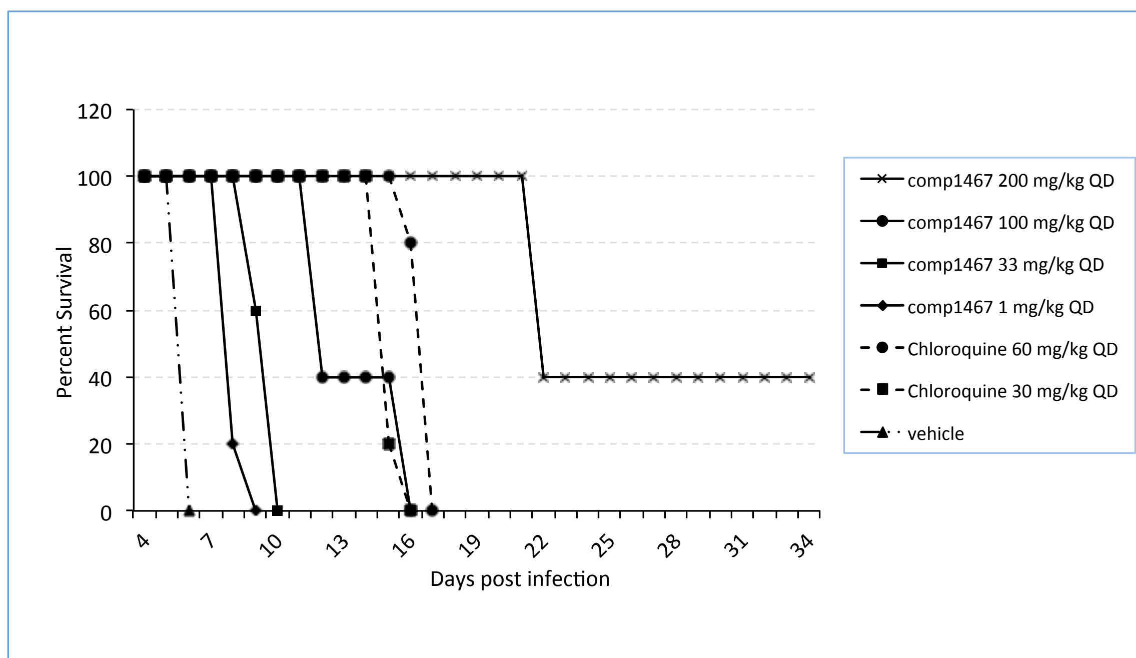
Figure 3.1. Chemical structure of antimalarial benzoxaboroles

Table 3.1: *In vitro* and *in vivo* antimalarial activities of compounds 1467 and 1474

	Lab *Strains* IC$_{50}$ (µM)			**Cell Line CC$_{50}$ (µM)**	***In vivo P. berghei*** **ED$_{90}$ (mg/kg)**
Compounds	**3D7**	**W2**	**Dd2**	**Jurkat**	
1467	0.19 ± 0.05	0.28 ± 0.14	0.42 ± 0.15	>100	7.44
1474	0.28 ± 0.01	0.36 ± 0.14	0.49 ± 0.18	>100	16.19

IC$_{50}$ results (±SD) are means from at least 3 experiments, each with duplicate readings. In vivo ED$_{90}$ values are based on comparisons of parasitemias between treated and control animals on the fourth day after inoculation of parasites and initiation of treatment.

A



B

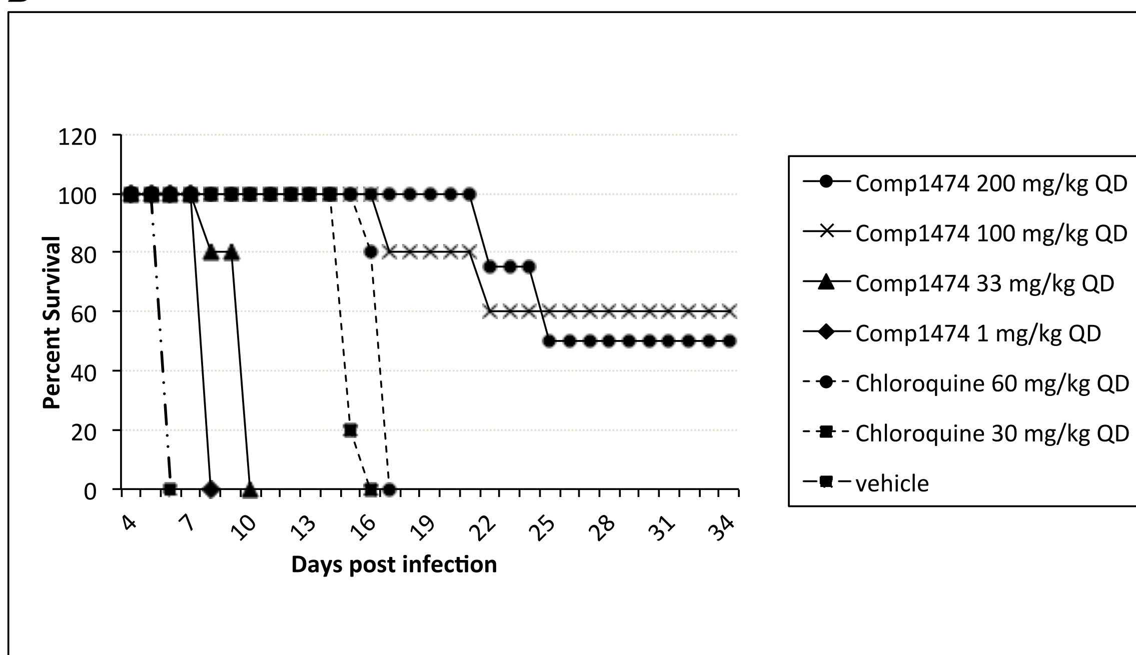


Figure 3.2: Survival curves of *P. bergei*- infected mice treated with 1467 (A) or 1474 (B). Animals were inoculated intraperitoneally with 6 x 10^6 *P. berghei* infected erythrocytes, followed one h later by initiation of once daily oral treatment with multiple dosages of test compounds, choloroquine, or vehicle control. Results are based on two independent experiments, each including five mice per dosing regimen.

3.2 *In vitro* morphological and stage-specific effects of 1467 on RBC stage *P. falciparum.*

Synchronized parasites were treated with 1467, beginning at the early ring stage, and examined over the course of the life cycle (Figure 3.3). The parasites had no obvious morphological abnormalities during the ring and trophozoite stages. However, treated parasites were unable to progress beyond this stage; they did not develop into multinucleated schizonts or new ring stage parasites (Figure 3.3). To consider the stage specificity of action of 1467, synchronized W2-strain parasites were incubated with the compound or chloroquine for 8 hour intervals across the life cycle, the compounds were removed by washing at the end of each interval, cultures were maintained until untreated control parasites had reached the ring stage, and the parasitemias of control and treated cultures were compared. For both compound 1467 and chloroquine, some activity was seen across the life cycle, but activity was greatest against trophozoites (Fig 3.4).

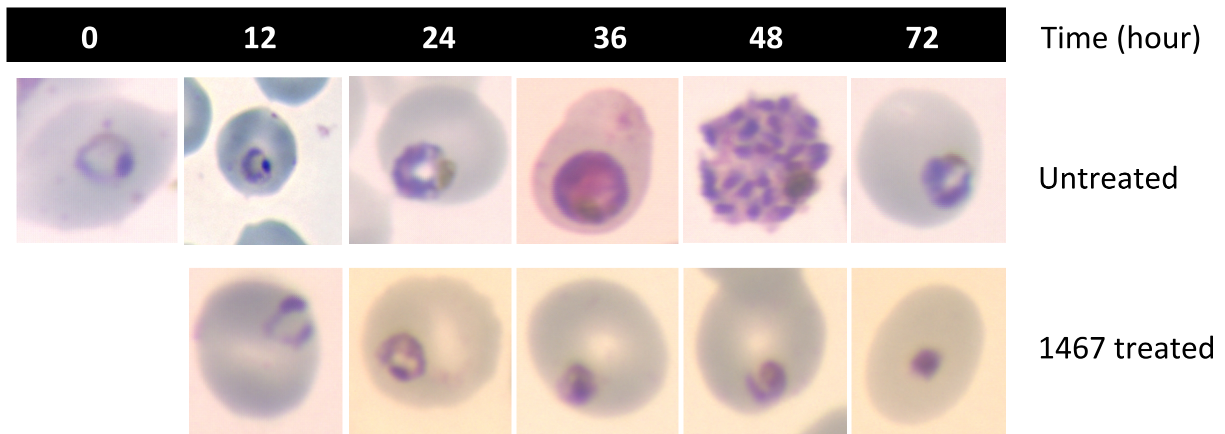


Figure 3.3. Light microscopic imaging of Giemsa-stained *P.falciparum* - infected erythrocytes after treatment with 4 μM compound1467, beginning at the early ring stage

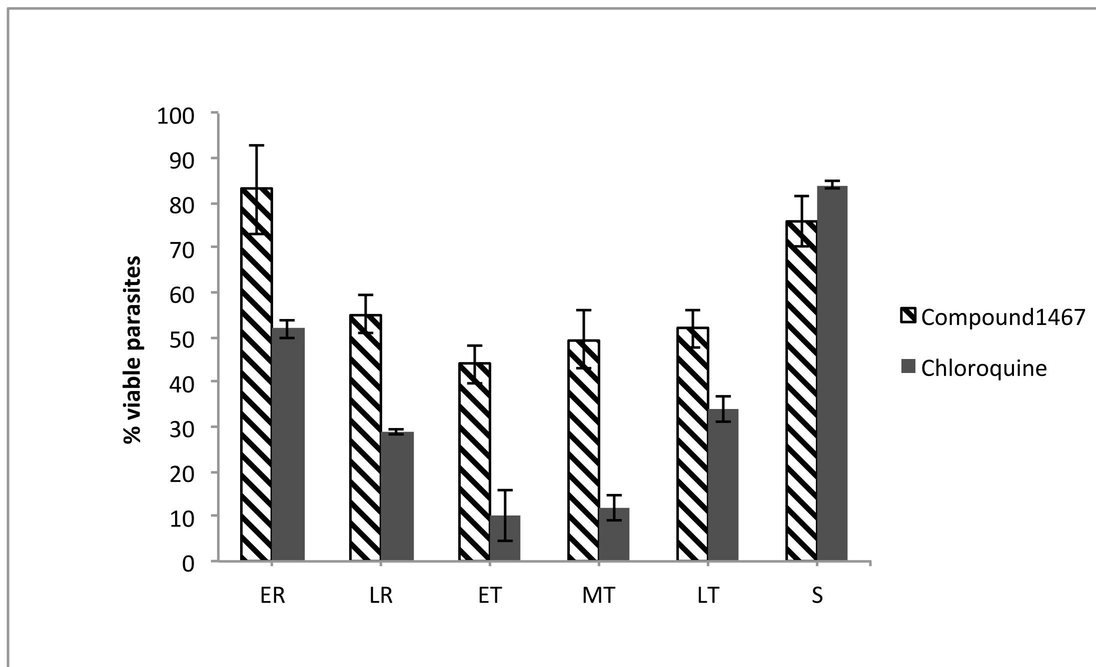


Figure 3.4. Stage specificity. Parasites were incubated with 2 μM 1467 or 1.3 μM chloroquine for 8 hour intervals across the life cycle. Parasites were then continued in culture until the following ring stage, and parasitemias were counted by FACS analysis and compared wth those of untreated controls. ER, LR (Early and Late Rings respectively); ET, MT, LT (Early, Mid and Late Trophozoites respectively), S (Schizonts)

3.3 Selection and analysis of parasites with decreased 1467 sensitivity

In an attempt to identify the antimalarial mechanism of action of compound 1467, we cultured Dd2 strain *P. falciparum* in step-wise increasing concentrations of 1467, assessed parasite sensitivity after each step, and characterized cloned parasites with decreased sensitivity by whole genome sequencing. Parasites were incubated with 0.4 μM compound for four weeks, 1μM compound for ten weeks, and then 10μM compound for fourteen weeks (Fig. 3.5). For each step of selection, parasites were initially not seen on Giemsa-stained smears, but after about two weeks they reappeared, suggesting slow regrowth of parasites with alterations allowing growth under drug pressure. Parasites from each step of selection were cloned by limiting dilution, and DNA from multiple clones was analyzed by whole genome sequencing. After three steps of selection, resulting in three generations with increasing levels of resistance, parasite sensitivity to 1467 decreased about 50-fold (Fig. 3.5). As expected, 1467-resistant parasites were similarly resistant to 1474, indicative of a shared mechanism of resistance. In contrast, selected parasites were not more resistant to the antimalarials chloroquine, mefloquine, lumefantrine, piperaquine, or dihydroartemisinin. Indeed, after three steps of selection parasites were about 5-fold more sensitive to mefloquine and lumefantrine (Table 3.4).

Sequencing identified numerous genetic changes between parental and 1467-resistant parasites, including SNPs and CNVs. Relevant SNPs were determined according to standard parameters (Guler et al 2013) outlined in Table 3.2. For each of the 1467 resistant generations analyzed, the top 200 SNPs per chromosome were selected, leading to about 2800 total SNPs (1st generation – 2834 SNPs, 2nd generation – 2803 SNPs and 3rd generation – 2833 SNPs) per clone. Similar to the approach described previously (Guler et al. 2013), the SNPs were subsequently filtered to exclude SNPs in non-coding regions and introns, and also SNPs located in genes that code for well-characterized *P. falciparum* variant surface antigens [rifin, strevor, erythrocyte membrane protein 1 (pfemp1)]. SNPs within the genes that code for *P. falciparum* variant surface proteins were excluded because they are naturally highly polymorphic; thus including them will increase the rate of false-positive SNPs with regard to 1467 resistance. Additionally, synonymous SNPs, SNPs in low coverage exonic regions (≤ 20 sequence reads covering the

genomic position of a SNP) and SNPs where < 80% of reads have the SNP in question were also disqualified. Although it is possible that SNPs in the non-coding and intronic genomic regions (for example, at a promoter, 3' UTR, or splice junction) could play important roles in resistance, we anticipate that SNPs in these regions are less likely to mediate drug resistance than those in coding regions. Furthermore, non-coding and intronic regions in *P. falciparum* tend to be highly A-T rich (~ 90% AT) (Gardner et al. 2002), thereby increasing the propensity to acquire non-specific SNPs in these regions. Synonymous SNPs are SNPs that lead to no amino acid change at the protein level; hence their disqualification..

Based on the described SNP filtration parameters, SNPs in two genes in the first generation, three genes in the second generation and four genes in the third generation clones were deemed potentially relevant for mediating resistance (Table 3.5). Upon comparison of the sequences of multiple clones, SNPs in only two genes (PF3D7_0622800 and PF3D7_1218100) were deemed relevant to 1467 resistance due to their consistency in multiple clones from all three generations. These genes are PF3D7_0622800, which encodes a homolog of LeuRS from other organisms and PF3D7_1218100, which encodes a protein with unknown function.

There are two predicted LeuRS genes in the chromosomal genome of *P. falciparum*; one is predicted to be the cytoplasmic LeuRS gene (PF3D7_0622800) located on chromosome 6. The other is a putative apicoplast-targeted LeuRS gene (PF3D7_0828200) on chromosome 8. Parasites selected for resistance to 1467 had 4 different SNPs in PF3D7_0622800, but none in PF3D7_0828200, arguing that alterations in the cytoplasmic LeuRS play a role in resistance to 1467. All 4 SNPs mapped to the predicted editing domain of the LeuRS, with the SNPs identified in second and third generation resistant parasites predicted to be in highly conserved active site residues based on comparisons with the LeuRS genes of humans, *S. cerevisiae* and *E. coli* (Table 3.5 and Fig 3.7).

The other gene that was consistently altered in 1467-resistant parasites was PF3D7_1218100. A single SNP (M416T) was present in all generations of 1467 resistant parasites (Table 3.5). Little is known about this gene, except that its product is classified as a membrane protein based on homology and a database from the gene ontology consortium (Ashburner et al. 2000).

With regard to copy number variation, amplification of two clusters of genes on chromosome 12 was observed in all generations of 1467 resistant parasites (Fig 3.6 and Table 3.3). The two clusters contain eight and seven

predicted open reading frames, respectively. The copy number of most of the genes (seven out of eight) in Cluster 1 increased additionally with increasing 1467 resistance. Four of the eight genes are plasmodium-conserved genes with unknown function including PF3D7_1218100 – the gene with the M416T SNP in resistant parasites discussed above. The other genes in cluster 1 are annotated as follows: adaptor protein subunit, hexose phosphate translocator, arginine tRNA ligase, secreted ookinete protein. Cluster 2 genes are annotated as leucine-rich repeat protein, NIMA related Kinase 1, putative RNA pseudouridylate synthetase and merozoite surface protein 9. Contrary to the cluster 1 genes, the copy number of most of the genes (five out of seven) in cluster 2 decreased with increasing 1467 resistance, with the exception of two genes that retained a constant copy number (Table 3.3).

Parasites selected for the third generation of resistance were subsequently cultured without drug pressure for five months. These parasites showed partial reversion to sensitivity, with a change from ~50 fold to ~11 fold decreased sensitivity compared to wild type, but they retained mutations in both PF3D7_0622800 (V568L) and PF3D7_1218100 (M416T) (Table 3.6).

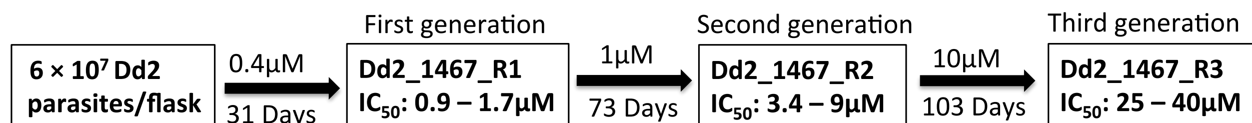


Figure 3.5: Analysis of 1467-resistant parasites. Resistance was selected against Dd2-strain *P. falciparum* with step-wise increases in concentration of 1467, leading to 3 generations of resistant parasites, as indicated in the schematic.

Table 3.2: SNP filtration flow chart showing the criteria and corresponding number of SNPs excluded from further analysis.

	Number of SNPs per criteria listed		
	1st generation	2nd generation	3rd generation
Total SNPs	2834	2803	2833
SNP filtration criteria			
Non-coding regions	1978	1958	1630
Introns	263	263	225
Hyper-variable genes (pfemp1, rifin, strevor)	187	189	191
< 80% of reads have SNP**	379	373	747
Read coverage* < 10	15	6	21
Synonymous SNP	4	5	9
SNPs common with 3661 resistant clones	6	6	6
Sum***	2832	2800	2829
SNPs of interest (Total SNPs - Sum)	2	3	4

SNPs were disqualified from further analysis based on the listed criteria.
*Number of reads (sequence fragments of about 60 to 90 base pairs) that align to any particular nucleotide position.
** At least 80% agreement between the reads that align to the SNP position in question.
******* Summation of the number of SNPs filtered based on the listed criteria

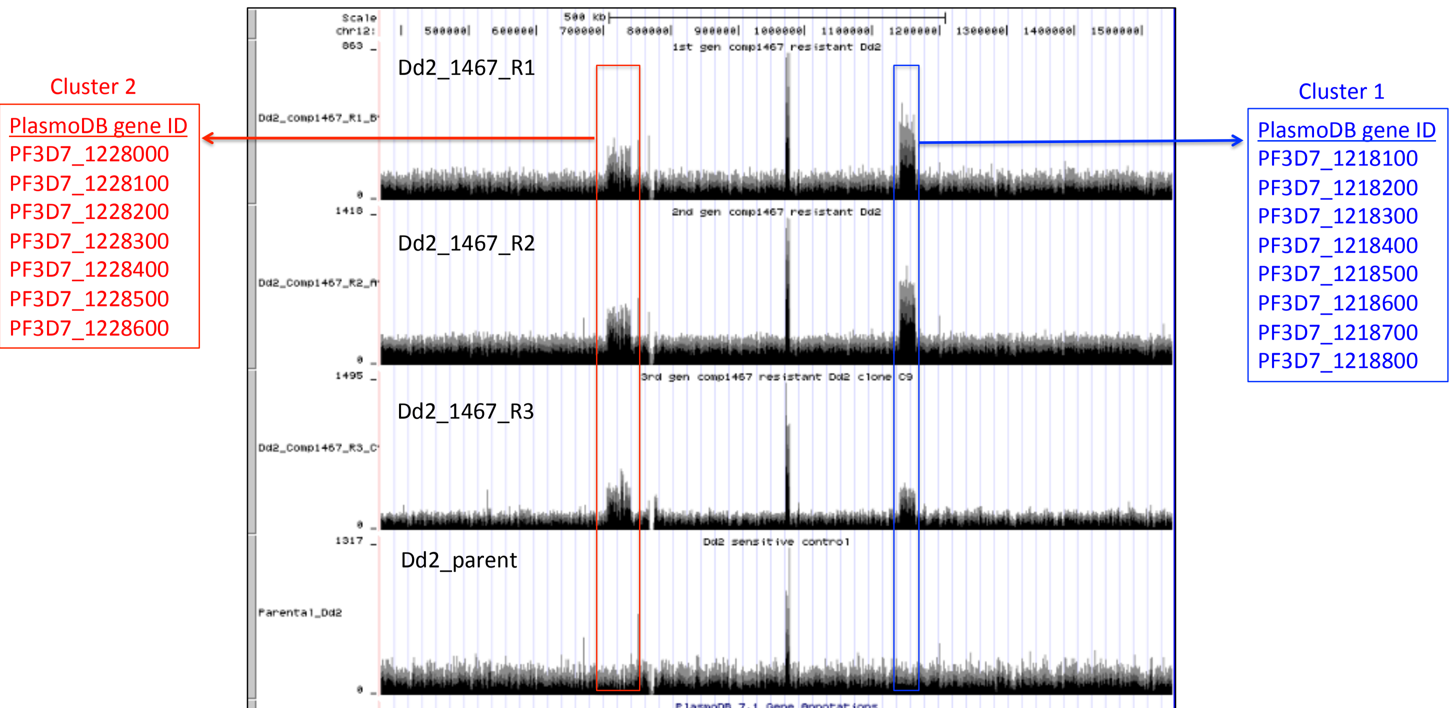


Figure 3.6: Histogram showing read coverage of a region of chromosome 12, comparing Dd2 parent (bottom panel) and 3 generations of 1467 resistant clones (Dd2_1467_R1, R2 and R3). The x-axis represents nucleotide positions along a section of chromosome 12, from position 1 to 1500 kb.The Y-axis represents number of reads per nucleotide position (read coverage). Sequencing showed that three amplification events occured within this region of chromosome 12. Two amplifications (red and blue boxes) were unique to 1467 resistant clones. Annotation of the genes in each cluster along with fold amplification seen are provided in Table 3.3

Table 3.3: Detailed list of genes amplified on chromosome 12 of 1467 resistant clones

	PlasmoDB Gene ID	Encoded Protein	R1	R2	R3
cluster 1	PF3D7_1218100	conserved protein, unknown function	2X	2X	3X
	PF3D7_1218200	conserved protein, unknown function	2X	2X	3X
	PF3D7_1218300	Adaptor protein subunit, putative	2X	2X	4X
	PF3D7_1218400	triose or hexose phosphate/phosphate translocator	2X	2X	2X
	PF3D7_1218500	conserved protein, unknown function	2X	2X	4X
	PF3D7_1218600	arginine tRNA ligase, putative	2X	2X	3X
	PF3D7_1218700	conserved protein, unknown function	2X	2X	3X
	PF3D7_1218800	secreted ookinete protein, putative	2X	2X	3X
cluster 2	PF3D7_1228000	conserved protein, unknown function	4X	4X	3X
	PF3D7_1228100	leucine-rich repeat protein	4X	3X	3X
	PF3D7_1228200	conserved protein, unknown function	3X	3X	3X
	PF3D7_1228300	NIMA related kinase 1	4X	3X	3X
	PF3D7_1228400	conserved protein, unknown function	3X	3X	3X
	PF3D7_1228500	RNA pseudouridylate synthetase, putative	4X	3X	3X
	PF3D7_1228600	merozoite surface protein 9	4X	4X	3X

Fold amplification in first (R1), second (R2), and third (R3) generation 1467 resistant clones, indicative of differences in sequencing coverage levels between amplified and surrounding regions, is shown.

Table 3.4: Activity of antimalarials against 2nd and 3rd generation 1467 resistant Dd2 *P. falciparum*

	IC_{50} Values (nM)		
	Wild type Dd2	2nd gen 1467 R	3rd gen 1467 R
Compound 1467	420 ± 150	6,660 ± 60.0	21,500 ± 1,280
Compound 1474	490 ± 180	6,360 ± 198	18,250 ± 627
Compound 3661	28.5 ± 6.32	34.4 ± 7.01	32.1 ± 6.47
Chloroquine	40.9 ± 0.0132	20.2 ± 6.85	9.46 ± 3.95
Lumefantrine	5.55 ± 0.0914	0.952 ± 1.12	0.975 ± 0.322
Mefloquine	2.16 ± 0.433	0.673 ± 0.104	0.228 ± 0.0712
Piperaquine	14.9 ± 0.0323	11.3 ± 2.54	5.73 ± 1.99
Dihydroartemisinin	7.99 ± 1.67	7.02 ± 3.11	3.58 ± 1.04

Error bars represent standard deviation between at least 3 independent experiments, each with 2 replicates.

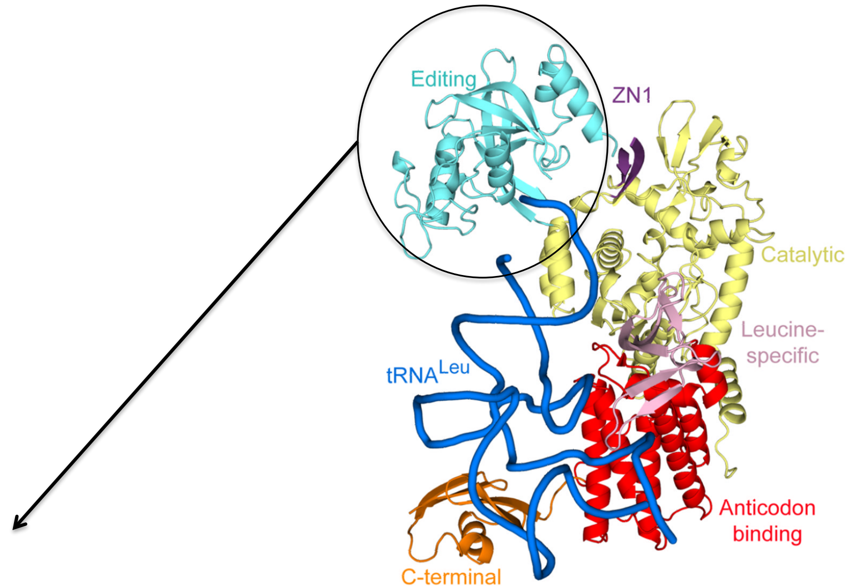
Table 3.5: Relevant SNPs identified in compound1467-resistant *P. falciparum* by whole genome sequencing

Clone	protein ID	amino acid Δ	Read coverage	SNP agreement (%)
Dd2_1467_R1 Clone 1	Leucyl tRNA ligase, putative	Val-630-Leu	89	100
	conserved Plasmodium protein, unknown function	Met-416-Thr	110	86
Dd2_1467_R2 Clone 1	Leucyl tRNA ligase, putative	Thr-400-Ile	178	100
	conserved Plasmodium protein, unknown function	Met-416-Thr	236	86
	conserved plasmodium protein, unknown function	Gln-1610-Leu	172	98
Dd2_1467_R3 Clone 1	Leucyl tRNA ligase, putative	Thr-568-Ile	166	99
	conserved Plasmodium protein, unknown function	Met-416-Thr	89	93
	dynein-related AAA-type ATPase, putative	Phe-1629-Val	109	98
	conserved plasmodium membrane protein, unknown function	Val-806-ile	44	88

Table 3.6: Detailed listing of the SNPs observed in PF3D7_0622800 and PF3D7_1218100 of 1467 resistant Dd2 clones.

		PF3D7_0622800				PF3D7_1218100
Clones	**IC50 (μM)**	**T400I**	**V568L**	**E628G**	**V630L**	**M416T**
Parental Dd2	0.42 ± 0.15					
Dd2_1467_R1_Clone 1	0.70 ± 0.15				✔✔	✔✔
Dd2_1467_R1_Clone 2	0.63 ± 0.20			✔		✔
Dd2_1467_R2_Clone 1	6.66 ± 0.06	✔✔				✔✔
Dd2_1467_R2_Clone 2	8.60 ± 1.19		✔			✔
Dd2_1467_R3_Clone 1	21.50 ± 1.30		✔✔			✔✔
Dd2_1467_R3_Clone 2	24.70 ± 1.00		✔✔			✔✔
5 month_Revertant_Dd2_1467_R3_Clone 2	4.68 ± 0.52		✔			✔

Two clones from each generation were analyzed by whole genome sequencing and/or dideoxy sequencing.
✔ = analyzed by dideoxy sequencing, ✔✔ = analyzed by dideoxy and whole genome sequencing




```
328  NNKINNNKINNNKSNNNKSNNNNNCGSSANISNTFFTDFEKGEEDLKNKIWNEDFFVKDK  387  P. falciparum
279  -----------------------------------------------------LSGLKGK  285  H. sapiens
216  ----------------VKTMQRNWIGRSEGVEI--------------------TFNVNDY  239  E. coli
297  --------------------------------------------------DSSSDLDKSK  306  S. cerevisiae
                2nd generation SNP: T400I
388  KVIFLGSTLKPETAYGQNYTFINPNEYYYLTLGFDKQNLHYGDKSYVNNIMTKEEIINSC  447  P. falciparum
286  NIFLVAATLRPETMFGQTNCWVRPDMKYIG---FET----------------------VN  320  H. sapiens
240  DNTLTVYTTRPDTFMGCTYLAVAAGHPLAQK-----------------------------  270  E. coli
307  KFYFVAATLRPETMYGQTCCFVSPTIEYGI---FDA-----------------------G  340  S. cerevisiae

448  PNIYVCSENSLYNLAYQGIIPLLKNKNIKNVQIPKSEDNTNDDDTLEKKNNDVITKNTNN  507  P. falciparum
321  GDIFICTQKAARNMSYQGFTKDNG------------------------------------  344  H. sapiens
271  --------AAENNPELAAFIDECRNTKVAEAEM----------ATMEKKGVD--------  304  E. coli
341  DSYFITTERAFKNMSYQKLTPKRG------------------------------------  364  S. cerevisiae

508  NNNENNNNMNNVYNLDDVFILNKIKGEHFVGLETYTNISKIKNLYILPMTTIKMNISTGI  567  P. falciparum
345  ----------------VVPVVKELMGEEILGASLSAPLTSYKVIYVLPMLTIKEDKGTGV  388  H. sapiens
305  ---------------TGFKAVHPLTGE---------------EIPVWAANFVLMEYGTGA  334  E. coli
365  ----------------FYKPIVTVPGKAFIGTKIHAPQSVYPELRILPMETVIATKGTGV  408  S. cerevisiae
     2nd and 3rd generations SNP: V568L
568  VPCVSSDSTDDYACLEDIRKKKNYYCEKYNLKEE---QLKNNSESCIELPEIGNNTGKYY  624  P. falciparum
389  VTSVPSDSPDDIAALRDLKKKQALR-AKYGIRDD--MVLPFEPVPVIEIPGFGNLSAVTI  445  H. sapiens
335  VMAVPGHDQRDYEFA-----------SKYGLNIKPVILAADGSEPDLS------------  371  E. coli
409  VTCVPSNSPDDYITTKDLLHKPE----YYGIKPE---WIDHEIVPIMHTEKYGDLTAKAI  461  S. cerevisiae
        1st generation SNPs: E628G, V630L
625  YEKEKVSSYKD-VKLQKIKEVLYKKQYFEGIMTVDPYKGMKTFNCRKLAKQNIIRNLDGF  683  P. falciparum
446  CDELKIQSQNDREKLAEAKEKIYLKGFYEGIMLVDGFKGQKVQDVKKTIQKKMIDAGDAL  505  H. sapiens
372  ------------QQALTEKGVLFNSGEFNGLDHEAAFNA---------IADKLTA-----  405  E. coli
462  VEEKKIQSPKDKNLLAEAKKIAYKEDYYTGTMIYGPYKGEKVEQAKNKVKADMIAAGEAF  521  S. cerevisiae

684  LYSEPEVMVIDRNNVKCIAALCNQWYINYGNMEFKKDVLIQLKKNNFQTYNDVLYKQLQH  743  P. falciparum
506  IYMEPEKQVMSRSSDECVVALCDQWYLDYGEENWKKQTSQCLK--NLETFCEETRRNFEA  563  H. sapiens
406  -----------------------------------------------------MGVGERK  412  E. coli
522  VYNEPESQVMSRSGDDCIVSLEDQWYVDYGEESWKKQAIECLE--GMQLFAPEVKNAFEG  579  S. cerevisiae
```

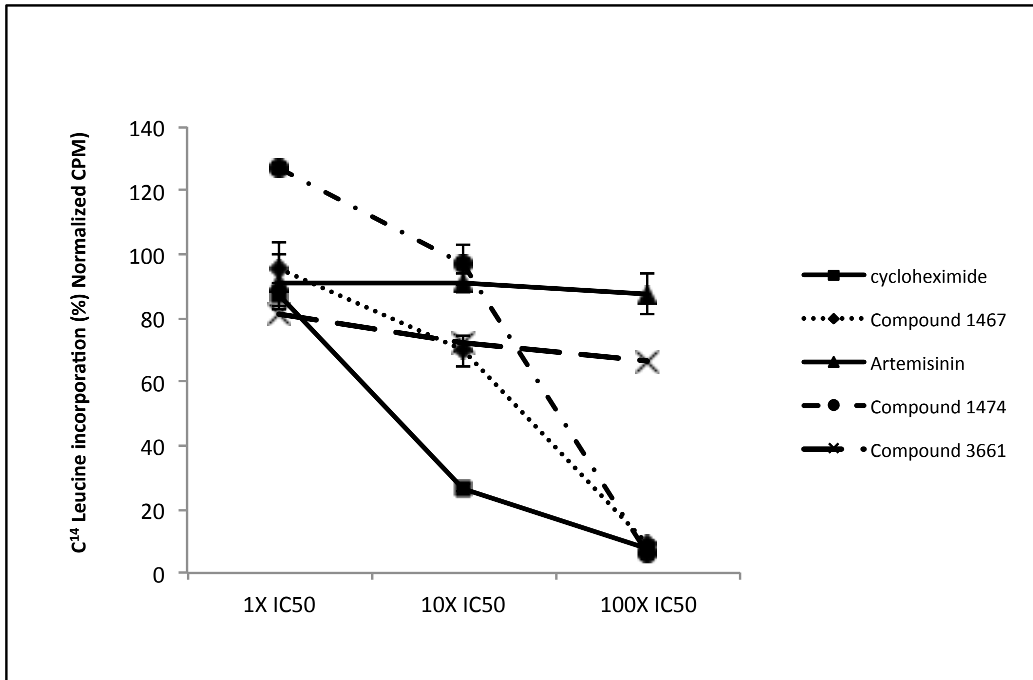
Figure 3.7: LeuRS sequence alignments. A. Structure of *E. coli* LeuRS-tRNA^{Leu} complex reproduced from Palencia et al. 2012. The editing domain is circled. B. Alignment of the predicted editing domain sequences of *P. falciparum* cytoplasmic LeuRS (amino acids 375 – 704) and the characterized LeuRS editing domains in homologs from *H. sapiens, S. cerevisiae* and *E. coli.* Positions

at which a SNP was identified in 1467-resistant parasites are highlighted in yellow. Three highly conserved active-site regions required for LeuRS editing activity in *S. cerevisiae* and *E. coli* are indicated as follows: Threonine Rich Region (Red), GTG region (blue) and Catalytic region (Fuchsia). The SNPs associated with second and third generation 1467 resistance (T400I, V568L) are located in the predicted active sites of the LeuRS editing domain, whereas the first generation SNPs (E628G, V630L), though located within the editing domain, are not within the predicted active sites.

3.4 Compound 1467 appear to inhibit LeuRS editing activity

The identification of *P. falciparum* LeuRS as a likely target of compounds 1467 and 1474 was intriguing, as other tRNA synthetases have recently received attention as potential drug targets (J.S Pham et al. 2014), including antimalarial targets (Hoepfner et al. 2013), and as other oxaboroles were recently characterized as inhibitors of LeuRS of bacteria and yeast (Rock et al. 2007, Ding et al. 2011, Hernandez et al. 2013 and Hu et al. 2013). To attempt to validate LeuRS as the target for the recently identified oxaboroles, we characterized the effect of compounds1467 and 1474 on *P. falciparum* protein synthesis. Wild type Dd2 strain parasites were cultured with varying concentrations of compounds in the presence of radiolabelled leucine. Treatment of cultured parasites with a 2 hour pulse of the compounds led to a block in parasite incorporation of [^{14}C]leucine, indicative of a block in protein synthesis, as also seen with the control cycloheximide, but not seen with compound 3661 and artemisinin, which is a potent antimalarial that does not act against protein synthesis (Fig. 3.8A). In contrast, 1467 failed to inhibit [^{14}C]leucine incorporation in the second and third generations of 1467 resistant parasites, but inhibition was observed for first generation 1467 resistant parasites at comparable concentrations (Fig 3.8B). These results indicate that the LeuRS SNPs in the second and third generation resistant clones are more relevant to 1467 resistance and/or action compared to the first generation LeuRS SNPs. Interestingly, this corroborates our genetic data, as the locations of the second and third generation resistant LeuRS SNPs map to the predicted editing active sites, unlike the first generation resistant LeuRS SNPs, which although within the editing domain, were not located within any of the identified active sites (Fig 3.7). Considering the predicted LeuRS target, we further tested the effect of 1467 and 1474 on the incorporation of [^{14}C]leucine in soluble *P. falciparum* crude extracts presumed to contain active LeuRS. Unlike 3661, both 1467 and 1474 inhibited LeuRS activity in soluble *P. falciparum* extracts, with inhibition of incorporation of [^{14}C]leucine into either prokaryotic (*E. coli*; Fig 3.9B) or eukaryotic (*S. cerevisiae*) Leu-tRNA (Fig 3.9A). On the contrary, 1467did not inhibit presumed ILeRS and ValRS activities in *P. falciparum* crude extract, as inhibition of incorporation of [^{14}C]isoleucine (Fig 3.10A)or [^{14}C]valine (Fig 3.10B) was not seen at inhibitor concentrations up to 300 μM. Taken together, our results suggest that compounds 1467 and 1474 act against *P. falciparum* by inhibiting the activity of LeuRS.

A



B

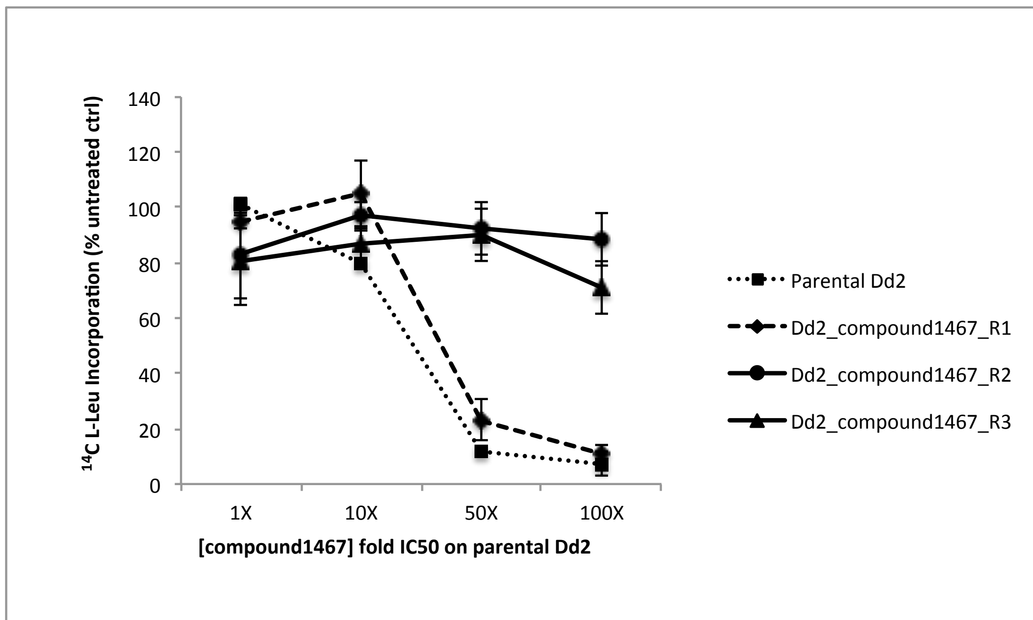
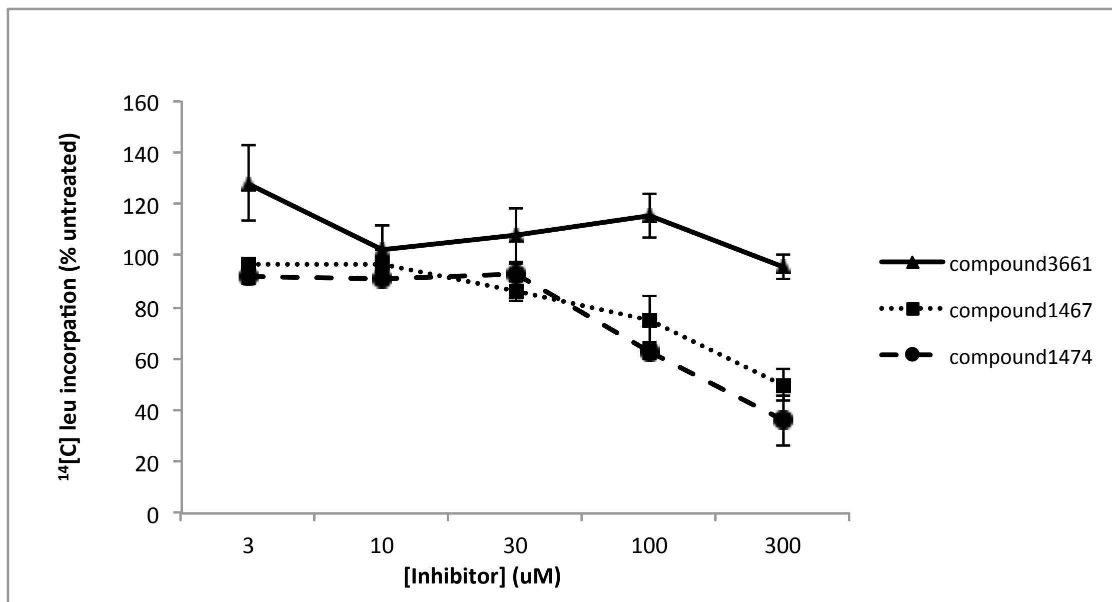


Fig 3.8. Effect of 1467 on [^{14}C]leucine incorporation in *P. falciparum* parasites. A) Effects on wild-type W2 strain *P. falciparum*. B) Effects on Dd2 strain *P. falciparum* selected for resistance. X-axis concentrations are based on multiples of IC_{50}s. Cycloheximide IC_{50} = 130nM (n = 3), artemisnin IC_{50} = 8.7nM (n = 3).

A



B

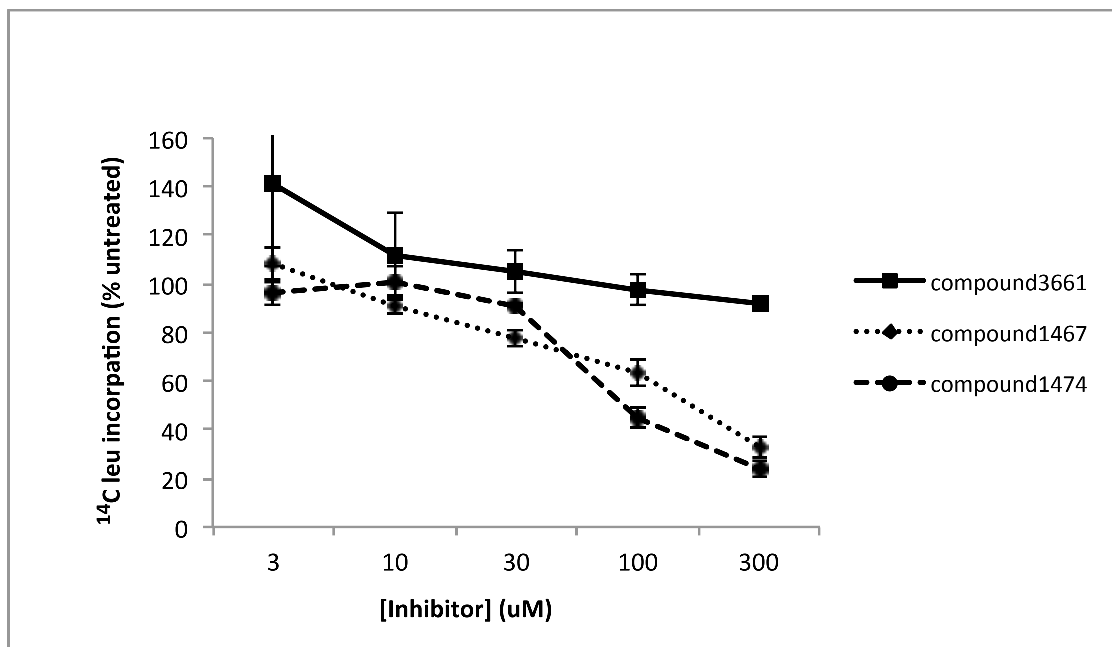
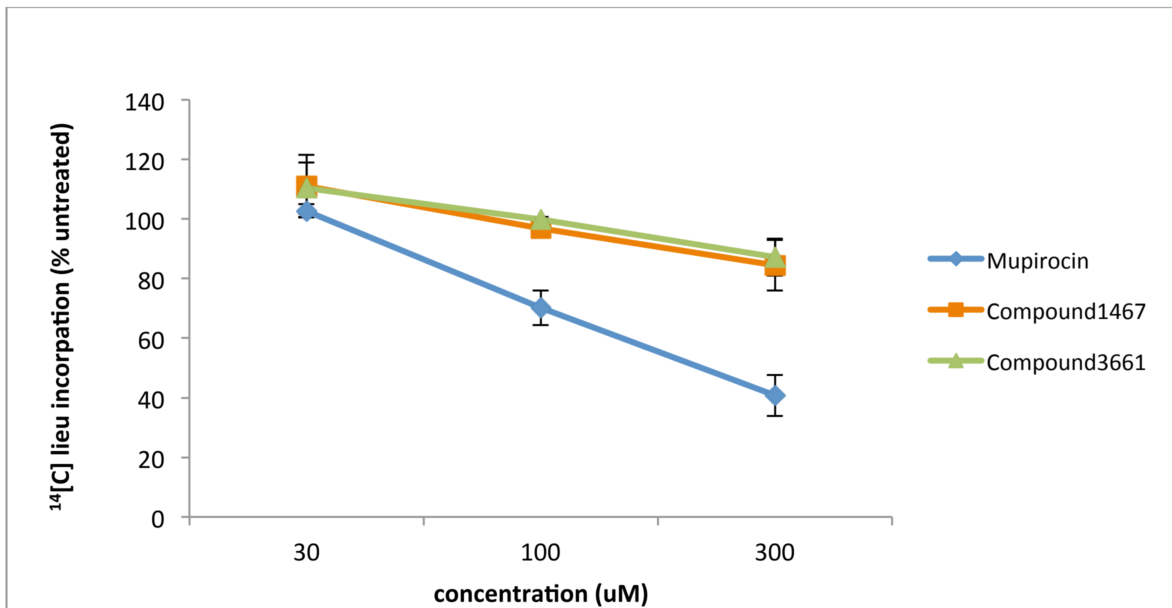


Figure 3.9: Effect of benzoxaboroles on *P. falciparum* LeuRS activity. Results are for incorporation of [^{14}C]leucine in the presence of exogenous *S. cerevisiae* tRNA (A) or *E.coli* tRNA (B).

A



B

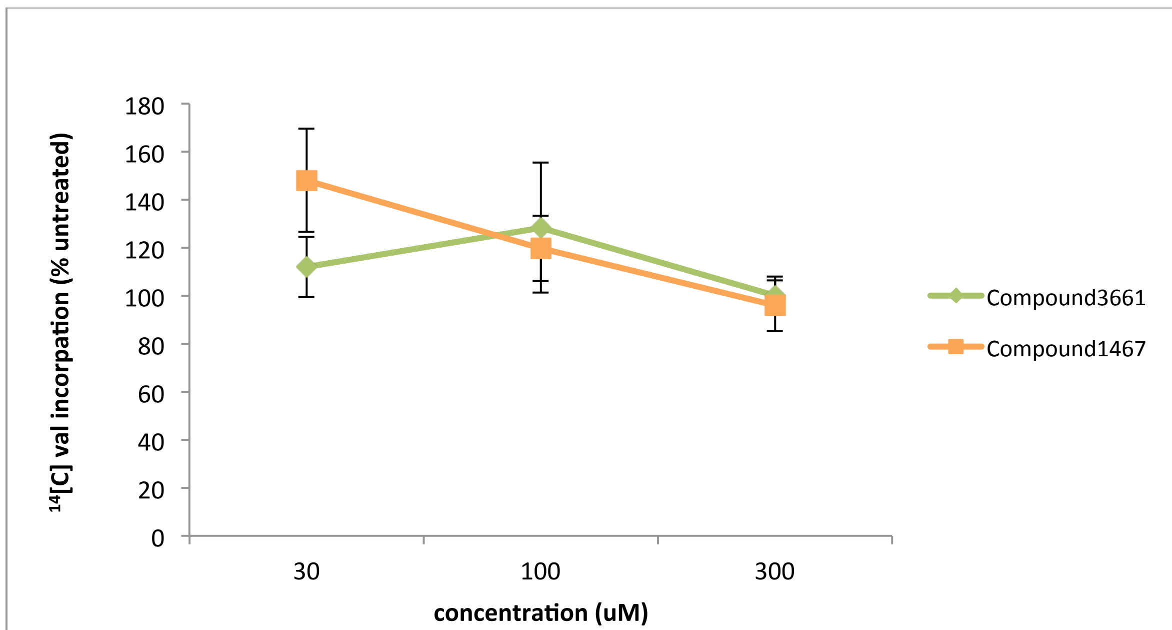
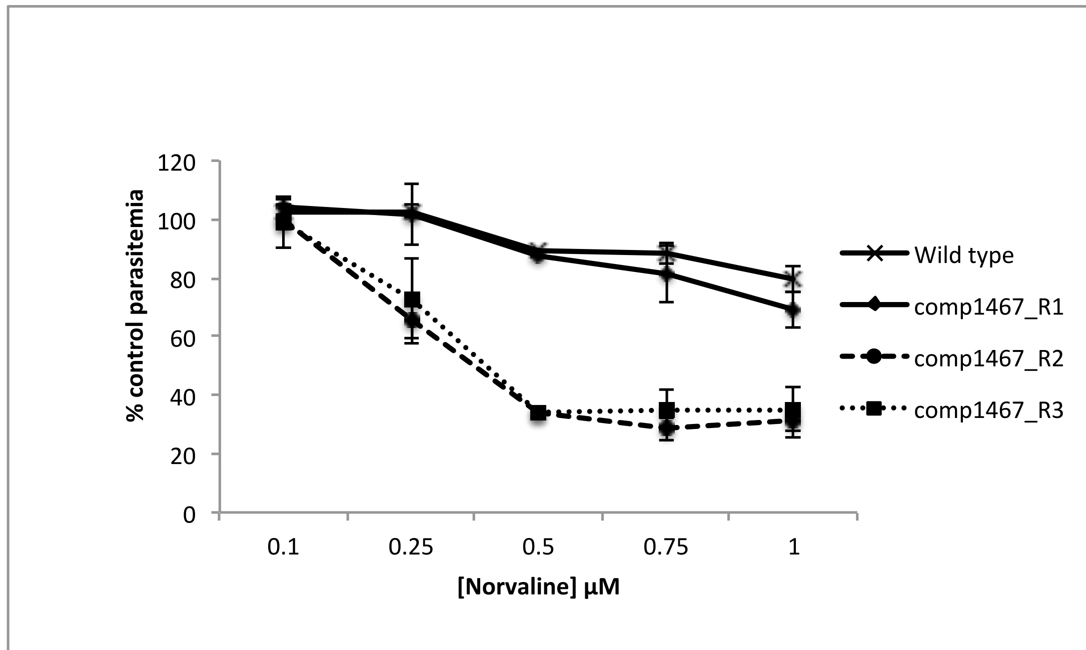


Figure 3.10: Effect of benzoxaboroles on *P. falciparum* (A) IleRS activity in the presence of exogenous *E. coli* tRNA and (B) ValRS activity in the presence of exogenous *S. cerevisiae* tRNA. Results are based on incorporation of [^{14}C]isoleucine and [^{14}C]valine respectively.

3.5 Impaired editing in 1467 resistant *P. falciparum*

Parasites selected for resistance to 1467 contained multiple mutations in LeuRS, and all identified mutations were in the predicted editing domain of the enzyme. Of note, other benzoxaboroles inhibited the editing activity of LeuRS in yeast (Rock et al. 2007) and bacteria (Hernandez et al. 2013, Hu et al. 2013). Biochemical confirmation of impaired LeuRS editing comes from growth in norvaline, which will be toxic to organisms with impaired LeuRS editing, due to misincorporation of norvaline in proteins. Indeed, growth impairment was seen in benzoxaborole-resistant *S. cerevisiae* in the presence of norvaline (Rock et al. 2007). We predicted that, similarly, 1467-resistant parasites have faulty LeuRS editing, and therefore would be particularly sensitive to incubation with the non-native leucine analog - norvaline due to inability to remove (edit) norvalines that are incorrectly attached to $tRNA^{Leu}$. Wild type and 1467 resistant Dd2-strain parasites were cultured in the presence of varying concentrations of norvaline with or without exogenous leucine for 48 hours, after which parasitemias were determined. Norvaline was not toxic to wild type and first generation 1467 resistant parasites at concentrations up to 1 μM. In contrast, norvaline markedly inhibited the growth of second and third generation resistant parasites in the absence of exogenous leucine (Fig 3.11A). This effect was reversed with exogenous high concentrations of leucine (Fig 3.11B). We posit that the lack of effect of norvaline on first generation 1467 resistant parasites, but marked inhibition of second and third generations can be attributed to differences in the mechanisms of resistance in the different generations of resistant parasites.

A



B

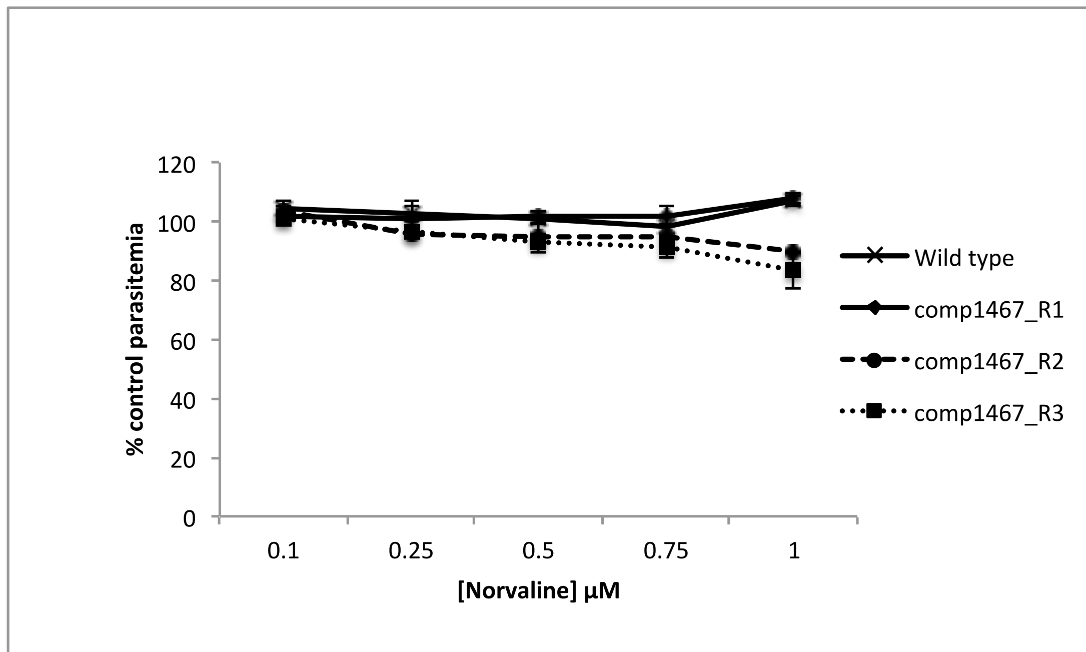


Figure 3.11. Sensitivity to norvaline of parasites selected for resistance to 1467. Shown are the effects of norvaline on growth of parasites selected for varied levels of resistance in the absence (A) or presence (B) of exogenous 0.025g/L leucine.

3.6 Discussion

Development of new antimalarial drugs, ideally acting by novel mechanisms of action, is needed. We identified two benzoxaboroles, 1467 and 1474, with excellent *in vitro* activity against *P. falciparum* and potent action in a rodent malaria model. The determination of the mechanisms of action of these compounds is an important priority. To determine the mechanism of action of compound 1467 in *P. falciparum*, parasites with markedly decreased sensitivities to 1467 were selected and characterized by whole genome sequencing. Resistant clones showed several SNPs compared to wild type, but only SNPs in a LeuRS gene (PF3D7_0622800) and another gene (PF3D7_1218100) that codes for a protein of unknown function were consistently seen in multiple clones from multiple passages. Additionally, two amplified gene clusters on chromosome 12 were observed in resistant clones. Furthermore, we tested the effects of 1467 and 1474 on protein synthesis and on leucine incorporation and editing by *P. falciparum* LeuRS. Compounds 1467 and 1474 elicited a dose dependent inhibition of intracellular protein synthesis and inhibited the LeuRS activity of a *P. falciparum* extract. Parasites selected for 1467 resistance lost the ability to edit the inappropriate incorporation of amino acids. Taken together, our data strongly suggest that *P. falciparum* LeuRS is a target of the antimalarial benzoxaboroles 1467 and 1474.

Malaria drug discovery is especially challenging. In addition to general drug requirements, in particular efficacy and safety, antimalarial drug candidates should meet additional challenging criteria, as described by MMV, which funds and directs several antimalarial drug discovery projects. Criteria for new antimalarial agents include rapid clinical response, requirement for no more than 3 days treatment, oral bioavailability, low tendency to select for drug resistance, lack of cross resistance with existing antimalarials, safety in children < 6 months of age and in pregnancy, and low cost of production. Although data to address each of these criteria are not yet available for1467, we can make some inferences based on our available results. First, the compounds are highly potent, with killing of cultured *P. falciparum*, including fresh clinical isolates, at mid-nanomolar concentrations. Second, the compounds were effective *in vivo*, with ED_{90}s at day 4 after 4 daily oral doses to *P. berghei*-infected mice ~ 10 mg/kg per day. These *in vivo* data suggest that the compounds are orally bioavailable and relatively non-toxic. Of note, the

safety of other oxaboroles has been demonstrated, with extensive *in vitro* and *in vivo* toxicology studies and progression of two compounds to trials in humans (Alley et al. 2007, Akama et al. 2009, Ciaravino et al. 2013, Bowers et al. 2013). Third, our results suggest that 1467 has a novel mechanism of action, the inhibition of *P. falciparum* LeuRS. Cross-resistance with the very similar compound 1474 suggests a shared mechanism for these two compounds, and the lack of cross resistance with known antimalarial drugs supports a novel mechanisms for 1467 and 1474. Actually, for unknown reasons, about 5-fold increase in sensitivity to mefloquine and lumefantrine was observed in the third generation 1467 resistant parasites (Table 3.4).

Considering resistance selection, we were able to select for high-level 1467 resistance over 104 days of continuous *in vitro* drug pressure. However, resistance can be selected against most antimalarials *in vitro*, and clinical studies will be required to gain insight into the ease of selection of resistance against this class of compounds. Considering cost of production, benzoxaboroles are easy to synthesize, requiring a four to six step scheme starting from cheap ingredients (Zhang et al. 2011). In summary, although much more data are needed for advancement to a clinical candidate, available data suggest that compounds 1467 and 1474 have many of the attributes desired in a new antimalarial drug.

Due to the observation that parasites selected for various levels of resistance to 1467 contained a total of 4 SNPs, all mapped to the editing domain of LeuRS, plus previous results showing targeting of the LeuRS editing function of other microbes by different benzoxaboroles (Rock et al. 2007, Ding et al. 2011, Hu et al. 2013, Hernandez et al., 2013), we sought to test whether LeuRS is a target of 1467 in *P. falciparum*. First, we first explored the effects of 1467 on the uptake of radiolabeled leucine, a marker for parasite protein synthesis, comparing effects on wild type and resistant clones. Dose dependent protein synthesis inhibition was observed for 1467 and the related compound 1474, but not for artemisinin or other benzoxaboroles tested. Protein synthesis inhibition required quite high concentrations (50-100X IC_{50}) of 1467 and 1474, likely due to the brief (2 hour) incubation used in the standard assay (as longer incubations would allow non-specific inhibition by any compound toxic to malaria parasites), but similarly high concentrations of artemisinin did not inhibit this process. Cycloheximide was active against leucine uptake at concentrations closer to its antiparasitic IC_{50}, presumably because cycloheximide binds the ribosome to inhibit elongation (Schneider-

Poetsch et al. 2010), a process very central to protein synthesis. In contrast, inhibition of LeuRS editing may not have as profound an effect on cellular protein synthesis. A direct test of inhibition of *P. falciparum* LeuRS is not yet possible, as active purified enzyme is not available. To measure activity against the enzyme we evaluated effects of benzoxaboroles on the ability of parasite extracts to incorporate radiolabeled leucine into either prokaryotic (*E. coli*) or eukaryotic (*S. cerevisiae*) tRNA. Both 1467 and 1474 caused dose dependent, albeit modest (mid-micromolar) inhibition of leucine incorporation by *P. falciparum* extracts into both bacterial and fungal tRNA. The control tRNA synthetase inhibitor mupirocin, an inhibitor of IleRS, also required micromolar concentrations to inhibit isoleucine incorporation (Fig 3.10A). Presumably, as with the *P. falciparum* leucine incorporation assay, brief incubation with tRNA synthetase inhibitors requires high concentrations to exert potent enzyme inhibition. We studied incorporation by both prokaryotic and eukaryotic tRNAs in an attempt to distinguish activities of the predicted *P. falciparum* cytoplasmic LeuRS target, which is a typical eukaryotic enzyme, and a second *P. falciparum* LeuRS that is active in the apicoplast, a prokaryote-like organelle with an independent protein synthesis machinery. However, both activities were inhibited with similar potency. This result suggests that the *P. falciparum* cytoplasmic LeuRS is capable of charging both prokaryotic and eukaryotic tRNA. A less likely explanation is that compounds 1467 and 1474 inhibit the two quite different enzymes with similar potency. Some early studies suggested that the aminoacylation reaction is dependent on the source of both the aaRS enzymes and their cognate tRNAs (Rendi and Ochoa 1962, Doctor and Mudd 1963). For example, prokaryotic aaRS enzymes would have a much higher specificity for charging a tRNA from another prokaryotic organism compared to a similar tRNA from a eukaryotic source and vice versa. However, other studies showed that this alleged specificity pattern is not universal but dependent on the particular amino acid and species in question (Benzer and Weisblum 1961, Clark and Eyzaguirre 1962). Thus, the observation that 1467 equally inhibited C14 leucine incorporation by either *S. cerevisiae* or *E. coli* tRNA could mean that the cytoplasmic LeuRS is non-discriminatory between prokaryotic and eukaryotic tRNAs, charging both equally well. In any event, taken together our evidence strongly suggests that the *P. falciparum* cytoplasmic LeuRS is the target of our compounds of interest, as parasites selected for resistance to 1467 contained mutations in the gene encoding this enzyme, but not that encoding the apicoplast LeuRS (PF3D7_0828200).

All mutations that developed in parasites selected for resistance to 1467 were predicted, based on comparison with better characterized LeuRSs from other organisms, to be located within the enzyme editing domain. To test whether 1467 specifically inhibited the LeuRS editing function, we incubated wild type and 1467-resistant parasites with the non-native amino acid norvaline. Parasites selected for high level resistance to 1467 had markedly impaired growth when cultured with norvaline, and not leucine. Wild type parasites had normal growth under these conditions, presumably because they successfully removed inappropriately incorporated norvaline, and acquired adequate quantities of leucine from hemoglobin hydrolysis. Thus, these results strongly support the hypothesis that a principal target for the antimalarial benzoxoboroles 1467 and 1474 in *P. falciparum* is the cytoplasmic LeuRS.

CHAPTER 4

STUDY OF THE MECHANISM OF ACTION OF THE BENZOXABOROLE COMPOUND 3661

4.1 Compound 3661 has excellent *in vitro* and *in vivo* antimalarial activity

Compound 3661 (Fig 4.1A) is one the most potent antimalarial benzoxaborole candidates identified in our library to date, with an IC50 of ~30nM againstlaboratory strains of *P. falciparum* both sensitive and resistant to standard antimalarial drugs (Table 4.1). When tested against a human cell line, 3661 did not exert apparent toxicity against cultured Jurkat cells at concentrations up to 100 μM. Compound 7334 (Fig 4.1B) is related to 3661 and, as expected, shared similar *in vitro* activity against *P. falciparum*. When administered orally once daily to *P. berghei*-infected mice for four days, 3661 was highly efficacious, with a day four ED_{90} of 0.34 mg/kg/day. With longer (28 day) follow up 100% of infected mice treated with 200 mg/kg/day of 3661were cured (Figure 4.2). With these excellent preliminary *in vitro* and *in vivo* antimalarial activities, we sought to investigate the mechanisms of action of 3661 and related compounds against *P. falciparum*.

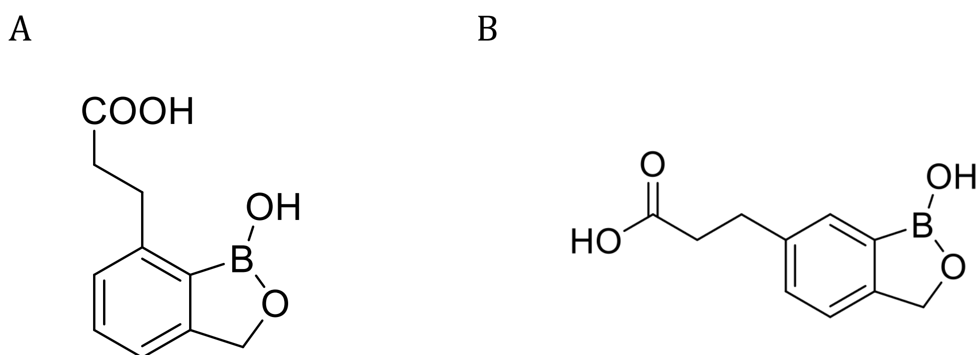


Fig 4.1. Structure of (A) compound 3661. MW = 206 and (B) compound 7334. MW = 206

Table 4.1: *In vitro* and *in vivo* antimalarial activities of compounds 3661 and 7334

Compounds	**Lab strains IC_{50} (μM)**			**Jurkat cell line CC_{50} (μM)**	***In vivo P. berghei* ED_{90} (mg/kg)**
	3D7	**W2**	**Dd2**		
3661	0.034 ± 0.008	0.032 ± 0.011	0.028 ± 0.006	>100.000	0.340
7334	0.038 ± 0.010	0.054 ± 0.012	0.055 ± 0.011	ND	ND

Table 4.2: Activity of antimalarials (IC_{50}s: nM) against 2nd and 4th generation 3661 resistant W2 *P. falciparum*

	Wild type	**2nd generation**	**4th generation**
Compound 3661	32.3 ± 1.1	390.4 ± 8.2	6900.2 ± 190.4
Compound 7334	54.2 ± 3.8	1057.3 ± 69.4	4748.3 ± 101.6
Compound 1467	280.1 ± 140.3	328. 7 ± 87.3	311.5 ± 119.3
CQ	42.5 ± 2.6	15.3 ± 3.2	16.5 ± 1.9
Piperaquine	7.6 ± 0.8	7.8 ± 1.1	4.6 ± 0.6
Mefloquine	0.3 ± 0.1	1.1 ± 0.1	0.6 ± 0.1
Desethylamodiaquine	9.1 ± 1.8	20.2 ± 2.3	21.5 ± 4.8
Lumefantrine	6.8 ± 1.1	6.05 ± 0.8	1.5 ± 0.1
Dihydroartemisin	8.7 ± 2.1	9.1 ± 1.7	3.5 ± 0.9

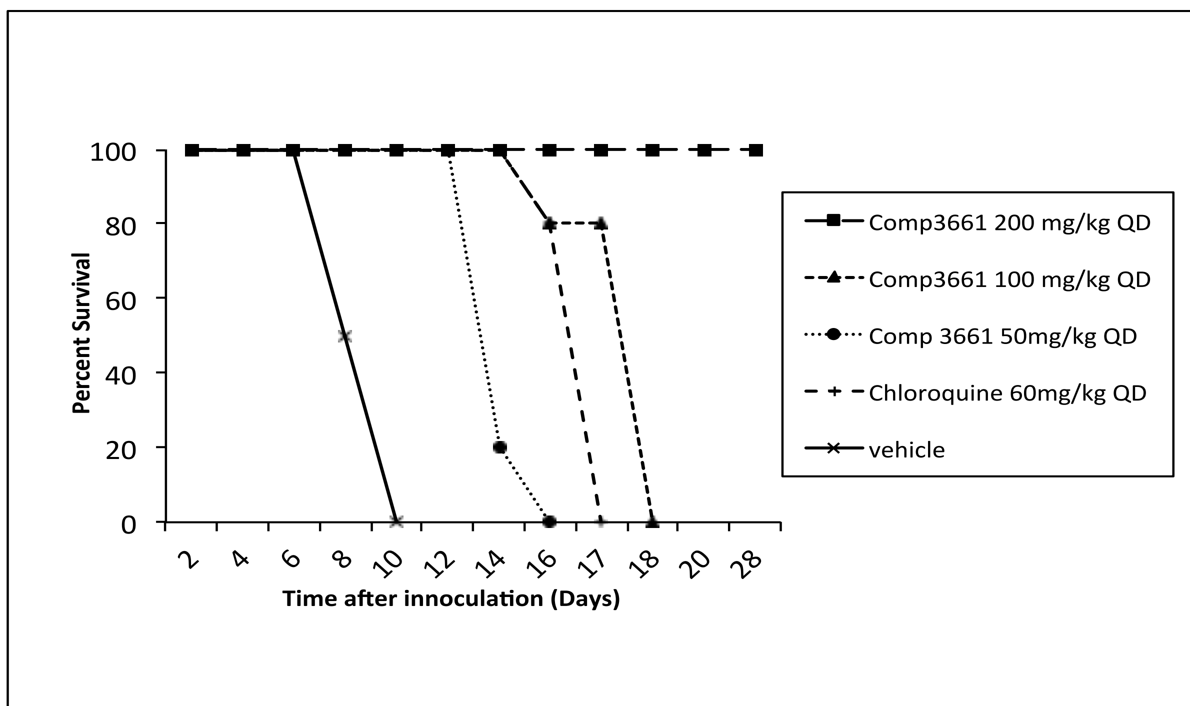


Figure 4.2: Survival curves of *P. bergei*- infected mice treated with 3661. Animals were inoculated intraperitoneally with 6 x 10^6 *P. berghei* infected erythrocytes, followed one hour later by initiation of once daily oral treatment with multiple dosages of test compounds, choloroquine, or vehicle control. Results are based on two independent experiments, each including five mice per dosing regimen.

4.2 *In vitro* morphological and stage-specific effects of 3661 on RBC stage *P. falciparum.*

To look for the presence of signature morphological effects of 3661 on *P. falciparum*, synchronized W2-strain parasites were treated with 3661 over the course of the RBC life cycle, beginning at the early ring stage. Although no obvious morphological abnormalities were observed, treated parasites were unable to progress beyond the trophozoite stage; they did not develop into multinucleated schizonts or new ring stage parasites (Fig 4.3). For stage specificity analysis, synchronized W2-strain parasites were incubated with 3661 or chloroquine for 8 hour intervals across the life cycle, the compounds were removed by washing at the end of each interval, cultures were maintained until untreated control parasites had reached the ring stage, and the parasitemias of control and treated cultures were compared. Similar to chloroquine, 3661 had some activity across the life cycle, but activity was greatest against trophozoites (Fig 4.4).

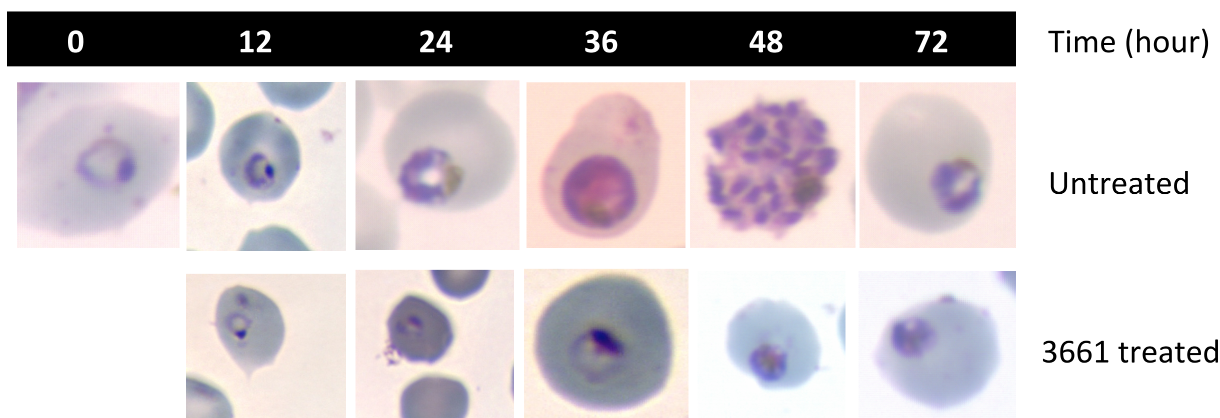


Figure 4.3. Light microscopic imaging of Giemsa-stained *P.falciparum* - infected erythrocytes after treatment with 370nM compound 3661, beginning at the early ring stage

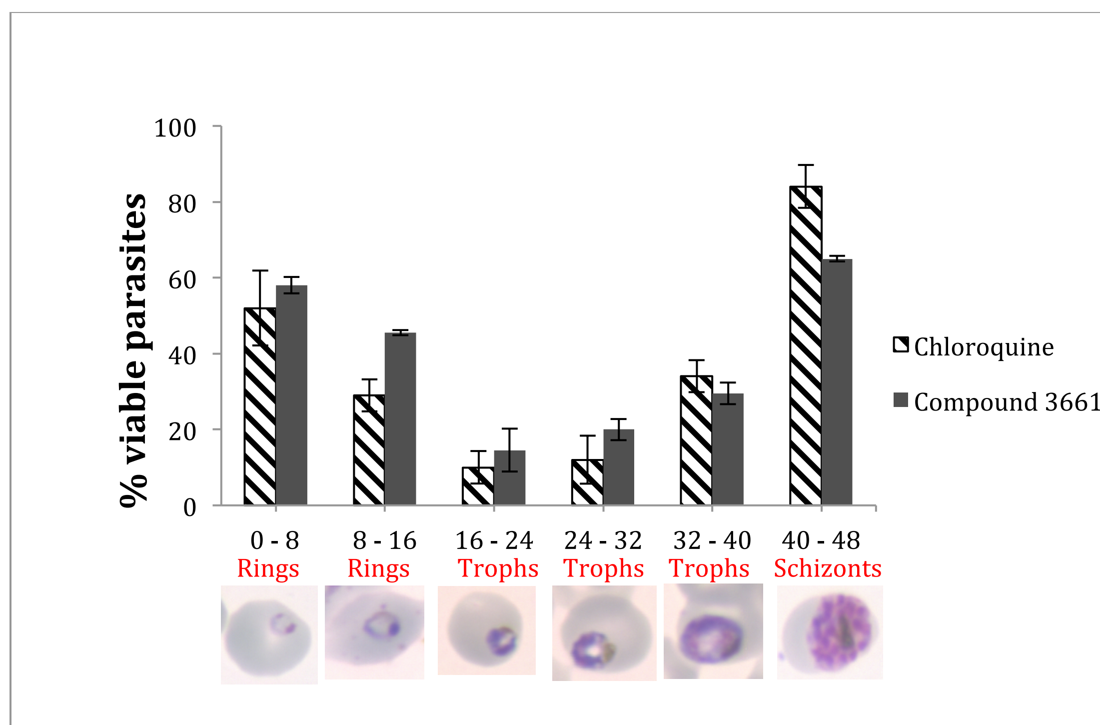


Figure 4.4. Stage specificity. Parasitemias were compared between untreated control parasites and parasites incubated with 370 nM 3661 or 1.3 μM chloroquine for 8 hour intervals across the life cycle and then continued in culture until the following ring stage. Incubation time intervals are represented on the x-axis along with corresponding stages in red, with photos taken at the beginning of each interval.

4.3 Selection and analysis of parasites with decreased 3661 sensitivity

To gain insight into the mechanism of action of compound 3661, three different approaches were used for the selection of 3661-resistant parasites. For the first and second approaches, we subjected two different laboratory strains of *P. falciparum* (W2 and Dd2) to step-wise increasing 3661 concentrations beginning at 37 nM (Figure 4.5A & B). For each step, parasite sensitivity to 3661 was assessed and parasites were cloned and stored for future analysis. For the third approach, 3661 resistant Dd2-strain *P. falciparum* was selected using a one-step *in vitro* selection approach (Fig 4.5C). Here, a larger population of Dd2-strain parasites (6×10^9) was cultured in the presence of a high concentration of 3661 (0.17 μM). Initially, no parasite growth was observed, but after 27 to 45 days parasites were identified in cultures, *in vitro* sensitivity was determined, and parasites were cloned. Parasites from all generations of resistance to 3661 were characterized by whole genome sequencing to determine genetic changes associated with resistance. For W2-strain parasites, five steps of continuous selection in three independent culture flasks over 11 months led to a 500 – 1000 fold change in the IC_{50}. For the most resistant parasites (fifth generation), the resistant phenotype was unstable, with reversion from an IC_{50} of 30 μM to an IC_{50} of 1.68 μM after two weeks of culture in drug free media (Fig 4.5A). For Dd2-strain parasites , after two steps of selection we identified a ~ 40 – 50 fold increase in IC_{50} (Fig 4.5B). As a third approach, three independent Dd2-strain parasite cultures subjected to a constant high concentration of 0.17 μM 3661 led to ~ 7-14 fold IC_{50} increase over 1 – 1.5 months (Fig 4.5C), however only two (Dd2_ANR1 and Dd2_ANR2) were successfully cloned. As expected, 3661 resistant parasites were similarly resistant to 7334, suggestive of a shared mechanism of resistance. In contrast, selected parasites were not more resistant to 1467, another antimalarial benzoxaborole described in this dissertation (Chapter 3) or antimalarials including chloroquine, mefloquine, lumefantrine, piperaquine and dihydroartemisinin (Table 4.2).

Whole genome sequencing identified numerous genetic changes between parental and 3661-resistant parasites, including SNPs and CNVs. Similar to the 1467 resistant clones analyzed, about 2800 top SNPs (200 SNPs per chromosome) were selected per 3661 resistant clone based on quality score. SNP filtration was done as previously detailed (Section 3.3; Fig 3.6), disqualifying synonymous SNPs, SNPs in non-coding regions and introns, and SNPs located in genes that code for variant surface antigens. Additionally,

SNPs in low coverage exonic regions (≤ 20 sequence reads covering the genomic position of a SNP) and SNPs where < 80% of reads had the SNP in question were disqualified.

Although 3661-resistant clones showed several SNPs compared to wild type, only SNPs in PF3D7_1438500, which encodes a homolog of human cleavage and polyadenylation specificity factor (CPSF), were seen consistently in multiple clones from multiple passages (Table 4.3 and 4.4). A total of eleven 3661 resistant clones were analyzed by whole genome sequencing. A total of 4 SNPs were identified in PF3D7_1438500 in nine out of the eleven resistant clones sequenced (Table 4.5), including all clones except the first and second generations of W2 3661 resistant clones (W2_3661_R1 and R2 clones).

The fact that SNPs in the CPSF gene (PF3D7_1438500) were seen in two different parasite strains selected for resistance to 3661 using three independent resistance selection approaches suggests that alterations in CPSF play a primary role in 3661 resistance. With regard to copy number variations, amplification of a cluster of genes on chromosome 5, including the *pfmdr1* gene, was observed in the 3661 resistant W2-clones (Table 4.5). At the early stages of selection, there was an increase in *pfmdr1* copy number in the W2-strain parasites, from a copy number of one at baseline to four to five copies in the first and second generations. With additional selection, *pfmdr1* copy number decreased to three in the fifth generation. Dd2 strain parasites differ from W2, in having three to four copies of pfmdr1. No *pfmdr1* copy number changes were observed in Dd2 3661 resistant clones (Table 4.5). Third and fourth generation W2 3661 resistant clones were subsequently cultured without drug pressure for five and four months, respectively. While the sensitivity of the third generation resistant clone reverted fully to wild type levels along with complete loss of the corresponding PF3D7_1438500 SNP (D470N), the fourth generation resistant clones had a partial reversion, decreasing to ~100-fold decreased sensitivity compared to wild type and retained both CPSF mutations (H36Y and D470N) (Table 4.5).

Table 4.3: Relevant SNPs identified in step-wise selected 3661 resistant *P. falciparum* clones

3661 resistant Clone (IC_{50} µM)	plasmoDB ID	protein ID	read coverage	SNP agreement (%)
W2_AN3661_R1	PF3D7_1144500	ubiquitin activating enzyme (E1) subunit Aos1 putative	196	100
clone 1	PF3D7_1250800	DNA repair protein rhp16 putative	178	98.8
(0.05µM)	PF3D7_1367500	NADH-cytochrome b5 reductase putative	185	100
	PF3D7_0214300	conserved Plasmodium protein unknown function	228	100
W2_AN3661_R2	PF3D7_1228500	RNA pseudouridylate synthase, putative	37	84
clone 1	PF3D7_1449700	Rrp6 homologue, putative	41	95
(0.12µM)	PF3D7_050330	Ser/Arg-rich splicing factor, putative	65	94
W2_AN3661_R3	PF3D7_1438500	CPSF	33	100
clone 1	PF3D7_0302900	exportin1, putative	32	97
(0.55µM)	PF3D7_0617300	conserved Plasmodium protein unknown function	34	100
W2_AN3661_R4	PF3D7_1438500	CPSF	224	100
clone 1	PF3D7_1438500	CPSF	231	100
(6.90µM)	PF3D7_0319100	ubiquitin-protein ligase putative	197	100
W2_AN3661_R5	PF3D7_1438500	CPSF	141	100
clone 1	PF3D7_1438500	CPSF	134	100
(1.68µM)	PF3D7_0319100	ubiquitin-protein ligase putative	154	100
Dd2_AN3661_R1	PF3D7_1438500	CPSF	311	100
clone 1	PF3D7_0302900	exportin1 putative	187	100
(0.40µM)	PF3D7_0319100	ubiquitin-protein ligase putative	182	100
Dd2_AN3661_R2	PF3D7_1438500	CPSF	96	100
clone 1	PF3D7_0302900	exportin1 putative	77	100
(0.93µM)	PF3D7_0319100	ubiquitin-protein ligase putative	102	100

Table 4.4: Relevant SNPs identified in one-time selected 3661 resistant *P. falciparum* clones

3661 Resistant clone (IC_{50} µM)	gene ID	protein ID	coverage	% coverage with SNP
Dd2_ANR1_clone 1 (0.34µM)	PF3D7_1438500	CPSF	127	100.0
	PFF0490w	conserved plasmodium protein (unknown function)	100	86.0
	PFF0765c	conserved plasmodium protein (unknown function)	53	92.4
	MAL7P1.84	conserved plasmodium protein (unknown function)	98	86.6
	PF07_0095	tRNAHis guanylyltransferase	69	89.8
	PFI0975c	conserved plasmodium protein (unknown function)	78	85.9
	PFC10_API0036	apicoplast ribosomal protein L14	60	100.0
	PF10_0058	DnaJ protein	93	91.0
Dd2_ANR1_clone 2 0.26µM)	PF3D7_1438500	CPSF	107	100.0
	PF13_0235	transcription factor with AP2 domain	70	82.8
	PF10_0058	DnaJ protein	74	87.8
	MAL7P1.84	conserved plasmodium protein (unknown function)	98	85.7
	PFC10_API0036	apicoplast ribosomal protein L14	207	100.0
Dd2_ANR2_clone 1 (0.45µM)	PF3D7_1438500	CPSF	103	100.0
	PF13_0161	conserved plasmodium protein (unknown function)	130	81.5
	PF10_0058	DnaJ protein	133	98.1
	PFF0765c	conserved plasmodium protein (unknown function)	78	89.7
	PFF0490w	conserved plasmodium protein (unknown function)	79	84.8
	PF13_0235	transcription factor with AP2 domain	47	82.9
	MAL7P1.84	conserved plasmodium protein (unknown function)	108	87.0
	PF07_0095	tRNAHis guanylyltransferase	83	86.7
	PFC10_API0036	apicoplast ribosomal protein L14	91	100.0
Dd2_ANR2_clone 2 (0.44µM)	PF3D7_1438500	CPSF	81	100.0
	PFF0765c	conserved plasmodium protein (unknown function)	73	90.4
	PF10_0058	DnaJ protein	98	92.8
	PFF0490w	conserved plasmodium protein (unknown function)	81	91.3
	MAL7P1.84	conserved plasmodium protein (unknown function)	88	88.6
	PF07_0095	tRNAHis guanylyltransferase	81	96.2
	PFC10_API0036	apicoplast ribosomal protein L14	40	100.0

Table 4.5: Detailed listing of the SNPs observed in PF3D7_1438500 of 3661 resistant clones.

Strain/Clones	IC50 (µM)	PF3D7_1438500 SNPs				*pfmdr1* CNV	
		H36Y	D470N	T406I	Y408S	WGS	qPCR
Parental W2	0.03 ± 0.01					1	1
Parental Dd2	0.03 ± 0.01					3	4
W2_3661_R1_clone 1	0.05 ± 0.01					3	4
W2_3661_R2_clone 1	0.12 ± 0.02					4	5
W2_3661_R2_clone 2	0.39 ± 0.07						
W2_3661_R3_clone 1	0.55 ± 0.26		✔✔			4	3
W2_3661_R3_clone 2	0.89 ± 0.09		✔				
W2_3661_R4_clone 1	6.90 ± 4.38	✔✔	✔✔			4	4
W2_3661_R4_clone 2	7.94 ± 1.25	✔	✔				
W2_3661_R5_clone 1	1.68 ± 0.63	✔✔	✔✔			3	3
Dd2_3661_R1_clone 1	0.40 ± 0.12		✔✔			3	3
Dd2_3661_R1_clone 2	ND		✔				
Dd2_3661_R2_clone 1	0.93 ± 0.24		✔✔			3	3
Dd2_3661_R2_clone 2	ND		✔				
Dd2_ANR1_clone 1	0.34 ± 0.07			✔✔		3	ND
Dd2_ANR1_clone 2	0.26			✔✔		3	ND
Dd2_ANR2_clone 1	0.45 ± 0.03				✔✔	3	ND
Dd2_ANR2_clone 2	0.44 ± 0.03				✔✔	3	ND
3month_revertant_W2_3661_R4 clone 1	3.12 ± 0.72	✔	✔			ND	ND
5month_revertant_W2_3661_R3 clone 1	0.022 ± 0.007					ND	ND

Two clones from each generation were analyzed by whole genome sequencing and/or dideoxy sequencing. ✔ = analyzed by dideoxy sequencing, ✔✔ = analyzed by dideoxy and whole genome sequencing

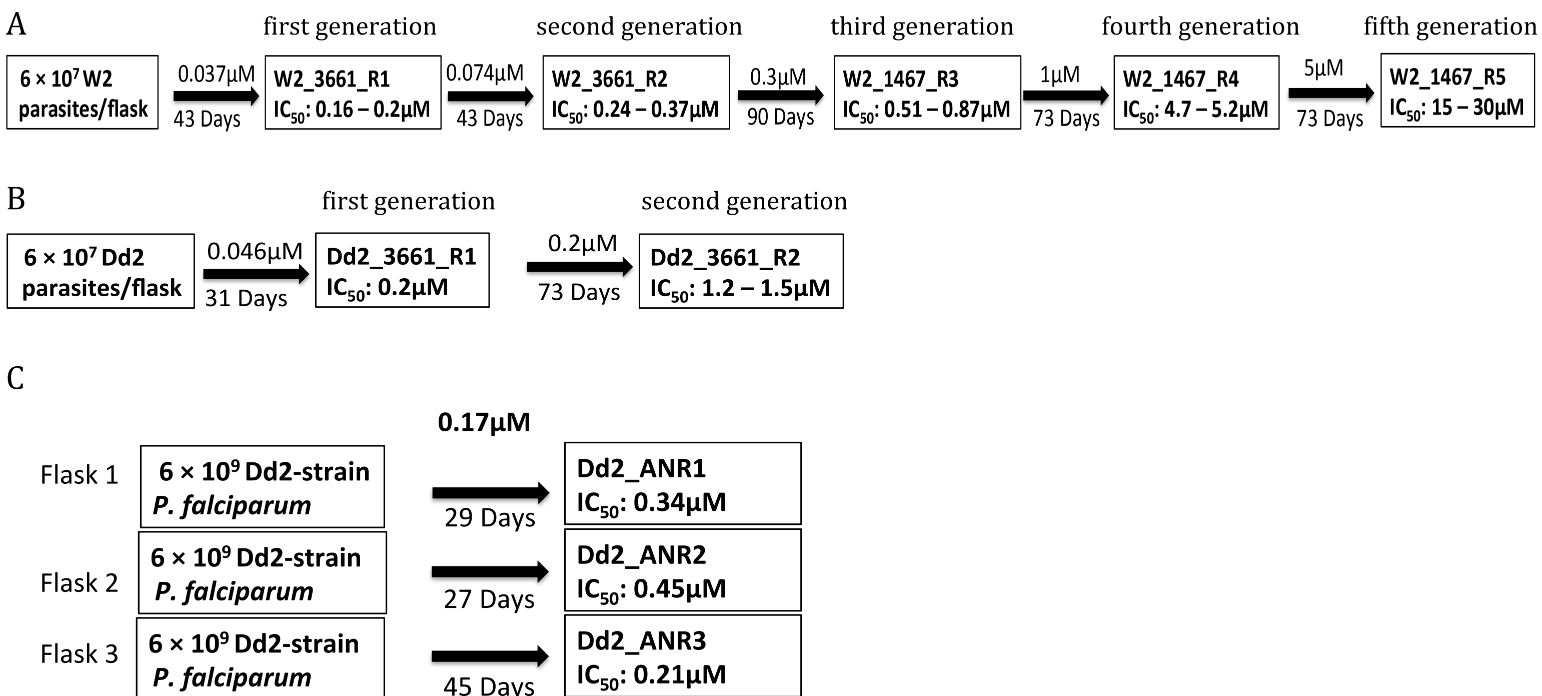


Figure 4.5: Schematic of 3661 resistance selection. Resistance was selected against A) W2 and B) Dd2-strain *P. falciparum* with step-wise increases in concentration of 3661, leading to five and two generations of 3661 resistant parasites respectively. C) One-step selection of 3661 resistance in Dd2-strain *P. falciparum* leading to three independent 3661 resistant populations

4.4 Discussion

In our screen of benzoxaboroles for antimalarial activity, 3661 had exceptional *in vitro* activity against *P. falciparum* and potent action against *P. berghei* in a rodent malaria model. To determine the mechanism of action of compound 3661 in *P. falciparum*, parasites with markedly decreased sensitivities to 3661 were selected using two different techniques – culture in step-wise increasing concentrations of 3661 and one-step selection using a single high concentration of 3661. Resistant parasites were cloned and characterized by whole genome sequencing. Resistant clones showed several SNPs compared to wild type, but only SNPs in PF3D7_1438500, which encodes a homolog of human cleavage and polyadenylation specificity factor (CPSF) were consistently seen in multiple clones from multiple passages. In terms of CNV results, one amplified cluster of genes on chromosome 5 including *pfmdr1* was observed in W2-strain resistant clones. In summary, our genetic data strongly suggest that mutations in *P. falciparum* CPSF (encoded by PF3D7_1438500), a protein predicted to be responsible for pre-mRNA 3' end cleavage and/or polyadenylation based on homology with proteins in mammalian cells and yeast, are implicated in the mechanism of 3661 resistance. More work is needed to validate these findings and to determine if pre-mRNA 3' end cleavage and/or polyadenylation are the antimalarial targets of 3661.

Although some compounds such as the adenosine analogue cordycepin and rifamycin derivatives have been reported to inhibit the 3' pre-mRNA polyadenylation process in eukaryotic cells (Jacob and Rose 1974, Rose et al. 1977, Kondrashov et al. 2012), none have been reported to target CPSF. Unlike in humans and yeast, the proteins of the 3' pre-mRNA polyadenylation complex have not been characterized in *Plasmodium* parasites. A search for "CPSF" on plasmoDB, a plasmodium genome database, yields three genes, two on chromosome 3 and one on chromosome 14 where our SNPs of interest were identified. This chromosome 14 CPSF (PF3D7_1438500) gene predicts a 101 kDa protein, and sequence alignment with human CPSF-73, the enzyme confirmed to be responsible for cleavage at the conserved AAUAAA site in human pre-mRNA (Mandel et al. 2006), shows 29% sequence identity and 56% similarity. PF3D7_1438500 also has homology (16% sequence

identity and 47% similarity) to CPSF-100, a CPSF-73 homolog that has been shown to lack endonuclease activity (Mandel et al. 2006). Hence, we cannot predict which of the two human CPSF homologs is functionally related to the predicted CPSF encoded by PF3D7_1438500 in *P. falciparum*.

All 3661 resistant *P. falciparum* parasites analyzed by whole genome sequencing had CPSF SNPs except for the early (1st and 2nd generation) W2 3661 resistant clones (Table 4.5). Of interest, these two generations had another polymorphism, increased copy number of the *pfmdr1* gene, which encodes a putative transporter of multiple antimalarial drugs. With selection of increasing levels of resistance, there was an initial spike in *pfmdr1* copy number (1st and 2nd generations) followed by a gradual decrease as SNPs accumulated in later generations, from a copy number of four and five in generations one and two respectively, to a copy number of three in generation five. Thus, W2-strain parasites, with only one copy of *pfmdr1* at baseline, elevated *pfmdr1* copy number to mediate low-level resistance. With increasing drug pressure, mutations were seen in PF3D7_1438500, and the copy number of pfmdr1 decreased. These results suggest that increased copy number of pfmdr1 may have mediated enhanced egress of 3661 from the site of drug action, and that mutations in the drug target, which we propose is *P. falciparum* CPSF, mediated higher level resistance. Interestingly, this trend in *pfmdr1* copy number was observed for the W2-strain, but not for the Dd2 strain, which contains multiple copies of *pfmdr1* at baseline.

Another interesting set of SNPs was identified in four clones in PF3D7_0319100, a gene that encodes a putative ubiquitin-protein ligase in *P. falciparum*. Interestingly, using population genome-wide methodologies, proteins of the ubiquitin system have been associated with *P. falciparum* resistance to antimalarial drugs including pyrimethamine and artemisinin (Park et al. 2012, Volkman et al. 2012, Hamilton et al. 2014). Although the particular role of the ubiquitin system proteins in *P. falciparum* drug resistances is yet to be validated, the ubiquitin system, as in other eukaryotic cells, may be important in parasite survival (Hamilton et al. 2014).

We have strong genetic evidence that PF3D7_1438500 is important in the mechanism of 3661 resistance. However, more work is needed to test whether the product of this gene is the direct target of

3661. Given the involvement of several proteins in the yeast and mammalian 3' pre-mRNA cleavage and polyadenylation process including other CPSF proteins, it is very possible that the protein encoded by PF3D7_1438500 may not be the direct target of 3661. In this case, the observed CPSF SNPs could be a secondary response to a direct targeting of another protein in the complex that interacts with CPSF.

CHAPTER 5

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Malaria, most especially *P. falciparum* malaria, is one of the most important infectious diseases globally. Although some control measures exist including use of insecticide treated bednets and indoor residual spraying, malaria incidence remains high. Nonetheless, chemotherapy has played a pivotal role in treating uncomplicated malaria, especially in highly endemic regions. However, there is increasing threat to the success of chemotherapy due the emergence of resistance to our current single most potent class of drugs – artemisinins, along with the longstanding resistance to most ACT partner drugs. As a result, the exploration of benzoxaboroles is very relevant as demand increases for novel non-artemisinin antimalarial drugs.

Our screen identified several benzoxaboroles, including compounds 1467 and 3661, with varying degrees of activity against P. falciparum. Using the *in vitro* resistance selection technique, we identified the mechanisms of resistance, and possibly mechanisms of action of our two benzoxaboroles of interest – 1467 and 3661. We adopted two different selection methods, comparing step-wise continuous selection and one-step selection. Although the techniques led similar results, our data highlight the advantage of the step-wise *in vitro* drug selection technique in giving insight into "behind-the-scenes" molecular events leading to drug resistance.

To validate the proteins encoded by the genes with observed mutations as the targets of 1467 or 3661, biochemical assays are necessary. For 1467, such assays were done and although we lack tests on active recombinant LeuRS, our biochemical data suggest that LeuRS is a likely target of 1467. For 3661, more work is needed.

Accordingly, more work is underway to confirm if the CPSF encoded by PF3D7_1438500 is the direct target of compound 3661. First, we plan to test the effect of knocking in the observed PF3D7_1438500 SNPs into wild-type *P. falciparum* on 3661 susceptibility. Second, in order to test the effect of 3661 on mRNA synthesis in cultured *P. falicaprum*, we plan to quantify the abundance of select transcripts using northern blots. Finally, we will test the effect of 3661 and related compounds on extra-cellular cleavage and polyadenylation reactions using both *P. falciparum* extracts and recombinant expressed CPSF (encoded by PF3D7_1438500).

CHAPTER 6

REFERENCES

1. Aikawa M, Miller LH, Johnson J, Rabbege J. Erythrocyte entry by malarial parasites. A moving junction between erythrocyte and parasite. J Cell Biol. 1978 Apr;77(1):72-82.

2. Akama T, Baker SJ, Zhang YK, Hernandez V, Zhou H, Sanders V, et al. Discovery and structure-activity study of a novel benzoxaborole anti-inflammatory agent (AN2728) for the potential topical treatment of psoriasis and atopic dermatitis. Bioorg Med Chem Lett. 2009 Apr 15;19(8):2129-32.

3. Akama T, Dong C, Virtucio C, Freund YR, Chen D, Orr MD, et al. Discovery and structure-activity relationships of 6-(benzoylamino)benzoxaboroles as orally active anti-inflammatory agents. Bioorg Med Chem Lett. 2013 Nov 1;23(21):5870-3.

4. Akama T, Dong C, Virtucio C, Sullivan D, Zhou Y, Zhang YK, et al. Linking phenotype to kinase: Identification of a novel benzoxaborole hinge-binding motif for kinase inhibition and development of high-potency rho kinase inhibitors. J Pharmacol Exp Ther. 2013 Dec;347(3):615-25.

5. Alekshun MN, Levy SB. Molecular mechanisms of antibacterial multidrug resistance. Cell. 2007 Mar 23;128(6):1037-50.

6. Alker AP, Lim P, Sem R, Shah NK, Yi P, Bouth DM, et al. Pfmdr1 and in vivo resistance to artesunate-mefloquine in falciparum malaria on the cambodian-thai border. Am J Trop Med Hyg. 2007 Apr;76(4):641-7.

7. Alley MR, Baker SJ, Beutner KR, Plattner J. Recent progress on the topical therapy of onychomycosis. Expert Opin Investig Drugs. 2007 Feb;16(2):157-67.

8. Amaratunga C, Sreng S, Suon S, Phelps ES, Stepniewska K, Lim P, et al. Artemisinin-resistant plasmodium falciparum in pursat province, western cambodia: A parasite clearance rate study. Lancet Infect Dis. 2012 Nov;12(11):851-8.

9. Aravind L. An evolutionary classification of the metallo-beta-lactamase fold proteins. In Silico Biol. 1999;1(2):69-91.

10. Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, et al. A molecular marker of artemisinin-resistant plasmodium falciparum malaria. Nature. 2014 Jan 2;505(7481):50-5.

11. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: Tool for the unification of biology. the gene ontology consortium. Nat Genet. 2000 May;25(1):25-9.

12. Ashley E, McGready R, Proux S, Nosten F. Malaria. Travel Med Infect Dis. 2006 May-Jul;4(3-4):159-73.

13. Assaraf YG, Abu-Elheiga L, Spira DT, Desser H, Bachrach U. Effect of polyamine depletion on macromolecular synthesis of the malarial parasite, plasmodium falciparum, cultured in human erythrocytes. Biochem J. 1987 Feb 15;242(1):221-6.

14. Assaraf YG, Kahana C, Spira DT, Bachrach U. Plasmodium falciparum: Purification, properties, and immunochemical study of ornithine decarboxylase, the key enzyme in polyamine biosynthesis. Exp Parasitol. 1988 Oct;67(1):20-30.

15. Aurrecoechea C, Brestelli J, Brunk BP, Dommer J, Fischer S, Gajria B, et al. PlasmoDB: A functional genomic database for malaria parasites. Nucleic Acids Res. 2009 Jan;37(Database issue):D539-43.

16. Avruch J, Long X, Ortiz-Vega S, Rapley J, Papageorgiou A, Dai N. Amino acid regulation of TOR complex 1. Am J Physiol Endocrinol Metab. 2009 Apr;296(4):E592-602.

17. Azcarate IG, Marin-Garcia P, Camacho N, Perez-Benavente S, Puyet A, Diez A, et al. Insights into the preclinical treatment of blood-stage malaria by the antibiotic borrelidin. Br J Pharmacol. 2013 Jun;169(3):645-58.

18. Babbitt SE, Altenhofen L, Cobbold SA, Istvan ES, Fennell C, Doerig C, et al. Plasmodium falciparum responds to amino acid starvation by entering into a hibernatory state. Proc Natl Acad Sci U S A. 2012 Nov 20;109(47):E3278-87.

19. Bacon DJ, Tang D, Salas C, Roncal N, Lucas C, Gerena L, et al. Effects of point mutations in plasmodium falciparum dihydrofolate reductase and dihydropterate synthase genes on clinical outcomes and in vitro susceptibility to sulfadoxine and pyrimethamine. PLoS One. 2009 Aug 26;4(8):e6762.

20. Baker SJ, Ding CZ, Akama T, Zhang YK, Hernandez V, Xia Y. Therapeutic potential of boron-containing compounds. Future Med Chem. 2009 Oct;1(7):1275-88.

21. Baker SJ, Tomsho JW, Benkovic SJ. Boron-containing inhibitors of synthetases. Chem Soc Rev. 2011 Aug;40(8):4279-85.

22. Barker JJ. Antibacterial drug discovery and structure-based design. Drug Discov Today. 2006 May;11(9-10):391-404.

23. Bathoorn E, Hetem DJ, Alphenaar J, Kusters JG, Bonten MJ. Emergence of high-level mupirocin resistance in coagulase-negative staphylococci associated with increased short-term mupirocin use. J Clin Microbiol. 2012 Sep;50(9):2947-50.

24. Beebe K, Ribas De Pouplana L, Schimmel P. Elucidation of tRNA-dependent editing by a class II tRNA synthetase and significance for cell viability. EMBO J. 2003 Feb 3;22(3):668-75.

25. Benzer S, Weisblum B. On the species specificity of acceptor rna and attachment enzymes. Proc Natl Acad Sci U S A. 1961 Aug;47(8):1149-54.

26. Benzer S, Weisblum B. On the species specificity of acceptor rna and attachment enzymes. Proc Natl Acad Sci U S A. 1961 Aug;47(8):1149-54.

27. Berk AJ, Sharp PA. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell. 1977 Nov;12(3):721-32.

28. Beshir K, Sutherland CJ, Merinopoulos I, Durrani N, Leslie T, Rowland M, et al. Amodiaquine resistance in plasmodium falciparum malaria in afghanistan is associated with the pfcrt SVMNT allele at codons 72 to 76. Antimicrob Agents Chemother. 2010 Sep;54(9):3714-6.

29. Beyer D, Kroll HP, Endermann R, Schiffer G, Siegel S, Bauser M, et al. New class of bacterial phenylalanyl-tRNA synthetase inhibitors with high potency and broad-spectrum activity. Antimicrob Agents Chemother. 2004 Feb;48(2):525-32.

30. Bhatt TK, Kapil C, Khan S, Jairajpuri MA, Sharma V, Santoni D, et al. A genomic glimpse of aminoacyl-tRNA synthetases in malaria parasite plasmodium falciparum. BMC Genomics. 2009 Dec 31;10:644.

31. Bondaryk M, Kurzatkowski W, Staniszewska M. Antifungal agents commonly used in the superficial and mucosal candidiasis treatment: Mode of action and resistance development. Postepy Dermatol Alergol. 2013 Oct;30(5):293-301.

32. Bowers GD, Tenero D, Patel P, Huynh P, Sigafoos J, O'Mara K, et al. Disposition and metabolism of GSK2251052 in humans: A novel boron-containing antibiotic. Drug Metab Dispos. 2013 May;41(5):1070-81.

33. Bozdech Z, Llinas M, Pulliam BL, Wong ED, Zhu J, DeRisi JL. The transcriptome of the intraerythrocytic developmental cycle of plasmodium falciparum. PLoS Biol. 2003 Oct;1(1):E5.

34. Brennand A, Gualdron-Lopez M, Coppens I, Rigden DJ, Ginger ML, Michels PA. Autophagy in parasitic protists: Unique features and drug targets. Mol Biochem Parasitol. 2011 Jun;177(2):83-99.

35. Brochet M, Couve E, Zouine M, Poyart C, Glaser P. A naturally occurring gene amplification leading to sulfonamide and trimethoprim resistance in streptococcus agalactiae. J Bacteriol. 2008 Jan;190(2):672-80.

36. Brody T, Yavatkar AS, Lin Y, Ross J, Kuzin A, Kundu M, et al. Horizontal gene transfers link a human MRSA pathogen to contagious bovine mastitis bacteria. PLoS One. 2008 Aug 27;3(8):e3074.

37. Burrows JN, Burlot E, Campo B, Cherbuin S, Jeanneret S, Leroy D, et al. Antimalarial drug discovery - the path towards eradication. Parasitology. 2014 Jan;141(1):128-39.

38. Callebaut I, Moshous D, Mornon JP, de Villartay JP. Metallo-beta-lactamase fold within nucleic acids processing enzymes: The beta-CASP family. Nucleic Acids Res. 2002 Aug 15;30(16):3592-601.

39. Carrara VI, Zwang J, Ashley EA, Price RN, Stepniewska K, Barends M, et al. Changes in the treatment responses to artesunate-mefloquine on the northwestern border of thailand during 13 years of continuous deployment. PLoS One. 2009;4(2):e4551.

40. Carter CW,Jr. Cognition, mechanism, and evolutionary relationships in aminoacyl-tRNA synthetases. Annu Rev Biochem. 1993;62:715-48.

41. Cheeseman IH, Miller BA, Nair S, Nkhoma S, Tan A, Tan JC, et al. A major genome region underlying artemisinin resistance in malaria. Science. 2012 Apr 6;336(6077):79-82.

42. Chen X, Ma JJ, Tan M, Yao P, Hu QH, Eriani G, et al. Modular pathways for editing non-cognate amino acids by human cytoplasmic leucyl-tRNA synthetase. Nucleic Acids Res. 2011 Jan;39(1):235-47.

43. Chopra I, Roberts M. Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial

resistance. Microbiol Mol Biol Rev. 2001 Jun;65(2):232,60 ; second page, table of contents.

44. Chou AC, Fitch CD. Control of heme polymerase by chloroquine and other quinoline derivatives. Biochem Biophys Res Commun. 1993 Aug 31;195(1):422-7.

45. Ciaravino V, Plattner J, Chanda S. An assessment of the genetic toxicology of novel boron-containing therapeutic agents. Environ Mol Mutagen. 2013 Jun;54(5):338-46.

46. CLARK JM,Jr, EYZAGUIRRE JP. Tyrosine activation and transfer to soluble ribonucleic acid. I. purification and study of the enzyme of hog pancreas. J Biol Chem. 1962 Dec;237:3698-702.

47. CLARK JM,Jr, EYZAGUIRRE JP. Tyrosine activation and transfer to soluble ribonucleic acid. I. purification and study of the enzyme of hog pancreas. J Biol Chem. 1962 Dec;237:3698-702.

48. Colgan DF, Manley JL. Mechanism and regulation of mRNA polyadenylation. Genes Dev. 1997 Nov 1;11(21):2755-66.

49. Collins WE, Jeffery GM. Plasmodium malariae: Parasite and disease. Clin Microbiol Rev. 2007 Oct;20(4):579-92.

50. Collins WE, Jeffery GM. Plasmodium ovale: Parasite and disease. Clin Microbiol Rev. 2005 Jul;18(3):570-81.

51. Cook GC. Malaria prophylaxis. mefloquine toxicity should limit its use to treatment alone. BMJ. 1995 Jul 15;311(6998):190,1; author reply 192.

52. Cordel H, Cailhol J, Matheron S, Bloch M, Godineau N, Consigny PH, et al. Atovaquone-proguanil in the treatment of imported uncomplicated plasmodium falciparum malaria: A prospective observational study of 553 cases. Malar J. 2013 Nov 7;12:399,2875-12-399.

53. Cowman AF, Galatis D, Thompson JK. Selection for mefloquine resistance in plasmodium falciparum is linked to amplification of the

pfmdr1 gene and cross-resistance to halofantrine and quinine. Proc Natl Acad Sci U S A. 1994 Feb 1;91(3):1143-7.

54. Cowman AF, Morry MJ, Biggs BA, Cross GA, Foote SJ. Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthase gene of plasmodium falciparum. Proc Natl Acad Sci U S A. 1988 Dec;85(23):9109-13.

55. Cui L, Wang Z, Miao J, Miao M, Chandra R, Jiang H, et al. Mechanisms of in vitro resistance to dihydroartemisinin in plasmodium falciparum. Mol Microbiol. 2012 Oct;86(1):111-28.

56. Cui ZL, Jing FX, Hu ZY, Wu ZR, Zhao JL. The use of gene chip in detecting mycobacterium tuberculosis resistant to rifampin and isoniazid. Zhonghua Jie He He Hu Xi Za Zhi. 2004 Jul;27(7):439-41.

57. Cusack S. Aminoacyl-tRNA synthetases. Curr Opin Struct Biol. 1997 Dec;7(6):881-9.

58. Cusack S, Berthet-Colominas C, Hartlein M, Nassar N, Leberman R. A second class of synthetase structure revealed by X-ray analysis of escherichia coli seryl-tRNA synthetase at 2.5 A. Nature. 1990 Sep 20;347(6290):249-55.

59. Cusack S, Hartlein M, Leberman R. Sequence, structural and evolutionary relationships between class 2 aminoacyl-tRNA synthetases. Nucleic Acids Res. 1991 Jul 11;19(13):3489-98.

60. Cusack S, Yaremchuk A, Tukalo M. The 2 A crystal structure of leucyl-tRNA synthetase and its complex with a leucyl-adenylate analogue. EMBO J. 2000 May 15;19(10):2351-61.

61. Cvetesic N, Perona JJ, Gruic-Sovulj I. Kinetic partitioning between synthetic and editing pathways in class I aminoacyl-tRNA synthetases occurs at both pre-transfer and post-transfer hydrolytic steps. J Biol Chem. 2012 Jul 20;287(30):25381-94.

62. Dahl EL, Rosenthal PJ. Apicoplast translation, transcription and genome replication: Targets for antimalarial antibiotics. Trends Parasitol. 2008 Jun;24(6):279-84.

63. Dahl EL, Rosenthal PJ. Multiple antibiotics exert delayed effects against the plasmodium falciparum apicoplast. Antimicrob Agents Chemother. 2007 Oct;51(10):3485-90.

64. Dahl EL, Shock JL, Shenai BR, Gut J, DeRisi JL, Rosenthal PJ. Tetracyclines specifically target the apicoplast of the malaria parasite plasmodium falciparum. Antimicrob Agents Chemother. 2006 Sep;50(9):3124-31.

65. Dantonel JC, Murthy KG, Manley JL, Tora L. Transcription factor TFIID recruits factor CPSF for formation of 3' end of mRNA. Nature. 1997 Sep 25;389(6649):399-402.

66. Das Gupta R, Krause-Ihle T, Bergmann B, Muller IB, Khomutov AR, Muller S, et al. 3-aminooxy-1-aminopropane and derivatives have an antiproliferative effect on cultured plasmodium falciparum by decreasing intracellular polyamine concentrations. Antimicrob Agents Chemother. 2005 Jul;49(7):2857-64.

67. Das BC, Thapa P, Karki R, Schinke C, Das S, Kambhampati S, et al. Boron chemicals in diagnosis and therapeutics. Future Med Chem. 2013 Apr;5(6):653-76.

68. del Pilar Crespo M, Avery TD, Hanssen E, Fox E, Robinson TV, Valente P, et al. Artemisinin and a series of novel endoperoxide antimalarials exert early effects on digestive vacuole morphology. Antimicrob Agents Chemother. 2008 Jan;52(1):98-109.

69. Del Rosso JQ, Plattner JJ. From the test tube to the treatment room: Fundamentals of boron-containing compounds and their relevance to dermatology. J Clin Aesthet Dermatol. 2014 Feb;7(2):13-21.

70. Dharia NV, Sidhu AB, Cassera MB, Westenberger SJ, Bopp SE, Eastman RT, et al. Use of high-density tiling microarrays to identify mutations globally and elucidate mechanisms of drug resistance in

plasmodium falciparum. Genome Biol. 2009 Feb 13;10(2):R21,2009-10-2-r21.

71. Ding D, Meng Q, Gao G, Zhao Y, Wang Q, Nare B, et al. Design, synthesis, and structure-activity relationship of trypanosoma brucei leucyl-tRNA synthetase inhibitors as antitrypanosomal agents. J Med Chem. 2011 Mar 10;54(5):1276-87.

72. Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, et al. A molecular marker for chloroquine-resistant falciparum malaria. N Engl J Med. 2001 Jan 25;344(4):257-63.

73. DOCTOR BP, MUDD JA. Species specificity of amino acid acceptor ribonucleic acid and aminoacyl soluble ribonucleic acid synthetases. J Biol Chem. 1963 Nov;238:3677-81.

74. DOCTOR BP, MUDD JA. Species specificity of amino acid acceptor ribonucleic acid and aminoacyl soluble ribonucleic acid synthetases. J Biol Chem. 1963 Nov;238:3677-81.

75. Dominski Z. Nucleases of the metallo-beta-lactamase family and their role in DNA and RNA metabolism. Crit Rev Biochem Mol Biol. 2007 Mar-Apr;42(2):67-93.

76. Dominski Z, Marzluff WF. Formation of the 3' end of histone mRNA: Getting closer to the end. Gene. 2007 Jul 15;396(2):373-90.

77. Dominski Z, Yang XC, Marzluff WF. The polyadenylation factor CPSF-73 is involved in histone-pre-mRNA processing. Cell. 2005 Oct 7;123(1):37-48.

78. Dominski Z, Yang XC, Purdy M, Wagner EJ, Marzluff WF. A CPSF-73 homologue is required for cell cycle progression but not cell growth and interacts with a protein having features of CPSF-100. Mol Cell Biol. 2005 Feb;25(4):1489-500.

79. Dondorp AM, Lee SJ, Faiz MA, Mishra S, Price R, Tjitra E, et al. The relationship between age and the manifestations of and mortality associated with severe malaria. Clin Infect Dis. 2008 Jul 15;47(2):151-7.

80. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, et al. Artemisinin resistance in plasmodium falciparum malaria. N Engl J Med. 2009 Jul 30;361(5):455-67.

81. Dorsey G, Kamya MR, Singh A, Rosenthal PJ. Polymorphisms in the plasmodium falciparum pfcrt and pfmdr-1 genes and clinical response to chloroquine in kampala, uganda. J Infect Dis. 2001 May 1;183(9):1417-20.

82. Eastman RT, Dharia NV, Winzeler EA, Fidock DA. Piperaquine resistance is associated with a copy number variation on chromosome 5 in drug-pressured plasmodium falciparum parasites. Antimicrob Agents Chemother. 2011 Aug;55(8):3908-16.

83. Eastman RT, White J, Hucke O, Bauer K, Yokoyama K, Nallan L, et al. Resistance to a protein farnesyltransferase inhibitor in plasmodium falciparum. J Biol Chem. 2005 Apr 8;280(14):13554-9.

84. Eldred EW, Schimmel PR. Rapid deacylation by isoleucyl transfer ribonucleic acid synthetase of isoleucine-specific transfer ribonucleic acid aminoacylated with valine. J Biol Chem. 1972 May 10;247(9):2961-4.

85. Eriani G, Delarue M, Poch O, Gangloff J, Moras D. Partition of tRNA synthetases into two classes based on mutually exclusive sets of sequence motifs. Nature. 1990 Sep 13;347(6289):203-6.

86. Espedido BA, Gosbell IB. Chromosomal mutations involved in antibiotic resistance in staphylococcus aureus. Front Biosci (Schol Ed). 2012 Jan 1;4:900-15.

87. Eziefula AC, Bousema T, Yeung S, Kamya M, Owaraganise A, Gabagaya G, et al. Single dose primaquine for clearance of plasmodium falciparum gametocytes in children with uncomplicated malaria in uganda: A randomised, controlled, double-blind, dose-ranging trial. Lancet Infect Dis. 2014 Feb;14(2):130-9.

88. Farnert A, Ursing J, Tolfvenstam T, Rono J, Karlsson L, Sparrelid E, et al. Artemether-lumefantrine treatment failure despite adequate

lumefantrine day 7 concentration in a traveller with plasmodium falciparum malaria after returning from tanzania. Malar J. 2012 May 25;11(1):176.

89. Fernando D, Rodrigo C, Rajapakse S. Primaquine in vivax malaria: An update and review on management issues. Malar J. 2011 Dec 12;10:351,2875-10-351.

90. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, et al. Mutations in the P. falciparum digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol Cell. 2000 Oct;6(4):861-71.

91. Fisher N, Abd Majid R, Antoine T, Al-Helal M, Warman AJ, Johnson DJ, et al. Cytochrome b mutation Y268S conferring atovaquone resistance phenotype in malaria parasite results in reduced parasite bc1 catalytic turnover and protein expression. J Biol Chem. 2012 Mar 23;287(13):9731-41.

92. Foote SJ, Kyle DE, Martin RK, Oduola AM, Forsyth K, Kemp DJ, et al. Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in plasmodium falciparum. Nature. 1990 May 17;345(6272):255-8.

93. Ford LP, Bagga PS, Wilusz J. The poly(A) tail inhibits the assembly of a 3'-to-5' exonuclease in an in vitro RNA stability system. Mol Cell Biol. 1997 Jan;17(1):398-406.

94. Ganesan K, Ponmee N, Jiang L, Fowble JW, White J, Kamchonwongpaisan S, et al. A genetically hard-wired metabolic transcriptome in plasmodium falciparum fails to mount protective responses to lethal antifolates. PLoS Pathog. 2008 Nov;4(11):e1000214.

95. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, et al. Genome sequence of the human malaria parasite plasmodium falciparum. Nature. 2002 Oct 3;419(6906):498-511.

96. Gassis S, Rathod PK. Frequency of drug resistance in plasmodium falciparum: A nonsynergistic combination of 5-fluoroorotate and

atovaquone suppresses in vitro resistance. Antimicrob Agents Chemother. 1996 Apr;40(4):914-9.

97. Gaston MA, Jiang R, Krzycki JA. Functional context, biosynthesis, and genetic encoding of pyrrolysine. Curr Opin Microbiol. 2011 Jun;14(3):342-9.

98. Gavin AC, Bosche M, Krause R, Grandi P, Marzioch M, Bauer A, et al. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature. 2002 Jan 10;415(6868):141-7.

99. Georghiou SB, Magana M, Garfein RS, Catanzaro DG, Catanzaro A, Rodwell TC. Evaluation of genetic mutations associated with mycobacterium tuberculosis resistance to amikacin, kanamycin and capreomycin: A systematic review. PLoS One. 2012;7(3):e33275.

100. Gething PW, Elyazar IR, Moyes CL, Smith DL, Battle KE, Guerra CA, et al. A long neglected world malaria map: Plasmodium vivax endemicity in 2010. PLoS Negl Trop Dis. 2012;6(9):e1814.

101. Gething PW, Patil AP, Smith DL, Guerra CA, Elyazar IR, Johnston GL, et al. A new world malaria map: Plasmodium falciparum endemicity in 2010. Malar J. 2011 Dec 20;10:378,2875-10-378.

102. Gilmartin GM, McDevitt MA, Nevins JR. Multiple factors are required for specific RNA cleavage at a poly(A) addition site. Genes Dev. 1988 May;2(5):578-87.

103. Gniadkowski M. Evolution of extended-spectrum beta-lactamases by mutation. Clin Microbiol Infect. 2008 Jan;14 Suppl 1:11-32.

104. Golightly LM, Mbacham W, Daily J, Wirth DF. 3' UTR elements enhance expression of Pgs28, an ookinete protein of plasmodium gallinaceum. Mol Biochem Parasitol. 2000 Jan 5;105(1):61-70.

105. Greenwood B. The use of anti-malarial drugs to prevent malaria in the population of malaria-endemic areas. Am J Trop Med Hyg. 2004 Jan;70(1):1-7.

106. Gregson A, Plowe CV. Mechanisms of resistance of malaria parasites to antifolates. Pharmacol Rev. 2005 Mar;57(1):117-45.

107. Guerra CA, Howes RE, Patil AP, Gething PW, Van Boeckel TP, Temperley WH, et al. The international limits and population at risk of plasmodium vivax transmission in 2009. PLoS Negl Trop Dis. 2010 Aug 3;4(8):e774.

108. Guler JL, Freeman DL, Ahyong V, Patrapuvich R, White J, Gujjar R, et al. Asexual populations of the human malaria parasite, plasmodium falciparum, use a two-step genomic strategy to acquire accurate, beneficial DNA amplifications. PLoS Pathog. 2013;9(5):e1003375.

109. Guo M, Schimmel P. Essential nontranslational functions of tRNA synthetases. Nat Chem Biol. 2013 Mar;9(3):145-53.

110. Gurney R, Thomas CM. Mupirocin: Biosynthesis, special features and applications of an antibiotic from a gram-negative bacterium. Appl Microbiol Biotechnol. 2011 Apr;90(1):11-21.

111. Hamilton MJ, Lee M, Le Roch KG. The ubiquitin system: An essential component to unlocking the secrets of malaria parasite biology. Mol Biosyst. 2014 Jan 30.

112. Han JM, Jeong SJ, Park MC, Kim G, Kwon NH, Kim HK, et al. Leucyl-tRNA synthetase is an intracellular leucine sensor for the mTORC1-signaling pathway. Cell. 2012 Apr 13;149(2):410-24.

113. Handel A, Regoes RR, Antia R. The role of compensatory mutations in the emergence of drug resistance. PLoS Comput Biol. 2006 Oct;2(10):e137.

114. Happi CT, Gbotosho GO, Folarin OA, Bolaji OM, Sowunmi A, Kyle DE, et al. Association between mutations in plasmodium falciparum chloroquine resistance transporter and P. falciparum multidrug resistance 1 genes and in vivo amodiaquine resistance in P. falciparum malaria-infected children in nigeria. Am J Trop Med Hyg. 2006 Jul;75(1):155-61.

115. Hayashi H. Species specificity of tyrosine transfer RNA's from escherichia coli and yeast. J Mol Biol. 1966 Aug;19(1):161-73.

116. Haynes JD, Diggs CL, Hines FA, Desjardins RE. Culture of human malaria parasites plasmodium falciparum. Nature. 1976 Oct 28;263(5580):767-9.

117. Hayward R, Saliba KJ, Kirk K. pfmdr1 mutations associated with chloroquine resistance incur a fitness cost in plasmodium falciparum. Mol Microbiol. 2005 Feb;55(4):1285-95.

118. Hazbon MH, Brimacombe M, Bobadilla del Valle M, Cavatore M, Guerrero MI, Varma-Basil M, et al. Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant mycobacterium tuberculosis. Antimicrob Agents Chemother. 2006 Aug;50(8):2640-9.

119. Hazbon MH, Motiwala AS, Cavatore M, Brimacombe M, Whittam TS, Alland D. Convergent evolutionary analysis identifies significant mutations in drug resistance targets of mycobacterium tuberculosis. Antimicrob Agents Chemother. 2008 Sep;52(9):3369-76.

120. Heal WP, Wright MH, Thinon E, Tate EW. Multifunctional protein labeling via enzymatic N-terminal tagging and elaboration by click chemistry. Nat Protoc. 2011 Dec 22;7(1):105-17.

121. Helmling S, Zhelkovsky A, Moore CL. Fip1 regulates the activity of poly(A) polymerase through multiple interactions. Mol Cell Biol. 2001 Mar;21(6):2026-37.

122. Henry KW, Nickels JT, Edlind TD. Upregulation of ERG genes in candida species by azoles and other sterol biosynthesis inhibitors. Antimicrob Agents Chemother. 2000 Oct;44(10):2693-700.

123. Hernandez V, Crepin T, Palencia A, Cusack S, Akama T, Baker SJ, et al. Discovery of a novel class of boron-based antibacterials with activity against gram-negative bacteria. Antimicrob Agents Chemother. 2013 Mar;57(3):1394-403.

124. Hoen R, Novoa EM, Lopez A, Camacho N, Cubells L, Vieira P, et al. Selective inhibition of an apicoplastic aminoacyl-tRNA synthetase from plasmodium falciparum. Chembiochem. 2013 Mar 4;14(4):499-509.

125. Hoepfner D, McNamara CW, Lim CS, Studer C, Riedl R, Aust T, et al. Selective and specific inhibition of the plasmodium falciparum lysyl-tRNA synthetase by the fungal secondary metabolite cladosporin. Cell Host Microbe. 2012 Jun 14;11(6):654-63.

126. Hopkins J, Fowler R, Krishna S, Wilson I, Mitchell G, Bannister L. The plastid in plasmodium falciparum asexual blood stages: A three-dimensional ultrastructural analysis. Protist. 1999 Oct;150(3):283-95.

127. Hu QH, Liu RJ, Fang ZP, Zhang J, Ding YY, Tan M, et al. Discovery of a potent benzoxaborole-based anti-pneumococcal agent targeting leucyl-tRNA synthetase. Sci Rep. 2013;3:2475.

128. Huang Y, Carmichael GG. Role of polyadenylation in nucleocytoplasmic transport of mRNA. Mol Cell Biol. 1996 Apr;16(4):1534-42.

129. Humphrey T, Christofori G, Lucijanic V, Keller W. Cleavage and polyadenylation of messenger RNA precursors in vitro occurs within large and specific 3' processing complexes. EMBO J. 1987 Dec 20;6(13):4159-68.

130. Hwang J, Bitarakwate E, Pai M, Reingold A, Rosenthal PJ, Dorsey G. Chloroquine or amodiaquine combined with sulfadoxine-pyrimethamine for uncomplicated malaria: A systematic review. Trop Med Int Health. 2006 Jun;11(6):789-99.

131. Ilan J, Ilan J. Aminoacyl transfer ribonucleic acid synthetases from cell-free extract of plasmodium berghei. Science. 1969 May 2;164(3879):560-2.

132. Inselburg J, Banyal HS. Plasmodium falciparum: Synchronization of asexual development with aphidicolin, a DNA synthesis inhibitor. Exp Parasitol. 1984 Feb;57(1):48-54.

133. Istvan ES, Dharia NV, Bopp SE, Gluzman I, Winzeler EA, Goldberg DE. Validation of isoleucine utilization targets in plasmodium falciparum. Proc Natl Acad Sci U S A. 2011 Jan 25;108(4):1627-32.

134. Jacob ST, Rose KM. Inhibition of poly(A) polymerase by rifamycin derivatives. Nucleic Acids Res. 1974 Nov;1(11):1549-59.

135. Jacobs RT, Nare B, Wring SA, Orr MD, Chen D, Sligar JM, et al. SCYX-7158, an orally-active benzoxaborole for the treatment of stage 2 human african trypanosomiasis. PLoS Negl Trop Dis. 2011 Jun;5(6):e1151.

136. Jacobs RT, Plattner JJ, Keenan M. Boron-based drugs as antiprotozoals. Curr Opin Infect Dis. 2011 Dec;24(6):586-92.

137. Jacobs RT, Plattner JJ, Nare B, Wring SA, Chen D, Freund Y, et al. Benzoxaboroles: A new class of potential drugs for human african trypanosomiasis. Future Med Chem. 2011 Aug;3(10):1259-78.

138. Jagannathan P, Muhindo MK, Kakuru A, Arinaitwe E, Greenhouse B, Tappero J, et al. Increasing incidence of malaria in children despite insecticide-treated bed nets and prompt anti-malarial therapy in tororo, uganda. Malar J. 2012 Dec 28;11:435,2875-11-435.

139. Jakubowski H, Goldman E. Editing of errors in selection of amino acids for protein synthesis. Microbiol Rev. 1992 Sep;56(3):412-29.

140. Jiang H, Patel JJ, Yi M, Mu J, Ding J, Stephens R, et al. Genome-wide compensatory changes accompany drug- selected mutations in the plasmodium falciparum crt gene. PLoS One. 2008 Jun 25;3(6):e2484.

141. Jiang X, Zhang W, Zhang Y, Gao F, Lu C, Zhang X, et al. Assessment of efflux pump gene expression in a clinical isolate mycobacterium tuberculosis by real-time reverse transcription PCR. Microb Drug Resist. 2008 Mar;14(1):7-11.

142. Johnson DJ, Fidock DA, Mungthin M, Lakshmanan V, Sidhu AB, Bray PG, et al. Evidence for a central role for PfCRT in conferring plasmodium

falciparum resistance to diverse antimalarial agents. Mol Cell. 2004 Sep 24;15(6):867-77.

143. Kalanon M, McFadden GI. Malaria, plasmodium falciparum and its apicoplast. Biochem Soc Trans. 2010 Jun;38(3):775-82.

144. Kalidas S, Cestari I, Monnerat S, Li Q, Regmi S, Hasle N, et al. Genetic validation of aminoacyl tRNA synthetases as drug targets in trypanosoma brucei. Eukaryot Cell. 2014 Feb 21.

145. Kamya MR, Yeka A, Bukirwa H, Lugemwa M, Rwakimari JB, Staedke SG, et al. Artemether-lumefantrine versus dihydroartemisinin-piperaquine for treatment of malaria: A randomized trial. PLoS Clin Trials. 2007 May 18;2(5):e20.

146. Kaufmann I, Martin G, Friedlein A, Langen H, Keller W. Human Fip1 is a subunit of CPSF that binds to U-rich RNA elements and stimulates poly(A) polymerase. EMBO J. 2004 Feb 11;23(3):616-26.

147. Keller TL, Zocco D, Sundrud MS, Hendrick M, Edenius M, Yum J, et al. Halofuginone and other febrifugine derivatives inhibit prolyl-tRNA synthetase. Nat Chem Biol. 2012 Feb 12;8(3):311-7.

148. Keller W, Bienroth S, Lang KM, Christofori G. Cleavage and polyadenylation factor CPF specifically interacts with the pre-mRNA 3' processing signal AAUAAA. EMBO J. 1991 Dec;10(13):4241-9.

149. Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, et al. The human genome browser at UCSC. Genome Res. 2002 Jun;12(6):996-1006.

150. Khan S, Garg A, Camacho N, Van Rooyen J, Kumar Pole A, Belrhali H, et al. Structural analysis of malaria-parasite lysyl-tRNA synthetase provides a platform for drug development. Acta Crystallogr D Biol Crystallogr. 2013 May;69(Pt 5):785-95.

151. Kim S, Lee SW, Choi EC, Choi SY. Aminoacyl-tRNA synthetases and their inhibitors as a novel family of antibiotics. Appl Microbiol Biotechnol. 2003 May;61(4):278-88.

152. Kolev NG, Yario TA, Benson E, Steitz JA. Conserved motifs in both CPSF73 and CPSF100 are required to assemble the active endonuclease for histone mRNA 3'-end maturation. EMBO Rep. 2008 Oct;9(10):1013-8.

153. Kondrashov A, Meijer HA, Barthet-Barateig A, Parker HN, Khurshid A, Tessier S, et al. Inhibition of polyadenylation reduces inflammatory gene induction. RNA. 2012 Dec;18(12):2236-50.

154. Konishi M, Nishio M, Saitoh K, Miyaki T, Oki T, Kawaguchi H. Cispentacin, a new antifungal antibiotic. I. production, isolation, physico-chemical properties and structure. J Antibiot (Tokyo). 1989 Dec;42(12):1749-55.

155. Konrad I, Roschenthaler R. Inhibition of phenylalanine tRNA synthetase from bacillus subtilis by ochratoxin A. FEBS Lett. 1977 Nov 15;83(2):341-7.

156. Krogstad DJ, Gluzman IY, Kyle DE, Oduola AM, Martin SK, Milhous WK, et al. Efflux of chloroquine from plasmodium falciparum: Mechanism of chloroquine resistance. Science. 1987 Nov 27;238(4831):1283-5.

157. Laishram RS, Anderson RA. The poly A polymerase star-PAP controls 3'-end cleavage by promoting CPSF interaction and specificity toward the pre-mRNA. EMBO J. 2010 Dec 15;29(24):4132-45.

158. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009;10(3):R25,2009-10-3-r25. Epub 2009 Mar 4.

159. Le Roch KG, Zhou Y, Blair PL, Grainger M, Moch JK, Haynes JD, et al. Discovery of gene function by expression profiling of the malaria parasite life cycle. Science. 2003 Sep 12;301(5639):1503-8.

160. Leatherbarrow RJ, Fersht AR, Winter G. Transition-state stabilization in the mechanism of tyrosyl-tRNA synthetase revealed by protein engineering. Proc Natl Acad Sci U S A. 1985 Dec;82(23):7840-4.

161. Leberman R, Hartlein M, Cusack S. Escherichia coli seryl-tRNA synthetase: The structure of a class 2 aminoacyl-tRNA synthetase. Biochim Biophys Acta. 1991 Jul 23;1089(3):287-98.

162. Lee KS, Kim TH, Kim ES, Lim HS, Yeom JS, Jun G, et al. Short report: Chloroquine-resistant plasmodium vivax in the republic of korea. Am J Trop Med Hyg. 2009 Feb;80(2):215-7.

163. Lee SW, Cho BH, Park SG, Kim S. Aminoacyl-tRNA synthetase complexes: Beyond translation. J Cell Sci. 2004 Aug 1;117(Pt 17):3725-34.

164. Leshem E, Meltzer E, Stienlauf S, Kopel E, Schwartz E. Effectiveness of short prophylactic course of atovaquone-proguanil in travelers to sub-saharan africa. J Travel Med. 2014 Mar;21(2):82-5.

165. Levin BR, Perrot V, Walker N. Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. Genetics. 2000 Mar;154(3):985-97.

166. Levitt A. RNA processing in malarial parasites. Parasitol Today. 1993 Dec;9(12):465-8.

167. Li L, Palencia A, Lukk T, Li Z, Luthey-Schulten ZA, Cusack S, et al. Leucyl-tRNA synthetase editing domain functions as a molecular rheostat to control codon ambiguity in mycoplasma pathogens. Proc Natl Acad Sci U S A. 2013 Mar 5;110(10):3817-22.

168. Li X, Zhang YK, Liu Y, Zhang S, Ding CZ, Zhou Y, et al. Synthesis of new acylsulfamoyl benzoxaboroles as potent inhibitors of HCV NS3 protease. Bioorg Med Chem Lett. 2010 Dec 15;20(24):7493-7.

169. Lin L, Schimmel P. Mutational analysis suggests the same design for editing activities of two tRNA synthetases. Biochemistry. 1996 Apr 30;35(17):5596-601.

170. Lincecum TL,Jr, Tukalo M, Yaremchuk A, Mursinna RS, Williams AM, Sproat BS, et al. Structural and mechanistic basis of pre- and

posttransfer editing by leucyl-tRNA synthetase. Mol Cell. 2003 Apr;11(4):951-63.

171. Ling J, Peterson KM, Simonovic I, Soll D, Simonovic M. The mechanism of pre-transfer editing in yeast mitochondrial threonyl-tRNA synthetase. J Biol Chem. 2012 Aug 17;287(34):28518-25.

172. Ling J, Reynolds N, Ibba M. Aminoacyl-tRNA synthesis and translational quality control. Annu Rev Microbiol. 2009;63:61-78.

173. Ling YH, Liebes L, Ng B, Buckley M, Elliott PJ, Adams J, et al. PS-341, a novel proteasome inhibitor, induces bcl-2 phosphorylation and cleavage in association with G2-M phase arrest and apoptosis. Mol Cancer Ther. 2002 Aug;1(10):841-9.

174. Llinas M, Bozdech Z, Wong ED, Adai AT, DeRisi JL. Comparative whole genome transcriptome analysis of three plasmodium falciparum strains. Nucleic Acids Res. 2006 Feb 21;34(4):1166-73.

175. Looareesuwan S, Viravan C, Webster HK, Kyle DE, Hutchinson DB, Canfield CJ. Clinical studies of atovaquone, alone or in combination with other antimalarial drugs, for treatment of acute uncomplicated malaria in thailand. Am J Trop Med Hyg. 1996 Jan;54(1):62-6.

176. Lund E, Dahlberg JE. Proofreading and aminoacylation of tRNAs before export from the nucleus. Science. 1998 Dec 11;282(5396):2082-5.

177. Lymans'ka OI, Lymans'kyi OP. Molecular technologies in detecting sensitive and isoniazid-resistant mycobacterium tuberculosis. Tsitol Genet. 2011 Nov-Dec;45(6):34-47.

178. Maeno Y, Toyoshima T, Fujioka H, Ito Y, Meshnick SR, Benakis A, et al. Morphologic effects of artemisinin in plasmodium falciparum. Am J Trop Med Hyg. 1993 Oct;49(4):485-91.

179. malERA Consultative Group on Drugs. A research agenda for malaria eradication: Drugs. PLoS Med. 2011 Jan 25;8(1):e1000402.

180. Mandel CR, Bai Y, Tong L. Protein factors in pre-mRNA 3'-end processing. Cell Mol Life Sci. 2008 Apr;65(7-8):1099-122.

181. Mandel CR, Kaneko S, Zhang H, Gebauer D, Vethantham V, Manley JL, et al. Polyadenylation factor CPSF-73 is the pre-mRNA 3'-end-processing endonuclease. Nature. 2006 Dec 14;444(7121):953-6.

182. McCracken S, Fong N, Yankulov K, Ballantyne S, Pan G, Greenblatt J, et al. The C-terminal domain of RNA polymerase II couples mRNA processing to transcription. Nature. 1997 Jan 23;385(6614):357-61.

183. McFadden GI. The apicoplast. Protoplasma. 2011 Oct;248(4):641-50.

184. McMaster GK, Carmichael GG. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc Natl Acad Sci U S A. 1977 Nov;74(11):4835-8.

185. McNamara CW, Lee MC, Lim CS, Lim SH, Roland J, Nagle A, et al. Targeting plasmodium PI(4)K to eliminate malaria. Nature. 2013 Dec 12;504(7479):248-53.

186. Melton DA, Krieg PA, Rebagliati MR, Maniatis T, Zinn K, Green MR. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. 1984 Sep 25;12(18):7035-56.

187. Menendez-Arias L, Matamoros T, Cases-Gonzalez CE. Insertions and deletions in HIV-1 reverse transcriptase: Consequences for drug resistance and viral fitness. Curr Pharm Des. 2006;12(15):1811-25.

188. Meshnick SR, Yang YZ, Lima V, Kuypers F, Kamchonwongpaisan S, Yuthavong Y. Iron-dependent free radical generation from the antimalarial agent artemisinin (qinghaosu). Antimicrob Agents Chemother. 1993 May;37(5):1108-14.

189. Miller LH, Ackerman HC, Su XZ, Wellems TE. Malaria biology and disease pathogenesis: Insights for new treatments. Nat Med. 2013 Feb;19(2):156-67.

190. Miller LH, Mason SJ, Clyde DF, McGinniss MH. The resistance factor to plasmodium vivax in blacks. the duffy-blood-group genotype, FyFy. N Engl J Med. 1976 Aug 5;295(6):302-4.

191. Miller LH, Su X. Artemisinin: Discovery from the chinese herbal garden. Cell. 2011 Sep 16;146(6):855-8.

192. Minvielle-Sebastia L, Winsor B, Bonneaud N, Lacroute F. Mutations in the yeast RNA14 and RNA15 genes result in an abnormal mRNA decay rate; sequence analysis reveals an RNA-binding domain in the RNA15 protein. Mol Cell Biol. 1991 Jun;11(6):3075-87.

193. Mirande M, Cirakoglu B, Waller JP. Macromolecular complexes from sheep and rabbit containing seven aminoacyl-tRNA synthetases. III. assignment of aminoacyl-tRNA synthetase activities to the polypeptide components of the complexes. J Biol Chem. 1982 Sep 25;257(18):11056-63.

194. Moore CL, Chen J, Whoriskey J. Two proteins crosslinked to RNA containing the adenovirus L3 poly(A) site require the AAUAAA sequence for binding. EMBO J. 1988 Oct;7(10):3159-69.

195. Moore CL, Sharp PA. Site-specific polyadenylation in a cell-free reaction. Cell. 1984 Mar;36(3):581-91.

196. Mueller I, Zimmerman PA, Reeder JC. Plasmodium malariae and plasmodium ovale--the "bashful" malaria parasites. Trends Parasitol. 2007 Jun;23(6):278-83.

197. Muller IB, Das Gupta R, Luersen K, Wrenger C, Walter RD. Assessing the polyamine metabolism of plasmodium falciparum as chemotherapeutic target. Mol Biochem Parasitol. 2008 Jul;160(1):1-7.

198. Mungthin M, Khositnithikul R, Sitthichot N, Suwandittakul N, Wattanaveeradej V, Ward SA, et al. Association between the pfmdr1

gene and in vitro artemether and lumefantrine sensitivity in thai isolates of plasmodium falciparum. Am J Trop Med Hyg. 2010 Nov;83(5):1005-9.

199. Murthy KG, Manley JL. The 160-kD subunit of human cleavage-polyadenylation specificity factor coordinates pre-mRNA 3'-end formation. Genes Dev. 1995 Nov 1;9(21):2672-83.

200. Nangle LA, De Crecy Lagard V, Doring V, Schimmel P. Genetic code ambiguity. cell viability related to the severity of editing defects in mutant tRNA synthetases. J Biol Chem. 2002 Nov 29;277(48):45729-33.

201. Nankabirwa JI, Wandera B, Amuge P, Kiwanuka N, Dorsey G, Rosenthal PJ, et al. Impact of intermittent preventive treatment with dihydroartemisinin-piperaquine on malaria in ugandan schoolchildren: A randomized placebo-controlled trial. Clin Infect Dis. 2014 Mar 12.

202. Nass G, Poralla K, Zahner H. Effect of the antibiotic borrelidin on the regulation of threonine biosynthetic enzymes in E. coli. Biochem Biophys Res Commun. 1969 Jan 6;34(1):84-91.

203. Nedea E, He X, Kim M, Pootoolal J, Zhong G, Canadien V, et al. Organization and function of APT, a subcomplex of the yeast cleavage and polyadenylation factor involved in the formation of mRNA and small nucleolar RNA 3'-ends. J Biol Chem. 2003 Aug 29;278(35):33000-10.

204. Nirmalan N, Sims PF, Hyde JE. Translational up-regulation of antifolate drug targets in the human malaria parasite plasmodium falciparum upon challenge with inhibitors. Mol Biochem Parasitol. 2004 Jul;136(1):63-70.

205. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM, et al. Evidence of artemisinin-resistant malaria in western cambodia. N Engl J Med. 2008 Dec 11;359(24):2619-20.

206. Noedl H, Teja-Isavadharm P, Miller RS. Nonisotopic, semiautomated plasmodium falciparum bioassay for measurement of

antimalarial drug levels in serum or plasma. Antimicrob Agents Chemother. 2004 Nov;48(11):4485-7.

207. Nsanzabana C, Rosenthal PJ. In vitro activity of antiretroviral drugs against plasmodium falciparum. Antimicrob Agents Chemother. 2011 Nov;55(11):5073-7.

208. Nureki O, Vassylyev DG, Tateno M, Shimada A, Nakama T, Fukai S, et al. Enzyme structure with two catalytic sites for double-sieve selection of substrate. Science. 1998 Apr 24;280(5363):578-82.

209. Obonyo CO, Juma EA. Clindamycin plus quinine for treating uncomplicated falciparum malaria: A systematic review and meta-analysis. Malar J. 2012 Jan 4;11:2,2875-11-2.

210. Obrig TG, Culp WJ, McKeehan WL, Hardesty B. The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. J Biol Chem. 1971 Jan 10;246(1):174-81.

211. Ogilvie A, Wiebauer K, Kersten W. Inhibition of leucyl-transfer ribonucleic acid synthetasymol. Biochem J. 1975 Dec;152(3):511-5.

212. Oguariri RM, Dunn JM, Golightly LM. 3' gene regulatory elements required for expression of the plasmodiumfalciparum developmental protein, Pfs25. Mol Biochem Parasitol. 2006 Apr;146(2):163-72.

213. O'Meara WP, Bejon P, Mwangi TW, Okiro EA, Peshu N, Snow RW, et al. Effect of a fall in malaria transmission on morbidity and mortality in kilifi, kenya. Lancet. 2008 Nov 1;372(9649):1555-62.

214. Osei-Akoto A, Orton L, Owusu-Ofori SP. Atovaquone-proguanil for treating uncomplicated malaria. Cochrane Database Syst Rev. 2005 Oct 19;(4)(4):CD004529.

215. Otoguro K, Ui H, Ishiyama A, Kobayashi M, Togashi H, Takahashi Y, et al. In vitro and in vivo antimalarial activities of a non-glycosidic 18-membered macrolide antibiotic, borrelidin, against drug-resistant strains of plasmodia. J Antibiot (Tokyo). 2003 Aug;56(8):727-9.

216. Otto TD, Wilinski D, Assefa S, Keane TM, Sarry LR, Bohme U, et al. New insights into the blood-stage transcriptome of plasmodium falciparum using RNA-seq. Mol Microbiol. 2010 Apr;76(1):12-24.

217. Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D, et al. High-level semi-synthetic production of the potent antimalarial artemisinin. Nature. 2013 Apr 25;496(7446):528-32.

218. Palencia A, Crepin T, Vu MT, Lincecum TL,Jr, Martinis SA, Cusack S. Structural dynamics of the aminoacylation and proofreading functional cycle of bacterial leucyl-tRNA synthetase. Nat Struct Mol Biol. 2012 Jun 10;19(7):677-84.

219. Park DJ, Lukens AK, Neafsey DE, Schaffner SF, Chang HH, Valim C, et al. Sequence-based association and selection scans identify drug resistance loci in the plasmodium falciparum malaria parasite. Proc Natl Acad Sci U S A. 2012 Aug 7;109(32):13052-7.

220. Peters PJ, Thigpen MC, Parise ME, Newman RD. Safety and toxicity of sulfadoxine/pyrimethamine: Implications for malaria prevention in pregnancy using intermittent preventive treatment. Drug Saf. 2007;30(6):481-501.

221. Peterson DS, Milhous WK, Wellems TE. Molecular basis of differential resistance to cycloguanil and pyrimethamine in plasmodium falciparum malaria. Proc Natl Acad Sci U S A. 1990 Apr;87(8):3018-22.

222. Pham JS, Dawson KL, Jackson KE, Lim EE, Pasaje CF, Turner KE, et al. Aminoacyl-tRNA synthetases as drug targets in eukaryotic parasites. Int J Parasitol Drugs Drug Resist. 2013 Nov 11;4(1):1-13.

223. Pham JS, Sakaguchi R, Yeoh LM, De Silva NS, McFadden GI, Hou YM, et al. A dual-targeted aminoacyl-tRNA synthetase in plasmodium falciparum charges cytosolic and apicoplast tRNACys. Biochem J. 2014 Mar 15;458(3):513-23.

224. Phyo AP, Nkhoma S, Stepniewska K, Ashley EA, Nair S, McGready R, et al. Emergence of artemisinin-resistant malaria on the western border

of thailand: A longitudinal study. Lancet. 2012 May 26;379(9830):1960-6.

225. Pickard AL, Wongsrichanalai C, Purfield A, Kamwendo D, Emery K, Zalewski C, et al. Resistance to antimalarials in southeast asia and genetic polymorphisms in pfmdr1. Antimicrob Agents Chemother. 2003 Aug;47(8):2418-23.

226. Ponts N, Yang J, Chung DW, Prudhomme J, Girke T, Horrocks P, et al. Deciphering the ubiquitin-mediated pathway in apicomplexan parasites: A potential strategy to interfere with parasite virulence. PLoS One. 2008 Jun 11;3(6):e2386.

227. Preiss T, Hentze MW. Dual function of the messenger RNA cap structure in poly(A)-tail-promoted translation in yeast. Nature. 1998 Apr 2;392(6675):516-20.

228. Preker PJ, Lingner J, Minvielle-Sebastia L, Keller W. The FIP1 gene encodes a component of a yeast pre-mRNA polyadenylation factor that directly interacts with poly(A) polymerase. Cell. 1995 May 5;81(3):379-89.

229. Price RN, Cassar C, Brockman A, Duraisingh M, van Vugt M, White NJ, et al. The pfmdr1 gene is associated with a multidrug-resistant phenotype in plasmodium falciparum from the western border of thailand. Antimicrob Agents Chemother. 1999 Dec;43(12):2943-9.

230. Price RN, Douglas NM, Anstey NM. New developments in plasmodium vivax malaria: Severe disease and the rise of chloroquine resistance. Curr Opin Infect Dis. 2009 Oct;22(5):430-5.

231. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, et al. Mefloquine resistance in plasmodium falciparum and increased pfmdr1 gene copy number. Lancet. 2004 Jul 31-Aug 6;364(9432):438-47.

232. Price RN, Uhlemann AC, van Vugt M, Brockman A, Hutagalung R, Nair S, et al. Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant

plasmodium falciparum malaria. Clin Infect Dis. 2006 Jun 1;42(11):1570-7.

233. Prichard RK. Ivermectin resistance and overview of the consortium for anthelmintic resistance SNPs. Expert Opin Drug Discov. 2007 Oct;2(s1):S41-52.

234. Proudfoot N. New perspectives on connecting messenger RNA 3' end formation to transcription. Curr Opin Cell Biol. 2004 Jun;16(3):272-8.

235. Ramaswamy SV, Reich R, Dou SJ, Jasperse L, Pan X, Wanger A, et al. Single nucleotide polymorphisms in genes associated with isoniazid resistance in mycobacterium tuberculosis. Antimicrob Agents Chemother. 2003 Apr;47(4):1241-50.

236. Rathod PK, McErlean T, Lee PC. Variations in frequencies of drug resistance in plasmodium falciparum. Proc Natl Acad Sci U S A. 1997 Aug 19;94(17):9389-93.

237. RENDI R, OCHOA S. Species specificity in activation and transfer of leucine from carrier ribonucleic acid to ribosomes. J Biol Chem. 1962 Dec;237:3707-10.

238. RENDI R, OCHOA S. Species specificity in activation and transfer of leucine from carrier ribonucleic acid to ribosomes. J Biol Chem. 1962 Dec;237:3707-10.

239. Ribas de Pouplana L, Schimmel P. Aminoacyl-tRNA synthetases: Potential markers of genetic code development. Trends Biochem Sci. 2001 Oct;26(10):591-6.

240. Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, et al. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature. 2006 Apr 13;440(7086):940-3.

241. Roca-Feltrer A, Kwizombe CJ, Sanjoaquin MA, Sesay SS, Faragher B, Harrison J, et al. Lack of decline in childhood malaria, malawi, 2001-2010. Emerg Infect Dis. 2012 Feb;18(2):272-8.

242. Rock FL, Mao W, Yaremchuk A, Tukalo M, Crepin T, Zhou H, et al. An antifungal agent inhibits an aminoacyl-tRNA synthetase by trapping tRNA in the editing site. Science. 2007 Jun 22;316(5832):1759-61.

243. Rodwell TC, Valafar F, Douglas J, Qian L, Garfein RS, Chawla A, et al. Predicting extensively drug-resistant mycobacterium tuberculosis phenotypes with genetic mutations. J Clin Microbiol. 2014 Mar;52(3):781-9.

244. Rose KM, Bell LE, Jacob ST. Specific inhibition of chromatin-associated poly(A) synthesis in vitro by cordycepin 5'-triphosphate. Nature. 1977 May 12;267(5607):178-80.

245. Rosenthal PJ. The interplay between drug resistance and fitness in malaria parasites. Mol Microbiol. 2013 Sep;89(6):1025-38.

246. Rosenthal PJ. Antimalarial drug discovery: Old and new approaches. J Exp Biol. 2003 Nov;206(Pt 21):3735-44.

247. Rosenthal PJ, McKerrow JH, Aikawa M, Nagasawa H, Leech JH. A malarial cysteine proteinase is necessary for hemoglobin degradation by plasmodium falciparum. J Clin Invest. 1988 Nov;82(5):1560-6.

248. Rosenthal PJ, Meshnick SR. Hemoglobin catabolism and iron utilization by malaria parasites. Mol Biochem Parasitol. 1996 Dec 20;83(2):131-9.

249. Rottmann M, McNamara C, Yeung BK, Lee MC, Zou B, Russell B, et al. Spiroindolones, a potent compound class for the treatment of malaria. Science. 2010 Sep 3;329(5996):1175-80.

250. Ruff M, Krishnaswamy S, Boeglin M, Poterszman A, Mitschler A, Podjarny A, et al. Class II aminoacyl transfer RNA synthetases: Crystal structure of yeast aspartyl-tRNA synthetase complexed with tRNA(asp). Science. 1991 Jun 21;252(5013):1682-9.

251. Ruvolo V, Altszuler R, Levitt A. The transcript encoding the circumsporozoite antigen of plasmodium berghei utilizes

heterogeneous polyadenylation sites. Mol Biochem Parasitol. 1993 Jan;57(1):137-50.

252. Sachanonta N, Chotivanich K, Chaisri U, Turner GD, Ferguson DJ, Day NP, et al. Ultrastructural and real-time microscopic changes in P. falciparum-infected red blood cells following treatment with antimalarial drugs. Ultrastruct Pathol. 2011 Oct;35(5):214-25.

253. Sachs AB, Sarnow P, Hentze MW. Starting at the beginning, middle, and end: Translation initiation in eukaryotes. Cell. 1997 Jun 13;89(6):831-8.

254. Sanchez CP, Dave A, Stein WD, Lanzer M. Transporters as mediators of drug resistance in plasmodium falciparum. Int J Parasitol. 2010 Aug 15;40(10):1109-18.

255. Schmidt E, Schimmel P. Residues in a class I tRNA synthetase which determine selectivity of amino acid recognition in the context of tRNA. Biochemistry. 1995 Sep 5;34(35):11204-10.

256. Schmidt E, Schimmel P. Mutational isolation of a sieve for editing in a transfer RNA synthetase. Science. 1994 Apr 8;264(5156):265-7.

257. Schneider-Poetsch T, Ju J, Eyler DE, Dang Y, Bhat S, Merrick WC, et al. Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. Nat Chem Biol. 2010 Mar;6(3):209-17.

258. Schwartz IK, Lackritz EM, Patchen LC. Chloroquine-resistant plasmodium vivax from indonesia. N Engl J Med. 1991 Mar 28;324(13):927.

259. Seah C, Alexander DC, Louie L, Simor A, Low DE, Longtin J, et al. MupB, a new high-level mupirocin resistance mechanism in staphylococcus aureus. Antimicrob Agents Chemother. 2012 Apr;56(4):1916-20.

260. Seiradake E, Mao W, Hernandez V, Baker SJ, Plattner JJ, Alley MR, et al. Crystal structures of the human and fungal cytosolic leucyl-tRNA

synthetase editing domains: A structural basis for the rational design of antifungal benzoxaboroles. J Mol Biol. 2009 Jul 10;390(2):196-207.

261. Shekalaghe S, Drakeley C, Gosling R, Ndaro A, van Meegeren M, Enevold A, et al. Primaquine clears submicroscopic plasmodium falciparum gametocytes that persist after treatment with sulphadoxine-pyrimethamine and artesunate. PLoS One. 2007 Oct 10;2(10):e1023.

262. Shenai BR, Semenov AV, Rosenthal PJ. Stage-specific antimalarial activity of cysteine protease inhibitors. Biol Chem. 2002 May;383(5):843-7.

263. Shenai BR, Sijwali PS, Singh A, Rosenthal PJ. Characterization of native and recombinant falcipain-2, a principal trophozoite cysteine protease and essential hemoglobinase of plasmodium falciparum. J Biol Chem. 2000 Sep 15;275(37):29000-10.

264. Sidhu AB, Verdier-Pinard D, Fidock DA. Chloroquine resistance in plasmodium falciparum malaria parasites conferred by pfcrt mutations. Science. 2002 Oct 4;298(5591):210-3.

265. Sijwali PS, Koo J, Singh N, Rosenthal PJ. Gene disruptions demonstrate independent roles for the four falcipain cysteine proteases of plasmodium falciparum. Mol Biochem Parasitol. 2006 Nov;150(1):96-106.

266. Silvian LF, Wang J, Steitz TA. Insights into editing from an ile-tRNA synthetase structure with tRNAile and mupirocin. Science. 1999 Aug 13;285(5430):1074-7.

267. Singh A, Rosenthal PJ. Selection of cysteine protease inhibitor-resistant malaria parasites is accompanied by amplification of falcipain genes and alteration in inhibitor transport. J Biol Chem. 2004 Aug 20;279(34):35236-41.

268. Singh B, Kim Sung L, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, et al. A large focus of naturally acquired plasmodium knowlesi infections in human beings. Lancet. 2004 Mar 27;363(9414):1017-24.

269. Singh N, Singh RT, Sundar S. Novel mechanism of drug resistance in kala azar field isolates. J Infect Dis. 2003 Aug 15;188(4):600-7.

270. Sinka ME, Bangs MJ, Manguin S, Rubio-Palis Y, Chareonviriyaphap T, Coetzee M, et al. A global map of dominant malaria vectors. Parasit Vectors. 2012 Apr 4;5:69,3305-5-69.

271. Smirnova EV, Lakunina VA, Tarassov I, Krasheninnikov IA, Kamenski PA. Noncanonical functions of aminoacyl-tRNA synthetases. Biochemistry (Mosc). 2012 Jan;77(1):15-25.

272. Smith WL, Edlind TD. Histone deacetylase inhibitors enhance candida albicans sensitivity to azoles and related antifungals: Correlation with reduction in CDR and ERG upregulation. Antimicrob Agents Chemother. 2002 Nov;46(11):3532-9.

273. Smithuis F, Kyaw MK, Phe O, Win T, Aung PP, Oo AP, et al. Effectiveness of five artemisinin combination regimens with or without primaquine in uncomplicated falciparum malaria: An open-label randomised trial. Lancet Infect Dis. 2010 Oct;10(10):673-81.

274. Spies FS, Ribeiro AW, Ramos DF, Ribeiro MO, Martin A, Palomino JC, et al. Streptomycin resistance and lineage-specific polymorphisms in mycobacterium tuberculosis gidB gene. J Clin Microbiol. 2011 Jul;49(7):2625-30.

275. Strasfeld L, Chou S. Antiviral drug resistance: Mechanisms and clinical implications. Infect Dis Clin North Am. 2010 Sep;24(3):809-33.

276. Streit S, Michalski CW, Erkan M, Kleeff J, Friess H. Northern blot analysis for detection and quantification of RNA in pancreatic cancer cells and tissues. Nat Protoc. 2009;4(1):37-43.

277. Su X, Hayton K, Wellems TE. Genetic linkage and association analyses for trait mapping in plasmodium falciparum. Nat Rev Genet. 2007 Jul;8(7):497-506.

278. Su X, Kirkman LA, Fujioka H, Wellems TE. Complex polymorphisms in an approximately 330 kDa protein are linked to chloroquine-resistant

P. falciparum in southeast asia and africa. Cell. 1997 Nov 28;91(5):593-603.

279. Sugawara A, Tanaka T, Hirose T, Ishiyama A, Iwatsuki M, Takahashi Y, et al. Borrelidin analogues with antimalarial activity: Design, synthesis and biological evaluation against plasmodium falciparum parasites. Bioorg Med Chem Lett. 2013 Apr 15;23(8):2302-5.

280. Sugiura I, Nureki O, Ugaji-Yoshikawa Y, Kuwabara S, Shimada A, Tateno M, et al. The 2.0 A crystal structure of thermus thermophilus methionyl-tRNA synthetase reveals two RNA-binding modules. Structure. 2000 Feb 15;8(2):197-208.

281. Sullivan DJ,Jr, Gluzman IY, Russell DG, Goldberg DE. On the molecular mechanism of chloroquine's antimalarial action. Proc Natl Acad Sci U S A. 1996 Oct 15;93(21):11865-70.

282. Takagaki Y, Ryner LC, Manley JL. Separation and characterization of a poly(A) polymerase and a cleavage/specificity factor required for pre-mRNA polyadenylation. Cell. 1988 Mar 11;52(5):731-42.

283. Tan KR, Magill AJ, Parise ME, Arguin PM, Centers for Disease Control and Prevention. Doxycycline for malaria chemoprophylaxis and treatment: Report from the CDC expert meeting on malaria chemoprophylaxis. Am J Trop Med Hyg. 2011 Apr;84(4):517-31.

284. Tanaka K, Tamaki M, Watanabe S. Effect of furanomycin on the synthesis of isoleucyl-tRNA. Biochim Biophys Acta. 1969 Nov 19;195(1):244-5.

285. Thomas CM, Hothersall J, Willis CL, Simpson TJ. Resistance to and synthesis of the antibiotic mupirocin. Nat Rev Microbiol. 2010 Apr;8(4):281-9.

286. Trager W, Jensen JB. Human malaria parasites in continuous culture. Science. 1976 Aug 20;193(4254):673-5.

287. Tran TH, Nguyen TT, Nguyen HP, Boni MF, Ngo VT, Nguyen TN, et al. In vivo susceptibility of plasmodium falciparum to artesunate in binh phuoc province, vietnam. Malar J. 2012 Oct 26;11:355,2875-11-355.

288. Trape JF, Tall A, Diagne N, Ndiath O, Ly AB, Faye J, et al. Malaria morbidity and pyrethroid resistance after the introduction of insecticide-treated bednets and artemisinin-based combination therapies: A longitudinal study. Lancet Infect Dis. 2011 Dec;11(12):925-32.

289. Tsuruta H, Paddon CJ, Eng D, Lenihan JR, Horning T, Anthony LC, et al. High-level production of amorpha-4,11-diene, a precursor of the antimalarial agent artemisinin, in escherichia coli. PLoS One. 2009;4(2):e4489.

290. Veiga MI, Ferreira PE, Malmberg M, Jornhagen L, Bjorkman A, Nosten F, et al. pfmdr1 amplification is related to increased plasmodium falciparum in vitro sensitivity to the bisquinoline piperaquine. Antimicrob Agents Chemother. 2012 Jul;56(7):3615-9.

291. Vinciguerra P, Stutz F. mRNA export: An assembly line from genes to nuclear pores. Curr Opin Cell Biol. 2004 Jun;16(3):285-92.

292. Vinetz JM, Li J, McCutchan TF, Kaslow DC. Plasmodium malariae infection in an asymptomatic 74-year-old greek woman with splenomegaly. N Engl J Med. 1998 Feb 5;338(6):367-71.

293. Volkman SK, Neafsey DE, Schaffner SF, Park DJ, Wirth DF. Harnessing genomics and genome biology to understand malaria biology. Nat Rev Genet. 2012 Apr 12;13(5):315-28.

294. von Seidlein L, Olaosebikan R, Hendriksen IC, Lee SJ, Adedoyin OT, Agbenyega T, et al. Predicting the clinical outcome of severe falciparum malaria in african children: Findings from a large randomized trial. Clin Infect Dis. 2012 Apr;54(8):1080-90.

295. Vondenhoff GH, Van Aerschot A. Aminoacyl-tRNA synthetase inhibitors as potential antibiotics. Eur J Med Chem. 2011 Nov;46(11):5227-36.

296. Wellems TE, Panton LJ, Gluzman IY, do Rosario VE, Gwadz RW, Walker-Jonah A, et al. Chloroquine resistance not linked to mdr-like genes in a plasmodium falciparum cross. Nature. 1990 May 17;345(6272):253-5.

297. Wellems TE, Walker-Jonah A, Panton LJ. Genetic mapping of the chloroquine-resistance locus on plasmodium falciparum chromosome 7. Proc Natl Acad Sci U S A. 1991 Apr 15;88(8):3382-6.

298. Werner RG, Thorpe LF, Reuter W, Nierhaus KH. Indolmycin inhibits prokaryotic tryptophanyl-tRNA ligase. Eur J Biochem. 1976 Sep;68(1):1-3.

299. Wesseling JG, Snijders PJ, van Someren P, Jansen J, Smits MA, Schoenmakers JG. Stage-specific expression and genomic organization of the actin genes of the malaria parasite plasmodium falciparum. Mol Biochem Parasitol. 1989 Jun 15;35(2):167-76.

300. White NJ. Primaquine to prevent transmission of falciparum malaria. Lancet Infect Dis. 2013 Feb;13(2):175-81.

301. White NJ, Pukrittayakamee S, Hien TT, Faiz MA, Mokuolu OA, Dondorp AM. Malaria. Lancet. 2013 Aug 15.

302. Wickens M, Anderson P, Jackson RJ. Life and death in the cytoplasm: Messages from the 3' end. Curr Opin Genet Dev. 1997 Apr;7(2):220-32.

303. Wilson CM, Serrano AE, Wasley A, Bogenschutz MP, Shankar AH, Wirth DF. Amplification of a gene related to mammalian mdr genes in drug-resistant plasmodium falciparum. Science. 1989 Jun 9;244(4909):1184-6.

304. Wilson CM, Serrano AE, Wasley A, Bogenschutz MP, Shankar AH, Wirth DF. Amplification of a gene related to mammalian mdr genes in drug-resistant plasmodium falciparum. Science. 1989 Jun 9;244(4909):1184-6.

305. Wilson DW, Langer C, Goodman CD, McFadden GI, Beeson JG. Defining the timing of action of antimalarial drugs against plasmodium falciparum. Antimicrob Agents Chemother. 2013 Mar;57(3):1455-67.

306. Wolfe CL, Warrington JA, Treadwell L, Norcum MT. A three-dimensional working model of the multienzyme complex of aminoacyl-tRNA synthetases based on electron microscopic placements of tRNA and proteins. J Biol Chem. 2005 Nov 18;280(46):38870-8.

307. Wong YY, Moon A, Duffin R, Barthet-Barateig A, Meijer HA, Clemens MJ, et al. Cordycepin inhibits protein synthesis and cell adhesion through effects on signal transduction. J Biol Chem. 2010 Jan 22;285(4):2610-21.

308. Woodrow CJ, Krishna S. Antimalarial drugs: Recent advances in molecular determinants of resistance and their clinical significance. Cell Mol Life Sci. 2006 Jul;63(14):1586-96.

309. Wu Y, Sifri CD, Lei HH, Su XZ, Wellems TE. Transfection of plasmodium falciparum within human red blood cells. Proc Natl Acad Sci U S A. 1995 Feb 14;92(4):973-7.

310. Xia Y, Cao K, Zhou Y, Alley MR, Rock F, Mohan M, et al. Synthesis and SAR of novel benzoxaboroles as a new class of beta-lactamase inhibitors. Bioorg Med Chem Lett. 2011 Apr 15;21(8):2533-6.

311. Yayon A, Cabantchik ZI, Ginsburg H. Identification of the acidic compartment of plasmodium falciparum-infected human erythrocytes as the target of the antimalarial drug chloroquine. EMBO J. 1984 Nov;3(11):2695-700.

312. Yayon A, Ginsburg H. Chloroquine inhibits the degradation of endocytic vesicles in human malaria parasites. Cell Biol Int Rep. 1983 Nov;7(11):895.

313. Yayon A, Vande Waa JA, Yayon M, Geary TG, Jensen JB. Stage-dependent effects of chloroquine on plasmodium falciparum in vitro. J Protozool. 1983 Nov;30(4):642-7.

314. Yeh E, DeRisi JL. Chemical rescue of malaria parasites lacking an apicoplast defines organelle function in blood-stage plasmodium falciparum. PLoS Biol. 2011 Aug;9(8):e1001138.

315. Zhang F, Du J, Wang Q, Hu Q, Zhang J, Ding D, et al. Discovery of N-(4-sulfamoylphenyl)thioureas as trypanosoma brucei leucyl-tRNA synthetase inhibitors. Org Biomol Chem. 2013 Aug 28;11(32):5310-24.

316. Zhang Y, Garcia MJ, Lathigra R, Allen B, Moreno C, van Embden JD, et al. Alterations in the superoxide dismutase gene of an isoniazid-resistant strain of mycobacterium tuberculosis. Infect Immun. 1992 Jun;60(6):2160-5.

317. Zhang YK, Plattner JJ, Freund YR, Easom EE, Zhou Y, Gut J, et al. Synthesis and structure-activity relationships of novel benzoxaboroles as a new class of antimalarial agents. Bioorg Med Chem Lett. 2011 Jan 15;21(2):644-51.

318. Zhao Y, Wang Q, Meng Q, Ding D, Yang H, Gao G, et al. Identification of trypanosoma brucei leucyl-tRNA synthetase inhibitors by pharmacophore- and docking-based virtual screening and synthesis. Bioorg Med Chem. 2012 Feb 1;20(3):1240-50.