


11-23-2016

Mitochondrial Heteroplasmy Contributes to the Dynamic Atovaquone Resistance Response in *Plasmodium falciparum*

Sasha Victoria Siegel

University of South Florida, svsiegel@gmail.com

Follow this and additional works at: <http://scholarcommons.usf.edu/etd>

 Part of the [Bioinformatics Commons](#), [Molecular Biology Commons](#), and the [Parasitology Commons](#)

Scholar Commons Citation

Siegel, Sasha Victoria, "Mitochondrial Heteroplasmy Contributes to the Dynamic Atovaquone Resistance Response in *Plasmodium falciparum*" (2016). *Graduate Theses and Dissertations*.
<http://scholarcommons.usf.edu/etd/6586>

This Dissertation is brought to you for free and open access by the Graduate School at Scholar Commons. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact scholarcommons@usf.edu.

Mitochondrial Heteroplasmy Contributes to the Dynamic Atovaquone Resistance
Response in *Plasmodium falciparum*

by

Sasha Victoria Siegel

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Molecular Medicine
College of Medicine
University of South Florida

Major Professor: Dennis E. Kyle, Ph.D.
John H. Adams, Ph.D.
Burt Anderson, Ph.D.
Rays Jiang, Ph.D.
Michael White, Ph.D.
Pradipsinh K. Rathod, Ph.D.

Date of Approval:
October 12, 2016

Keywords: Malaria, Electron Transport Chain, Drug Resistance

Copyright © 2016, Sasha Victoria Siegel

ACKNOWLEDGMENTS

I have many people to thank that helped me along the way; this project was truly an effort that spanned decades. First, and above all others, I would like to thank Dr. Dennis Kyle, for not only conducting the clinical trials and preliminary work that made my efforts possible, but even more for his unwavering support of me throughout my doctoral education at the University of South Florida. For anyone who knows him, he is passionate about science, parasitology, and the education of young scientists and I could not have asked for a better role model and mentor. My achievements to date were never without his guiding presence, and I will be forever grateful.

I would like to thank past and present members of the Kyle Lab for their contributions to this project. First, to Andrea Rivero-Bracho, a wonderfully talented student that has truly been a pleasure to teach over the last few years who contributed countless hours working by my side. Tina Mutka was instrumental in passing down so much knowledge and insight and helping generate preliminary data. I owe an incredible amount of gratitude to Swamy Rakesh-Adapa, Charley Wang and Dr. Rays Jiang, who elevated my project immeasurably. I would like to thank the Kyle Lab as a whole for providing feedback, suggestions, and moral support during my time here. My committee: John Adams, Rays Jiang, Burt Anderson, and Michael White deserve thanks for seeing me through my doctoral experience; their expertise and guidance has truly been invaluable.

Last, I need to thank all of my family and friends for trying to keep me positive throughout this process, it was a hard job! To Beatrice, Adonis, Debbie, Marvin, Vivian, Jenna, and again Andrea: you are truly life-long friends that I've come to depend on for over-estimating my intelligence and also keeping me sane every single day of this journey; I owe this dissertation to these daily lab warriors and co-conspirators. Finally, a thank you to the most important people in my life: to my dad, who was so confident I'd get my Ph.D. that he bought me post-it notes with a heading that said "Dr. Sasha V. Siegel" three years early, to my brother who always had way more faith in me than I had in myself, to my sister for always being there with a word of encouragement and food if that failed, and to Brian who learned how to cook just so I kept eating while this dissertation was being written.

TABLE OF CONTENTS

List of Tables	v
List of Figures	vi
Abstract	viii
Chapter 1: Introduction	1
Malaria: An Ancient and Persisting Global Health Problem	1
Discovery and Historical Overview	3
<i>Plasmodium</i> Life Cycle	4
<i>Plasmodium</i> in the Mosquito.....	5
<i>Plasmodium</i> in Humans	7
Clinical Signs, Symptoms, and Diagnosis	9
Uncomplicated Malaria	9
Severe Malaria.....	10
Current Control and Eradication Efforts.....	11
Prophylaxis	12
Treatment.....	14
Uncomplicated Malaria	14
Severe Malaria	15
Vaccines	15
Transmission-blocking Vaccines.....	17
Pre-erythrocytic Vaccines	18
Blood Stage Vaccines	20
Vector Control.....	20
Insecticide-treated Nets	21
Indoor Residual Spraying.....	22
Historical Antimalarial Drugs and Resistance.....	23
Arylamino Alcohols	24
Quinine.....	24
Mefloquine	25
Lumefantrine	25
Mechanism of Action/Resistance to Arylamino Alcohols	26
4-aminoquinolines.....	26
Chloroquine.....	26
Amodiaquine	29
Piperaquine.....	29
Mannich Bases	29
Pyronaridine	29

8-aminoquinolines.....	30
Primaquine.....	30
Antibiotics.....	31
Sesquiterpene Lactone Endoperoxides (Artemisinins).....	32
Artemisinin Resistance.....	32
2-hydroxynaphthoquinones	33
Atovaquone.....	34
Antifolates	36
Proguanil and Cycloguanil	36
Sulfadoxine and Pyrimethamine	37
Experimental Antimalarials in Clinical Development	38
Phase I Clinical Trials	38
P218.....	38
SJ733.....	38
MMV048.....	38
Phase II Clinical Trials	39
KAE609 + KAF156.....	39
OZ439 + Piperaquine/Ferroquine	39
Fosmidomycin + Piperaquine.....	39
DSM265	40
Methylene Blue	40
Arterolane + Piperaquine	41
Phase III Clinical Trials	41
Trimethoprim.....	41
Tafenoquine	42
<i>Plasmodium</i> Mitochondria	44
Metabolism.....	44
Structure	45
Genome	46
Mitochondrial Diversity, Replication, and Inheritance	47
Mitochondrial Electron Transport Chain	49
Enzymes and Their Functions	51
Type I NADH Dehydrogenase.....	52
Glycerol 1-Phosphate Dehydrogenase	52
Malate Quinone Oxidoreductase.....	52
Dihydroorotate Dehydrogenase	53
Succinate Dehydrogenase	53
Cytochrome <i>bc1</i> Complex.....	53
Cytochrome <i>c</i> Oxidase.....	55
ATP Synthase	55
The mtETC Inhibitors and Resistance	55
Atovaquone.....	55
Atovaquone Resistance	56
Myxothiazol and Antimycin A	58
HDQ	58
GSK93121A	58

ICI56,780 and P4Q-391	58
DSM1	59
Preliminary/Relevant Studies from the Kyle Laboratory	59
Phase II Clinical Trials of Atovaquone, Thailand (1991).....	59
Chemotype Screen Identifies Extreme Pan-Resistance to mtETC Inhibitors	60
Focus of Study.....	61
 Chapter Two: Phenotypic and Genotypic Characterization of Admission and Recrudescent Patient Isolates (Specific Aim 1)	62
Rationale of Study	62
Materials and Methods	65
Parasites and <i>In Vitro</i> Culture	65
<i>In Vitro</i> Drug Susceptibility Testing	65
Genomic DNA Sequencing.....	66
DNA Extraction from Parasites	66
Sequencing for SNPs in Mitochondrial Genes	66
Pyrosequencing of Cytochrome <i>b</i> Y268S Allele	68
Establishment of Pyrosequencing Y268S Assay Standard Curve.....	69
Phenotype/Genotype Stability Assessment of TM92-C1086.....	69
Results.....	73
Sequencing of Mitochondrial Genes in Patient Isolates	73
Establishment of a Y268S Pyrosequencing Assay.....	75
Extreme mtETC Resistance Parasites Gradually Lose Resistance	77
Pyrosequencing the Y268S Allele in Patient Isolates	80
Discussion	83
 Chapter Three: Induction of Atovaquone and DSM1 Resistance in Admission And Recrudescent Isolate Clones (Specific Aim 2)	86
Rationale of Study	86
Materials and Methods	87
Cloning by Limiting Dilution of Patient Isolates	87
Development of Resistant Parasite Lines to Atovaquone/Menoctone <i>in vitro</i>	88
Development of DSM1 Resistance <i>in vitro</i>	89
DHODH Copy Number Quantitative PCR (qPCR).....	89
Results.....	89
Parasites Develop Resistance to Atovaquone/Menoctone <i>in vitro</i>	89
Sequencing of Atovaquone/Menoctone Resistant Parasites Reveals Clinically Relevant Y268S Mutations.....	92
Pyrosequencing Confirms Y268S Populations in Resistant Parasites.....	94
C2A+10xATOV Parasites Display Variable Phenotypes	94
TM90-C2B Cannot Generate DSM1 Resistance at 10xEC ₅₀ Concentrations.....	97

Initial Characterization Shows Several Compensatory Mechanisms to DSM1 Pressure	98
Low-grade Resistance to DSM1 Causes Loss of Y268S Mutation in TM90-C2B	102
Discussion	104
Chapter Four: Genotypic Characterizations of Admission/Recrudescence Isolates and <i>in vitro</i> Drug Selected Parasites (Specific Aim 3)	107
Rationale of Study	107
Materials and Methods	108
Mitochondrial Deep-Sequencing of Patient Isolates and Drug Selections	108
Estimation of mtDNA Copy Number in MalariaGEN Pf3K Parasite Collection	109
Estimation of mtDNA, DHODH, and Y268S mt-Copy Number in Patient Isolates/Drug Selections	110
Worldwide Distribution of Y268S Cryptic Heteroplasmy	111
Results	112
Pyrosequencing Detects the Y268S Allele in Global Populations	112
The MalariaGEN Pf3K Project is Used to Estimate the Scope of Global Mitochondrial Diversity	116
Mitochondrial Deep-Sequencing of Patient Isolates/Drug Selections Shows Cryptic Y268S and Common Heteroplasmic Loci	122
Discussion	126
Chapter Five: Summary	136
Chapter Six: List of References	141
Appendix A: Publication Copyright	158

LIST OF TABLES

Table 1.1: Parasite History for Paired (Admission and Recrudescence) <i>P. falciparum</i> Isolates from Phase II Studies in Thailand	60
Table 1.2: Low, Moderate, and Extreme Resistance to mtETC Inhibitors in <i>P. falciparum</i>	61
Table 2.1: PCR Primers and Programs Used in mtETC Sequencing	70
Table 2.2: Sequencing of Candidate mtETC Resistance Genes in Patient Isolates of <i>P. falciparum</i>	73
Table 3.1: Genotypes of Atovaquone/Menoctone Drug Selections.....	92
Table 3.2: Resistance to mtETC Inhibitors in Atovaquone-Selected Populations of <i>P. falciparum</i>	93
Table 3.3: Resistance to mtETC Inhibitors in Menoctone-Selected Populations of <i>P. falciparum</i>	94
Table 3.4: Fluctuations of Moderate and Elevated mtETC Resistance in C2A+10xATOV-2B.....	96
Table 3.5: Summary of <i>P. falciparum</i> <i>in vitro</i> Drug Selections and Corresponding Mutations	96
Table 3.6: Genotypes of DSM1 Drug Selections Immediately Following Recovery	100
Table 3.7: Earliest Drug Susceptibility Profiles of DSM1-resistant <i>P. falciparum</i>	100
Table 3.8: Gradual pan-mtETC Elevated Resistance Decline in C2B+2xDSM1	103
Table 4.1: Mitochondrial Deep-Sequencing of Patient Isolates/Drug Selections	125

LIST OF FIGURES

Figure 1.1: Worldwide Map of Malaria Endemic Regions	2
Figure 1.2: Malaria Life Cycle in Mosquito and Human Hosts	5
Figure 1.3: Global Distribution of Dominant or Potentially Important Malaria Vectors	6
Figure 1.4: Asexual Stage Durations of <i>Plasmodium</i> Species Coincide with Febrile Spikes in Humans	10
Figure 1.5: Vaccines Strategies Attempt to Interrupt the <i>Plasmodium</i> Life Cycle	17
Figure 1.6: The Development of Anti-parasitic Naphthoquinones	35
Figure 1.7: Experimental Compound Structures in Clinical Development in 2015	43
Figure 1.8: Mitochondrial Electron Flow in <i>Plasmodium falciparum</i>	51
Figure 1.9: The <i>Plasmodium</i> Cytochrome <i>bc</i> ₁ Complex Reactions in the Q-cycle	54
Figure 2.1: The mtETC and Candidate Resistance Genes	74
Figure 2.2: Standard Curve of Y268S Cytochrome <i>b</i> Pyrosequencing Assay	76
Figure 2.3: TM90-C2B Retains Stable Y268S Genotype	79
Figure 2.4: TM92-C1086 Gradually Loses Y268S Genotype	80
Figure 2.5: Detection of Y268S Mutants in Patient Isolates	82
Figure 3.1: Schematic of Drug Selections Using Atovaquone/Menoctone <i>in vitro</i>	91
Figure 3.2: Pyrosequencing of Atovaquone/Menoctone-Selected Parasites	93
Figure 3.3: Summary of DSM1 Selections in Atovaquone Sensitive And Resistant Backgrounds	98

Figure 3.4: Pyrosequencing of DSM1-Selected Parasites Shows Y268S Heteroplasmy is Responsible for Combined Atovaquone/DSM1 Resistance-Spectrum Phenotypes	101
Figure 3.5: C2B+2xDSM1 Cultures Gradually Convert Their Heteroplasmic State Under Continuous DSM1 Pressure.	104
Figure 4.1: Pyrosequencing Detection of Cryptic Y268S Mutants in Africa	113
Figure 4.2: Pyrosequencing Detection of Cryptic Y268S Mutants in Brazil.....	114
Figure 4.3: Pyrosequencing Detection of Cryptic Y268S Mutants in S.E. Asia	115
Figure 4.4: Maximum Likelihood Estimation of Mitochondrial Heteroplasmy	118
Figure 4.5: Estimating the mtDNA Copy Number in the MalariaGEN Pf3K Project	119
Figure 4.6: Homoplasmy and Heteroplasmy Contribute to the Mitochondrial Diversity in a Single <i>Pf</i> -SNP	120
Figure 4.7: Both Homoplasmic and Heteroplasmic SNPs Vary by Region	121
Figure 4.8: Y268S Status of Patient Isolates/Drug Selections Using Mitochondrial Deep-Sequencing.....	124
Figure 4.9: Substoichiometric Shifting in Plants with Class 1 and Class 2 Sublimons	130

ABSTRACT

Of the considerable challenges researchers face in the control and elimination of malaria, the development of antimalarial drug resistance in parasite populations remains a significant hurdle to progress worldwide. Atovaquone is used in combination with proguanil (Malarone) as an antimalarial treatment in uncomplicated malaria, but is rendered ineffective by the rapid development of atovaquone resistance during treatment. Previous studies have established that *de novo* mutant parasites confer resistance to atovaquone with a substitution in amino acid 268 in the cytochrome *b* gene encoded by the parasite mitochondrial genome, yet much is still unknown about how this resistance develops, and whether parasites are inherently predisposed to resistance development. Here we report phenotypic characterization of isolates from patients that failed treatment in the original atovaquone Phase II studies in Thailand by using a diverse series of chemotypes that target mitochondrial functions. We defined their structure-activity relationships and observed broad resistance (5-30,000 fold in atovaquone), suggesting that cytochrome *b* mutations alone are not sufficient to explain this spectrum of resistance. We also report the first known *in vitro* selection that recapitulates the clinical Y268S mutation using the TM90-C2A genetic background, the pre-treatment parent for TM90-C2B. Selection of the Y268S mutation in TM90-C2A and others indicates that the parasite genetic background is critical in the selection of clinical atovaquone resistance, since selection attempts in multiple other genetic backgrounds

results in mutations at positions other than amino acid 268. We implicate mitochondrial heteroplasmy in the development of sporadic, rapid resistance to atovaquone, where pre-existing low-level mutations in the multi-copy mitochondrial DNA can be quickly selected for in parasite populations. High-coverage mitochondrial deep-sequencing data showed that low-level Y268S mutants were present in admission parasites from the atovaquone Phase II clinical trials in Thailand, and recrudescence parasites either maintained high level Y268S mutation frequencies or gradually returned to cryptic Y268S levels. The phenomenon of gradual heteroplasmic conversion back to wild-type was noted in some *ex vivo* patient isolated parasites as well as some *in vitro* selected lines, which suggests that other factors are at play that influence heteroplasmy stability. In addition to mitochondrial heteroplasmy, the total mtDNA copy number is likely influencing phenotypes in a gene dose-dependent fashion. Further, pressure on the DHODH enzyme that results in DHODH copy number amplifications/mutations has been shown to influence mitochondrial heteroplasmy directly. Last, mitochondrial diversity was shown to be vastly underestimated without heteroplasmic loci being taken into account, as seen in the re-analysis of the Pf3K MalariaGEN genome dataset we performed. The complex interactions between these drug resistance mechanisms reveal the phenotypic and genotypic plasticity that the *Plasmodium falciparum* parasite utilizes are a clear fitness advantage in the face of mitochondrial stress, and further studies are required to determine the impact of this wide-ranging phenotype on the development of new mitochondria-targeted drugs.

CHAPTER 1

INTRODUCTION

Malaria: An Ancient and Persisting Global Health Problem

Malaria remains a significant pathogen in the modern world, retaining its foothold that predates the evolutionary divergence of *Homo sapiens*, and has resulted in more deaths than any other infectious disease. Some estimate that half of all the people who ever lived were killed by this ancient pathogen [1, 2]. The evolutionary race between the *Plasmodium* species and humans can be seen in our genetic imprints, as evidenced by the *Plasmodium*-protective human adaptations such as sickle cell anemia, thalassemia, the altered red blood cell surface antigen expression in the case of *P. falciparum* erythrocytic membrane protein (*PfEMP1*), and erythrocytes that lack the Duffy antigen [3-5].

The malarial fever has been described by human societies as early as the fourth century B.C. that was attributed to spending time in swamps [6]. The gasses rising out of swamps, or miasmatas, were the original suspect of the cause of the intermittent malarial fevers known as 'rigors.' The word malaria translates directly from these early assumptions in Italian to mean 'bad air.' Today, malaria continues to cause substantial morbidity and mortality in South America, Africa, and Asia, caused by the obligate intracellular protozoan parasites of the *Plasmodium* genus. While there are six malaria parasites known to infect man (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale curtisi*, *P.*

ovale wallikeri, *P. cynomolgi*, and *P. knowlesi*), these represent a minority of the over 200 known species that infect diverse vertebrate hosts, such as other mammals, birds, and lizards [7, 8].

Roughly half the world's population, or 3.3 billion people, is at risk of getting malaria. In 2015, there were an estimated 214 million cases of malaria (range 149-303 million), and 306,000 deaths, with 78% of deaths being in children under 5 years old. About 90% of all deaths occur in Africa, and malaria is the fourth leading cause of death among children which accounts for 10% of child deaths [9]. The regions of endemicity are shown in Figure 1.1 [10].

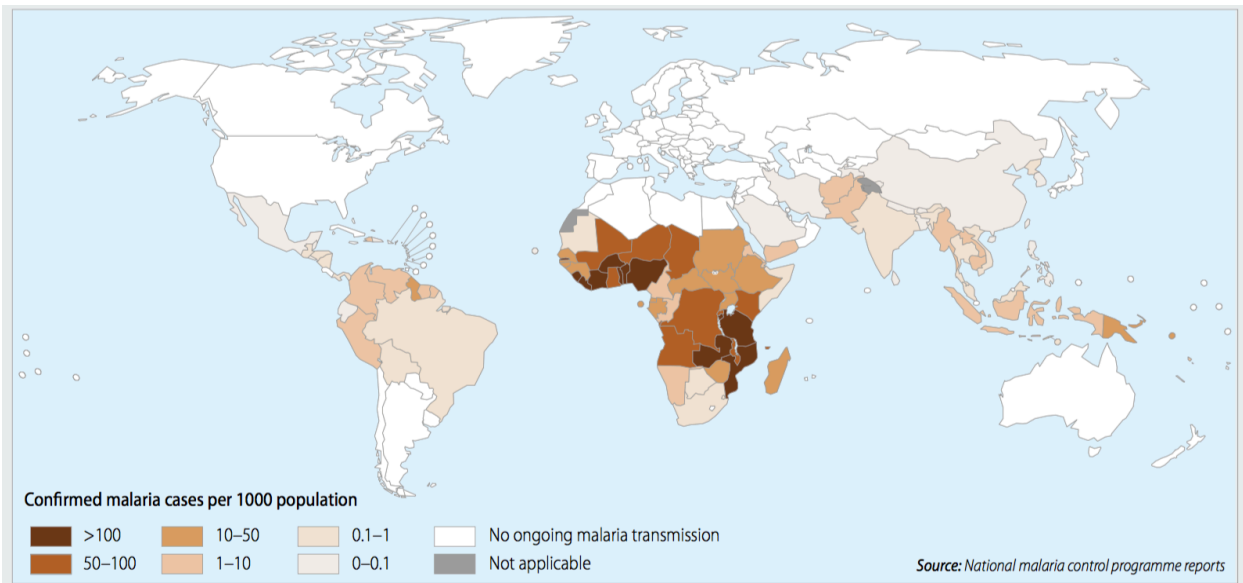


Figure 1.1. Worldwide Map of Malaria Endemic Regions, World Health Organization, *World Malaria Report 2014* [10]. Countries with ongoing transmission of malaria in 2013, where Africa contains 90% of worldwide cases, and Central and South America and Southeast Asia experiencing lower malaria incidence.

P. falciparum is dominant in Africa, and is by far more deadly. *P. vivax* is capable of developing in the *Anopheles* mosquito at lower temperatures and climates, as well as higher altitudes, which gives it a wider geographic range than *P. falciparum*. The burden of malaria disproportionately affects the poorest countries in the world, which have the highest risk of malaria and the least access to healthcare [9].

Discovery and Historical Overview

In the nineteenth century, it was suggested by Tomasi-Crudeli that malaria was caused by a bacterium, hence named *Bacillus malariae*. Johann Meckel first noted a sign indicating malaria infection in 1847 as a dark pigmentation present in the blood of malaria patients, now known as the refractive hemozoin pigment which is manufactured by the parasite and stored within the food vacuole. At this time, this pigmentation was incorrectly described as being produced by the body in response to malaria infection, not made by the parasite itself. Nearly 40 years later, Charles Laveran, a physician of the French Army, was credited with the discovery of the malaria parasite while seeing pigment in the spleens and erythrocytes of infected patients [11]. At about the same time as Laveran, a Russian physician named Vassily Danilewsky identified similar protozoan parasites in blood samples of both birds and reptiles [12]. With the development of the ability to stain the parasites within red blood cells with an eosin-based stain developed by Romanowsky in 1891, study of the malaria parasite was revolutionized because prior to that, full visualization of the parasite was not possible. In 1897, MacCallum was credited with observing the exflagellation of gametocytes into microgametes and ensuing macrogamete fertilization resulting in the formation of an

ookinete from the closely related *haemoproteus* parasite that infected the blood cells of crows [13].

Following this discovery, Laveran and Manson were the first to suggest that mosquitoes transmitted malaria, coming from evidence that mosquitos transmitted filarial worms as well. Upon Manson's suggestions, Ronald Ross, an army surgeon, carried out transmission experiments with *Plasmodium relictum* to demonstrate that the avian *Plasmodium* could be transmitted to chickens from mosquitoes. He observed rupture of parasites from the red blood cells, fertilization and development of the midgut oocyst wall, and sporozoites in the salivary glands of the mosquito. He then confirmed parallel results in humans, completing the life cycle of the *Plasmodium* parasite. In 1948, the liver was discovered to be the primary tissue tropism of asexual replication before release into circulation by Shortt and Garnham [11]. Nearly 100 years were required to elucidate the *Plasmodium* life cycle, which shows how far the malaria scientific community has come in the years following these discoveries.

Plasmodium Life Cycle

The life cycle of *Plasmodium falciparum* is shown in Figure 1.2 [14]. The life cycle is comprised of morphological forms that exist in the definitive host (female *Anopheles* spp.) in which the sporogonic cycle occurs, and in the human intermediate host where asexual reproduction in the liver hepatocytes occurs, followed by erythrocytic schizogony.

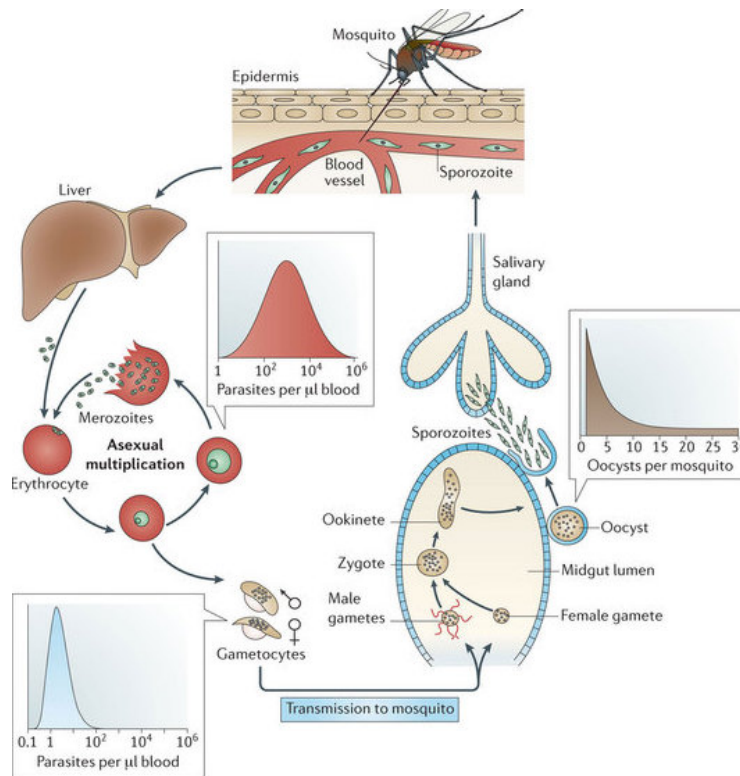


Figure 1.2. Malaria Life Cycle in Mosquito and Human Hosts, from Bousema *et al.* 2014 *Nature Reviews Microbiology*, 12(12): 833-40 [14]. Parasite densities at each successive stage are outlined in boxes. The transmission between mosquito and man represents a population bottleneck in the life cycle, where oocyst numbers per mosquito are low, typically > 5 . Another bottleneck occurs at the gametocyte stage, where gametocytes represent $> 1\%$ of parasites per microliter (μL) of blood compared to asexual forms.

***Plasmodium* in the Mosquito**

Anopheles mosquitoes are the definitive host of *Plasmodium* spp., and approximately 30-40 species are capable of transmitting malaria [15]. Among those, several species stand out in their ability to efficiently transmit *Plasmodium falciparum*, which are *Anopheles gambiae* and *Anopheles funestus*. The geographic distribution of mosquito species worldwide can be seen in Figure 1.3 [16]. The typical mosquito life span is 7-14 days. When a female *Anopheles* mosquito takes a blood meal on an

infected human and ingests gametocytes, the cycle begins. Male gametocytes exflagellate and fertilize female gametocytes, which then forms the zygote. Zygotes achieve motility and elongate themselves, becoming ookinetes, at which point they invade the midgut wall of the mosquito, converting to an oocyst. As the oocysts release sporozoites, they then migrate to the salivary glands of the mosquito, ready to be injected through the proboscis of the mosquito during the next blood meal. The entire process of development in the definitive host takes from 10-18 days [16].

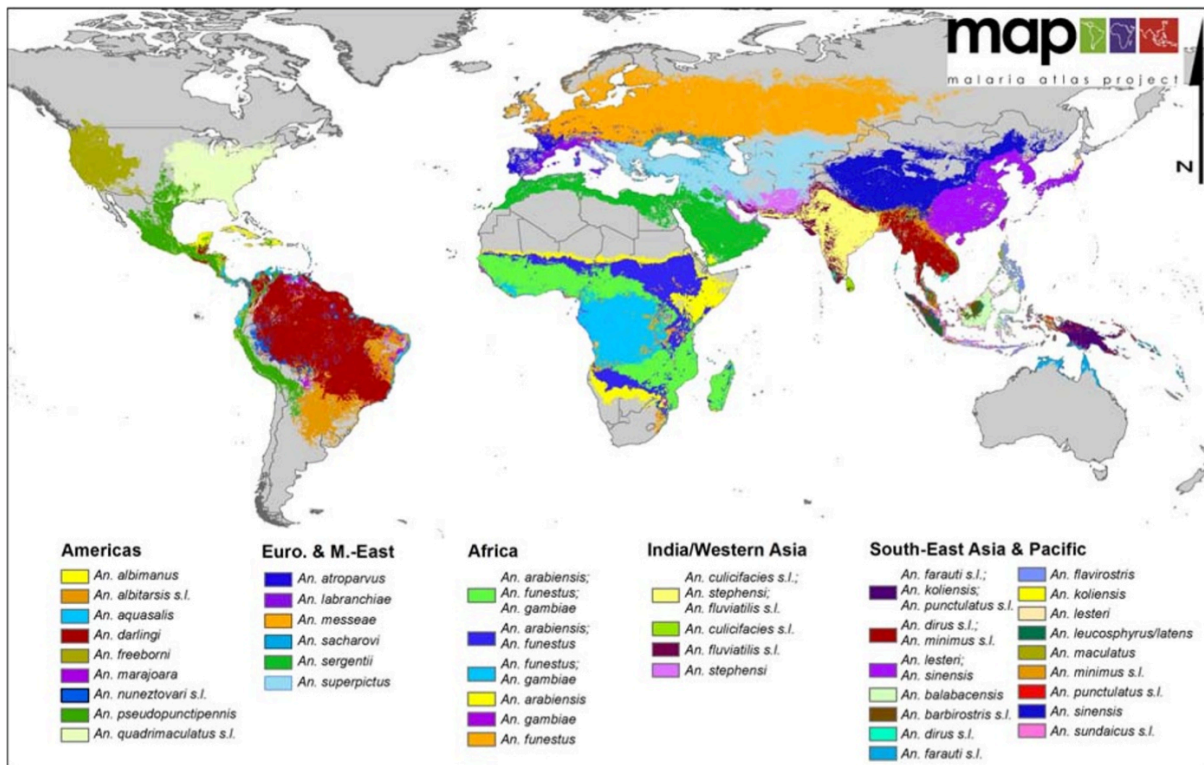


Figure 1.3. Global Distribution of Dominant Malaria Vectors, from Sinka *et al.* 2012, *Parasites & Vectors*, 5(1):69 [17]. This map was created using compiled occurrence data from 4800 sources to create this vector distribution which mapped the dominant *Anopheles* species. There were 34 dominant vectors worldwide, with the two most dominant species being *Anopheles gambiae* and *Anopheles funestus*.

***Plasmodium* in humans**

Once sporozoites have been injected into the skin of a human intermediate host by a female mosquito during a blood meal, approximately 100 sporozoites injected will begin to traverse through multiple cell types using gliding motility to reach circulation [18]. Once in circulation, the sporozoites eventually arrive at the liver, traversing liver sinusoidal epithelial cells and Kupffer cells, to eventually reside in hepatocytes during which they undergo multiple rounds of asexual replication. At this time, many rounds of asexual replication produce an estimated 30,000 merozoites produced per single sporozoite, and continues between 6-10 days [19].

Merozoites are subsequently released from the liver, where the invasion of erythrocytes begins. Merozoites bind to the surface of the erythrocyte via surface receptor-ligand interactions of several merozoite surface proteins (MSP family), apical membrane antigen-1 (AMA-1), and the erythrocyte-binding antigen EBA-175. At the apical end of the merozoite, a tight junction is formed which the parasite begins to squeeze through, forming a parasitophorous vacuole [19]. At the completion of invasion, the fledgling parasite progresses through ring stage for approximately 24 hours, followed by trophozoite stage (hours 24-36) and finally schizont stage (hours 40-48) [20].

The stages have a rough representation to the standard cell cycle format seen in other cell types, where the merozoite exists in a G_0 state, the main purpose being erythrocyte invasion. Once inside the erythrocyte, ring stage follows, which can be correlated to G_1 of the cell cycle, where the preparation for DNA replication and stockpiling of molecule precursors and RNA levels increase [20]. The red blood cell is

remodeled by the ring stage parasite, which involves the breakdown of hemoglobin with the aid of the plasmepsins and falcipain families of proteases in the digestive vacuole, obtaining amino acids in the process [21]. The parasites also intake and sequester the glucose and hypoxanthine needed for synthesis of purines and glycolysis during this stage [22].

Trophozoites represent the S phase during which DNA replication occurs. Further, the knob-associated proteins such as KARP and *PfEMP1* are shuttled to the surface of the red blood cell, which function to bind to the lining of the smooth endothelial cells of the blood vessels via interactions with ICAM-1 and others to sequester the trophozoite and schizont stages. This sequestration event prevents the more mature stages from being removed from circulation via splenic clearance mechanisms [23]. The parasite utilizes antigenic variation to disguise the highly immunogenic *PfEMP1* protein, which is known to have some 60 *var* genes that encode *PfEMP1* variants [24].

The segmentation of DNA brings the schizont stage of development, which can segment into as many as 30 daughter merozoites. The schizont stage concludes after nuclear segmentation and when the mitochondria have segmented as well, coupling each daughter nuclei with a single mitochondria and plastid directly prior to cytokinesis [20]. The mature merozoites then rupture from the host cells collectively in a synchronized fashion within the host, ready to invade new erythrocytes. The asymptomatic incubation period (time between infection and clinical presentation of symptoms) of infection is typically 9-14 days for *Plasmodium falciparum*. A small

proportion of newly ruptured merozoites in circulation differentiate into male and female gametocytes, typically induced by high parasitemias.

Clinical Signs, Symptoms, and Diagnosis

Uncomplicated Malaria

According to the World Health Organization, clinical presentation of uncomplicated malaria consists of fever, headache, chills, malaise, perspiration, abdominal discomfort, muscle and joint aches, anorexia, vomiting, and is diagnosed with an evaluation of patient blood smears looking for presence of parasites or a confirmation of infection using a rapid diagnostic test (RDT).

The periodic fever is caused by *Plasmodium* infection and is a direct effect of the asexual stages of the life cycle. Schizont rupture of infected erythrocytes coincides with expulsion of red blood cell debris, hemozoin, and toxins directly into the blood stream, causing an inflammatory immunological response that spikes fever [25]. The length of time between periodic fevers is dependent on the length of the asexual cycle duration, which is species specific. A fever that occurs every 48 hours is consistent with infections of *P. vivax*, *P. falciparum*, *P. cynomolgi* and *P. ovale*. Fevers occurring every 72 hours are associated with *P. malariae*, and *P. knowlesi* has an incredibly short 24-hour periodicity. Figure 1.4 shows the comparisons of parasite asexual cycle duration between various mammalian *Plasmodium* species [26].

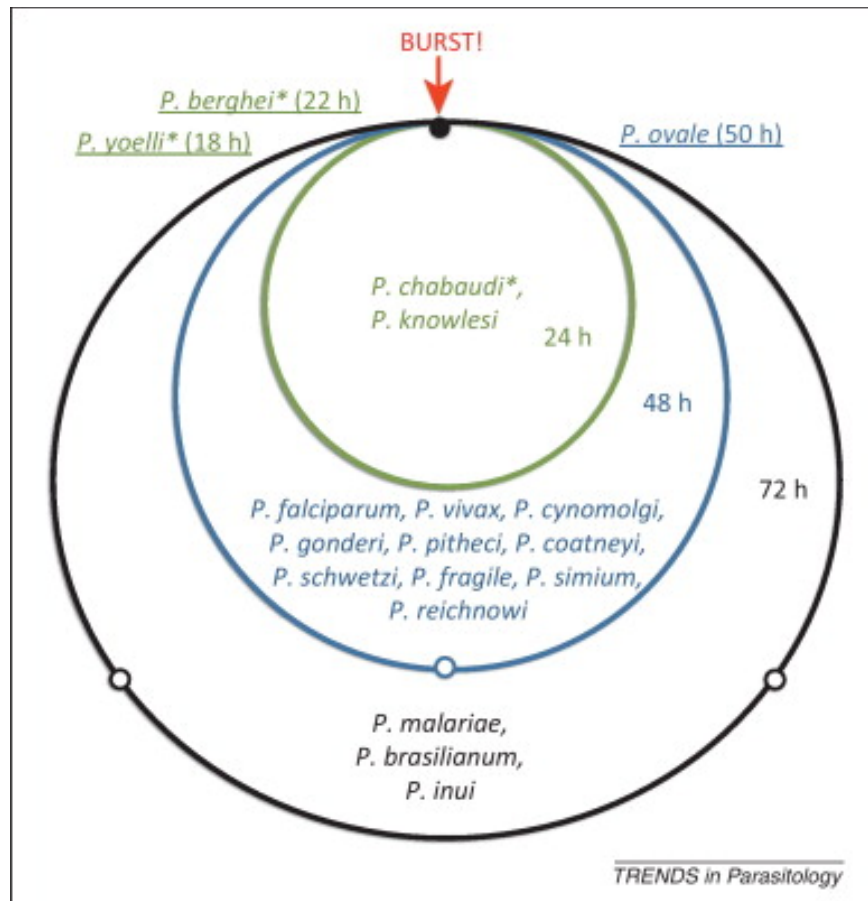


Figure 1.4. Asexual Stage Durations of *Plasmodium* Species Coincide with Febrile Spikes in Humans, from Mideo *et al.* 2012, *Trends in Parasitology*, 29(1) [26]. Circle size indicates the parasite cell cycle length in their mammalian hosts. Asterisks indicate rodent malaria species, and all others infect primates. Parasites underlined have non-24 hour cycles. The diversity of mammalian *Plasmodium* intraerythrocytic cycle duration coincides with the periodicity of febrile spike seen in the mammalian host.

Severe Malaria

If left untreated, severe *Plasmodium falciparum* malaria can progress with severe anemia, respiratory distress, or cerebral malaria, which is most commonly seen in *Plasmodium falciparum* infection. Severe malaria is indicated in patients that experience organ failure, and is accompanied by a high mortality rate between 10-20% with

effective antimalarial treatment and supportive care, but approaches 100% when untreated [27]. Signs of severe malaria as indicated by the World Health Organization include both clinical and laboratory features. Clinical signs include impaired consciousness (coma), prostration, convulsions, respiratory distress, significant bleeding, shock, and pulmonary edema. Laboratory signs are severe anemia, hypoglycemia, jaundice, renal impairment, hemoglobinuria, metabolic acidosis, and hyperparasitemia.

The majority of severe malaria cases are those of cerebral malaria caused by *Plasmodium falciparum*, which account for about 10% of hospitalizations, and 80% of malaria deaths. The definition of severe malaria is broad, and some symptoms are associated with lower mortality rates (severe anemia, for example) and others with subsequently higher mortality risk, such as metabolic acidosis. The risk of death magnifies with the presentation of multiple complications. Pregnant women in their second and third trimesters are more likely to have severe malaria than other adults, with a maternal mortality of 50%. Premature labor and fetal death are common outcomes [27].

Current Control and Eradication Efforts

Malaria eradication is a goal to which the global malaria research community aspires, and renewed vigor comes in large part from the Bill and Melinda Gates Foundation, who classified several priority aims to march toward eradication in 2007. With their desire to fund projects in transmission blocking, chemoprotection, and new drug development, the landscape of malaria research has transformed. Several new

promising classes of drugs have been discovered because of massive collaborative efforts to speed up the malaria drug discovery pipeline. Malaria eradication is possible if: i) new drugs are consistently in development to combat resistance, ii) funding is maintained for the public health focus in malarious countries, iii) insecticides, larvicides and insecticide treated bed nets are used extensively, and iv) drugs for chemoprotection of vulnerable populations are administered. Many additional goals for eradication seem critical for success as well, such as the desire for a drug that can provide a single-dose radical cure (includes transmission blocking ability), which would eliminate the problem of patient compliance to finish drug regimens that last multiple days. Further, those that have long *in vivo* half-lives are attractive, since they would be incredibly beneficial to help patients stay protected longer post-treatment in high-transmission areas, who can be bitten by mosquitoes on a nightly basis. New chemopreventative drugs are also badly needed, since many drugs are unsafe for pregnant women, and the standard seasonally distributed sulfadoxine-pyrimethamine chemoprevention is experiencing failure due to resistance [28]. Chemoprotection has been an incredibly successful campaign in Africa, with monthly chemoprotection during the four months of seasonal malaria costing only US\$0.60 per child [29].

Prophylaxis

Malaria can be prevented by taking causal prophylactic drugs that target liver stage parasites (e.g. malarone and primaquine), which means they only need to be taken while in malaria endemic areas of the world. Suppressive prophylactic drugs that singularly kill blood stages are a less convenient approach that requires treatment for at least four weeks after leaving an endemic area to clear any possible parasites emerging

from the liver into circulation. Several prophylactic drugs are indicated for non-immune travelers to malaria endemic areas of the world by the Centers for Disease Control and Prevention [30], which are atovaquone/proguanil (Malarone®), chloroquine, doxycycline, mefloquine, and primaquine. Malarone, doxycycline and primaquine are contraindicated for pregnant women. Chloroquine and mefloquine have widespread resistance, reducing their efficacy. Finally, doxycycline, mefloquine and primaquine are all known to have serious side effects, with primaquine possibly causing life-threatening hemolysis in people with glucose-6-phosphatase dehydrogenase deficiency (G6PD), and mefloquine causing severe psychiatric events [30].

Intermittent preventative treatment (IPT) is used in endemic areas of Africa for women in their first or second pregnancy, given once a month in the form of sulfadoxine/pyrimethamine tablets (SP). SP administration during pregnancy was shown to increase mean birth weight by about 56 grams, reduced low-birth weight infants by 20%, lower placental parasitemias by 50%, and maternal parasitemias by 33%. Infants under one year of age in moderate-to-high transmission areas of Africa are recommended to receive SP along with standard vaccination schedules, which provides protection for approximately 35 days following administration. Administration was shown to have overall 30% protection in the first year of life, 21.3% protection against anemia, and 22.9% protection in malaria related hospitalization. Children under the age of six years of age in regions with seasonal transmission are recommended to receive amodiaquine + SP monthly during transmission season. Trials indicated that administration prevented 75% of malaria episodes [27].

Treatment

Uncomplicated malaria. Currently for the treatment of uncomplicated malaria, the front line therapy is an ACT, or artemisinin combination therapy. The five ACTs with recommended dosing in mg/kg (milligrams per kilogram) of body weight per day recommended by the World Health Organization are: artemether + lumefantrine (5-24 mg/kg, 29-144 mg/kg, 2x daily for 3 days), artesunate + amodiaquine (2-10 mg/kg, 7.5-15 mg/kg, 1x daily for 3 days), artesunate + mefloquine (2-10 mg/kg, 5-11 mg/kg, 1x daily for 3 days), artesunate + SP (2-10 mg/kg 1x daily for 3 days, 25-70 mg/kg + 1.25-3.5 mg/kg single administration on day 1), and dihydroartemisinin + piperaquine (2-10 mg/kg, 16-27 mg/kg 1x daily for 3 days). In the first trimester of pregnancy, women are treated with 7 days of quinine + clindamycin, and women in the second and third trimester are given standard ACT treatment.

These combinations of drugs were chosen to pair a short acting, but potent artemisinin derivative, with a long half-life drug. These combinations are all recommended for three days of treatment [27]. Unfortunately, ACTs have begun to fail clinically, and longer courses of treatment or new partner medicines (e.g. artesunate + pyronaridine) are being evaluated. Single dose primaquine is strongly recommended as an addition to treatment regimens in areas with established artemisinin resistance. In low transmission settings, an additional recommendation of a single dose of primaquine (0.25 mg base/kg of body weight) is used to reduce transmission as part of a pre-elimination or elimination program. This dose is unlikely to cause significant complications regardless of G6PD status, therefore requires no G6PD testing, and is aimed at acting as a gametocytocide.

Recommendations for *P. ovale*, *P. vivax*, *P. malariae*, or *P. knowlesi* have the same recommendations of ACT treatment, with the exception of artesunate + SP, where resistance to SP compromises the efficacy significantly. To prevent relapse of infection from dormant liver hypnozoites with *P. vivax* and *P. ovale*, an additional recommendation is to give a 14-day course of primaquine in G6PD normal individuals (0.25-0.5 mg/kg body weight per day). The rate of relapse in *P. vivax* infections varies geographically, between 8-80%, making the administration of primaquine of vital importance in the overall treatment regimen of *P. vivax* [27].

Severe malaria. For the treatment of severe malaria, adults, children, and pregnant women are given either intravenous or intramuscular artesunate for at least 24 hours until they are able to tolerate oral medication, at which point a complete three-day course of an ACT is given, with added single dose primaquine in areas of low transmission. Children weighing less than 20 kg are recommended to receive a higher parenteral dose of artesunate (3 mg/kg/dose) compared with larger children or adults (2.4 mg/kg/dose) to optimize optimal drug exposure. With severe malaria cases in regions where artemisinin resistance has been established, parenteral artesunate and quinine are to be given together in full doses. Substantial supportive care is required in instances of severe malaria, and treatment is dependent on the complications that present themselves [27].

Vaccines

If malaria is to be eliminated around the world, an effective vaccine will likely have to be part of a successful elimination effort. Significant progress has been made in the development of vaccines in recent years, with three candidate vaccines in Phase 2B

clinical trials, one that has recently completed Phase III clinical trials, and 25 potential total projects in development [31]. There are several challenges to the development of a malaria vaccine capable of providing sterilizing immunity to patients, many of which are a result of the highly-adapted evasive responses the parasite utilizes to conceal itself from host immune responses. First, malaria is unlike many other infectious diseases that have successful vaccines; people who get malaria do not acquire immunity from the disease after infection and recovery. While in many instances, the severity of disease is lessened by previous exposures to the malaria parasite, this partial immunity is not completely protective. Second, there are ten different transitions in morphology throughout the two host life cycle that are present in five different tissue types, making the development of a single vaccine capable of inhibiting all the parasitic forms difficult. However, hope for an efficacious vaccine is seen in studies with mice that show sterile protective immunity after being introduced to attenuated sporozoite injections of *P. berghei* [32, 33].

Therefore, a perfect malaria vaccine would accomplish three key goals: generate a robust immune response to multiple stages in *Plasmodium* development, overcome antigenic variation, and induce both cell-mediated and humoral immune responses in the host. There are three main types of vaccine approaches being attempted that focus on different stages of *Plasmodium* development: transmission-blocking vaccines, pre-erythrocytic vaccines, and blood stage vaccines. Figure 1.5 shows where each vaccine type attempts to intervene in the *Plasmodium* life cycle [34].

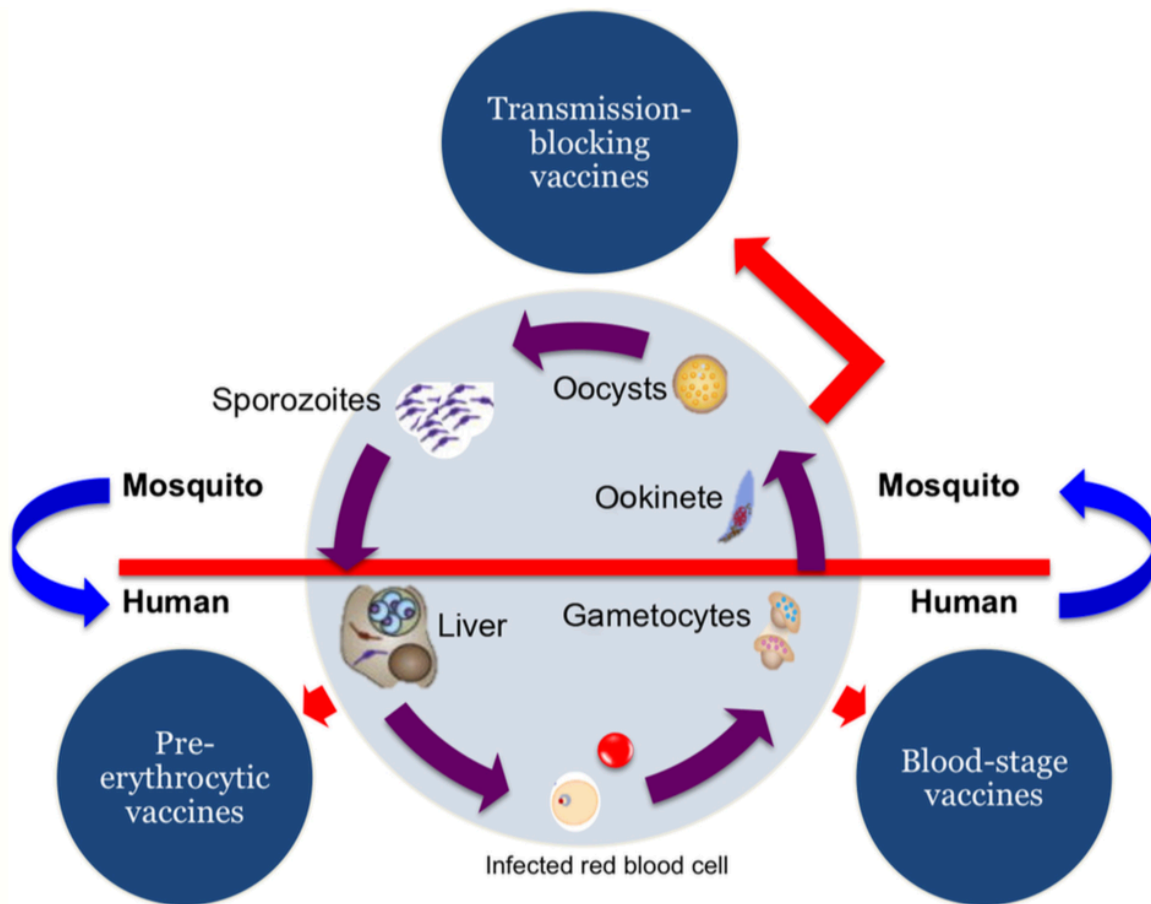


Figure 1.5. Vaccines Strategies Attempt to Interrupt the *Plasmodium* Life Cycle, from Arama *et al.* 2014, *Journal of Internal Medicine*, 275(5): 456-66 [34]. Target sites of the malaria life cycle that have vaccine potential, which occur at population bottlenecks. The three vaccine strategies are shown in blue circles: transmission blocking vaccines, blood-stage vaccines, and pre-erythrocytic vaccines.

Transmission-blocking Vaccines. Transmission-blocking vaccines are designed to train the host immune response to develop antibodies against antigens present on gametes, zygotes, and ookinetes, which would prevent the ookinete-oocyst

transformation. This strategy subsequently prevents the formation of viable sporozoites. While this strategy does not prevent humans from getting malaria, it does prevent the infected individual from transmitting malaria to others, therefore providing protection to the community. The vaccine furthest in development for transmission blocking is *Pfs25/Pfs28*, which is in Phase I trials. The *Pfs25/Pfs28* vaccine uses a nanoparticle delivery technique to target the *P. falciparum* ookinete surface antigens Pfs25 and Pfs28, as well as the *P. vivax* homologues of those antigens [34].

Pre-erythrocytic Vaccines. The pre-erythrocytic vaccine strategy targets the liver stage to prevent malaria in the human host, and occurs at a biological choke point in parasite development that would disrupt the *Plasmodium* life cycle before the parasite could propagate in circulation. Pre-erythrocytic vaccines include those using attenuated sporozoite preparations, as well as truncated circumsporozoite protein (CSp). The most developed vaccine in this category is Glaxo-Smith Kline's RTS,S vaccine, which had Phase III clinical trial results released in 2014. The RTS,S vaccine, known commercially as Mosquirix®, utilizes a formulation of CSp that is fused to the highly immunogenic hepatitis B surface antigen. The mechanism of protection in this vaccine is not well understood, but it is proposed to reduce the parasite load of newly emerged merozoites, which may in turn provide time for the immune system to obtain naturally acquired immunity against blood stages. RTS,S showed modest protection in children first vaccinated between 5-17 months old with 36% protection against clinical malaria, and 32% protection against severe malaria. In the younger age group that received the first vaccination at 6-12 weeks of age, there was an overall 26% protection against clinical malaria, but no protection against severe malaria over the course of the 38-month study

[35]. The World Health Organization is set to review what, if any, policy recommendations will be made regarding implementation of this vaccine, and will be released as early as 2015. The *PfSPZ* vaccine candidate, another promising pre-erythrocytic vaccine manufactured by Sanaria, induces immunity using *P. falciparum* sporozoites in various preparations. The advantage with this approach is that no target has to be identified, and allows a complete immune response to develop in the host. The *PfSPZ* vaccine has been shown to provide complete protection against a controlled human malaria infection when 6.75×10^5 sporozoites were given by IV injection [36]. The vaccine has several additional trials planned for 2015-2016, with objectives aimed at testing the vaccine in elderly and HIV positive groups, as well as malaria-exposed and malaria-naïve subjects 6 months and older [35].

Because the use of irradiated sporozoites has the risk of potential malaria infection if batches of sporozoites are improperly irradiated, the use of genetically attenuated sporozoites for vaccination are increasingly attractive options [37]. Another alternative uses wild-type sporozoites co-administered with azithromycin, which has shown superior protection compared to the irradiated sporozoite preparation. Major challenges still face this approach, since the large-scale production of sporozoites (either irradiated, genetically attenuated, or wild-type preparations) will be costly and labor intensive, since mosquito rearing and dissection is required to obtain sporozoites. Further, direct venous inoculation is required, so obtaining large amounts of safe, sterile, and stable sporozoites will be a necessity to bring this vaccine through clinical trials and into the global malaria eradication arsenal.

Blood Stage Vaccines. Blood stage vaccines attempt to intervene in the malaria cycle by blocking merozoites from erythrocyte invasion. There are several blood stage erythrocyte surface antigens that are being evaluated as potential vaccine candidates in clinical trials: AMA1, EBA-175, MSP1, MSP2, MSP3, GLURP, and SERA5 [38-44].

Other vaccine candidates of this type that show particularly promising immunogenicity include the rhoptry-associated leucine zipper-like protein 1 (RALP1), and the *Pf* reticulocyte-binding protein homologue 5 (*PF*RH5) [34]. In order for surface antigen-based vaccines to be a realistic option, researchers will have to address the polymorphic nature of these antigens and overcome the challenges that genetic diversity presents to vaccine efficacy. Studies on AMA1 and MSP1 demonstrated lack of efficacy in African parasite populations, which shows the need to diversify this vaccine strategy for ‘vaccine resistant’ malaria [41]. Addressing these hurdles will likely involve the combination of multiple antigens to develop an additive or synergistic immune response.

Vector Control

The goal of vector control is to reduce transmission of infection from the mosquito to human, and from human to mosquito. Historically, vector control is the most effective weapon against malaria; countries that succeeded in malaria elimination achieved it largely with the power of larvicides and pesticides such as Paris Green and DDT (dichlorodiphenyltrichloroethane), although systematically eliminating standing water sources where female mosquitoes lay eggs proved critical as well [45, 46]. The primary method of vector control is the widespread use of insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRS). These two interventions combined

represent nearly 60% of global funding toward malaria control, with approximately 300 million long-lasting insecticidal nets (LLINs) distributed in Africa between 2008-2010 alone. Further, approximately 185 million people were protected by IRS in Africa in 2010, indicating that aggressive vector control efforts are making a substantial impact in these endemic areas [47].

While the use of larvicides and pesticides had a very successful campaign against malaria, their potential toxicity became a serious global concern. DDT use was largely discontinued after being classified as a persistent organic pollutant in the 2001 Stockholm Convention based on concerns that chemical residues of DDT are stored in fat deposits and vital organs, as well as ecological concerns of the harm to wildlife. In spite of the concerns related to DDT use and the development of DDT resistance, in 2006 the World Health Organization continued to recommend the use of DDT in regions of the world where the mosquito remains DDT-susceptible [48].

Insecticide-treated Nets. The use of ITNs was based on the suggestion of Ronald Ross in 1910 when he thought nets would provide a physical barrier between the nighttime feeding behaviors of mosquitoes and sleeping humans to reduce malaria transmission. Today, ITNs are used to not only create that physical barrier that Ross described, but also to kill the mosquitoes that come to rest on the insecticide-impregnated nets. Pyrethroids are the insecticides of choice for use on bed nets, chosen for their comparatively low toxicity to humans and long-lasting residual activity. In comparison with conventional untreated nets, ITNs are twice as effective in malaria prevention [49]. Net washing and lack of retreatment every six months compromise ITN efficacy; long-lasting insecticidal nets (LLINs) have largely replaced them. LLINs are

typically polyester nets that have insecticide resin bound to the fibers, or polyethylene/polypropylene that incorporate insecticide into the fibers directly. These can withstand repeated washing (> 20) and last several years. When compared directly in tests of mosquito mortality, LLINs were able to kill > 80% of mosquitoes that landed on them after up to 20 hand washes, in comparison to a conventionally treated ITN, which only had comparable mortality for three washes [50]. LLINs have shown strong reductions in mosquito densities, with a demonstrated 79% reduction in *A. gambiae* and 38% in *A. arabiensis* in studies in Tanzania. In Kenya, reports show 40-48% reductions in children under six, and 78% reduction in all age groups in Zanzibar [48]. The World Health Organization gives full recommendations for the use of Duranet® (alpha-cypermethrin incorporated into polyethylene), Interceptor® (alpha-cypermethrin incorporated into polyethylene), MAGNet® (alpha-cypermethrin incorporated into polyethylene), Olyset Net® (permethrin incorporated into polyethylene), PermaNet® 2.0 (deltamethrin coated on polyester), Royal Sentry® (alpha-cypermethrin incorporated into polyethylene) and Yorkool® LN (deltamethrin coated on polyester) although there are several others that only have interim recommendations as well [51].

Indoor Residual Spraying. IRS refers to the application of long-lasting insecticide residue to interior walls of domestic structures with the intention of vector contact with residual insecticide. When a female mosquito is seeking a blood meal, she often exhibits endophilic (indoor resting) behaviors, and tends to reside on walls and ceilings of homes in her search. When the mosquito lands on surfaces covered with residual insecticide, it absorbs the lethal insecticide, killing the mosquito and therefore reducing the transmission of malaria. This type of control method is dependent on the

majority (over 85%) of dwellings to have received residual spraying, otherwise there is minimal protection obtained for an individual home. According to the World Health Organization, IRS is appropriate in regions where the vector *i*) primarily feeds and rests indoors, *ii*) are insecticide susceptible, *iii*) have a malaria transmission pattern that can be covered by one or two IRS applications per year, *iv*) have structures that suit spraying adequately, and *v*) have dwellings that are condensed in an appropriately small area. The WHO-recommended insecticides for IRS against malaria vectors based on human safety and efficacy, and include the following chemical classes: carbamates (bendiocarb, propoxur), organochlorines (DDT), organophosphates (malathion, fenitrothion, pirimiphos-methyl), and the pyrethroids (alpha-cypermethrin, deltamethrin, lambda-cyhalothrin, etofenprox, bifenthrin, cyfluthrin) [47].

Historical Antimalarial Drugs and Resistance

Quinoline-containing antimalarials are the foundation of the historical fight against malaria, and are suspected to disrupt hemoglobin digestion in the erythrocytic stage of parasite development. As the parasite degrades hemoglobin within the food vacuole, byproducts in the form of reactive oxygen radicals and free heme, both of which are rendered inert by the parasite via heme polymerization and antioxidant mechanisms, respectively. The quinoline-containing antimalarials can be separated into three main groups: the arylamino alcohols (quinine, mefloquine, halofantrine, lumefantrine), 4-aminoquinolines (chloroquine, amodiaquine, piperaquine), Mannich bases (pyronaridine) and the 8-aminoquinolines (primaquine).

Arylamino Alcohols

Quinine. The first of the quinolone antimalarials was quinine, isolated from the chinchona tree, a South American native. The chinchona tree was given its name according to the legend of the Countess of Chinchon, who was cured of malaria in 1630 using the crude powdered bark of the “fever tree.” This crude preparation was distributed throughout Europe in the 17th century brought by Jesuits and the isolation of quinine came from Pelletier and Caventou in 1820; the synthesis of quinine was not established until 1944 by Woodward and von Doering [52]. The complexity of quinine synthesis prevented commercial production. Following WWII, quinine was widely discontinued in favor of chloroquine and pyrimethamine for routine malaria treatment and prophylaxis due to toxicity concerns, despite the fact that it is still used today for multi-drug resistant malaria infections and severe malaria in situations where an ACT is unavailable. The side effects of quinine are observed in nearly all patients, and are collectively known as chinchonism (hearing impairment, nausea, headache, blurred vision, tinnitus, and dysphoria). Because quinine use was not used continuously to treat malaria, resistance to quinine developed slowly [53-57]. Resistance was first reported in Brazil, soon after in Southeast Asia, and is linked to polymorphisms in several transporter genes, including *pfmdr1*, *pfcr1*, *pfmrp1*, and possibly even *pfhhe1*, a sodium proton exchanger [58]. *Pfmdr1*, a multidrug resistance gene that encodes the *Plasmodium* P-glycoprotein homolog, is implicated in the development of drug resistance for several antimalarials. The function of *PfMDR1* is not known, but it is thought to act as a drug transporter; the protein localizes to the digestive vacuole membrane [59]. As a whole, changes in *pfmdr* sequence or copy number modify the

transport of several drugs, with polymorphisms that mediate opposite effects on different drugs. Additionally, *pfmdr* gene amplification has been shown to decrease sensitivity to quinine [60]. In allelic exchange experiments with *pfhhe1*, some strains, but not all were shown to return some quinine sensitivity [58]. The *Plasmodium falciparum* multidrug resistance protein-1 (*PfMRP1*) is an ABC transporter that has developed mutations conferring resistance to both quinine and chloroquine [61].

Mefloquine. Mefloquine is post-WWII era antimalarial developed at the Walter Reed Army Institute of Research, which was developed along with many other structural analogs of quinine in response to challenges with malaria during the Vietnam war and began therapeutic use in 1985. Mefloquine shows activity in chloroquine-resistant parasites, and has been used extensively despite severe neuropsychiatric side effects, including depression, panic attacks, insomnia, hallucinations, anxiety, and suicidal ideation. Mefloquine was used for nearly 40 years, particularly on chloroquine-resistant populations, but has decreased in recent years because of resistance and serious side effects [62]. Mefloquine was used extensively in Asia, and resistance developed accordingly. Because of the widespread development of mefloquine resistance, it is given only as part of an ACT in combination with artesunate.

Lumefantrine. Lumefantrine was developed by the Chinese in the 1970's at the Academy of Military Sciences, and is structurally related to halofantrine, but slightly less effective. Absorption is varied with this very lipophilic drug, and approaches consistency when taken with a fatty meal. Lumefantrine separates itself from halofantrine with a lack of cardiac complications, and has shown synergism with artemether, of which it is currently used in combination. The ACT combination artemether-lumefantrine is known

to select for the N86 and D1246 wild type alleles in the *pfmdr1* gene, causing modest increases in sensitivity to chloroquine and amodiaquine [63].

Mechanism of Action/Resistance to Arylamino Alcohols. The mechanism of action for the arylamino alcohols is not precisely known but it is thought to be distinct from the 4-aminoquinolines. The arylamino alcohols are presumed to work in an antagonistic fashion to the 4-aminoquinolines by preventing Ca^{2+} release, which would subsequently shut down vesicle-digestive vacuole fusion needed to transport hemoglobin [64]. More is known about the mechanism of resistance to the arylamino alcohols, but is still a matter of serious debate. *PfMDR1*, a membrane transport protein analog of ABC multidrug resistance transporters in mammals, contributes to parasite resistance in the arylamino alcohols quinine, mefloquine, halofantrine and lumefantrine as well as chloroquine and artemisinin [65, 66]. An increase in wild-type *pfmdr1* copies accompanies a 2-4 fold decreased susceptibility to these compounds, and shows a positive correlation with treatment failure risk for mefloquine monotherapy as well as mefloquine + artesunate combination therapy [60, 65-71].

4-aminoquinolines

Chloroquine. German scientists from Bayer laboratories who developed primaquine were also responsible for the development of an acridine derivative called quinacrine, also named atebrine and mepacrine. Allied scientists successfully synthesized quinacrine with the aid of German patent information, and quinacrine was distributed widely to allied forces in the Pacific, despite the characteristic yellowing of the skin that the drug caused. In the German surrender of Tunis in 1943, the allied forces obtained German research of a compound called resochin, a 4-aminoquinoline

that was renamed chloroquine (CQ), which is the single most successful antimalarial drug in the history of malaria [72]. Chloroquine is a safe and affordable dibasic compound that is known to accumulate significantly in the parasite's food vacuole, killing with a mechanism of assisted-suicide, where the parasite can no longer sufficiently detoxify radicals and polymerize free heme, becoming a victim of its own waste products.

Chloroquine remained effective until the 1960's, when misuse/overuse of the drug contributed to the development of resistance independently in four different areas of the world, with the vast majority of field isolates being CQ-resistant today [73]. Considerable blame for CQ resistance is shouldered by the inclusion of CQ in table salt, providing low-dose CQ that established suboptimal dosing environments for rapid CQ resistance to develop and spread [74]. In areas where CQ was abandoned, there have been indications of parasites returning to CQ-sensitivity [75]. The CQ treatment failure rate can be drastically reduced with twice per day administration by raising plasma levels, which retains activity against resistant parasites. However, this is complicated by the fact that the therapeutic window for CQ is quite narrow, with 10 mg/kg b.w. being therapeutic, 20 mg/kg being toxic, and 30 mg/kg being lethal [76].

The molecular mechanism of CQ resistance involves mutations in the chloroquine resistance transporter protein (*PfCRT*), which was identified by progeny analysis in a genetic cross between a CQ-resistant and sensitive strain [77, 78]. *PfCRT* is a membrane-embedded protein that resides in the membrane of the parasite food vacuole. All CQ-resistant parasites have the K76T mutation in the *pfcr*t gene, which is thought to allow CQ binding, therefore permitting CQ to be pumped out of the food

vacuole so that it cannot accumulate [79]. There are several other mutations that accompany K76T to restore viability to *PfCRT*'s native function and improve fitness, which may be used for small peptide/amino acid transport from the food vacuole to the cytoplasm [80]. The exact function of *PfCRT* is unknown, but it is essential, considering gene disruption attempts proved unsuccessful [81]. It is important to note that CQ-resistant strains under pressure with halofantrine have resulted in a S163R *pfcr*t mutation that induces halofantrine resistance and restores CQ-sensitivity, presumably from the restoration of positive charge in *PfCRT* [82, 83]. This finding is in concurrence with the return of CQ-sensitivity using verapamil exposure [82, 83]. The K76T mutation confers resistance to both CQ and amodiaquine, and increased sensitivity to mefloquine, suggesting a reciprocal resistance/sensitivity relationship between CQ-like drugs and the arylamino alcohols, which is also seen in the *pfmdr1* relationship as well [84].

Mutations at N86Y and D1246Y in the *pfmdr1* gene accompany the K76T *pfcr*t mutation, and leads to the increased sensitivity with mefloquine, halofantrine, lumefantrine and dihydroartemisinin [65, 66]. Additionally, *pfmdr1* mutations S1034C and N1042D occur in CQ-resistant strains of South American origin, all of which result in modest increased sensitivity to the arylamino alcohols [85]. It is thought that *pfmdr1* mutations in CQ-resistant parasites compensate for the decrease in fitness caused by mutations in *pfcr*t [86].

Mutations in *pfmrp1* have indicated reduced susceptibilities to chloroquine, mefloquine, pyronaridine and lumefantrine in parasites from Myanmar, yet it is unclear what role *pfmrp1* has in resistance [87]. In genetic disruption experiments with *pfmrp1*,

reduced growth and increased CQ-sensitivity were observed, which suggests it plays a role in drug efflux that affects parasite fitness [88].

Amodiaquine (AQ). The chloroquine analog amodiaquine resulted from improved lipophilicity of the side chain via aromatic structure incorporation, and has been used for prophylaxis for about 60 years despite its low bioavailability and relatively high rate of treatment failures in Southeast Asia. Some cross-resistance occurs between AQ and CQ, which renders AQ only somewhat effective against CQ-resistant parasites. Rare but life-threatening side effects of hepatotoxicity and agranulocytosis resulted in amodiaquine being largely discontinued therapeutically for long-term use (prophylaxis), but has been deemed sufficiently safe for short-term use. These side effects led to the discontinuation of AQ use in western countries, however WHO recommends amodiaquine as an effective ACT partner drug [27]. Amodiaquine-containing ACT exposures select for 86Y and 1246Y mutations in *pfmdr1*, which confer decreased sensitivity to chloroquine and amodiaquine [63].

Piperaquine. Piperaquine was once widely used in China and was developed there in the 1980's, but increasing drug resistance resulted in tapering use of this drug for monotherapy. In recent years piperaquine was revived when it was combined with dihydroartemisinin as another ACT, and is being administered extensively in Southeast Asia. Piperaquine is highly active against chloroquine-resistant parasites, and is also known to induce 86Y and 1246Y mutations in *pfmdr1* [89].

Mannich Bases

Pyronaridine. Pyronaridine was developed in the 1980's in China, and is considered a Mannich base schizonticide but has an unconventional replacement of its

quinolone heterocycle with an azaacridine and two Mannich base side chains. Western quality requirements prevent pyronaridine's use in the west as the compound shows toxicity in neutrophils [90]. Further, clinical trials indicated high recrudescence rates (~25%) with 30-day follow-up periods. However, it has shown utility as an effective partner drug in combination therapy, and is currently being combined with artesunate, called Pyramax®.

8-aminoquinolines

Primaquine (PQ). The 8-aminoquinoline drugs, including primaquine, were derived from an initial observation by Paul Ehrlich who noted that methylene blue was an excellent *Plasmodium* stain and postulated that methylene blue could be used as a drug as well [72]. In 1891, Ehrlich and Guttman tested this and successfully treated two people using methylene blue, showing curative results as the first synthetic drug for malaria. While methylene blue was not introduced therapeutically at this point, the syntheses of several 8-aminoquinolines were derived, such as pamaquine and primaquine. German researchers from Bayer laboratories under IG Farben created pamaquine and primaquine in the 1920's. While pamaquine was first used in 1926 and provided radical cure, it was considered too toxic for use, spurring the development of a pamaquine analog called primaquine. WWII activities cut off the world supply of quinine to allied forces during the Japanese occupation of Indonesia in 1942 making the development of synthetic antimalarials a top US priority, which is when the US Army began the development of primaquine. Veterans coming home from the Korean War battled relapsing *P. vivax* malaria following cessation of chloroquine prophylaxis, so the

US Army began to bring primaquine into efficacy and safety studies in the 1950's, and late in the Korean War soldiers were given primaquine on their return voyage [91].

Primaquine sets itself apart from other antimalarials with potent activity against liver stages and some activity against asexual stages. Primaquine remains the only antimalarial used for radical cure of *P. vivax* and *P. ovale* infections to kill hepatic forms; it is also known to eliminate gametocytes, reducing transmission back to the mosquito vector. The mechanism of action for the 8-aminoquinolines is unknown. Primaquine shows some ability to restore CQ-resistant parasites to sensitivity. However, primaquine is dangerous for G6PD-deficient individuals and can cause severe hemolysis; this is particularly troubling since this deficiency is relatively common in both Africa and Asia [92].

Antibiotics

Doxycycline, clindamycin, and tetracycline are part of the tetracycline class of antibiotics known to have antimalarial action in binding to ribosomes, which then leads to protein synthesis inhibition. The tetracyclines act on liver and blood stage schizonts. The elimination time for doxycycline is long, making it well suited for use as a chemoprophylaxis agent except in pregnant women and children. Doxycycline is also known to cause gastrointestinal distress and photosensitivity. The Walter Reed Army Institute of Research conducted the Thailand 1992 Phase II studies that led to FDA approval for doxycycline to be used for chemoprophylaxis [93]. Clindamycin is a safer alternative, yet its elimination time is unsuitably short for prophylaxis and needs to be combined with a faster acting antimalarial, as it is with quinine or artesunate currently.

Sesquiterpene Lactone Endoperoxides (Artemisininins)

The current gold standard of malaria treatment is the ACT, which includes a combination of a fixed-dose artemisinin derivative (artemether, AM; artesunate, AS; or dihydroartemisinin, DHA) and either an arylamino alcohol or 4-aminoquinoline. One of the biggest issues with artemisinin derivatives is that the plant *Artemisia annua* is needed for its derivation, and has an 18-month lag between the demand and supply which leads to wide fluctuations in drug pricing. Because of this, attempts to make inexpensive and fully-synthetic endoperoxide drugs became a priority. Significant progress has been made in the efficiency of endoperoxide synthesis, yet it is still not as cost effective as plant-based derivations [94, 95]. Artemisinin itself is poorly soluble in both water and oil, therefore semisynthetic derivatives were created as an alternative. Neurotoxicity is a concern with all artemisinin-derivatives because of the formation of the primary metabolite, DHA, which is thought to be neurotoxic especially in the brain stem [96], although the use of artemisinin for many years in China has not mirrored these findings.

Artemisinin Resistance. In response to the potential loss of efficacy of the artemisinin drugs, systemic surveillance was employed to track and prevent the spread of artemisinin resistance. It was not until 2008 that an actual treatment failure was observed; the initial manifestation of worry came from delayed parasite clearance times. This delayed parasite clearance phenotype did not result in EC_{50} shifts with standard 72-hour *in vitro* drug susceptibility assays, so alternative methods had to be used to assess the development of resistance in real time [97, 98]. *Pfmdr1* was once again implicated in the development of antimalarial drug resistance, with *pfmdr1* amplification

and antioxidant processes being upregulated [99, 100]. A genome-wide association study implicated regions on chromosome 13 to be involved in the artemisinin delayed clearance phenotype, and then mutations in the kelch gene (K13) on chromosome 13 were then identified to be associated with resistance [101-103]. Introduction of K13 mutations in artemisinin-sensitive parasites have confirmed their role in artemisinin resistance [104].

Surveillance of artemisinin resistance indicates that areas of Thailand, Cambodia, Vietnam and Myanmar have regions of delayed parasite clearance and recrudescence from K13 mutations, but none so far have been observed in Africa [105]. Parasites with acquired K13 mutations also have prolonged ring stages of development, yet have the same overall cycle duration as sensitive isolates. Comparative transcriptomics studies revealed an upregulation of the unfolded protein response (UPR), which is thought to help resistant parasites withstand the exposure to artemisinin drugs. Artemisinins are activated by iron-containing moieties, which then cause destruction of intracellular structures with alkylation and oxidation events [106]. This upregulated UPR would theoretically aid the parasites repair and degradation responses to artemisinin. The kelch protein itself is thought to function as a negative regulator of signal transduction that can initiate the UPR in the event of damage to proteins. A resistant parasite with a K13 mutation may lead to constitutive action of the UPR pathway or potentially increased activation in the presence of artemisinin [107].

2-Hydroxynaphthoquinones

The development of quinone compounds for antiplasmodial activity began in the 1940's, but real interest in new antimalarials were stalled because chloroquine was both

inexpensive and effective. In the 1960's, the naphthoquinones were revisited as chloroquine failed and the need arose for new scaffolds that had antimalarial potential. One of the first naphthoquinones was called hydrolapachol, and the structure was further developed to create lapinone, a compound shown to successfully treat *P. vivax* at high doses [108]. Menoctone has the characteristic 2-hydroxynaphthoquinone structure and cyclohexyl group in the side chain, yet very high doses were required and made toxicity an issue, as the molecule was found to undergo biotransformation and it was subsequently abandoned [72]. Menoctone was shown to have broader antiparasitic activity so the cyclohexyl side chain was pursued further, leading eventually to parvaquone, BW58C80, and atovaquone [109-111]. A comparison of these compounds and their structures can be seen in Figure 1.6 [72]. Atovaquone is a costly compound to synthesize, though in recent years alternative atovaquone syntheses and the expiration of Malarone's patent led to more affordable generics [112].

Atovaquone (ATQ). Atovaquone is a hydroxy-1,4-naphthoquinone that functions as a ubiquinone analog and is a competitive inhibitor that binds to the Q_o site (quinol oxidation site) of the cytochrome *bc*₁ complex [113, 114]. Atovaquone binding leads to collapse of mitochondrial membrane potential, and a loss of mitochondrial function [115]. Because the primary function of the mitochondrial electron transport chain is to provide pyrimidine precursors for DNA synthesis via the action of the *P. falciparum* dihydroorotate dehydrogenase enzyme, the parasite is deprived of its ability to synthesize pyrimidines [116, 117]. ATQ is administered as a fixed-dose combination with proguanil (Malarone®), indicated for use in both children and adults for prophylaxis when travelling to malaria-endemic countries. 70% of all antimalarial travel prescriptions

in the United States from 2009-2011 were for Malarone [118]. Malarone obtained FDA approval in 2000 for treatment and prophylaxis of *P. falciparum* malaria, is known to have activity in blood and early liver stages. It is also indicated for the treatment of *Pneumocystis* pneumonia under the trade name Mepron [91]. While atovaquone potently inhibits *P. falciparum*, sporadic resistance developed in ~30% of patients in Phase II clinical trials in Thailand, leading it to be paired with its synergistic partner proguanil [119].

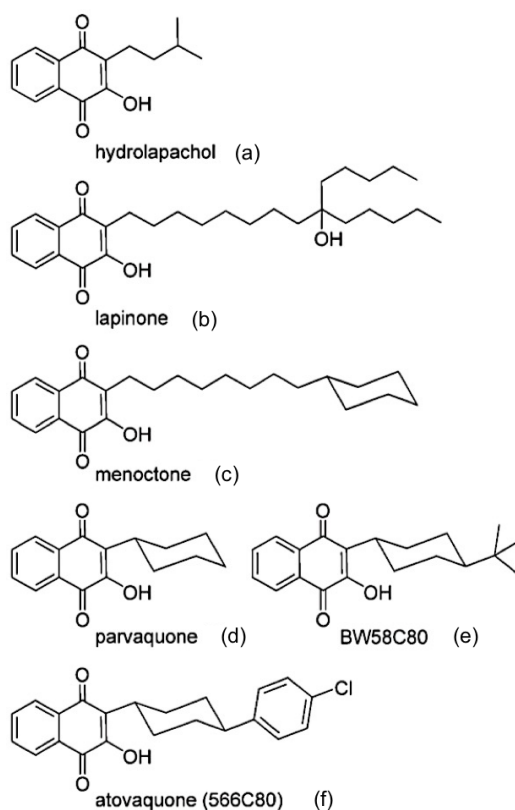


Figure 1.6. The Development of Antiparasitic Naphthoquinones, from Schlitzer 2007, *ChemMedChem*, 2(7) [72]. (a) Hydrolapachol, an early naphthoquinone developed in the 1940's. (b) Lapinone, the first naphthoquinone used to successfully cure human malaria. (c) Menoctone, toxic at therapeutic doses but shown to have broad antiparasitic activity against *Theileria parva*. (d) Parvaquone, used to treat cattle for *Theileria parva* infections. (e) BW58C80 has broad anti-protozoal activity but lost metabolic activity in humans. (f) Atovaquone, a (2-[trans-4-(48-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthoquinone) is a stable compound with high anti-protozoal activity against *Pneumocystis jiroveci*, *Toxoplasma gondii*, and *Babesia spp.* infections in humans.

Antifolates

Antifolates act on two important parasite enzymes, dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) which in turn inhibits nucleic acid synthesis [120, 121]. These parasitic enzymes can be exploited as drug targets based on their necessity to synthesize pyrimidines for nucleic acids and an absence of a pyrimidine salvage pathway. Resistance to antifolates is associated with several mutations in these enzymes, with substitution S108N conferring resistance to cycloguanil and pyrimethamine in Africa and Southeast Asia, and S108T also present in South America. [122-124]. Additional resistance-contributing mutations coincide with S108 mutations, most commonly C59R and N51I.

Proguanil and Cycloguanil. Proguanil (originally named Paludrine) is currently used in combination with atovaquone as the commercial drug Malarone for the treatment and prevention of malaria. Cycloguanil is the highly active metabolite of proguanil and is activated by cytochrome P450 [125]. The addition of proguanil with atovaquone profoundly enhances the ability of atovaquone to collapse mitochondrial membrane potential in a synergistic fashion [126]. While proguanil is known to bind to DHFR much like PYR, the primary action of proguanil in Malarone may not involve this pathway at all. The strong synergy seen with proguanil is not found when atovaquone is combined with the active metabolite cycloguanil—meaning the synergistic action relies on proguanil itself. Further, PYR and atovaquone do not have synergy, so proguanil likely acts elsewhere to create synergistic action. Studies have shown that proguanil does not collapse mitochondrial membrane potential on its own, yet when combined with atovaquone, it lowers the concentration of atovaquone needed to collapse

membrane potential [127]. The cause for this atovaquone-hypersensitization is not known and is responsible for keeping the treatment failures low when compared with atovaquone alone, since atovaquone monotherapy leads to atovaquone-resistant treatment failures in 30% of patients [119]. Atovaquone-proguanil synergy is lost in atovaquone-resistant parasites, rendering Malarone ineffective [128].

Sulfadoxine and Pyrimethamine (SP). Pyrimethamine was developed by George Hitchings with a group at Burroughs Wellcome in the US in 1950, originally meant to be an anticancer therapy. Pyrimethamine and sulfadoxine each inhibit DHFR and DHPS enzymes respectively, which in turn prevents the synthesis of folic acid in the parasite and therefore inhibits pyrimidine biosynthesis. Because each drug interferes with folic acid synthesis from different enzymes, SP combination results in synergistic action. SP, known as Fansidar, has been very successful in treating CQ-resistant malaria in the past. Pyrimethamine was originally given as monotherapy (Daraprim) for both *P. vivax* and *P. falciparum*, but within a year resistance was observed [129]. SP combination therapy also induced parasite resistance in Southeast Asia and South America, with the target enzymes developing several mutations in *dhfr* and *dhps* [120, 121]. Microsatellite surveillance of the spread of SP resistance points to *dhfr* resistance mutations crossing from Asia to Africa before SP was even introduced to African parasite populations [130]. Interestingly, *dhps* mutations in Southeast Asia and Africa are thought to occur *de novo* [131, 132]. While resistance limits the efficacy of SP to treat malaria, it is still implemented for use in intermittent preventative therapy in Africa for pregnant women and for seasonal chemoprevention in the sub-Saharan region of Africa. It is also used in ACT formulations widely in India [133].

Experimental Antimalarials in Clinical Development

The current landscape for antimalarials as of 2015 is promising, with several novel targets being exploited. Figure 1.7 shows the structures of some new experimental antimalarials in clinical development, as well as a few that were abandoned as clinical candidates early in 2016. Compounds that remain in development currently are discussed.

Phase I Clinical Trials

P218. P218 is a selective *Pf*-DHFR inhibitor from a partnership with Thai BIOTEC, Monash University, London School of Hygiene and Tropical Medicine and the Medicines for Malaria Venture. Concerns remain about DHFR inhibitors because of their historical propensity to fail because of rapid development and spread of resistance. However, P218 is a “next generation” DHFR inhibitor designed with affinity for both DHFR-resistant mutant and sensitive parasites alike, owing to a flexible side-chain capable of acting on both [134].

SJ733. SJ733 is a dihydroisoquinolone that came from St. Jude Children’s Research Hospital. SJ733 is an inhibitor of the *P. falciparum* sodium-transporting ATPase (*Pf*-ATP4), and is a fast-acting antimalarial with blood stage and transmission blocking ability [135]. SJ733 has potential to be part of a single dose radical cure.

MMV048. MMV048 is 2-aminopyridine compound from the University of Cape Town. Its molecular target is known to inhibit the *P. falciparum* phosphatidylinositol 4-kinase, and has blood stage, relapse prevention, and chemoprevention potential. Phase I trials for MMV390048 began in 2014, with completion of Phase I/II studies with a new formulation and submission for Phase IIa protocol in 2016.

Phase II Clinical Trials

KAE609 + KAF156. KAE609 is a spiroindolone compound developed by Novartis and the Swiss Tropical and Public Health Institute, which emerged from a phenotypic screen. The target of KAE609 was determined to be a sodium-ATPase 4 ion channel called *PfATP4*. *PfATP4* is a plasma membrane protein and is believed to be a sodium efflux pump [136]. Several classes are now known to target *PfATP4*, including the spiroindolone KAE609. Diverse chemotypes from the Malaria box library have indicated sodium and pH modulation that coincides with *PfATP4* inhibition [137]. KAE609 had very promising Phase I trials, with faster parasite killing than even artesunate [138]. KAF156 is a imidazolopiperazine that inhibits the cyclic amine resistance locus (CARL) [139]. It also showed promise pre-clinically in chemoprevention against liver stages. Both KAF156 and KAE609 have been tested in Phase II as monotherapies prior to being put in combination.

OZ439 + Piperaquine/Ferroquine. OZ439, also known as artefenomel, is currently in combination studies with piperaquine. Ferroquine is a chloroquine analog that is a ferrocene tethered to a 4-aminoquinoline, making it active in two locations. Ferroquine is also being paired with OZ439, and has promise as a combination therapy since it is active against both chloroquine and mefloquine-resistant parasites [140]. OZ439 contains the endoperoxide group found in artemisinin-derivatives, but is structurally distinct.

Fosmidomycin + Piperaquine. Fosmidomycin is an inhibitor of parasite isoprenoid biosynthesis, and was originally isolated from *Streptomyces lavendulae* for potential use as an antibacterial in the 1970's. Plasmodium uses a distinctive method to

produce isopentyl diphosphate (IPP) with a mevalonate-independent mechanism called the 1-desoxy-D-xylulose-5-phosphate pathway (DOXP). The enzymes involved are localized in the parasite apicoplast organelle, which represents a valuable antimalarial target unique from the human host [141]. Fosmidomycin was shown to inhibit the second enzyme in the DOXP pathway, 1-desoxy-D-xylulose-5-phosphate reductoisomerase (DXR) which catalyzes the transformation of DOXP to MEP (2-C-methyl-D-erythritol-4-phosphate) [142]. Fosmidomycin is a substrate analog that inhibits *Pf*DXR with an IC₅₀ of between 400-950 nM, but because the toxicity is so low, relatively high plasma levels are realistic [143]. The recrudescence rates were shown to be unacceptably high, which led to it being used as a partner drug [144]. Fosmidomycin shows additive effects with quinine and doxycycline, and synergy with clindamycin [143, 145]. Fosmidomycin showed poor efficacy in young children, so the partner drug was changed to piperazine, and clinical trials are in progress for that combination.

DSM265. DSM265 is an inhibitor of the dihydroorotate dehydrogenase (DHODH) enzyme of the mitochondrial electron transport chain, which has excellent selectivity for the *Pf*-DHODH enzyme when compared to the human DHODH enzyme. DSM265 has potential for both chemoprophylaxis and combination single dose radical cure. In 2015, DSM265 began Phase IIa trials in Peru for patients with either *P. falciparum* or *P. vivax*, and Phase Ib blood stage challenge in combination with OZ439 [146]. As of 2016, it is set to do Phase II combination studies with OZ439, direct mosquito bite challenge, and sporozoite challenge studies.

Methylene Blue. Methylene blue is a synthetic phenothiazin dye that was used to successfully treat malaria by Paul Ehrlich more than a century ago. Methylene blue is

redox active, thought to deliver electrons from flavoproteins to hemoglobin-associated or free Fe(III)-protoporphyrin IX [147]. Fe(III)-protoporphyrin IX reduction would then inhibit hemoglobin digestion/heme detoxification, leading to parasite death. Methylene blue has promise as a transmission-blocking compound, and is planned to be used in combination with ACTs. A clinical trial in Thailand is in progress to evaluate its use compared to single dose primaquine [146].

Arterolane + Piperaquine. Arterolane, also known as OZ277 is the result of a combined effort with the Medicines for Malaria Venture, Ranbaxy, and the Indian Government to develop the first somewhat cost-competitive fully-synthetic endoperoxide derivative. This combination is marketed as Synriam® and has been administered to over one million people in India; it is cheaper than any other ACT currently available there but is yet to receive stringent approval from the WHO.

Phase III Clinical Trials

Trimethoprim. Trimethoprim/sulfamethoxazole (Cotrimoxazole®) are well known antibacterial compounds that also show antimalarial activity, with trimethoprim being both a potent inhibitor of *Pf*-DHFR and a potentiator of sulfonamides [148]. Cotrimoxazole has effectively treated chloroquine-resistant parasites, but concerns of rapid resistance development because of its similarity in mechanism of action as well as resistance with SP prevented its use previously. However, the WHO recommends the use of Cotrimoxazole as prophylaxis against potential opportunistic bacterial infections in HIV-infected individuals. Its combined antibacterial and antimalarial properties make it an ideal candidate for prophylaxis in these populations, and a clinical trial in Malawi is ongoing to determine whether there is a reduction in morbidity/mortality in patients

taking anti-retrovirals, and if so, whether the benefits come from its antibacterial or antimalarial properties [149].

Tafenoquine. Tafenoquine is a derivative of primaquine with more lipophilic qualities, and is structurally distinct with its trifluoromethylphenoxy substituent that is responsible for its elevated blood, liver, and sporontocidal activity. However, tafenoquine is inactive against gametocytes [150]. Tafenoquine is yet another drug developed by the Walter Reed Army Institute of Research, and development of this drug candidate came from the support of GlaxoSmithKline and the Medicines for Malaria Venture (MMV). Tafenoquine showed rapid therapeutic effect, with a single dose being comparable to 14 days of primaquine dosing [151, 152]. Tafenoquine carries the same risk for hemolysis in G6PD-deficient individuals, but is generally better tolerated in patients when compared to primaquine. The mechanism of tafenoquine action is unknown, but it does appear to have similarities to primaquine in causing respiratory chain effects and heme polymerization defects within the parasite. Tafenoquine is on track for submission to regulatory authorities for approval some time in 2017, which could have a substantial impact in reducing relapse rates in patients infected with *Plasmodium vivax* considering there is a greater potential for mass-drug administration applications compared to primaquine.

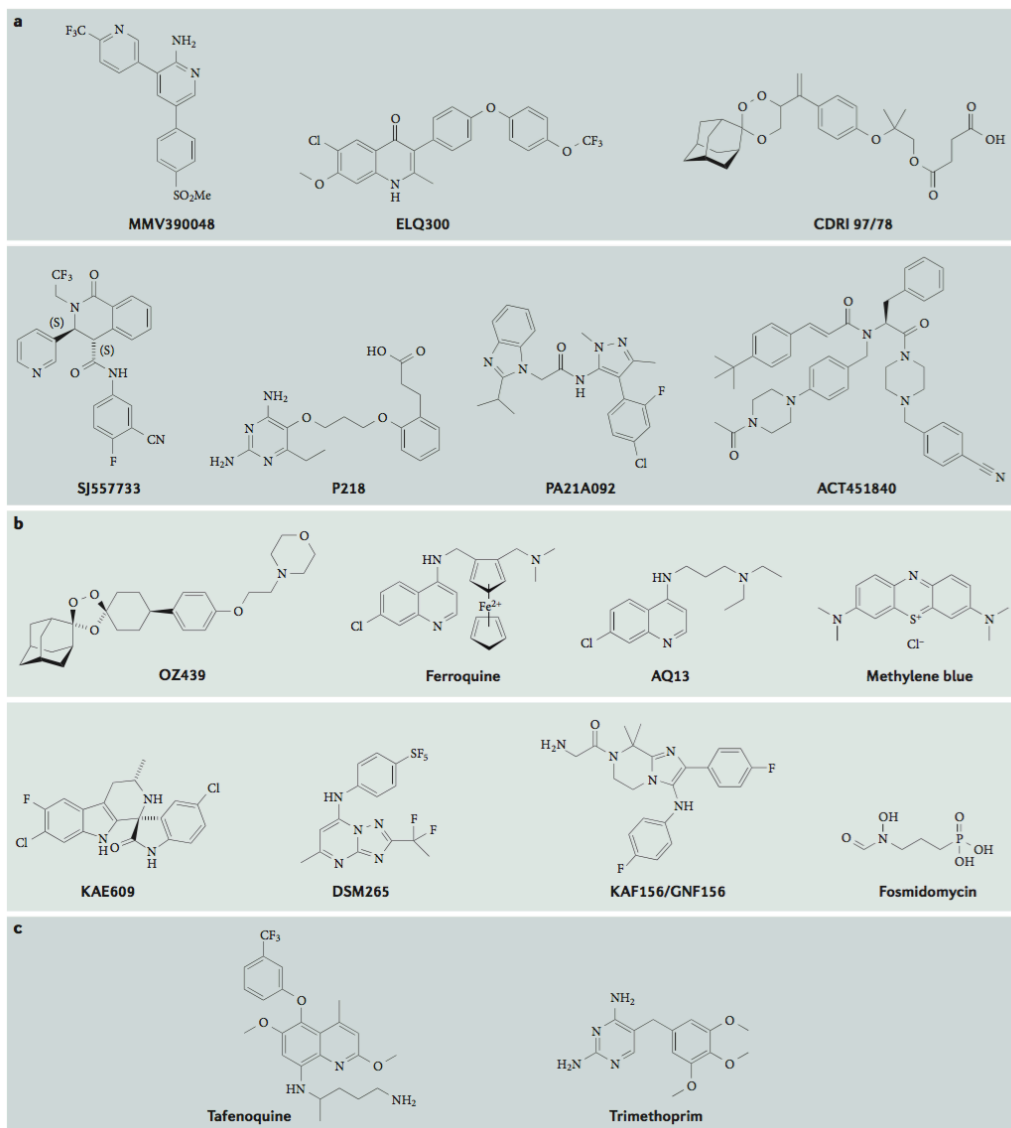


Figure 1.7. Experimental Compound Structures in Clinical Development in 2015, from Wells *et al.* 2015, *Nat Rev Drug Discov*, 14(6):424-442 [146]. A diverse set of antimalarials in various stages of clinical development: Phase I (a), Phase II (b), or Phase III (c) clinical trials represent new antimalarial chemistry from the Medicines for Malaria Venture. As of 2016, several of the Phase I studies in (a) have been put on hold or abandoned: ELQ300, CDRI 97/78, PA21A082, ACT451840, as well as AQ13 from Phase II (b). MMV048 inhibits phosphatidylinositol 4-kinase. OZ439 is a synthetic trioxolane with the active endoperoxide group found in artemisinin, but is otherwise structurally distinct. SJ733 and KAE609 target the *Pf* Na⁺-ATPase 4. P218 selectively inhibits *Pf*-DHFR. DSM265 inhibits mitochondrial DHODH. KAF156 inhibits the cyclic resistance amine locus. Older compounds in trials are also included: fosmidomycin and tafenoquine. Methylene blue and trimethoprim have been indicated in treatment of other medical conditions and are currently being evaluated for use in malaria.

Plasmodium Mitochondria

Metabolism

In canonical eukaryotic systems, glycolysis converts glucose into pyruvate and the tricarboxylic (TCA) cycle/oxidative phosphorylation in the mitochondria converts pyruvate into ATP, which is used for energy by cells. For this reason, mitochondria are considered the “powerhouse” of the cell, as oxidative phosphorylation results in abundant ATP production when compared to glycolysis. In *P.falciparum* asexual stages, however, it has been shown that the mtETC only converts 7% of host-scavenged glucose into ATP, with the remaining 93% being consumed in aerobic glycolysis; not surprisingly, lactic acidosis is a symptom of severe malaria [153]. Initially this hardly seems efficient, yet aerobic glycolysis is known to be a hallmark of rapidly dividing cells, such as with cancer cells, where glycolysis is used to convert glucose to lactate for ATP even in the presence of oxygen [154]. Since glucose is abundant and ATP can be derived quickly, it is preferred to the more efficient oxidative phosphorylation at this point in their development. Even though the mitochondria are not generating sufficient ATP in asexual stages, the parasite mtETC remains essential for the regeneration of ubiquinone and maintenance of membrane potential that the DHODH enzyme requires to synthesize pyrimidine precursors, as the parasite lacks a pyrimidine salvage pathway and must synthesize them *de novo* [117].

A small subpopulation of parasites in the blood stages convert to gametocytes in hopes of being taken up by a mosquito blood meal, and are now known to have very different energetic requirements. Recently, gametocytes were shown to utilize much more glucose, have increased glycolytic flux and TCA pyruvate catabolism compared to

asexual blood stages [153]. This transition from aerobic glycolysis to oxidative metabolism is an advantage for sexual parasites in several ways. Gametocyte conversion increases with disease severity in the human host, and hypoglycemia is common in severe malaria, making the switch to oxidative phosphorylation an advantage in glucose scarcity. Glucose scarcity is also likely in the mosquito hemolymph, where the female gametes will need more energy to prepare for fertilization and development [153].

Structure

In asexual stages, *P. falciparum* parasites contain a single metabolically active mitochondrion with a double membrane and mitochondrial activity that is abbreviated in function compared to the mitochondria of its human host, and have a simplified structure reflective of this [155]. The mitochondria transform throughout the asexual stage, with its single discrete organelle forming a larger elongated structure as the parasite transitions from trophozoite to schizont [156]. As schizonts, the parasite mitochondria form branched structures without distinct cristae (morphologically called 'type I' mitochondria) and with cytokinesis they segment into multiple organelles, couple with a nucleus and apicoplast, which become merozoites.

There is a noticeable difference in the mitochondrial structure between asexual and sexual stages, where mature gametocytes have 4-8 'type II' mitochondria that show presence of distinct tubular cristae. A subset of mature gametocytes were observed with more cristae that were electron-dense, which were termed 'type III' [155]. This is consistent with the sexual stages having more demanding energetic requirements compared to asexual stages. The ATPase complex of the mitochondria is structurally

responsible for the formation of the curved cristate structure of the inner mitochondrial membrane, so the observation that cristae structure is pronounced in sexual stages is consistent with their metabolic switching to TCA oxidative energy generation [157].

Genome

The malaria parasite has a truncated genome, which consists of highly conserved linear, tandemly repeated 6 kb units, and is the smallest known genome in eukaryotes [158-160]. The mitochondrial genome is maternally inherited, and has about 90% sequence identity within the human malaria species [161]. Estimates on the mtDNA copy number in *P. falciparum* vary between 20-30 copies, yet mtDNA copy number may not be conserved among the *Plasmodium spp.* as *Plasmodium yoelli* was estimated to have approximately 150 copies [162]. There is also evidence that copy number varies within members of the same species [162, 163]. Only three genes are encoded in the mtDNA which are mtETC subunits: *coxI* and *coxIII* of the cytochrome *c* oxidase enzyme (Complex IV), and cytochrome *b* which belongs to the cytochrome *bc1* enzyme (Complex III), as well as fragmented rRNA genes. All other participants in the mtETC are nuclear encoded and require localization to the mitochondria, including genes typically seen in the mitochondria: tRNA genes, NADH dehydrogenase subunits, and cytochrome *c* oxidase subunit II (*coxII*) [164].

In a comparison of sexual and asexual mitochondrial DNA/RNA with qPCR and q-RT-PCR, mtDNA copy number and mt-associated transcripts were 3-8 fold higher in sexual stages, illustrating that sexual stage mtDNA gene dose and mt-transcription are both elevated and dynamic [155].

Mitochondrial Diversity, Replication and Inheritance

The mitochondrial genome replication of the linear, concatamerized 6–kb elements of *P. falciparum* was described as having highly-branched networks of replication and recombination intermediates, some rolling circle lariat intermediate species characteristic of the T₄ phage, and a subpopulation of small circular form DNA [165]. These small circular forms of 6 kb mtDNA were shown to directly interact with the highly-branched recombination forms of the main mt-genome, and were said to account for less than 5% of the total mtDNA forms. Interestingly, this type of replication is common to plants, as they too have linear, concatamerized mtDNA molecules, form branching structures, and possess small single-unit circular forms; what follows is a discussion of relevant mitochondrial inheritance mechanisms and their important implications for *P. falciparum*.

The mitochondria are uniparentally maternally inherited generally in eukaryotes, and as a consequence mitochondrial genomes within an individual favor homoplasmy, which is when the mitochondrial genomes are all identical. This strict maternal inheritance (SMI) creates a bottleneck in oogenesis that reduces mtDNA copy number, and would therefore rid mtDNA of mutations because only a subset of mtDNA molecules will be passed on [166]. Current working knowledge of the inheritance of mtDNA has contributed to the following model governing organellar inheritance: (i) uniparental inheritance exists to both prevent the propagation of selfish cytoplasmic elements and to minimize mito-nuclear incompatibility; (ii) uniparental inheritance is evolutionarily unstable since mtDNA would be subject to Muller's ratchet (lack of recombination will eventually accumulate irreversible deleterious mutations) and (iii)

uniparental inheritance must be relaxed regularly or occasionally by various mechanisms [167]. Mechanisms of disruption have been noted in different organisms, such as temporary biparental transmission and occasional paternal leakage, and maternal inheritance of stable/constitutive heteroplasmy [166].

Mitochondrial heteroplasmy is the presence of multiple mitochondrial genomes present within a single organism. Mitochondrial heteroplasmy has distinguished itself as a wealth of mitochondrial diversity in many other systems, found in humans, *Trypanosoma cruzi*, and even *Plasmodium relictum* [168-171]. It is important to note that mitochondrial heteroplasmy was originally thought to be evolutionarily transient state, where homoplasmy is reinstated after several generations' time. There are notable examples of organisms that diverge from homoplasmy and stably maintain low levels of heteroplasmy that have been uncovered with next-generation sequencing, where heteroplasmy can be resolved at 0.1% frequencies at ~45,000x mtDNA coverage with strict read quality control measures [169].

Mitochondrial heteroplasmy has been implicated in severity of human disease, but also in sustained health and longevity. In a study of centenarians using ultra-deep sequencing, Giuliani *et al.* was able to show that maternal low-level heteroplasmies can be passed down and sustained in humans, and were part of a "rare variant pool" which may be involved in prolonging life [169]. The presence of a low-level variant pool of mitochondrial diversity can also be seen universally in humans, the product of both inherited and somatic processes, which can be beneficial in coping with various types of physiological or environmental stressors [172]. It is obvious how this would lend particular utility in the context of enzymes participating in oxidative phosphorylation, as

having low-level variants could allow for enzymatic isoforms that better handle stress conditions [173].

Heteroplasmy has also been shown to contribute to severity of resistance to drugs that target cytochrome *b* in other organisms. Indeed, the strawberry grey mold *B. cinerea* has been characterized with dynamic heteroplasmy against a cytochrome *b* Q_o site inhibitor in the strobilurin fungicide family [174]. Mitochondrial heteroplasmy has not been explored in *Plasmodium falciparum*, and has an mt genome that has been described as “incredibly intolerant of mutation” owing to the general lack of sequence divergence observed despite the fact that mitochondrial mutation rates in higher eukaryotes are orders of magnitude higher than their counterpart nuclear genomes [163]. Indeed, mitochondrial barcoding relies on the mtDNA mutation rate to discern speciation events, and is a standard utilized in population genetics.

Last, it is interesting to note that in genetic cross experiments with several *P. falciparum* clones, it was shown that in every cross there was unidirectional dominance of inheritance of the mitochondrial and apicoplast genomes, suggesting a “unidirectional parental incompatibility” in cross-fertilization, alluding to cytoplasmic incompatibility as a selective force [175]. It seems that while the *Pf* mitochondrial mutation rates might appear slow and well-conserved at the surface level, cryptic mitochondrial heteroplasmy could provide an explanation for the input of mitochondrial diversity that has been suspiciously lacking to date.

Mitochondrial Electron Transport Chain (mtETC)

As discussed earlier, in eukaryotic systems the mtETC is responsible for generating the protonmotive force needed for oxidative energy generation, but *P.*

falciparum asexual stages utilize glycolytic metabolism and it was unclear for some time whether the parasite even utilized oxidative phosphorylation at all, especially considering many canonical enzymatic subunits have still not been identified [176]. Genetic knock out studies illustrate that mtETC enzymes are nonessential in blood stage; the only requirement is a functional DHODH enzyme and ubiquinone regeneration [117, 177]. The four enzyme complexes characteristic of many inner mitochondrial membrane of eukaryotes are: NADH:ubiquinone oxidoreductase (Complex I), succinate:ubiquinone oxidoreductase (Complex II, SDH), ubiquinol:cytochrome *c* oxidoreductase (also known as cytochrome *bc*₁ or Complex III), and cytochrome *c* oxidase (Complex IV). Ubiquinone and cytochrome *c* carry electrons between complexes in this traditional system, with the protonmotive force being fueled by Complexes I, III and IV. The *Pf*-mtETC differs substantially from this canonical system, which are discussed in the descriptions of the *Pf*-mtETC components to follow. Briefly, Complex I is missing, yet electron flow is maintained in complexes II-IV. There are five non-protonmotive dehydrogenases that provide electrons to the downstream complexes: NADH:ubiquinone oxidoreductase (NDH2), SDH, glycerol-3-phosphate dehydrogenase (G3PDH), the malate:quinone oxidoreductase (MQO), and dihydroorotate dehydrogenase (DHODH). Neither DHODH nor MQO are found in human mitochondria, and the full enzymatic functional occupations remain somewhat elusive. The dehydrogenase activity converts dihydroorotate to orotate, essential for synthesizing nucleic acids, as well as to donate electrons to Complex III and then Complex IV, where ubiquinone and cytochrome *c* act to shuttle electrons among complexes, generating electrochemical potential. The ATP synthase at the end of the

chain (Complex V) is essential in sexual stages and utilizes the transmembrane proton gradient generated upstream to make ATP, but is not very active in blood stage [178, 179]. The locations of the respiratory enzymes and electron flow of the *Pf* mtETC can be seen in Figure 1.8.

Mitochondrial electron flow

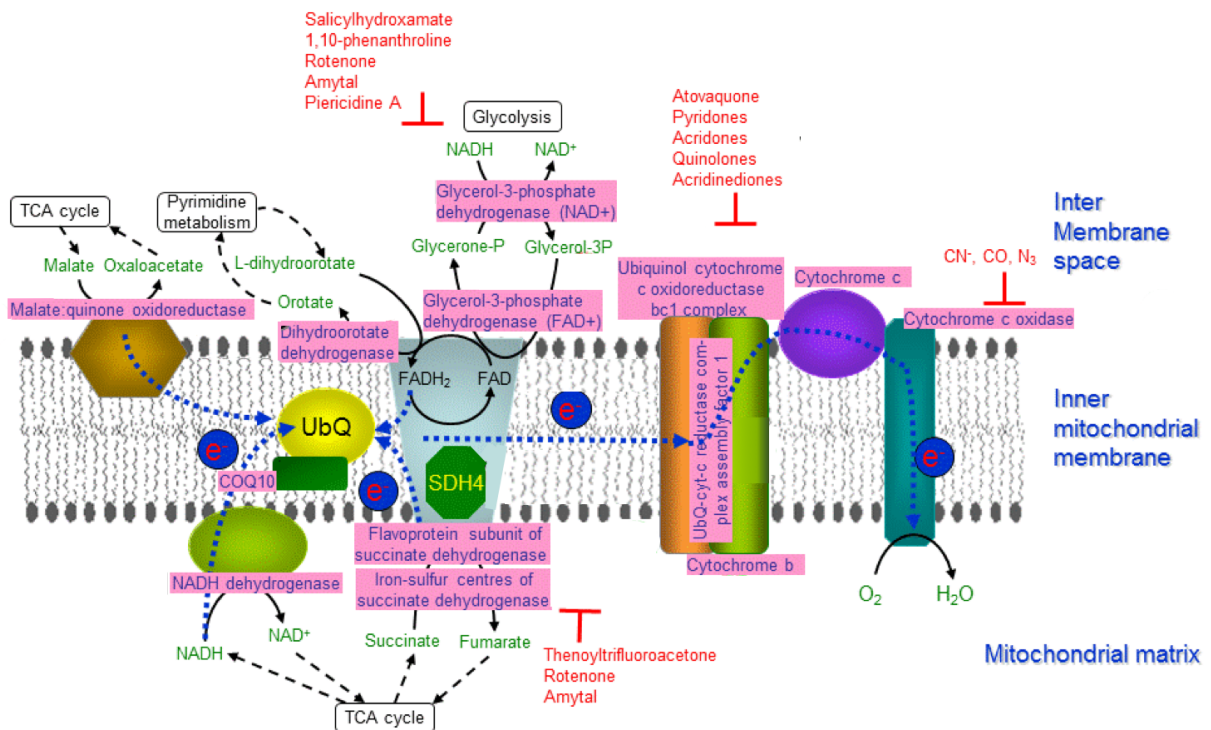


Figure 1.8. Mitochondrial Electron Flow in *Plasmodium falciparum*, adapted from Ginsburg, 2016, *Malaria Metabolic Pathways Database* [180]. Pink boxes show the name of enzyme complexes participating in the mtETC. Red text indicates specific enzyme inhibitors. Black boxes designate the direct link of mitochondrial electron flow to another branch of parasite metabolism.

Enzymes and Their Functions. *Type 1 NADH Dehydrogenase (NDH2).*

Complex I is absent in *Pf*, but instead utilizes a non-proton pumping NDH that reduces ubiquinone. *Pf*-NDH2 is available to function without being influenced by the electrochemical gradient, being a non-protonmotive enzyme, which is an advantage to maintain protonmotive force required for mitochondrial import especially since the asexual stages have minimal oxidative phosphorylation to maintain it. *Pf*-NDH2 additionally performs NADH/HAD⁺ cycling, with its NADH-dependent respiration being responsible for providing approximately half of the quinol flux to bc₁ [181]. *Pf*-NDH2 is inhibited by HDQ (1-hydroxy-2-dodecyl-4(1H)quinolone) which leads to a loss of membrane potential [182, 183].

Glycerol 3-Phosphate Dehydrogenase (G3PDH). G3PDH is a non-proton motive, FAD-dependent quinone reductase enzyme located on the inner mitochondrial membrane's outer surface. It acts to reoxidize glycerol-3-phosphate to DHAP (dihydroxyacetone phosphate), as well as shuttling electrons from cytosolic NADH and delivering them into the mitochondrial ubiquinone pool [164].

Malate:quinone oxidoreductase (MQO). MQO is a peripheral, membrane bound flavoprotein that is a non-protonmotive quinone reductase. MQO is responsible for catalyzing the oxidation of malate to oxaloacetate, feeding into the TCA cycle. The oxidation reaction then donates the electron generated to reduce ubiquinone [164]. The *Pf*-MQO is a replacement malate dehydrogenase in the TCA cycle, and is likely essential in blood stages as multiple knockout attempts were unsuccessful, however this is a puzzling finding since MQO enzymatic activity could be disrupted without consequence, suggesting it has structural essentiality of some kind [116].

Dihydroorotate Dehydrogenase. DHODH is another non-protonmotive quinone reductase shown to be essential in its function as the only redox reaction in the *de novo* pyrimidine biosynthesis pathway; its conversion of dihydroorotate to orotate is crucial to feed into pyrimidine metabolism as the parasite is incapable of pyrimidine salvage. DHODH functions as a dihydroorotate:quinone oxidoreductase with its active site oriented toward the intermembrane space [117]. The triazolopyrimidine class of compounds (DSM1, DSM76) are inhibitors of the DHODH enzyme [184].

Succinate Dehydrogenase (Complex II). Another non-protonmotive enzyme, SDH is composed of two active units: the flavoprotein subunit and the iron-sulfur center. Succinate from the TCA cycle is converted to fumarate by the iron-sulfur center, which may then provide electrons to ubiquinone.

Cytochrome bc_1 Complex (Complex III). Complex III has a three-subunit catalytic core consisting of cytochrome *b*, cytochrome c_1 and the Rieske iron-sulfur protein (ISP). Complex III possesses two active sites for quinone binding, Q_o (quinol oxidation site) and Q_i (quinone reduction site), which are located on opposite sides of the inner mitochondrial matrix but connected via transmembrane electron transfer pathway [185]. A basic schematic of electron transfer through Complex III can be seen in Figure 1.9 [186].

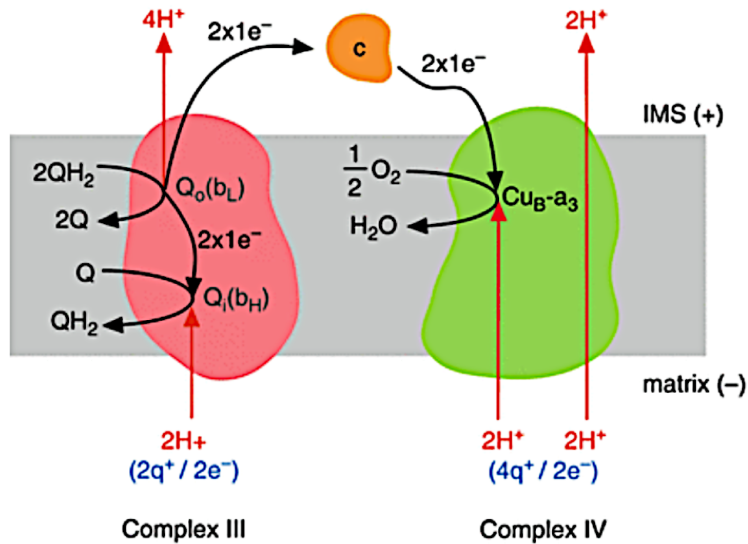


Figure 1.9. The *Plasmodium* Cytochrome bc_1 Complex Reactions in the Q-cycle, from Biagini 2014, *The Encyclopedia of Malaria*, Mitochondrial Electron Transport Chain of *Plasmodium falciparum* [186]. The *Plasmodium* Complex III (red) and Complex IV (green) participate in a series of reactions that result in the translocation of protons from the matrix to the intermembrane space. The schematic shows the ubiquinol substrate (QH_2) docking at the ubiquinol oxidation site (Q_o), and ubiquinone docking at the ubiquinone reduction site (Q_i), as well as the electron transfers that take place from ubiquinol \rightarrow cytochrome c \rightarrow cytochrome c oxidase.

Complex III is responsible for the transfer of electrons from ubiquinol (reduced form of ubiquinone) to cytochrome c , where one complete catalytic cycle is completed as follows: (2) ubiquinol molecules bind to the Q_o site and are reduced to ubiquinone, two electrons transfer to the ISP where it moves to transfer them to the c -type heme of cytochrome c_1 , then cytochrome c_1 gets oxidized by cytochrome c oxidase (2). The second electron pair gets transferred by two b -type heme molecules to the Q_i site of cytochrome b , and ubiquinone is reduced to (1) ubiquinol. The *Pf* mitochondria have a distinct ubiquinone homolog (CoQ_8) unique from that of their human host, making it theoretically a good target of inhibition [187, 188].

Cytochrome c Oxidase (Complex IV). Complex IV is the final electron acceptor, converting $O_2 \rightarrow H_2O$ through the oxidation of cytochrome *c* and subsequent transfer of protons through the electrochemical gradient. Cytochrome *c* oxidase strips four matrix protons for each 2-electron half-cycle as shown in Figure 1.9, where two protons are utilized by the oxygen reduction, and two are translocated through the enzyme's hydrophilic channel through the membrane and out into the intermembrane space [186].

ATP Synthase (Complex V). The function of the F_1F_0 -ATP synthase/hydrolase in *P. falciparum* utilizes the electrochemical gradient generated in the mtETC to couple proton transfer with ATP synthesis/hydrolysis. Complex V has a matrix facing external catalytic domain (F_1) that is responsible for the conversion of ATP to ADP and inorganic phosphate. The membrane domain (F_0) forms a proton pore through the membrane, which rotates to relocate protons through the inner mitochondrial membrane to the matrix. The ATP synthase (and TCA cycle) are essential in later sexual development and transmission through the mosquito, although there is modest growth impairment in asexual stages with ATPase knockouts [179].

The mtETC Inhibitors and Resistance

Atovaquone. As discussed earlier, atovaquone is a competitive inhibitor of ubiquinol in the bc_1 complex at the Q_o site, which is encoded in the cytochrome *b* gene in the mtDNA. Docking studies using yeast cytochrome *b* revealed atovaquone forming hydrogen bonds with the iron-sulfur domain as well as cytochrome *b*, tethered between the hydrogen bonding and inhibiting translocation of the ISP [189]. Inhibition by atovaquone causes a drop in mitochondrial membrane potential, as ubiquinol is not getting regenerated back to ubiquinone by Complex III [115]. The downstream impact of

inhibiting ubiquinone regeneration prevents the essential DHODH function of converting dihydroorotate to orotate, a key pyrimidine precursor that is essential since *Plasmodium* lack a pyrimidine salvage pathway. The mechanism of death by atovaquone in asexual stages is that of slow pyrimidine starvation, as DNA synthesis primarily occurs in trophozoites, explaining the delayed death [190]. Indeed, metabolite concentrations from the pyrimidine biosynthetic pathway are altered during atovaquone exposure [191]. Ubiquinone regeneration inhibition also inhibits the TCA cycle in asexual stages, since the TCA cycle enzymes MQO, SDH, and the ATPase of the ETC all rely on coupling their reactions to the donation of electrons to ubiquinone [116]. Atovaquone inhibitory concentrations vary substantially, with published findings of mean EC₅₀ values from 0.5 to 6.2 nM (0.4—22.6 nM range) *in vitro* for both lab strains and field isolates [67, 128].

Atovaquone Resistance. Atovaquone resistance was an issue from the very beginning of atovaquone's clinical use, where Phase II studies for atovaquone in Thailand reported parasite populations that came from patient treatment failures with atovaquone monotherapy that developed resistance mutations in the Q_o site of cytochrome b, with either Y268S or Y268N mutations. The most common atovaquone resistance mutations were found to be Y268S, Y268N, or Y268C [192]. This type of rapid resistance selection is a highly unusual event in terms of historical resistance development patterns, as the resistant parasite populations had to have been selected within the duration of a single patient infection. Typical resistance patterns require a heritable trait that must pop up and then spread through a region, but this is not the case for atovaquone. This sporadic resistance pattern led to atovaquone being

immediately paired with its highly synergistic partner drug proguanil, and has been used successfully for chemoprophylaxis and occasional treatment of uncomplicated malaria.

The resistance mutation at the Y268 position is thought to destabilize the Q_o pocket, which makes atovaquone inefficient at binding a pocket with a deformed conformation and allows the parasite to survive atovaquone exposure. Further, there are known fitness costs associated with this mutation, as the *bc₁* complex has been shown to have decreased catalytic turnover compared to wild-type enzymes [193]. Despite this decreased fitness, there have been no reports of resistant parasites reverting back to wild-type, which is partially explained by the parasite utilizing fitness compensatory mechanisms, such as reducing rieske-iron sulfur subunit protein expression as well as transcriptional upregulation of Complex III and IV enzymes [193].

Atovaquone resistance development *in vitro* has not resulted in clinically relevant atovaquone resistance mutations to date. Multiple reports of *in vitro* resistance generation in lab strains such as 3D7, AT200 and K1 link decreased susceptibility to several other mutations or mutation combinations in cytochrome *b*: M133I, M133V, F267V, and other peripheral mutations that accompany them [192, 194, 195]. In addition, the ability of parasites to generate atovaquone resistance varies among different strains, where 3D7, W2, and FCR3 easily generate atovaquone resistance compared to HB3 and D6, which are incapable of developing atovaquone resistance regardless of parasite population density. Interestingly, the same set of parasites were used in selection with 5-fluoroorotate, and only W2 was able to generate resistance at any parasite density, showing that there are variations in frequencies of drug resistance between parasite lines [196]. Since the molecular target for 5-fluoroorotate is likely

nuclear-encoded, perhaps it is possible that these two drug selection experiments also highlight the ability of certain parasites to develop resistance to mitochondrial-encoded genes more readily than those encoded in the nucleus.

Myxothiazol and Antimycin A. Myxothiazol is a generic inhibitor of the Q_o site of the cytochrome *bc1* complex, and is a competitive inhibitor of ubiquinol. Antimycin A is a generic inhibitor of the Q_i site in the *bc1* complex, which inhibits the oxidation of ubiquinone. Myxothiazol binding causes collapse of mitochondrial membrane potential similar to that of atovaquone, but its binding only forms the b-proximal region of the Q_o site, as compared with atovaquone which prevents movement of the ISP [183].

HDQ. 1-Hydroxy-2-dodecyl-4(1H)quinolone is a potent nanomolar inhibitor of *P. falciparum*. HDQ is structurally very similar to that of ubiquinol, and was found to inhibit the mitochondrial NDH2 enzyme, as well as a potent inhibitor of the cytochrome *bc1* complex, where inhibition is caused by binding the Q_i site. This is a novel mechanism of action capable of targeting two mitochondrial enzymes [182, 183].

GSK93121A. This GSK compound belongs to the 4(1H)pyridone class, and is an electron transport inhibitor that acts on the Q_i site of cytochrome *bc1*. It was initially a preclinical candidate but was discontinued based on safety data from a soluble phosphate prodrug of the candidate, leading to other related compounds being abandoned as well [197]. It has been suggested that the Q_i site binding was to blame for safety concerns [198].

ICI56,780 and P4Q-391. Both compounds are known to be very potent inhibitors of the cytochrome *bc1* complex. Both compounds remain effective against atovaquone-resistant parasite TM90-C2B, but to a slightly diminished extent which brings about

questions as to whether it is truly a Q_i site inhibitor or possibly acts to inhibit the bc_1 complex at a site other than Q_o or Q_i [199].

DSM1. DSM1 is a novel triazolopyrimidine inhibitor of the DHODH enzyme. Parasite resistance to this inhibitor has manifested as DHODH copy number amplifications, but resistance to the related triazolopyrimidine DSM74 has shown both DHODH copy number amplifications and mutations in DHODH [200, 201]. DSM74 selection yielded the following DHODH mutations: E182D, F188I, F188L, F227I, I263F, and L531F [201]. Interestingly, high levels of DSM1 pressure were shown to create atovaquone tolerance, though the cause for this is uncertain [202].

Preliminary/Relevant Studies from the Kyle Laboratory

Phase II Clinical Trials of Atovaquone, Thailand (1991)

Initial observations made in the Phase II studies of atovaquone in Thailand showed that roughly 30% of patients experienced treatment failure to atovaquone monotherapy [119]. The patient histories for the admission and recrudescence isolate pairs can be seen in Table 2.1, as well as the various treatment regimen dosing groups they belonged to. Parasites from the treatment failures with atovaquone alone or in combination with pyrimethamine were found to either have a Y268S/N mutation in the cytochrome *b* gene, or wild-type in the case of TM90-C6B. TM93-C1088 had an admission isolate but it was lost in a liquid nitrogen tank failure. TM92-C1086's paired admission isolate TM92-C1028 was found to be positive for mycoplasma contamination upon thawing and was treated with MRA (mycoplasma removal agent), but it failed to thrive in culture.

Table 1.1. Parasite History for Paired (Admission and Recrudescence) *Pf* Isolates from Phase II studies in Thailand (Looareesuwan *et al.*, 1996. *AJTMH* 54:62-66)

Treatment Regimen	Patient No.	Admission/ Recrudescent	Isolate	Cyt. <i>b</i> mutation
ATOV 750 mg q8h x 4	2	A	C2A	--
		R	C2B	Y268S
	6	A	C6A	--
		R	C6B	--
ATOV 750 mg q8h x 21	29	A	C40	--
		R	C50	Y268S
	32	R	C32B	Y268N
ATOV 1000 mg plus PYR 25 mg q24h x 3	210	A	C1028	--
		R	C1086	Y268S
	207	A	C1051	--
		R	C1090	Y268N
	206	R	C1088	Y268S

q8h = dosed every 8 hours, q24h = dosed every 24 hours

Chemotype Screens Identify Extreme Pan-Resistance to mtETC Inhibitors

In order to identify whether any cross-resistance was observed in patient isolate samples, [³H]hypoxanthine assays were performed using drugs that target diverse mitochondrial function. Any unique cross-resistance patterns may have given clues as to alternative mechanisms of resistance that contribute to the wide-ranging atovaquone resistance phenotype, considering Y268S mutations alone cannot explain it. As seen in Table 1.2 below, TM90-C6B (wt-treatment failure) only showed low-level resistance to

atovaquone. TM90-C2B and TM90-C50B5 (Y268S treatment failures) both display moderate levels of resistance to atovaquone and myxothiazol (both mtETC inhibitors that act on the Q_o site of Complex III), while their sensitive admission isolate pairs TM90-C2A and TM90-C40B2 are sensitive. TM92-C1086 and TM93-C1088 are Y268S recrudescence parasites that came from the atovaquone/pyrimethamine treatment regimen, and they displayed high-level resistance to atovaquone as well resistance to all electron transport chain inhibitors tested.

Table 1.2. Low, Moderate and Extreme Resistance to mtETC Inhibitors in *P. falciparum* (EC50, nM)

	Complex III								
	NDH2	DHODH	Q _o site (cyt <i>b</i>)		Q _i site (cyt <i>b</i>)			Unknown (cyt. <i>b</i>)	
	HDQ	DSM-1	ATOV	MYX	ANT	ELQ 300	GSK121	ICI56,780	P4Q-391
W2	55.0	52.4	0.41	10.8	165.5	2.72	3.39	0.03	11.1
TM90-C6B	ND	24.5	109	7.45	125	7.67	36.4	0.50	33.0
TM90-C2A	14.8	79.4	3.09	154	72.0	0.69	20.2	0.04	1.92
TM90-C2B	146	57.4	5290	428	152	4.61	77.5	14.3	55.5
TM90-C40B2	830	47.2	1.53	45.1	72.9	4.02	7.84	0.04	22.1
TM90-C50B5	ND	ND	3940	3010	ND	6.45	122	13.4	32.4
TM92-C1086	180	770	31400	4160	>18200	16300	2470	882	14160
TM90-C1088	252	540	29100	3990	>18200	21000	3470	219	>20200

Focus of Study

The primary aims of this project were to understand the mechanisms by which atovaquone resistance is generated, as well as to provide an explanation of the diverse parasite phenotypes that derived from atovaquone treatment failures, as well as *in vitro* selected parasite lines.

CHAPTER 2
PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF ADMISSION AND
RECRUDESCENT PATIENT ISOLATES
(SPECIFIC AIM 1)

Rationale of Study

Parasite resistance to all existing drugs endangers the global elimination campaign, with untreatable malaria as a potential consequence. The *Plasmodium falciparum* parasite has historically defeated several remarkably efficacious drugs, the most recent being the potent artemisinin class. Many new chemotherapeutic strategies are currently exploiting the parasite mitochondria as a drug target, since their divergent and severely attenuated mitochondrial functions provide an attractive biological choke point to kill the parasite with minimal host toxicity. Although the mtETC is abbreviated in *P. falciparum*, it is the source of the mitochondrial electrochemical gradient and is essential in its role to provide orotate for pyrimidine biosynthesis via activity of the dihydroorotate dehydrogenase enzyme (DHODH) [117, 155, 176].

Atovaquone, a naphthoquinone, along with the pyridones [197, 203, 204], acridones [205], acridinediones [206-208], tetrahydroacridines [206], and the 4(1H)-quinolones [190, 209-211] potently inhibit the cytochrome *bc1* complex of the mitochondrial electron transport chain (mtETC) within the mitochondria, with disruption

of pyrimidine biosynthesis and collapse of membrane potential leading to parasite death.

Despite the initial appeal of this strategy, there has been mounting evidence that targeting this pathway leads to rapid development of antimalarial resistance, beginning with the treatment failures first seen in the initial Phase II clinical trials for atovaquone between 1991-1994 in Thailand [119]. These studies demonstrated that atovaquone monotherapy resulted in clinical treatment failures and subsequent recrudescence of infection, prompting the use of atovaquone in combination with proguanil (Malarone) for malaria treatment and prophylaxis. Following the administration of Malarone in Thailand, sporadic treatment failures were reported soon after, and it is estimated that approximately 1 in 100 travelers treated with Malarone to treat *P. falciparum* infection experience treatment failure attributed to atovaquone resistance [212]. Clinical treatment failure was linked to an amino acid substitution at position Y268 in cytochrome *b* and was additionally confirmed in unpublished genotype data of the treatment failures from these initial Phase II studies [192, 213].

While it is thought that atovaquone monotherapy rapidly gives rise to *de novo* resistance within the duration of a single patient infection with the acquisition of the cytochrome *b* Y268 substitution, this clinically relevant mutation has yet to be seen with *in vitro* atovaquone resistance selection studies. It has been suggested that evolutionary fitness plays a role in limiting the diversity of resistance development, with repeated selection of Y268 substitutions accounting for parallel mutation development in clinical resistance outcomes [201]. However, this concept alone cannot explain why *in vitro* selections do not have the same mutation outcome of clinical atovaquone

resistance, with reports of mutations elsewhere in cytochrome *b* reported at peripheral amino acid positions, such as M133I, M133V, P275T, K272R, G280D, L283I, V284K, L144S and F267V [192, 194, 201]. Additionally it has been shown that there are strain-specific differences in the propensity of parasites to become resistant to atovaquone in any capacity, where HB3 and D6 parasites failed to generate resistance to 10^{-8} M atovaquone even with parasite densities of 10^8 /flask [196]. Taken together, these two studies suggest that parallel selection of the Y268 cytochrome *b* mutation will occur only in certain genetic backgrounds.

Here we show the phenotypic characterization of parasites isolated from patients that failed various dose regimens of atovaquone or atovaquone/pyrimethamine combination therapy. These patient isolates exhibited remarkable phenotypic diversity, with a range of 5-30,000 fold resistance to atovaquone. Parasites with exceptionally high resistance to atovaquone also revealed pan-mtETC resistance to all drugs we tested that target the mitochondria, as well as phenotypic plasticity with a gradual loss of high-grade resistance during continuous culture over time. In the field isolates, we observed considerable variation in resistance to atovaquone and other drugs that target diverse mitochondrial enzymatic functions. This spectrum of response to mitochondrial inhibitors cannot be explained by the Y268 mutation alone, leaving many questions about how parasites respond and overcome drug pressures in the mitochondrial setting, and how mitochondrial mutations emerge in parasite populations. These remarkable parasite adaptive strategies underscore a potential threat to many drugs in the pipeline that target mitochondrial function.

Materials and Methods

Parasites and *in vitro* Culture

Admission and recrudescence parasite samples were collected from patients in the Phase II clinical trials upon admission for treatment, and again following failure of the treatment regimen (various dose regimens of atovaquone monotherapy or atovaquone/pyrimethamine combination therapy) [119]. The parasite history for paired admission and recrudescence isolates are outlined in Table 1.1.

Parasites were adapted to *in vitro* culturing and maintained according to the methods previously described by Trager and Jensen, with modifications [214]. Parasites were maintained at 2% hematocrit in human O+ erythrocytes in RPMI 1640 (Invitrogen) medium containing 25 mM HEPES, 28 mM NaHCO₃, 10% human type A positive plasma and incubated at 37°C in 5% O₂, 5% CO₂, and 90% N₂ atmospheric conditions. Cultures were sustained with media changes three times per week and kept below 5% parasitemia with sub-culturing.

***In vitro* Drug Susceptibility Testing**

The methods used were performed as described previously [215] with a modification of a 72-hour incubation period. Briefly, sorbitol-synchronized ring stage parasites were diluted to 2% parasitemia, and seeded into 96-well plates in the presence of drug in a 1:3 dilution series. After 48 hours of incubation, [³H]hypoxanthine was added to the plates at 48 hours, and allowed to incorporate for an additional 24 hours. The radiolabeled cells were harvested at 72 hours, and 50% maximal response concentrations (EC₅₀) were calculated using Trifox. Several drugs were used that target mitochondrial function to perform a mini-structure activity study for a clearer

understanding of the broad spectrum atovaquone-resistance phenotypes (as seen in Table 1.2). The drugs atovaquone (ATOV; 2-hydroxynaphthoquinone), antimycin A [ANT], and myxothiazol [MYX] were purchased from Sigma (St. Louis, MO). The compounds ICI 56,780 [phenoxyethoxy-4(1H)-quinolone], P4Q-391 [4(1H)-quinolone], ELQ-300 [4(1H)-quinolone], and HDQ [1-hydroxy-2-dodecyl-4(1H)-quinolone] were synthesized and purified by the Manetsch laboratory at the University of South Florida, Department of Chemistry. DSM-1 [triazolopyrimidine] was generously provided by Pradipsinh Rathod (University of Washington). All compounds were used following dilution in DMSO with final concentrations no more than 0.5%.

Genomic DNA Sequencing

DNA Extraction from Parasites. For PCR and downstream candidate gene sequencing, *P. falciparum*-infected erythrocytes (8-10% parasitemia, predominantly trophozoite/schizont stages) were centrifuged to remove media, washed with 0.1% saponin in 1x PBS for 10 min, centrifuged for 10 min and washed again briefly in 0.1% saponin PBS. Following saponin lysis, two more PBS washes and spins were performed to remove any residual lysis products. The parasite pellet was resuspended in 10x of the original RBC pellet volume in 1x PBS, and incubated with 20 uL RNase A (20 mg/mL) at 37°C for 10 min. Genomic DNA (gDNA) was then extracted with the Qiagen DNeasy Kit according to manufacturer's protocols.

Sequencing for SNPs in Mitochondrial Genes. We wanted to sequence several candidate genes in the mtETC for possible additional SNPs to explain our drug resistance spectrum phenotypes, beginning with confirming the mutations in cytochrome *b*. All genes that were sequenced for patient isolates are denoted in closed

boxes in Figure 2.1, and all primer sets and programs can be found in Table 2.1. All PCRs were set up similarly to the sequencing of the cytochrome *b* gene below. Candidate genes included PFI0735c (NDH2), PFF0160c (DHODH), PFE1155c (Core 1), PFI1625c (Core 2), PF14_0597 (cyt. *c*₁), PF14_0373 (Rieske), PF14_0248 (QCR6), PF10_0120 (QCR7), and the three mt-encoded genes: MAL_MITO_3 (cyt. *b*), MAL_MITO_1 (*coxIII*), MAL_MITO_2 (*coxI*). For the primers in Table 2.1, the primers labeled PCR FOR and PCR REV were used in PCR amplifications, and SEQ PR denotes primers used to sequence the amplification in its entirety.

All *P. falciparum* cytochrome *b* PCR products were amplified using primers cytbFOR 5'—TGCCTAGACGTATTCCTG—3' and cytbREV 5'—GAAGCATCCATCTACAGC—3' with AccuPrime Taq DNA Polymerase HF (Invitrogen) as 50 µL reactions with 10X AccuPrime PCR Buffer II (5 µL), PCR primers (0.2 µM final concentration), template DNA (~20-50 ng), AccuPrime Taq HF (1 U), and brought to 50 µL total volume with nuclease-free water. The *P. falciparum* cytochrome *b* thermocycling conditions were as follows: initial denaturation of 94°C for 1 min, 32 cycles of 94°C for 30s, 54°C for 20s, and 68°C for 1:20s. All PCR products and no-template controls were run out on 1% agarose in 1X Tris-acetate-EDTA (TAE) gels stained with SYBR Safe DNA Gel Stain (Invitrogen) and visualized on a BioRad GelDoc imaging system. PCR products were purified with the QIAquick PCR purification kit according to kit protocols (Qiagen), and the concentration assessed using a NanoDrop spectrophotometer. Sequencing of PCR products was performed by Genewiz using sequencing primers for cytochrome *b* outlined in Table 2.2. Each parasite sample was PCR amplified and sequenced in duplicate to rule out any SNPs being introduced by

PCR polymerase. Sample sequences were analyzed and aligned using ApE (A Plasmid Editor) software, and mapped to the *Pf*-3D7 *cytochrome b* gene for mutation detection.

Pyrosequencing of Cytochrome *b* Y268S Allele

The Pyromark Q96 ID system was used for the detection of SNPs for Y268S detection in *Pf*-cytochrome *b*, with Qiagen Pyromark Gold Q96 reagents and buffers along with streptavidin sepharose beads (GE Healthcare). All template and reaction components were prepared according to manufacturer's protocols. Pyrosequencing primers were designed using Pyromark Assay Design Software. Primers for the initial PCR reaction were amplified with PFcytb_pyro_Biotin_FOR 5'—Biotin-ACCATGGGGTCAAATGAGTTAT—3' and PFcytb_pyro_REV 5'—AGCTGGTTTACTTGGAACAGTTTT—3' as 50 µL reactions with 25 µL 2X Phusion Hot Start II HF PCR Master Mix, 0.2 µM primer concentrations, ~10-50 ng template gDNA, brought to 50 µL total volume with nuclease-free water, with the following thermocycling conditions: initial denaturation of 98°C for 30s, 55 cycles of 98°C for 30s, 53°C for 5s, and 72°C for 8s. Subsequent PCRs were run on 1.5% agarose gels to confirm a single discrete band without excess primer present, as unconsumed primer has been shown to interact with the pyrosequencing primer to contribute to a background signal in no template controls commonly in pyrosequencing reactions. This was minimized by using low primer concentrations and using a high cycle number to exhaust any residual primers.

The Pyromark pyrosequencing assay was performed according to standard manufacturer's protocols with pyrosequencing primer PFcytb_seq_assay_REV 5'—TGGAACAGTTTTTAAACATTG—3'. Each parasite gDNA sample was initially amplified

independently in triplicate, and had two technical replicates per reaction (25 μ L PCR per pyrosequencing reaction) on the Pyromark Q96 ID for a total of at least 12 pyrosequencing runs per parasite gDNA template. Allele frequencies were analyzed by Pyromark ID software in allele quantification mode.

Establishment of Pyrosequencing Y268S Assay Standard Curve

Since all Y268S mutant genotypes still contained some small quantities of wild-type allele (wt), we chose to use the parasite with the highest percentage Y268S mutant, TM90-C50B5 gDNA (99.52% mutant) and D6 (0% mutant) were mixed at 10% increments from 0% wt gDNA + 100% wt gDNA, adding in additional increment mixtures at the lower 5% and upper 95%, with 1% increments to look at the sensitivity of detection. These ratio wild wt:mutant gDNA mixtures were made independently four times and then used in subsequent PCR reactions and pyrosequencing reactions to generate the standard curve seen in Figure 2.2. The standard curve was plotted as mean % wt frequencies, and error bars indicate SEM.

Phenotype/Genotype Stability Assessment of TM92-C1086

TM92-C1086 displayed an unstable phenotype with declining atovaquone EC_{50} over a period of two months. A fresh cryopreserve of TM92-C1086 and TM90-C2B were thawed, and parasites were monitored weekly with [3 H]hypoxanthine assays and Y268S pyrosequencing to see if the parasite was maintaining the Y268S mutation. This experiment was performed two times with the same outcome, though the week-to-week EC_{50} fluctuations in each flask was different, so for clarity a single two-month time course was plotted.

Table 2.1. PCR Primers and Programs Used in mtETC Sequencing.

Primer Type	Primer Name (Gene ID, Primer Use, Primer Orientation)	Primer Sequence	PCR Product Length	PCR Program
PCR FOR PCR REV SEQ PR SEQ PR SEQ PR SEQ PR	0120_PCR_FOR 0120_PCR_REV 0120_FOR_INT 0120_E1_REV 0120_E1_FOR 0120_REV_INT	5'—CTAACCGCGTTTGTCTAACC—3' 5'—CTGGTGGTATCGTGTATC—3' 5'—ATTCAGCTCCAAGCCTGTTC—3' 5'—TAAGAGCACCATATGAGAGATGG—3' 5'—CAAGGAGATATAGAAGGATGTTAAGAGGAAC—3' 5'—AGCAGCCATACCTCATTC—3'	2733 bp	94°-1:00 32 cycles: 94°-0:20 52°-0:20 58°-4:00
PCR FOR PCR REV SEQ PR SEQ PR SEQ PR SEQ PR	0735_PCR_FOR 0735_PCR_REV 0735_FOR_INT 0735_REV_INT 0735_FORINT2 0735_REVINT2	5'—ACCCTAATTCGCCTGCTC—3' 5'—GGTTCCTCAAATCACATGC—3' 5'—GTTCAGGAAATGTGGACAAG—3' 5'—CAAATGGTATGGGCGTCCTT—3' 5'—TATGGTCTTCTTATCTGGGCTAGTGG—3' 5'—CATGTAGCTGTTGTAGGAGGAGGTC—3'	5038 bp	94°-1:00 32 cycles: 94°-0:20 55°-0:20 58°-4:00
PCR FOR PCR REV SEQ PR SEQ PR	0248_PCR_FOR 0248_PCR_REV 0248_FOR_INT 0248_REV_INT	5'—CTTGACACATTCACCTGAAC—3' 5'—ACAGTACATTCTTGTGGGAC—3' 5'—GCAGTCAAATGTGTAAGACCAG—3' 5'—ACAGTACATTCTTGTGGGAC—3'	2707 bp	94°-1:00 32 cycles: 94°-0:20 52°-0:20 58°-4:00
PCR FOR PCR REV SEQ PR SEQ PR SEQ PR SEQ PR	1155_PCR_FOR 1155_PCR_REV 1155_REV_INT 1155_E1_REV 1155_E2_REV 1155_E2_FOR	5'—AGCATAGCACTGAGAACAAG—3' 5'—ACGGACAAGAGTTGATACTG—3' 5'—GAACCATCGAATACCTCTG—3' 5'—GCTTCACGTTTACCTATCGAACAC—3' 5'—GTTCTTAAATGAGATAAATGTGCCGTAATG—3' 5'—GAATCATGTATGGCCTTTAGTACTCAGCATTGAG—3'	3209 bp	94°-1:00 32 cycles: 94°-0:20 55°-0:20 58°-3:30

Table 2.1, continued. PCR Primers and Programs Used in mtETC Sequencing.

Primer Type	Primer Name (Gene ID, Primer Use, Primer Orientation)	Primer Sequence	PCR Product Length	PCR Program
PCR FOR PCR REV SEQ PR SEQ PR SEQ PR SEQ PR	1625_PCR_FOR 1625_PCR_REV 1625_FOR_INT 1625_FOR_INT2 1625_REV_INT 1625_REV_INT2	5'—TCCTGCCCTCTTCATTTG—3' 5'—CGAGCAATACAAACGGAC—3' 5'—CGAGCAATACAAACGGAC—3' 5'—TATGTGCCGTTGGTGATG—3' 5'—TGATGACTCAGGTCCAAATG—3' 5'—TCAGTACATCGACCTCAG—3'	3511 bp	94°-1:00 32 cycles: 94°-0:20 55°-0:20 58°-3:30
PCR FOR PCR REV SEQ PR	MM2_PCR_FOR MM2_PCR_REV MM2_REV_INT	5'—CTGGCCTACACTATAAGAAC—3' 5'—GAGAATTATGGAGTGGATGGTG—3' 5'—GGTATGATACACAGCTCTTC—3'	1809 bp	98°-0:30 32 cycles: 98°-0:10 53°-0:30 72°-7:00 72°-0:30FE
PCR FOR PCR REV	MM1_PCR_FOR MM1_PCR_REV	5'—TGCGATGAGACGACATGGAG—3' 5'—GCTATCAAATGGCGAGAAGGGAAG—3'	1008 bp	98°-0:30 32 cycles: 98°-30s 61°-0:30 72°-0:15 72°-3:00FE
PCR FOR PCR REV SEQ PR SEQ PR	MM3_PCR_FOR MM3_PCR_REV MM3_2B_REV MM3_2A_FOR	5'—TGCCTAGACGTATTCCTG—3' 5'—GCTGTAGATGGATGCTTC—3' 5'—CTGAGTATTGAGCGGAAC—3' 5'—GTGGAGGATATACTGTGAGTG—3'	1382 bp	98°-0:30 32 cycles: 98°-0:10 54°-0:40 72°-0:30 72°-7:00FE

Table 2.1, continued. PCR Primers and Programs Used in mtETC Sequencing.

Primer Type	Primer Name (Gene ID, Primer Use, Primer Orientation)	Primer Sequence	PCR Product Length	PCR Program
PCR FOR PCR REV SEQ PR	DHODH_FOR DHODH_REV DHODH_INT	5'—GATCCCTAGGATGATCTCTAAATTGAAACCTCAATTTATG—3' 5'—GATACTCGAGTTAACTTTTGCTATGCTTTTCGGCCAATG—3' 5'-CATTATTTGGATTATATGGGTTTTTTTTGAATCTTATAATCCTG—3'	1774 bp	94°-1:00 32 cycles: 94°-0:20 55°-0:20 58°-3:30
PCR FOR PCR REV SEQ PR	0597_PCR_FOR 0597_PCR_REV 0597_FOR_INT 0597_REV_INT2	5'—AAAAATGGCTGGTGGGGGAG—3' 5'—CCAACGTCCAAAAATAAGAACTAATCCA—3' 5'—TTCCTTGTCCACTGTGTAG—3' 5'—GGCAAAGATTCTTCTGGAC—3'	1428 bp	94°-1:00 32 cycles: 94°-0:20 56°-0:20 58°-2:10

Results

Sequencing of Mitochondrial Genes in Patient Isolates

In an attempt to find additional SNPs potentially involved in the observed differences between low, moderate, and extreme resistance, candidate genes were chosen from the mtETC to sequence. All Complex III subunits were sequenced, the NDH2 and DHODH enzymes, and the other two subunits encoded in the mtDNA: COXI and COXIII in Complex IV. As seen in figure 2.1 below, all enzyme subunits sequenced and their gene IDs are identified in boxes. Primers were initially designed for the Complex II subunits, as well as glycerol-3 phosphate dehydrogenase and malate quinone oxidoreductase, but they were unable to be amplified because of long repetitive AT stretches in the intronic regions of the DNA.

Table 2.2. Sequencing of Candidate mtETC Resistance Genes in Patient Isolates of *P. falciparum*

Gene ID	Parasite names (abbreviated)											
	W2	D6	C2A	C2B	C6A	C6B	C40B2	C50B5	C1051	C1090	C1086	C1088
PFI1625c (NDH2)	--	--	--	--	--	--	--	--	ND	ND	--	--
PFF0160c (DHODH)	--	--	--	--	--	--	--	--	--	--	--	--
MAL_MITO_1 (COXIII, Complex IV)	--	I239V	I239V	I239V	--	--	I239V	I239V	I239V	--	--	--
MAL_MITO_2 (COXI, Complex IV)	--	--	--	--	--	--	--	--	--	--	--	--
MAL_MITO_3 (Cyt. b)	--	--	--	Y268S	--	--	Y268S	Y268S	--	Y268N	Y268S	Y268S
PFE1155c (Core 1, Complex III)	--	--	--	--	--	--	--	--	ND	ND	--	--
PFI1625c (Core 2, Complex III)	--	--	--	--	--	--	--	--	ND	ND	--	--
PF14_0373 (Rieske, Complex III)	--	--	--	--	--	--	--	--	ND	ND	--	--
PF14_0248 (QCR6, Complex III)	--	--	--	--	--	--	--	--	ND	ND	--	--
PF10_0120 (QCR7, Complex III)	--	--	--	--	--	--	--	--	ND	ND	--	--
PF14_0597 (Cyt. c ₁)	--	--	--	--	--	--	--	--	ND	ND	ND	--

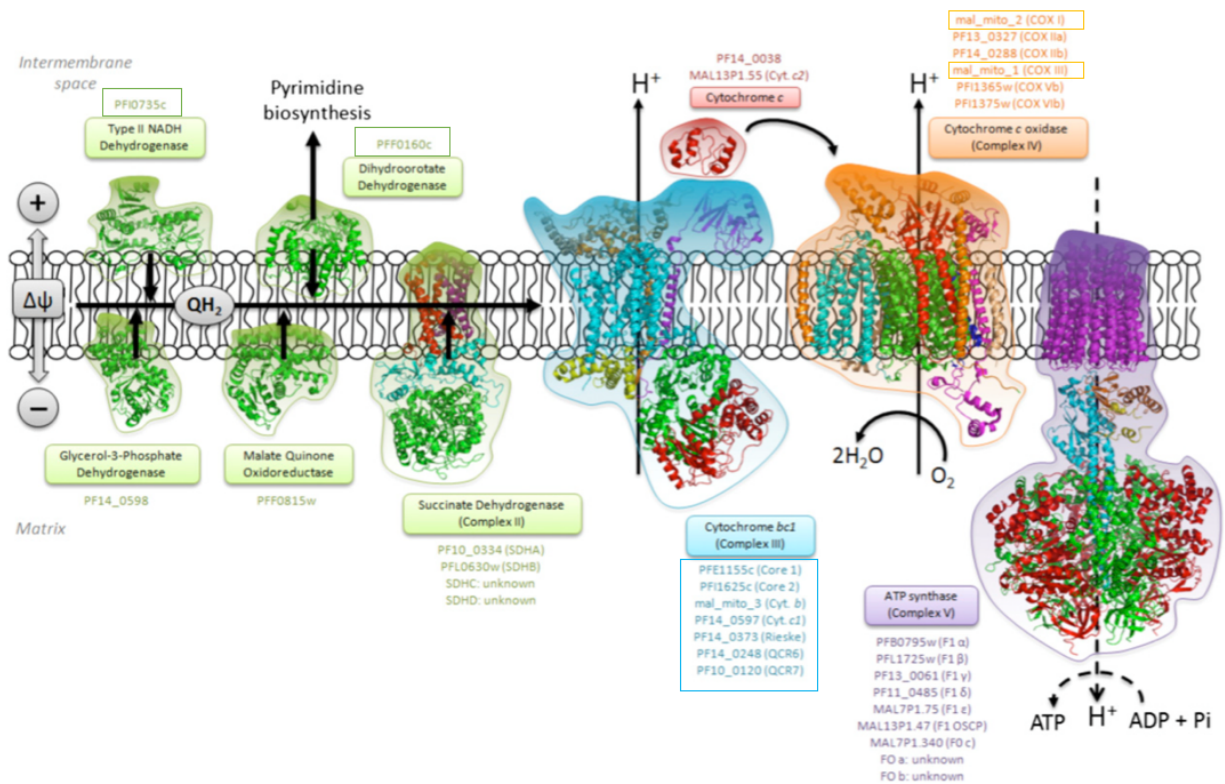


Figure 2.1. The mtETC and Candidate Resistance Genes (adapted from Biagini *et al.* 2012. *PNAS* 109(21): 8298-303) [190]. All genes enclosed in boxes were successfully amplified and sequenced to look for SNPs that may contribute to the atovaquone-resistance spectrum of phenotypes, including all subunits of the cytochrome *bc*₁ complex, DHODH, NDH2, and all mitochondrially-encoded genes (*cyt. b*, *coxI*, *coxIII*). Several other genes seen in this figure were attempted to be amplified, but were unsuccessful after primer optimization/redesigns.

As seen in Table 2.2 above, the only gene sequenced that contained SNPs was MAL_MITO_1 (*coxIII*), with D6, TM90-C2A, TM90-C2B, TM90-C40B2, TM90-C50B5, and TM93-C1090 having an I239V mutation. It seems unlikely that this mutation is of significance in extreme mtETC resistance, considering it is present in both admission and recrudescence isolates in moderate-resistant phenotypes like TM90-C2A/C2B.

Further, it is not a novel SNP as it can be found commonly in among parasites with available sequence data in PlasmoDB. However, it is of interest that TM93-C1051 has an I239V mutation, yet its recrudescence pair TM93-C1090 loses the mutation after atovaquone exposure, especially since TM93-C1090, TM92-C1086 and TM93-C1088 are all missing the mutation and were in the atovaquone/pyrimethamine treatment group. Further, no peripheral mutations were found that could explain the low-level resistance for TM90-C6B.

Establishment of a Y268S Pyrosequencing Assay

Since there are 20-30 mtDNA copies in the mitochondrial genome, we developed a pyrosequencing assay to detect the Y268S allele so that we could monitor the presence of Y268S in our populations of parasites that exhibited unstable drug response phenotypes. Additionally, we aimed to pyrosequence all of the admission and recrudescence isolates to see whether low levels of Y268S mutants could be seen in the pre-treatment admission isolates. It is a widely-held theory that the Y268S mutation is generated *de novo* in the parasite, and that this could be because atovaquone exerts a mutagenic effect on the parasite. However, it would then be expected to have this same mutagenic effect *in vitro*, yet many have generated *in vitro* atovaquone resistance without recapitulating the clinically relevant Y268S mutation, leaving questions about how Y268S mutations arise. Therefore, we speculated that the Y268S mutation could be a heritable trait, and that low levels of mutants could be cryptically present in sensitive parasites, since its theoretically possible that a 20-30 mtDNA copy parasite could have heterogeneity in these copies.

A standard curve was created to assess the ability of the assay to detect known percentages of wild-type and mutant DNA. Parasite wild-type DNA (D6) was combined at in 10% intervals with TM90-C50B5 (99.5% mutant) DNA from 0% wild-type to 100% wild-type, in addition to the lower 5% and upper 5% being mixed in 1% increments. All DNA mixtures were PCR amplified and pyrosequenced (PCRs in triplicate, and 2 runs per PCR product) on four separate occasions (n=24). Means and SEM were plotted to generate the standard curve in Figure 2.1. No false-positives were detected in the 100% D6 pyrosequencing runs that passed quality control in pyromark analysis (n=21). As seen in Figure 2.1, the assay slightly over-estimates the WT percentage in the lower 10% of the standard curve, and slightly underestimates it in the upper 10%, with an $R^2=0.9946$ calculated in Graphpad Prism Software. A combination of D6, W2, NF54, and 3D7 runs were used to calculate the false-positive rate for the assay, since D6 is known not to be capable of generating atovaquone resistance, and W2, NF54, and 3D7 are known to generate atovaquone resistance mutations other than Y268S [192, 194, 196, 201]. Out of 48 combined pyrosequencing runs, four gave false-positives (3 in D6, 1 in 3D7), for a false-positive rate of 8.3%.

Y268 cytochrome *b* pyrosequencing assay standard curve

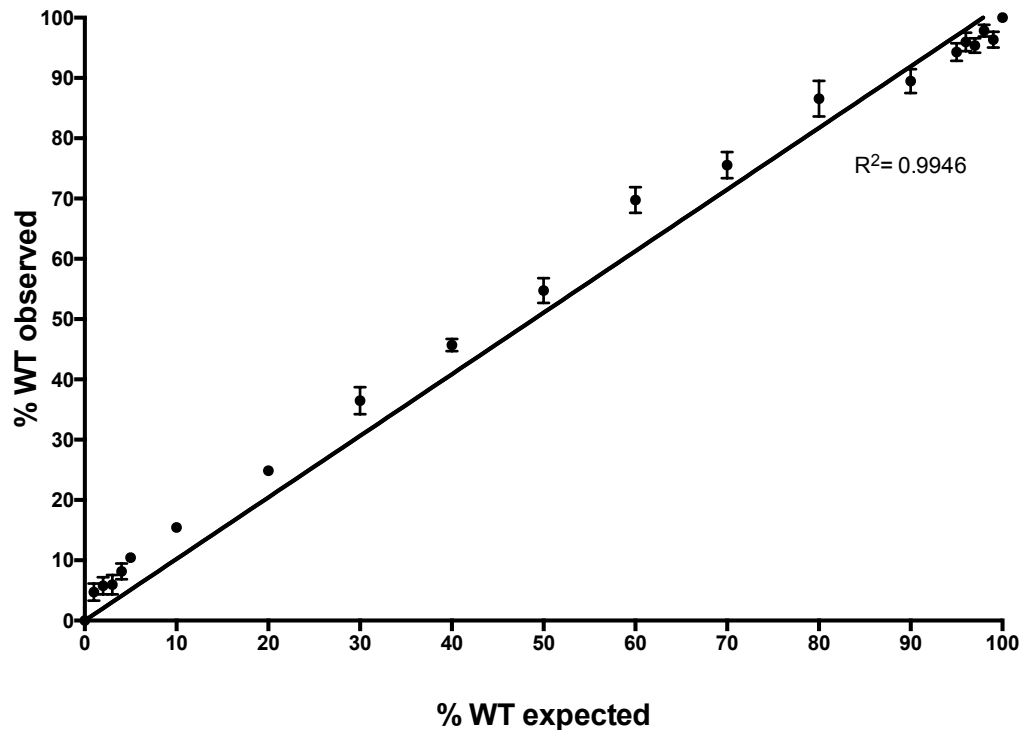


Figure 2.2. Standard Curve of Y268S Cytochrome *b* Pyrosequencing Assay. Genomic DNA from D6 (wt) and TM90-C50B5 (99.52% mutant) were mixed at various concentrations and used to create a standard curve. Data points were plotted as mean percent wt frequencies with at least 12 pyrosequencing runs per data point. Allele frequencies on the lower 10% and upper 10% were slightly overrepresented and underrepresented, respectively.

Extreme mtETC Resistance Parasites Gradually Lose Resistance

While culturing the extreme-resistant parasites TM92-C1086 and TM93-C1088, there was a general observation made that they did not thrive in culture like other culture-adapted strains such as D6 and W2, as noted by a reduced proliferation rate. However, over a period of several months in continuous culture, the parasites began to

grow better but that was accompanied with a fluctuating decline in EC_{50} value, until the parasite lost extreme resistance altogether. While TM90-C2B has been seen to fluctuate more than 3-fold in EC_{50} to atovaquone (generally 10-60 μ M), it has never experienced a sharp, steady decline or showed signs of reversion of the Y268S allele despite over 20 years of *in vitro* culturing. In order to better understand this unstable phenotype, a pyrosequencing assay was used to quantify the frequencies of the Y268S allele and observe the parasite phenotype/genotype relationship over time. Fresh cryopreserves of TM92-C1086 and TM90-C2B were thawed and cultured continuously, with gDNA samples and [3 H]hypoxanthine assays obtained weekly. As seen in Figure 2.3, TM90-C2B indeed showed fluctuating responses to atovaquone and DSM1 over time, but it remained steady in Y268S allele frequency. TM90-C2B spiked up to 68 μ M in atovaquone EC_{50} , and was seen as low as 15 μ M during this two-month period.

With TM92-C1086, the atovaquone and DSM1 EC_{50} values did not rise and fall in tandem, but fluctuated without any discernable pattern. During the course of the experiment, the Y268S frequency declined to nearly wild-type at the end of two months. This genotype reversion back to wild-type was accompanied with accelerated growth rates in the parasites, as initially the parasites did not grow to high parasitemias.

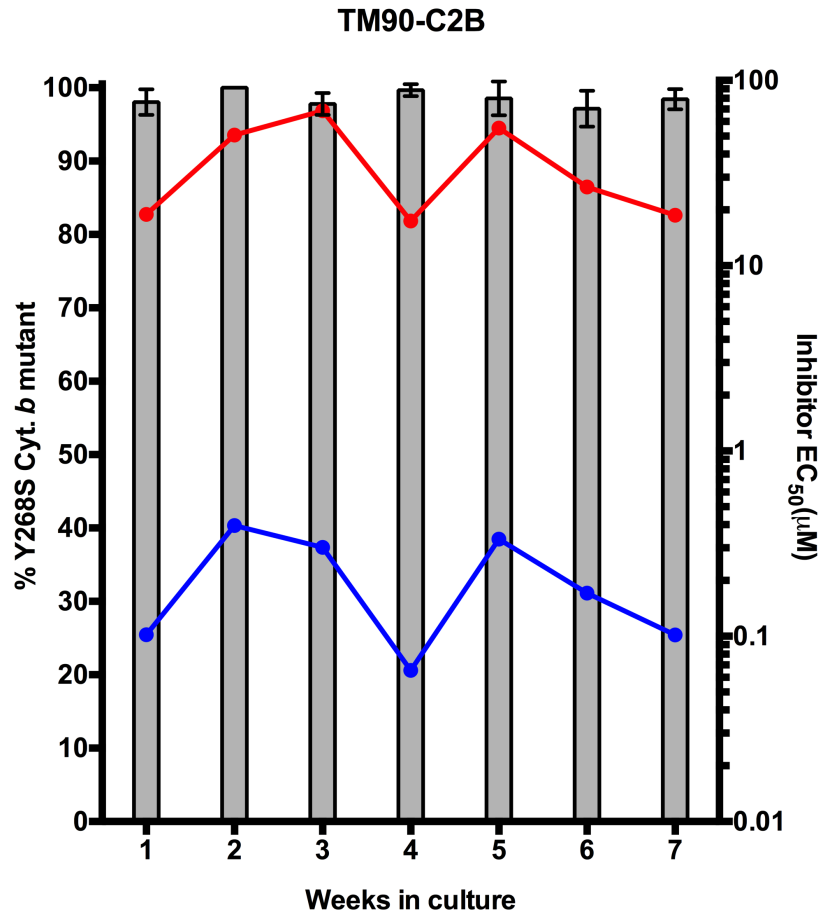


Figure 2.3. TM90-C2B Retains Stable Y268S Genotype. While EC₅₀ for both atovaquone (shown in red) and DSM1 (blue) fluctuate in parallel from week to week, there is no evidence in the loss of Y268S (shown in grey bars, plotted as mean frequency of Y268S and SEM). The left axis corresponds to the frequency of Y268S as % mutant, and the left axis corresponds to inhibitor EC₅₀ in μM for atovaquone and DSM1. Pyrosequencing mean Y268S frequencies are the average of three independent PCR reactions, each run in duplicate (6 pyrosequencing runs total). The error bar missing on week 2 indicates that it detected 100% mutant for all 6 pyrosequencing runs.

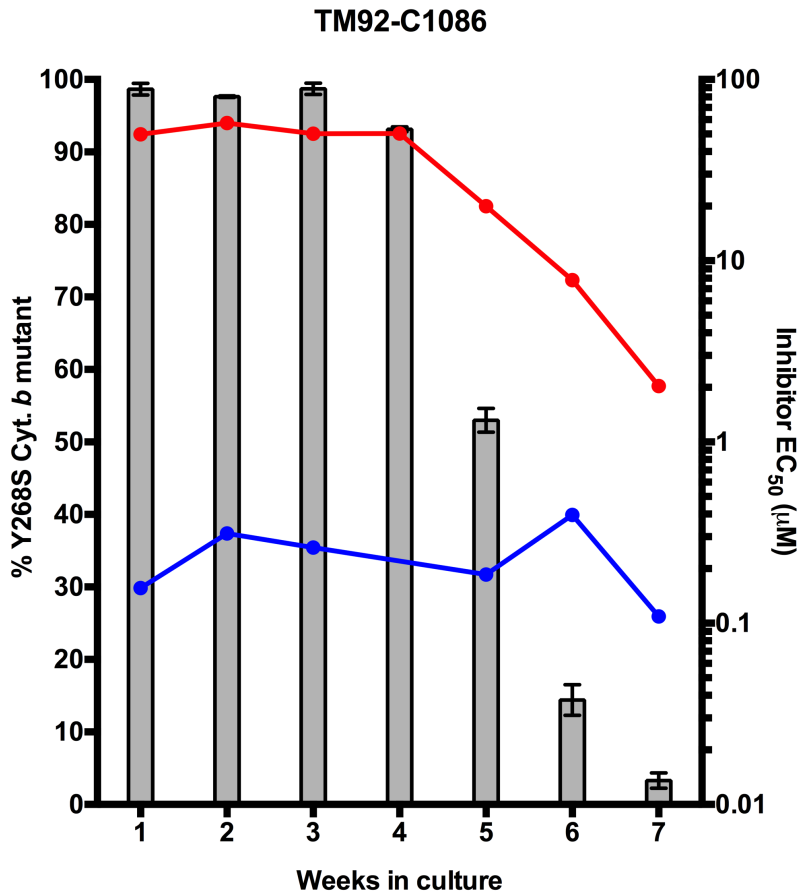


Figure 2.4. TM92-C1086 Gradually Loses Y268S Genotype. EC₅₀ values for atovaquone (shown in red) and DSM1 (blue) do not correlate from week to week, and Y268S genotype declines over a period of two months (shown in grey bars, plotted as mean frequency of Y268S and SEM). The left axis corresponds to the frequency of Y268S as % mutant, and the right axis corresponds to inhibitor EC₅₀ in μM for atovaquone and DSM1. Pyrosequencing mean Y268S frequencies are the average of three independent PCR reactions, each run in duplicate (6 pyrosequencing runs total). The data point for DSM1 missing on week 4 indicates DSM1 EC₅₀ was not obtained for that week.

Pyrosequencing the Y268S allele in Patient Isolates

All parasites were grown to harvest gDNA from earliest cryopreservation dates, and each parasite was pyrosequenced as seen in Figure 2.5. All admission isolates had

detectable Y268S, with TM90-C2A, TM90-C6A, and TM90-C40B2 having 1.0 ± 0.39 , 1.0 ± 0.30 , and 1.1 ± 0.40 percent mutant respectively.

TM90-C6B was a supposed 'wild-type treatment failure' from the Phase II studies, and it was found to be 2.3 ± 0.63 percent mutant. TM90-C6B was deposited into the MR4 repository to be a public research resource for parasite lines, but it was reported in publication as having atovaquone-resistant EC_{50} values from researchers that obtained it. MR4-C6B was included in this dataset because it was speculated that this parasite was actually TM90-C2B and was accidentally mislabeled. Another possibility is that our TM90-C6B parasite grew up from cryopreservation in one vial as wild-type, and another was mutant and got sent to MR4. MR4-C6B was $94 \pm 1.6\%$ mutant, while TM90-C2B is $97 \pm 0.81\%$ mutant, giving them comparable Y268S frequencies, but it is unsure what the identity of MR4-C6B is without more in-depth genotyping.

TM90-50B5 had the highest Y268S mutant frequency of any parasite sampled, with $99.5 \pm 0.32\%$ mutant, and extreme-resistant parasite TM93-C1088 had $99.3 \pm 0.45\%$ mutant. TM92-C1086 shows signs of beginning reversion in pyrosequencing, with two lower values being detected around 50%, with a mean of $88.9 \pm 6.09\%$ mutant.

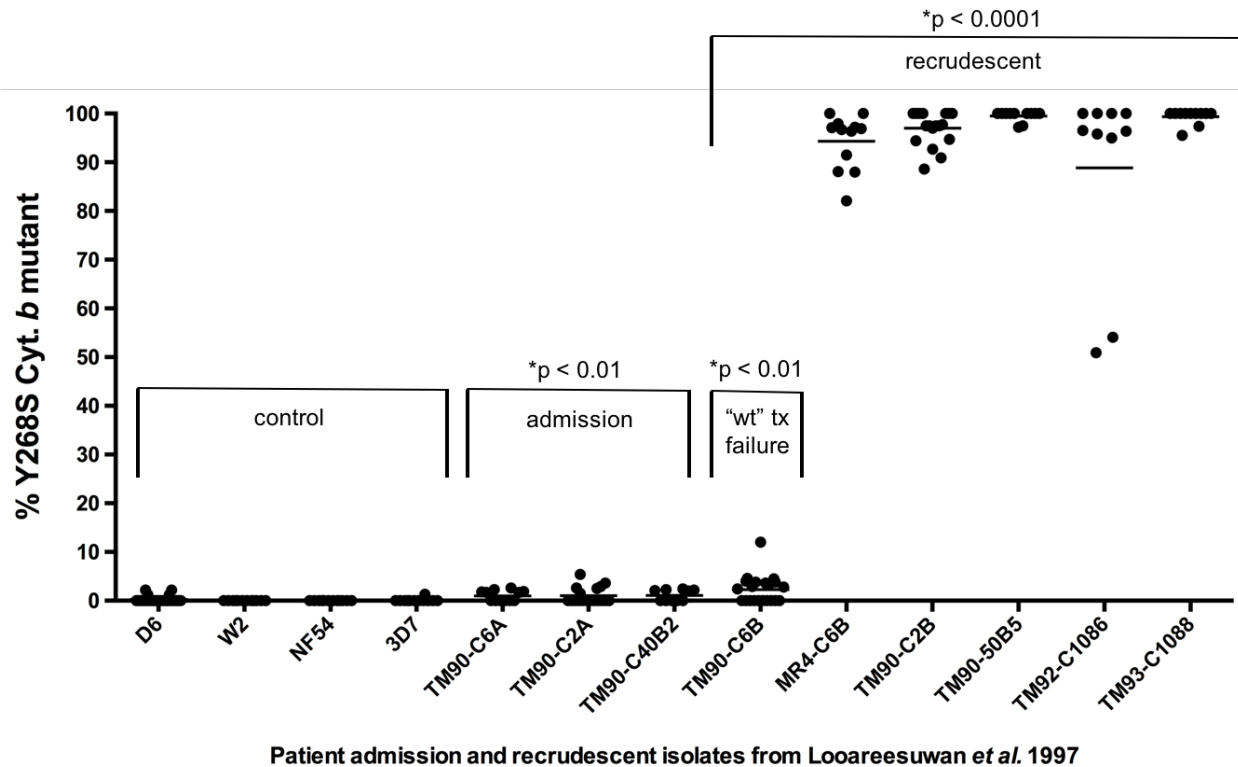


Figure 2.5. Detection of Y268S Mutants in Patient Isolates. For each pyrosequencing run, parasite gDNA sample was PCR amplified in triplicate and pyrosequenced in duplicate for $n=6$. Pyrosequencing runs were completed twice (total $n=12$) but some individual wells did not pass quality control, so all gDNAs have between 9-12 overall data points (single dots are individual runs plotted, line is population mean). Genotype frequencies were plotted as % mutant Y268S gDNA. D6, W2, NF54, and 3D7 were used as controls because they are either known to be incapable of developing atovaquone resistance (D6) or known to develop mutations other than Y268 mutations (NF54, W2, 3D7). P-values are significantly different than controls in a one-tailed t-test, with $p < 0.01$ in admission and “wt treatment failure” groups, and $p < 0.0001$ for other recrudescent isolates.

Discussion

While sequencing of the candidate mtETC genes was important to rule out as a possibility in contributing to the atovaquone resistance spectrum, only a single change was noted in the cytochrome *c* oxidase subunit III, coding for an I239V amino acid substitution. All of the mutations in *Pf*-cytochrome *b* were consistent with their initially reported genotypes, with the exception of the TM90-C6B in the MR4 repository. Interestingly, this is not the first time there has been a discrepancy between the genotypes of parasites stored there and our own collection. The MR4-stock of TM93-C1088 was sequenced and had a Y268S mutation as expected, but in addition, it also had a K272R mutation (data not shown). The K272R mutation has never been seen in isolation, but it has been seen to be co-selected with M133I during *in vitro* atovaquone selections, and it contributes to a substantial increase in EC₅₀ compared with an M133I mutation alone [192]. We can only speculate what causes the genotypic inconsistencies between parasites from the same parasite line (assuming they weren't mislabeled). It is unlikely that MR4-1088 was mislabeled considering we have not observed the K272R mutation in patient isolates before so it does not resemble any of the Thai patient genotypes, suggesting that these parasites are actively mutating without drug pressure. Along the same lines, it is possible that our TM90-C6B experienced spontaneous reversion of its Y268S genotype, while the MR4-C6B did not. Regardless, there is a dynamic process involved that is contributing to rapid genotypic changes in these parasites that warrants further investigation.

In the phenotype stability experiments with TM90-C2B and TM92-C1086, we were able to show that TM90-C2B has an unstable response to atovaquone, yet its

genotype remained stable (Figure 2.3). We are unsure what would cause such strong fluctuations in drug response, but it was observed in two separate studies. It is possible that the pyrosequencing assay is not sensitive enough to truly capture 5% differences in genotype frequencies, but another possibility is the parasites have unstable mtDNA copy number inheritance in each replication round. Plants that have similar mtDNA structure and replication strategies are known to have fluctuating mtDNA copy numbers, which would make sense in the context of being genotypically stable, yet having phenotypic fluctuations [216]. However, it cannot be ruled out that there are other elements acting on the phenotype of TM90-C2B, such as transcriptional expression of cytochrome *b*. Indeed, it has been shown that Y268S mutants have ~2-fold upregulated expression of both Complex III and Complex IV genes to compensate for the fitness costs associated with the Y268S mutated enzyme [193]. Our control parasites during this time frame did not experience more than 2-fold differences in their atovaquone EC₅₀ during this experiment, so we believe it is unlikely to be due to week-to-week assay inconsistencies. More experiments, such as using RT-PCR to investigate TM90-C2B atovaquone response fluctuations are warranted to better understand the unique phenotypes of mitochondrial mutants in this setting.

TM92-C1086 was monitored over a two-month time course and was found to revert its Y268S genotype in favor of wild-type (Figure 2.4). The evidence for the beginning of genotype reversion can be seen in the pyrosequencing of TM92-C1086 in Figure 2.5, where lower trailing values were detected and had an overly disperse detection plot. We can speculate that the extreme resistance phenotype has a higher

fitness cost to the parasite, causing a genotypic reversion event, though what causes extreme resistance remains unclear.

This is the first *in vitro* study implicating cryptic Y268S in parasites prior to atovaquone exposure, as seen in Figure 2.5. All admission isolates were shown to have significant Y268S frequencies around 1% compared to controls, and over 2% in the “wt treatment failure” TM90-C6B. While the assay is in the lower limit of detection for pyrosequencing technology, there are clear differences in detection between the control strains and low-level mutants. Other groups have looked at detection of mutants in admission isolates as well, but none have been detected [217]. This could be for many reasons, such as method of detection. Sanger sequencing, for example, will only detect minor allele frequencies starting between 20-30%, with low frequencies being completely missed. Further, strains may be different in the relative frequency of low-level mutants they may harbor, and ours were high enough to be detected. Traits conferring selective advantage are random events, and a selection pressure bottlenecks a population which allows for survival of a minor variant that otherwise would be less fit, so we see no reason why the mutagenic atovaquone theory is the best explanation for the development of atovaquone resistance, as shown in Aim 2.

CHAPTER THREE
INDUCTION OF ATOVAQUONE AND DSM1 RESISTANCE IN ADMISSION AND
RECRUDSCENT ISOLATE CLONES
(SPECIFIC AIM 2)

Rationale of Study

Evidence of cryptic Y268S heteroplasmy in admission isolates made us curious whether these cryptic mutants were capable of being selected *in vitro*. No *in vitro* selections with atovaquone have yielded the clinically relevant Y268 mutations, so we aimed to show that the parasite genetic background determines the type of resistance mutation that is selected upon atovaquone exposure. We included another cryptic Y268S mutant, ARC08-88-8A that was unrelated to the Thailand atovaquone studies, to see whether a wild, culture adapted clonal line with cryptic Y268S could develop atovaquone resistance. We also used menoctone (structurally related to atovaquone) in some drug selections to see whether developing the Y268S mutation is specific to atovaquone action.

In addition to selections with cryptic Y268S isolates, we wanted to attempt to select for an *in vitro* extreme resistance phenotype by taking an atovaquone resistant parasite (TM90-C2B clone) and exposing it to DSM1 pressure. DSM1 is an inhibitor of the DHODH enzyme, and is the only essential mtETC component in asexual stages, so

we speculated that a combination of atovaquone resistance and DSM1 resistance may create an extreme resistance phenotype. DSM1 resistance has been shown to manifest as either copy number amplifications or mutations in DHODH [201, 218]. Further, high levels of DSM1 resistance have shown to create “atovaquone-tolerant” parasites, although the cause for this is uncertain.

Materials and Methods

Cloning by limiting dilution of Patient Isolates

Parasites from Phase II studies (TM90-C2A, TM90-C2B) were thawed as cryopreserved infected erythrocytes with the earliest possible freeze date, and cultured until they reached 2% parasitemia. Parasites were then diluted in whole culture and erythrocytes were counted on a hemocytometer to determine the number of erythrocytes per mL of culture volume. Parasites were then diluted to 1 parasite/50 μ L concentration, and seeded into 96-well plates. Only plates with fewer than 50% positive wells were used, to increase confidence that they were single parasite clonal populations. Complete media (80%) was changed twice per week, and blood thick smears were made weekly of each well until parasites were sufficiently high to expand into larger culture volumes, and >20 clones were generated. A clone selected from TM90-C2A (TM90-C2A-F6) and TM90-C2B (TM90-C2B-A3) were used for subsequent resistance selection experiments. Sub-cloning the parasites prior to drug selections was necessary in order to provide an isogenic background as well as to maximize phenotypic stability, as many of these parasites experienced more widely fluctuating EC_{50} values to mitochondrial inhibitors (4-8 fold) than control parasites (> 3 fold). We

believe the phenotypic fluctuations are a function of the genotypic plasticity within the population as a whole, where successive replication rounds vary somewhat in Y268S frequency.

Development of Resistant Parasite Lines to Atovaquone/Menotcone *in vitro*

In order to evaluate whether the genetic cryptic heteroplasmy background of parasite strains is essential to the development of the Y268S mutation conferring atovaquone resistance, we assessed the resistance potential of admission isolate clones TM90-C2A-F6, TM90-C40B2, and ARC08-88-8A. TM90-C2A and TM90-C40 were taken from patients prior to treatment and later recrudesced with Y268S mutations in *cyt. b* following atovaquone monotherapy regimens (Table 1.1). TM90-C2A, TM90-C40, and ARC08-88 were sub-cloned by limiting dilution prior to any drug selections, and sub-clones TM90-C2A-F6, TM90-C40B2, and ARC08-88-8A were used for all drug selections. ARC08-88 was originally obtained from the World Health Organization Global Plan Artemisinin Resistance Containment group, and was used to demonstrate the development of atovaquone resistance from a parasite outside the Phase II studies of atovaquone in Thailand that had cryptic Y268S heteroplasmy. TM90-C2A, TM90-C40B2, and ARC08-88-8A were grown from earliest available cryopreserves to 10^8 and seeded into 25 ml flasks in triplicate. The complete medium contained approximately $\sim 10 \times EC_{50}$ atovaquone (10 nM) or $\sim 10 \times EC_{50}$ menotone (1.5 μ M) with media changed twice per week, and split 1:2 with fresh erythrocytes every 10 days to maintain 2% hematocrit. Parasites were considered “recovered” from drug selection when parasite densities reached 2% parasitemia and continued growth under continuous drug

pressure to freeze gDNA and cryopreserved samples. All parasites had the cytochrome *b* gene sequenced to look for possible mutations developed during drug pressure.

Development of DSM1 Resistance *in vitro*

DSM1 selection was carried out as described above, with using concentrations at $\sim 10\times EC_{50}$ of DSM1 for TM90-C2B-A3 (300 nM) and the same concentrations given to the atovaquone-sensitive clone TM90-C2A-F6. Initial EC_{50} values for these parasites was between 30-40 nM for DSM1, so the lower 30 nM was chosen for drug selection experiments. Because selection with the atovaquone-resistant population TM90-C2B was unsuccessful in three separate attempts at the $10\times EC_{50}$ concentration, a lower concentration was attempted at $\sim 2\times EC_{50}$ (60 nM) and was successful.

DHODH Copy Number Quantitative PCR (qPCR).

Pf-DHODH copy number was determined using the DHODH qPCR primers previously described by Guler *et al.* [218] and the LDH-T1 FOR/REV control primers from Chavchich *et al.* [219] using Brilliant II/III SYBR Green Master Mix with ROX and the Mx3005P qPCR machine (Applied Biosystems). The relative copy number of DHODH was determined for 0.1 ng of gDNA and normalized to the LDH gene using the $\Delta\Delta C_T$ method [220]. Copy number is described as mean values of 2 or 3 replicates, rounded to the nearest whole number.

Results

Parasites Develop Resistance to Atovaquone/Menoctone *in vitro*

The atovaquone/menoctone selections aimed to evaluate the possibility that the genetic background is responsible for the type of resistance that develops in

atovaquone exposure. Each parasite background was cloned prior to drug selection. Interestingly, clonal populations of TM90-C2A and TM90-C2B were much more stable in their initial EC_{50} , yet clonal populations have not been in continuous culture for comparable amounts of time to be compared directly about phenotype stability. On day three and four of all selections, many parasites were seen outside erythrocytes, and schizonts were observed that lacked significant DNA content. This morphological aberration is consistent with pyrimidine depletion in the parasites caused by atovaquone preventing ubiquinone generation in the parasite [117]. This was followed by parasites generally clearing from detection in blood thin smear. Parasites could still be seen sparingly in the thick smear, with one parasite visible for every 10-20 microscope fields of view. The notable exception to this is ARC08-88-8A, which showed evidence of crashing in the thin smear, but parasites were much more present in the thick smear, with 1-2 parasites still visible per field. As seen in Figure 3.1 below, there were considerable differences in recovery times between strains. TM90-C2A-F6 and TM90-C40B2 under atovaquone pressure had similar recovery times, with approximately 30 days until healthy parasites grew through continued atovaquone pressure. ARC08-88-8A was fully recovered and growing 7 to 10 days sooner than the other atovaquone selections. ARC08-88-8A is a cloned parasite from the Artemisinin Resistance Containment group, and is known to have a delayed clearance phenotype to artemisinin as well as being a cryptic Y268S mutant, so it is uncertain whether this is a contributing factor to its earlier recovery from atovaquone exposure.

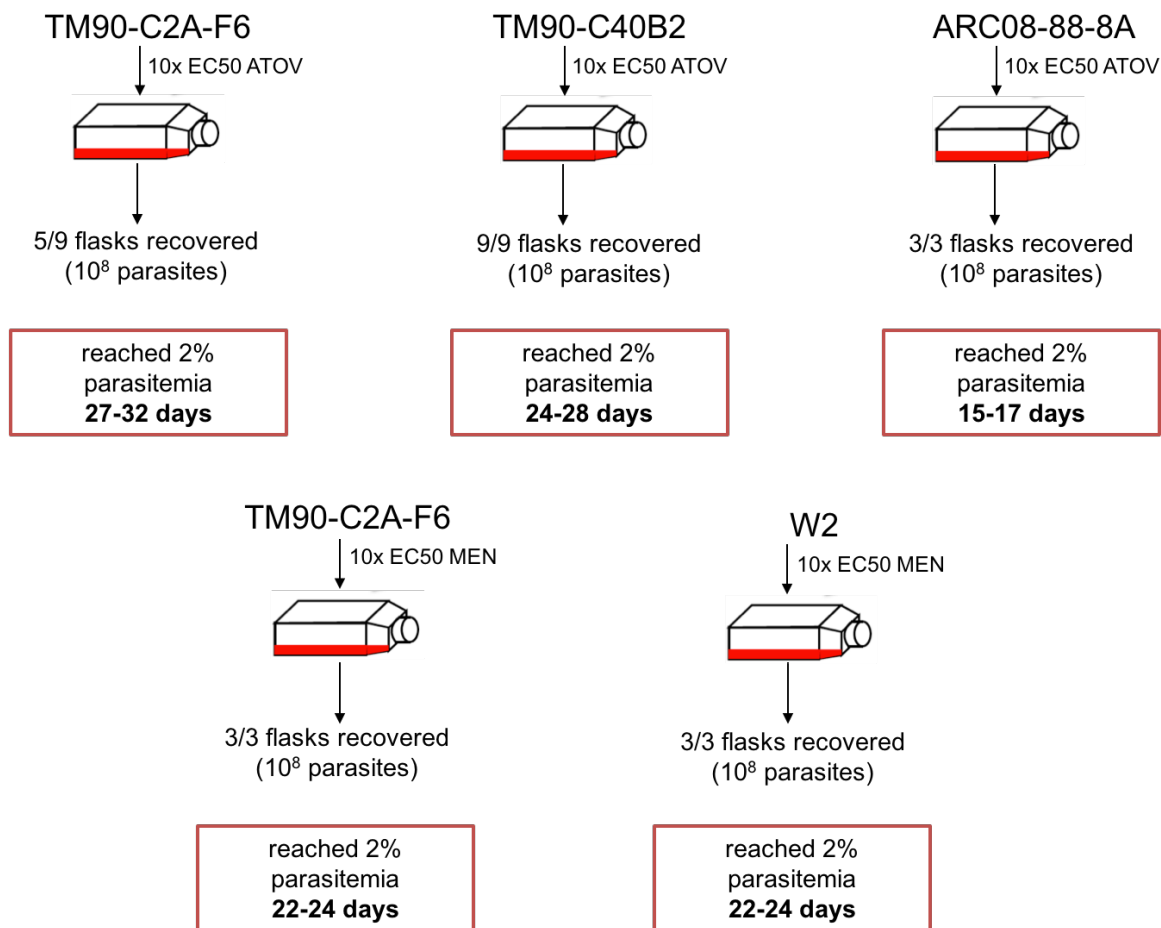


Figure 3.1. Schematic of Drug Selections using Atovaquone and Menoactone *in vitro*. All parasites were treated with 10x EC₅₀ (10 nM) drug pressure with 10^8 parasites. Clonal lines of TM90-C2A (TM90-C2A-F6), TM90-C40B2 and ARC08-88-8A were used to generate resistant parasites in cryptic Y268S backgrounds to see if low-level mutants are responsible for the development of Y268S resistance. The mechanism of action of menoactone is suspected to be similar to atovaquone, so menoactone selections were performed to see if menoactone could generate Y268S mutations as well. W2 does not have cryptic Y268S mutant frequencies and is not anticipated to generate Y268S mutants. All atovaquone-treated parasite groups successfully generated resistant mutants by day 32, but ARC08-88-8A developed resistance rapidly with recrudescence parasites at 2% parasitemia on day 15. In the menoactone-treated parasites, recovery times were the same at 22 days.

Sequencing of Atovaquone/Menoctone Resistant Selections Reveals Clinically Relevant Y268S Mutations

All parasite selections were PCR amplified and sequenced for mutations in *dhodh*, cytochrome *b*, and *coxIII*. All cryptic mutants were found to be Y268S mutants in cytochrome *b*, which is the first time the Y268S mutation has been successfully recapitulated *in vitro*. Menoctone, a naphthoquinone related to atovaquone, developed an M133I mutation, which is a common atovaquone-resistance mutation that has been selected *in vitro* in multiple genetic backgrounds, suggesting the mode of action/resistance is similar to that of atovaquone. C2A-F6+10xMEN resulted in a Y268S mutation, which furthers that notion. All parasites sequenced were wild-type *dhodh* upon sequencing as expected. The majority of the parasites retained the *coxIII* genotype of their parental lines, with the exception of ARC08-88-8A+10xATOV, which started out as wild-type prior to selection. The significance of this genotype switching is unknown.

Table 3.1. Genotypes of Atovaquone/Menoctone Drug Selections

Atovaquone-selected populations	PFF_0160C (<i>dhodh</i>)	MALMITO_3 (<i>cyt. b</i>)	MALMITO_1 (<i>coxIII</i>)
C2A-F6+10xATOV (n=5)	WT	Y268S	I239V
TM90-C40B2+10xATOV (n=9)	WT	Y268S	I239V
ARC08-88-8A+10xATOV (n=3)	WT	Y268S	I239V
C2A-F6+10xMEN (n=3)	WT	Y268S	I239V
W2+10xMEN (n=3)	WT	M133I	--

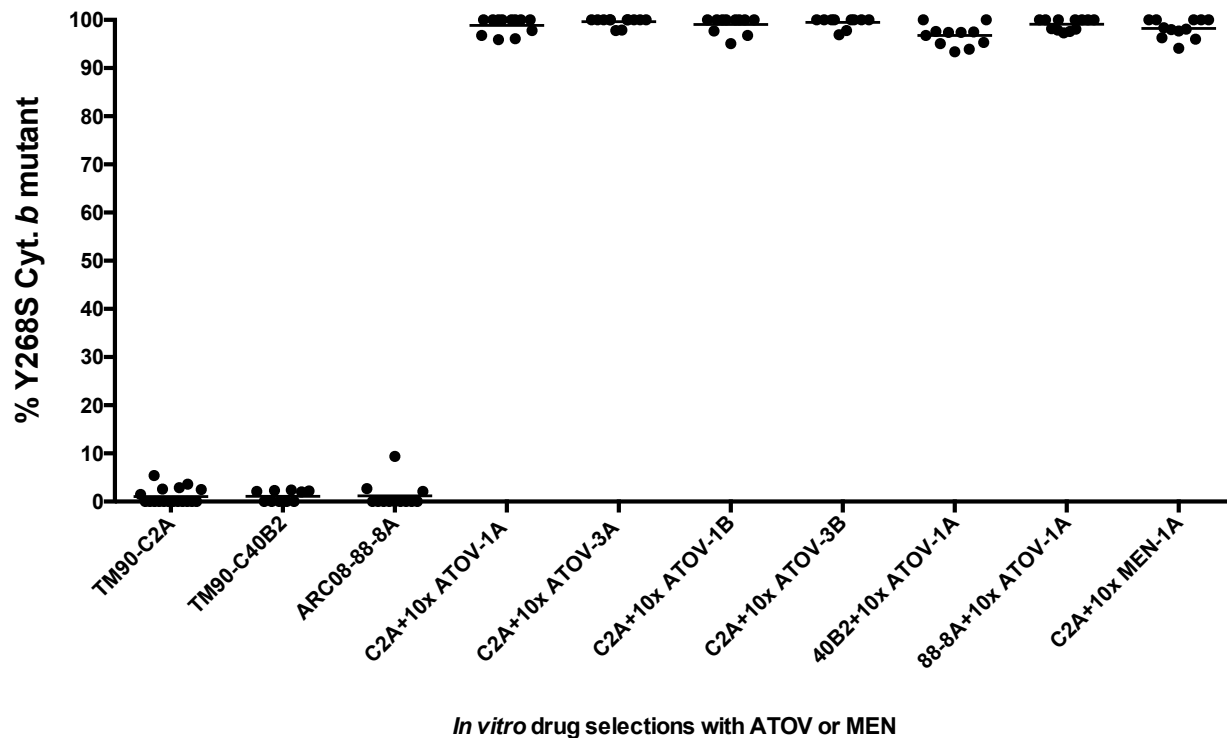


Figure 3.2. Pyrosequencing of Atovaquone/Menoctone Selected Parasites. Genomic DNA was harvested from parasite populations immediately following recovery and was used to evaluate the Y268S frequency.

Parasite	Q _o site inhibitors		DHODH inhibitor	cyt. <i>b</i> genotype
	ATOV	MYX	DSM-1	
C2A+10xATOV-3A	3.1 ± 0.73	0.45 ± 0.15	0.032 ± 0.00035	Y268S
C2A+10xATOV-3B	4.2 ± 1.3	0.29 ± 0.096	0.031 ± 0.0038	Y268S
C2A+10xATOV-2B high	38 ± 11	1.8 ± 0.76	0.067 ± 0.0018	Y268S
C2A+10xATOV-2B low	4.6 ± 0.17	0.28 ± 0.034	0.029 ± 0.0064	Y268S
ARC08-88-8A+10xATOV-1A	63 ± 5.0	ND	0.067 ± 0.042	Y268S
TM90-C2A-F6	0.0013 ± 0.0003	0.094 ± 0.023	0.12 ± 0.0072	--
TM90-C2B-A3	12 ± 3.0	1.2 ± 0.21	0.040 ± 0.0042	Y268S
TM92-C1086	55 ± 4.2	22 ± 4.8	0.45 ± 0.090	Y268S

Table 3.3. Resistance to mtETC Inhibitors in Menoctone-selected Populations of *P. falciparum* (EC₅₀ μ M), SEM

Parasite	Q _o site inhibitors		DHODH inhibitor	cyt. <i>b</i> genotype
	ATOV	MYX	DSM-1	
C2A+10xMEN-1A	54 \pm 8.9	ND	0.052 \pm 0.0023	Y268S
W2+10xMEN-1A	0.066 \pm 0.022	ND	0.16 \pm 0.028	M133I
TM90-C2A-F6	0.0013 \pm 0.00030	0.094 \pm 0.023	0.067 \pm 0.042	--
TM90-C2B-A3	12 \pm 3.0	1.2 \pm 0.21	0.040 \pm 0.0042	Y268S
TM92-C1086	55 \pm 4.2	22 \pm 4.8	0.45 \pm 0.090	Y268S

Pyrosequencing confirms Y268S populations in resistant parasites

To evaluate whether the parasites fully selected for the Y268S allele, pre-treatment and post-recovery parasite populations were pyrosequenced. All atovaquone-selected parasites as well as menoctone-selected C2A showed majority Y268S genotypes, although no parasite has shown complete 100% mutant frequencies to date, they hover between 98-99% mutant. Interestingly, ARC08-88-8A (pre-treatment) showed a single detection of 10% mutant in its population, which may indicate a subpopulation that had higher Y268S frequencies (above 1-2%) that allowed it to recover so much faster than either TM90-C2A or TM90-C40B2.

C2A+10xATOV Parasites Display Variable Phenotypes

Following recovery, all parasites were maintained on atovaquone pressure in the hopes of keeping stable phenotypes. As seen in Table 3.2, the majority of atovaquone-selected populations had stable EC₅₀ values such as C2A+10xATOV-3A and 3B, which were on the low-end of EC₅₀'s typical for TM90-C2B. ARC08-88-8A+10xATOV-1A was incredibly resistant to atovaquone immediately following drug selection, with an EC₅₀ of

66 μM to atovaquone but remained sensitive to DSM so did not exhibit an extreme resistance profile. In fact, this parasite was tested for drug susceptibility immediately following selection, and had not been in continuous culture for very long, so little can be said about whether this high EC_{50} is stable over time.

Menoctone resistance in W2 developed the common M133I mutation, which accompanies a very modest resistance profile to atovaquone as published elsewhere [192]. C2A+10xMEN-1A on the other hand, had high grade resistance to atovaquone similar to ARC08-88-8A+10xATOV-1A, which may indicate that the parasites show highest resistance profiles prior to being cryopreserved, as both of these were assessed for drug susceptibility prior to cryopreservation, where the others were not.

However, certain parasites did not keep stable phenotypes (like C2B) and could be cultured at different times and have periods of low and high EC_{50} s. An example of this is C2A+10xATOV-2B, which over time had EC_{50} 's similar to that of the other atovaquone-selected parasites, but would then go through periods with highly elevated resistance similar to that of TM90-C2B. There was no particular correlation to timing or duration of culture to explain these fluctuations, similar to that of TM90-C2B during the phenotype stability experiments shown in Aim 1. Additionally, the high levels of atovaquone resistance were accompanied by an elevated, but not extreme resistance to other electron transport chain inhibitors, as seen in comparisons of C2A+10xATOV-2B EC_{50} 's below. The lower and upper limits show the lowest and highest EC_{50} 's obtained respectively, and is the representation of four replicates, two while in high EC_{50} phenotype, and two in low EC_{50} phenotype.

Table 3.4. Fluctuations of Moderate and Elevated mtETC Resistance in (EC₅₀, nM)

	Complex III							
	NDH2	DHODH	Qo site (cyt <i>b</i>)		Qi site		Unknown <i>bc1</i>	
	HDQ	DSM-1	ATOV	MYX	ANT	GSK121	ICI56,780	P4Q-391
W2	55.0	52.4	0.41	10.8	165.5	3.39	0.03	11.1
Lower limit	7.23	11.9	4400	252	53.2	21.4	2.52	10.6
mean	107	43.2	21400	1050	172	133	49.6	163
Upper limit	305	69.0	49200	2570	383	286	156	562
TM90-C2B	146	57.4	5290	428	152	77.5	14.3	55.5
TM92-C1086	180	770	31400	4160	>18200	2470	882	14200

Table 3.5. Summary of *Pf in vitro* Drug Selections and Corresponding Mutations

Parasite line	cyt. <i>b</i> mutation(s)	Source
3D7	M133I M133I & P275T M133I and K272R M133I and G280D L283I & V284K	Korsinczky <i>et al.</i> 2000
3D7	M133V M133I M133I & L144S F267V	Bopp <i>et al.</i> 2013
K1	M133I M133I & G280D	Schwobel <i>et al.</i> 2003
AT200	M133I M133I & L271F	Schwobel <i>et al.</i> 2003
TM90-C2A	Y268S	This study
TM90-C40B2	Y268S	This study
ARC08-88-8A	Y268S	This study
W2*	M133I	This study
TM90-C2A*	Y268S	This study

*Starred parasites indicate menoctone selection

Overall, the atovaquone selections were successful in recapitulating the TM90-C2B genotype and phenotype, especially where the phenotypic plasticity is concerned. No changes were observed in *dhodh* copy number for these parasites (data not shown) so DSM1 involvement is unlikely to be involved in this phenotype. A summary chart of drug selection outcomes *in vitro* to date are seen below in Table 3.5.

TM90-C2B Cannot Generate DSM1 Resistance at 10x EC₅₀ Concentrations

In an effort to recreate extreme resistance, TM90-C2B was given DSM1, a drug known to target the DHODH enzyme, as we believed that double-mutant parasites would have pan-resistance to mtETC inhibitors. Following multiple (3) attempts at selecting DSM1-resistant C2B parasites and carrying it out for 90 days, it was determined that these two resistance mechanisms are intolerant of one another, where atovaquone-resistance conferred by Y268S mutations could not tolerate generating the DSM1-resistance mechanism simultaneously. This argument is strengthened by the simultaneous selection of TM90-C2A-F6 with 10x EC₅₀ DSM1 (300 nM), which was able to generate resistance readily at this high concentration. We then attempted a lower-level DSM1 selection for TM90-C2B, where ~2x EC₅₀ DSM1 was used (60 nM). Parasites readily tolerated this lower concentration, and while they dropped considerably in parasitemia and failed to grow for about two weeks, they didn't completely crash, as is typical in a drug selection experiment. C2B+2xDSM1 parasites recovered on average at day 21, while C2A+10xDSM1 took somewhat longer, about 24 days. A summary of DSM1 selection experiments can be seen below in Figure 3.3 below.

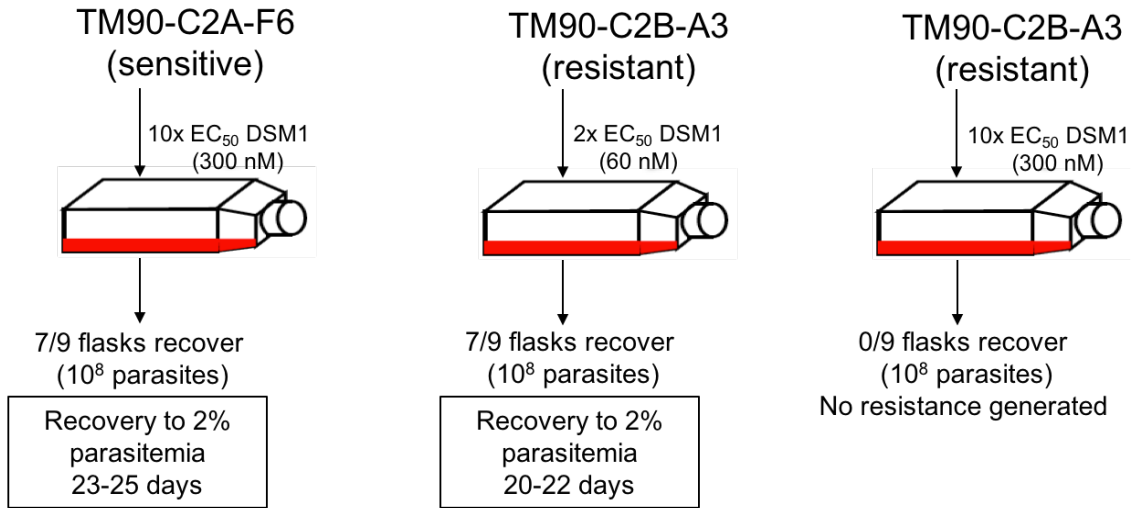


Figure 3.3. Summary of DSM1 Selections in Atovaquone Sensitive and Resistant Backgrounds. C2B parasites fail to recover at 10x EC₅₀ concentrations of DSM1, yet readily generate resistance at 2x EC₅₀ concentrations. Atovaquone sensitive TM90-C2A readily generates resistance to 10xEC₅₀ DSM1, suggesting these two resistance mechanisms are intolerant of one another.

Initial Characterization shows Several Compensatory mechanisms to DSM1 Pressure

Initial characterization of DSM1 selection parasites showed that parasite exposures in atovaquone-sensitive and atovaquone-resistant lines varied considerably in their chosen mechanism to combat DSM1 pressure. As the genotyping of DSM1 selections in Table 3.6 show, atovaquone-sensitive TM90-C2A responded to DSM1 pressure with either copy number amplifications in *dhodh* (which has been previously reported) as seen with C2A+10xDSM1-1A, or shockingly with low-level amplification of Y268S genotype combined with a mutation in *dhodh*, R265G, as seen in C2A+10xDSM1-2B. This R265G mutation in *dhodh* is a novel one, and the combination with partial Y268S/WT mutant, yielded a combined DSM1 and atovaquone-resistant

genotype as seen in Table 3.6. This is the first description of a Y268S mutation popping up from drug pressure on the DHODH enzyme. In order to quantify the percentages of Y268S mutant present in this parasite, all DSM1-selected lines were pyrosequenced as seen in Figure 3.4 below. Pyrosequencing results indicate that C2A+10xDSM1-2B was in fact 30% Y268S mutant at the time of sequencing, while C2A+10xDSM1-1A remained WT, as its chosen selection strategy was multiple (3) copies of the *dhodh* gene.

C2B+2xDSM1 selections sequenced immediately following recovery showed a mixture WT/Y268S mutant genotype, and initial EC₅₀ determinations are shown for the parasites in Table 3.7. Parasites show varying response to atovaquone and DSM1, with the highest atovaquone EC₅₀s accompanying the highest DSM1 EC₅₀s, such as in C2B+2xDSM1-1B, 3B, and 2C. The two lowest EC₅₀ pairs seen were in C2B-2xDSM1-1A. The C2B+2xDSM1 parasites all had various mixtures of the Y268S mutant along with copy number amplifications in DSM1 that corresponded to their spectrum of response to the two drugs. The parasites tolerated both mechanisms sufficiently at low pressure at the cost of reverting some of the Y268S copies back to wild-type. Pyrosequencing was used to determine the relative frequencies of these Y268S mutants as seen in Figure 3.4. These phenotypes show populations in flux, with varying amounts of *dhodh* copy number, Y268S copies, and WT copies.

Table 3.6. Genotypes of DSM1 Drug Selections Immediately Following Recovery

DSM1-selected populations	<i>dhodh</i> copy number	PFF_0160C (<i>dhodh</i>)	MALMITO_3 (<i>cyt. b</i>)	MALMITO_1 (<i>coxIII</i>)
C2A+10xDSM1-1A	3	WT	WT	I239V
C2A+10x DSM1-2B	1	R265G	Y268S/WT	I239V
C2B+2xDSM-1A	2	WT	Y268S/WT	I239V
C2B+2xDSM1-1B	2	WT	Y268S/WT	I239V
C2B+2xDSM1-3B	1.5	WT	Y268S/WT	I239V
C2B+2xDSM1-2C	1	WT	Y268S/WT	I239V

Table 3.7. Earliest Drug Susceptibility Profiles of DSM1-resistant *P. falciparum* (μM)

DSM1-selected populations	ATOV	MYX	DHODH
C2A+10xDSM-1A	0.0018	0.010	0.43
C2A+10xDSM-2B	5.8	0.56	0.22
C2B+2xDSM-1A	7.0	1.7	0.20
C2B+2xDSM-1B	31	0.62	0.55
C2B+2xDSM-3B	29	5.6	0.31
C2B+2xDSM-2C	19	1.7	0.37
TM90-C2A-F6	0.0013	0.094	0.067
TM90-C2B-A3	12	1.2	0.040

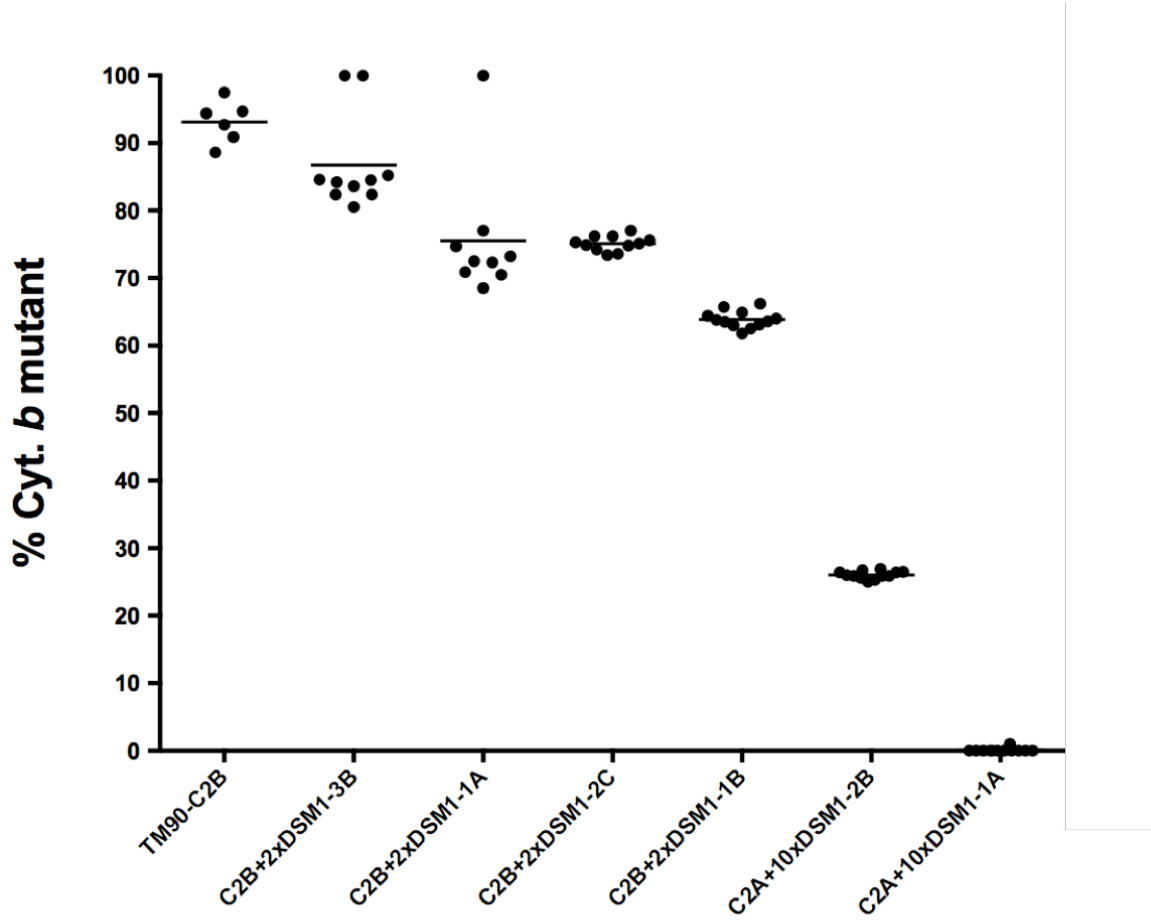


Figure 3.4. Pyrosequencing of DSM1-selected parasites shows Y268S heteroplasmy is responsible for combined atovaquone/DSM1 resistance spectrum phenotypes. Pyrosequencing was performed as previously described in Aim 1 with the gDNA of the parasites immediately following recovery from DSM1 treatment. Control parasite TM90-C2B (n=6) is representative of gDNA harvested right before selection experiments began. All other parasites had 6 PCRs run in duplicate (n=12) and all that passed quality control were plotted.

Low-grade Resistance to DSM1 Causes Loss of Y268S mutation in TM90-C2B

Initial phenotypic characterization following parasite recovery in the C2B+2xDSM1 selections showed was impeded by gradually declining EC_{50} s from week to week, despite being maintained by constant drug pressure. The highest and lowest EC_{50} values for each of the C2B+2xDSM1 parasites are shown in Table 3.8, where EC_{50} s started out moderately pan-resistant to all mtETC inhibitors, but exhibited a gradual decline until all EC_{50} s except DSM1 were sensitive. Because the parasite populations were a mixture of WT/Y268S mutant, it was speculated that these parasites were in the process of reversion of the Y268S genotype. Parasites were allowed to grow while under continuous drug pressure for a month following recovery, and parasite EC_{50} s for atovaquone and DSM1 were determined along with pyrosequencing of the Y268S allele as seen in Figure 3.5. All parasite populations completely reverted their Y268S genotype over time. Consistent with the notion that these two resistance mechanisms are incompatible, the continued DSM1 pressure gradually converts the Y268S copies back to WT, and losing their atovaquone resistance in the process. Once parasites reverted, they retained a modest 2-3 fold resistance to DSM1, indicative of a duplicated DHODH gene in exchange for atovaquone resistance. Considering they were on constant DSM1 pressure, this exchange of one resistance mechanism for another was very gradual.

Table 3.8. Gradual Pan-mtETC Elevated Resistance Decline in C2B+2xDSM1 (EC₅₀, nM)

	Complex III							
	NDH2	DHODH	Qo site (cyt <i>b</i>)		Qi site		Unknown <i>bc1</i>	
	HDQ	DSM-1	ATOV	MYX	ANT	GSK121	ICI56,780	P4Q-391
W2	55.0	52.4	0.41	10.8	165.5	3.39	0.03	11.1
1A Lower limit	6.94	43.6	1.53	111	83.8	6.71	0.126	2.63
1A Mean	33.7	110	3090	613	182	30.0	15.8	20.0
1A Upper limit	54.3	204	6890	1440	256	63.4	75.2	52.7
1B Lower limit	103	40.0	4430	398	93.0	37.6	7.39	16.2
1B Mean	472	195	14400	2060	596	97.0	30.0	73.4
1B Upper limit	796	545	31000	5470	1680	204	65.0	158
3B Lower limit	96.5	61.8	1.89	51.3	120	4.93	0.144	24.3
3B Mean	1070	179	13600	1990	916	98.7	16.5	202
3B Upper limit	3020	309	28800	5620	1740	202	29.5	569
2C Lower limit	235	58.1	1.07	18.5	31.0	4.70	0.286	6.08
2C Mean	1020	195	4380	654	946	46.5	12.7	70.7
2C Upper limit	2650	367	18700	1760	4590	113	32.7	204.5
TM90-C2B	146	57.4	5290	428	152	77.5	14.3	55.5
TM92-C1086	180	770	31400	4160	>18200	2470	882	14200

Means represent between 4 and 11 replicates of EC₅₀s for each parasite.

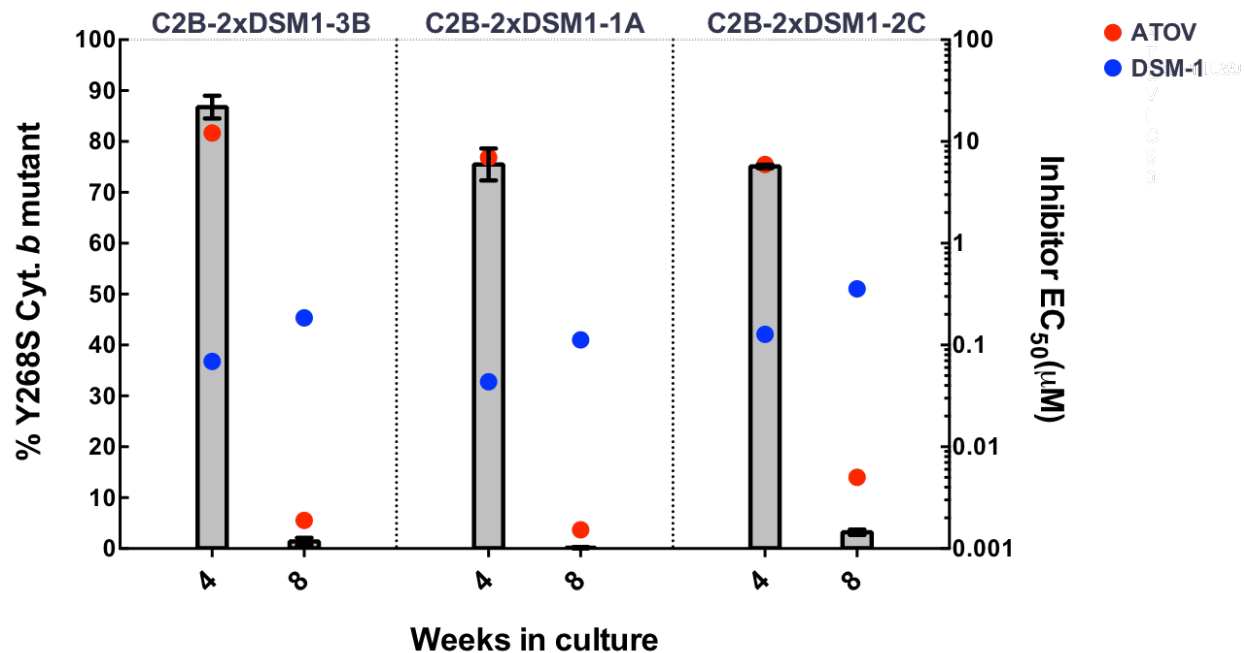


Figure 3.5. C2B+2xDSM1 Cultures Gradually Convert Their Heteroplasmic State Under Continuous DSM1 Pressure. Beginning with TM90-C2B parasites that successfully generated resistance to 2x EC₅₀ DSM1 and recovered to 2% parasitemia (~4 weeks), parasites were monitored after an additional 4 weeks for their heteroplasmic Y268S status using pyrosequencing, as well as their EC₅₀s for both ATOV (red) and DSM1 (blue). It took a total of 8 weeks from the beginning of DSM1 selection with DSM1 to fully convert TM90-C2B parasites to wild-type (ATOV-sensitive), despite growing normally after 4 weeks. Parasites maintained modest (~2-3 fold) resistance to DSM1 after full Y268S reversion. Error bars indicate SEM for averaged pyrosequencing frequencies (n=12).

Discussion

In Aim 2, we showed that cryptic Y268S presence can be selected out in several parasite backgrounds, in both admission isolates from the Phase II studies in Thailand (TM90-C2A, TM90-C40B2) as well as wild parasites (ARC08-88-8A). Further, we showed that the related naphthoquinone menoctone was able to select out the clinically relevant mutation Y268S in TM90-C2A, as well as the common *in vitro* selected M133I

mutation in W2. The phenomenon of mitochondrial heterogeneity is called heteroplasmy, which is when mitochondrial copies consist of multiple genotypes. Last, we showed that mitochondrial heteroplasmy can be induced in both atovaquone susceptible and atovaquone resistant backgrounds, with C2B+2xDSM1 parasites gradually experiencing heteroplasmic conversion from mutant Y268S to WT, and also in TM90-C2A+10xDSM1, which in one parasite selected for a partial Y268S mutant along with a *dhodh* R265G mutation.

The long-term incompatibility of atovaquone and DSM1 resistance suggests that while the parasites dislike having both mechanisms simultaneously, it is not completely lethal at low enough DSM1 levels. Increasing the copy number of *dhodh* to overcome DSM1 pressure likely means that the parasite wants a functional *bc₁* complex so that it is not compensating for two things at once. This is not the first time that these two mechanisms were shown to be incompatible. Indeed, the combination of 5-fluoroorotate with atovaquone was shown to be superior in suppression of atovaquone resistance *in vitro* [221]. Since atovaquone kills parasites with slow pyrimidine starvation in asexual stages, having a somewhat inefficient *bc₁* complex with reduced catalytic turnover as well as an inhibited DHODH enzyme is a huge survival disadvantage, and only suboptimal concentrations of DSM1 allowed for this resistance mechanism conversion to take place. For this reason, it is interesting that high levels of DSM1 (10x EC₅₀) induced a partial Y268S mutant. This may have something to do with the additional R265G mutation observed in this parasite, or it could be that the parasite tries out any adaptive strategy that could lend an advantage, and since it was only ~30% Y268S mutant, it could be unintended, unrelated, or transient in nature. The combination of

Y268S and *dhodh* amplifications in this transitional period conferred an enhanced resistance to all mtETC inhibitors, though not truly extreme resistance.

The cryptic Y268S presence was both detectable and able to be selected out *in vitro*, which is a sharp contrast to what is commonly believed about *Plasmodium* mitochondria. Current thought believes that the mitochondria are inherently intolerant of mutation, and further, that their recombination-dependent replication mechanism combined with strict maternal inheritance keeps diversity to a minimum [165, 222]. If generation of the Y268S mutant is a *de novo* occurrence and caused by a mutagenic effect of atovaquone, then how did these parasites manage to revert to WT when given DSM1 pressure? Considering DSM1 pressure causes an amplification in a nuclear-encoded gene, it seems far-fetched that DSM1 is also a mutagenic force on the mitochondrial DNA. Several labs have selected for mutants with triazolopyrimidines, yet none have seen any mt-encoded mutations develop [201, 202, 218]. It seems much more likely that these parasites are genotypically much more diverse and plastic than initially thought, since we have shown that parasites with cryptic Y268S heteroplasmy are never forced to 100% mutant or 100% WT, regardless of pressure. This lack of evidence for homoplasmy (100% homogeneous mtDNA copies) in the face of strong selective pressure suggests there are intrinsic mechanisms that maintain either low levels of Y268S or low levels of WT at all times. This work highlights the need to understand mitochondrial diversity, particularly how it is generated and how it is maintained in populations.

CHAPTER FOUR

**GENOTYPIC CHARACTERIZATIONS OF ADMISSION/RECRUDESCENT ISOLATES
AND IN VITRO DRUG SELECTED LINES (SPECIFIC AIM 3)**

Rationale of Study

Mitochondrial heteroplasmy is a phenomenon found to be common in many organisms such as humans, plants, fungi, trypanosomes, and even found in other *Plasmodium* species [166, 168-170, 172, 174]. We aimed to look at heteroplasmy with a more zoomed-out perspective in the hope of finding *Plasmodium* mitochondrial diversity on a global scale. In order to accomplish this, we used a combination of pyrosequencing and NGS technologies to show that mitochondrial diversity is not just a temporary reaction to drug pressure, but a common and much needed mechanism of maintaining parasite fitness in *Plasmodium falciparum*. Heteroplasmy is now commonly detected with deep-sequencing at high coverage (>10,000x) with strict requirements for base-calling, including double-strand validation and phred quality scores >30 to achieve confidence in calling low-level variants. Because sequencing error at specific loci is both predictable and reproducible in multiple samples, and usually only present on a single strand, errors can be distinguished from true low-level variants with this filtration method with confidence [169].

To discover low-level pre-existing heteroplasmy at known drug resistance alleles such as Y268S, we deep-sequenced paired admission and recrudescence isolates with high coverage (10-30,000x). Low-level Y268S heteroplasmy was successfully detected at 1-2% frequency in pre-treatment (wild-type, ATO susceptible) patient isolates, which agrees with pyrosequencing data that Y268S exists in parasites before drug pressure. Parasite lines generated with *in vitro* drug pressure were sequenced as well, and provide new insights into the mechanics of mitochondrial heteroplasmy. Further, to discover mitochondrial diversity in the worldwide parasite population, we re-analyzed the publicly available *P. falciparum* genome data from the MalariaGEN Pf3K project for minor allele frequencies (MAF) > 0.15 to uncover mitochondrial heteroplasmic diversity in the ~2600 parasite collection. We estimated the mt copy number to be between 10-27 copies and we found that mitochondrial genome diversity was underestimated at least 5-10 fold without taking heteroplasmy into consideration. Mitochondrial heteroplasmy and its role in providing genetic diversity to a parasite with strict maternal inheritance of mitochondrial DNA is a necessary response to maintain parasite viability and fitness.

Materials and Methods

Mitochondrial Deep-Sequencing of Patient Isolates and Drug Selections.

Parasites were all grown and maintained under their respective drug pressures while being propagated for gDNA for sequencing, or in the case of patient isolate parasites, they were not given drug pressure since they are normally maintained without it. DNA was harvested from parasites and extracted as previously described in Aim 1. Parasite DNA samples were sent for mitochondrial deep-sequencing at the Genomics

Core at the Oklahoma Medical Research Foundation to be run on the Illumina HiSeq 3000, as 150 cycle, paired-end reads.

Estimation of mtDNA Copy Number in MalariaGEN Pf3K Parasite Collection

For the 2640 samples of the MalariaGEN Pf3K parasite genome collection, we extracted the median coverage (DP) for each of the reported sites for nuclear chromosome 7 (N) and the mitochondrial genome (M). Then, the samples lying in the top and bottom 10% of DP were removed for both chromosome 7 and the mitochondrial genome, since these samples exhibit extremes in the higher end (> 1500) or lower end (< 5) of DP. This left 1784 remaining samples that were subsequently extracted to estimate the distribution of mtDNA copy number. The following equation was used to determine the estimated mtDNA copy number for each sample (i), where:

$$\hat{C}_i = \frac{\text{median}(DP_M^i)}{\text{median}(DP_N^i)}$$

A histogram composed of the estimated copy number for each sample (i) was plotted which displays the overall distribution of estimated copy number of all mitochondrial genomes, where the green curve indicates the background Gaussian distribution estimated by maximum likelihood. The yellow shadow represents the probability of 0.8 (integral area) under the Gaussian distribution curve, which estimates the mitochondrial copy number to be between 9.6 and 26.8 copies in the population.

Estimation of mtDNA, DHODH, and Y268S Copy Number of Patient Isolates/Drug Selections

The read depth of DNA sequencing is often used as an estimation of relative copy number, where the ratio of average depth of a genomic region compared to that of the whole genome can be used to infer copy number. Similar approaches have been used to estimate the mitochondrial copy number by comparing the average mtDNA depth as a whole to the average autosomal read depth [163]. Similar to above, the average mtDNA copy number was estimated:

$$\hat{C}_t = \frac{\text{mean}(DP_M^i)}{\text{mean}(DP_N^i)}$$

During asexual replication, the nuclear and mtDNA replicate at approximately the same rate, therefore this should reflect approximate the mtDNA copy number of the sequenced parasites at the time of sequencing [165]. Whether or not copy number fluctuates in the same population of parasites has not been determined. We used the mean for these calculations so that they could be compared to that of other studies [163], but either method is appropriate.

Similarly, the DHODH copy number of the parasites was estimated by dividing the average read depth of the DHODH gene to that of the nuclear genome with the same basic formula. These numbers were then compared to that of the DHODH qPCR results from Aim 2 to see whether the sequencing data was a good representation of DHODH copy number.

Once the mtDNA copy number was calculated for each parasite, if the parasite had Y268S heteroplasmy, the estimated mtDNA WT copy number and estimated mtDNA MUT copy number were determined by multiplying the % Y268S SNP by the mtDNA copy number. This is simply to provide an estimation of Y268S gene dose, since parasites with higher mtDNA copy numbers would correspondingly have higher numbers of Y268S mutant DNA copies.

Worldwide Distribution of Y268S Cryptic Heteroplasmy

Parasites that were pyrosequenced to look for cryptic Y268S heteroplasmy came from clinical sites in the 1990's in Peixoto, Brazil [223], Yaounde, Cameroon [224], and Bangkok, Thailand from the Bangkok Hospital for Tropical Diseases, and the Shoklo Malaria Research Unit along with the clinic in Pailin Province, Cambodia [119]. Any of the isolates labelled "Thai" or "Cam" were clinical isolates from Shoklo and Cambodia clinics, respectively in 2008. All parasites had gDNA extracted from non-viable cryovials, and were subsequently sequenced. In the group of S.E. Asian parasites, those from the Phase II studies were also included, since they were clinical isolates from that region and timeframe. All parasites were sequenced with three triplicate PCRs run in duplicate (n=12). All parasites were plotted as mean % mutant, and error bars indicate SEM. Only parasites that were statistically significant ($p < 0.01$) compared to controls in a student's one-tailed t-test were considered "cryptic Y268S positive," as the parasites we know to be cryptic mutants that develop resistance were in this p-value range. All graphs were made to the same scale for ease of comparison.

Results

Pyrosequencing Detects the Y268S Allele in Global Populations

Using the same Y268S pyrosequencing assay as Aim 1 and 2, we aimed to look at parasite populations in Africa (Figure 4.1), South America (Figure 4.2), and S.E. Asia (Figure 4.3) in the hopes of detecting Y268S cryptic heteroplasmy. Indeed, we were able to detect four heteroplasmic parasites from Yaounde, Cameroon, and four from Peixoto, Brazil. Interestingly, no new parasites were detected with a p-value < 0.01 in S.E. Asia, aside from the parasites we already knew to be cryptic mutants from the Phase II studies in Thailand although one parasite “thai 8” approached significance. Since we had a low sample number at all sites, it would be unwise to make statements about the frequency of Y268S at these locations, but we can say that Y268S was detected in 4/14 (28%) in Cameroon, 4/28 in Brazil (14%), and 3/31 (10%) in S.E. Asia. Overall, 11/73 (15%) of parasites sampled had cryptic mutants. It is interesting to note that Brazil had the highest proportion of parasites detecting heteroplasmy, as almost all of the samples were nonzero, but Africa had the highest detected frequencies of Y268S, with samples as high as 2-3% Y268S. We chose to use a stringent p-value in a student’s one-tailed t-test as a metric for detecting true heteroplasmy because our cryptic mutants from the Phase II studies were within this significance level and wanted to feel confident that these were not false positives, as our assay had a false positive detection rate as high as 8%. It is entirely possible that many other sampled parasites represent true Y268S detection frequencies, but a more conservative estimation was used.

Frequency of Y268S cyt. *b* in African *P. falciparum*

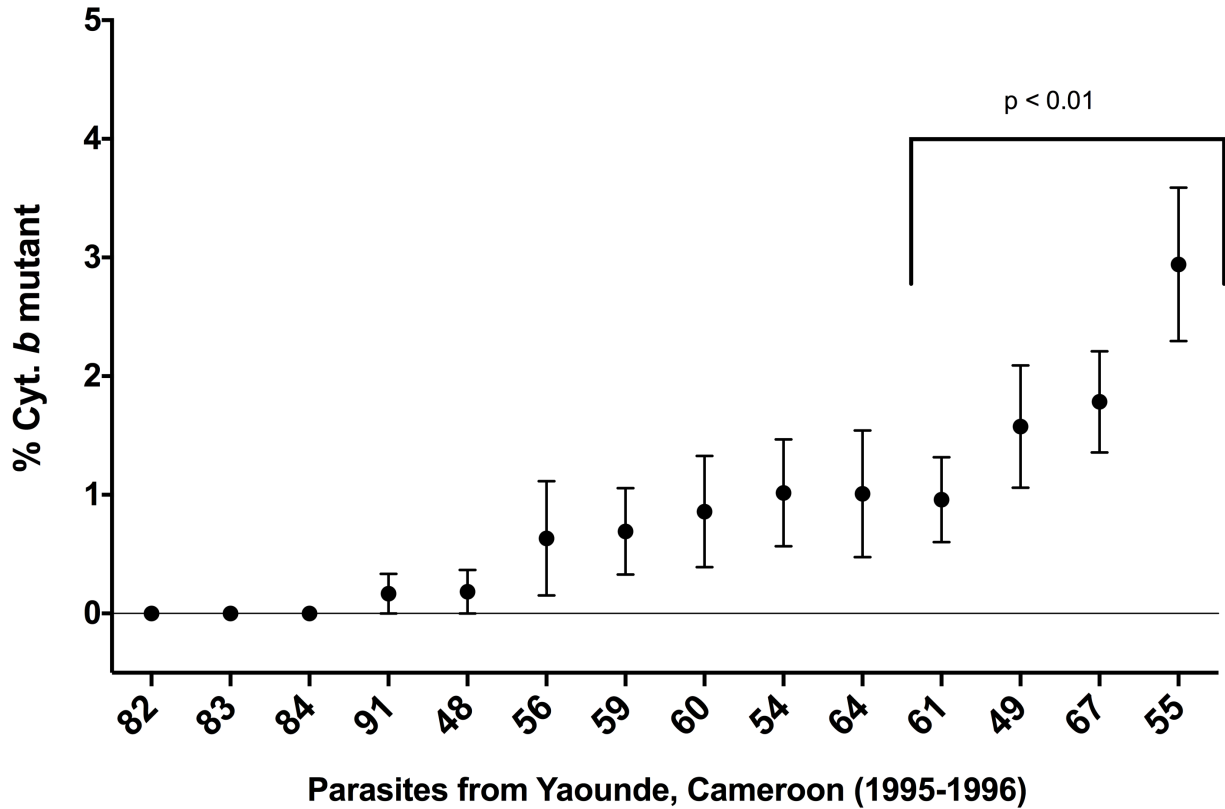


Figure 4.1. Pyrosequencing Detection of Cryptic Y268S Mutants in Africa. Parasites from sites in Yaounde, Cameroon (n=14) were collected between 1995-1996. Only four (28%) were found to be cryptic Y268S according to a cutoff p-value < 0.01, although these parasites had the highest mutant frequencies of any parasites sequenced to date. All parasites were sequenced with 6 individual PCRs and pyrosequenced in duplicate (n=12).

Frequency of Y268S *cyt. b* in Brazilian *P. falciparum*

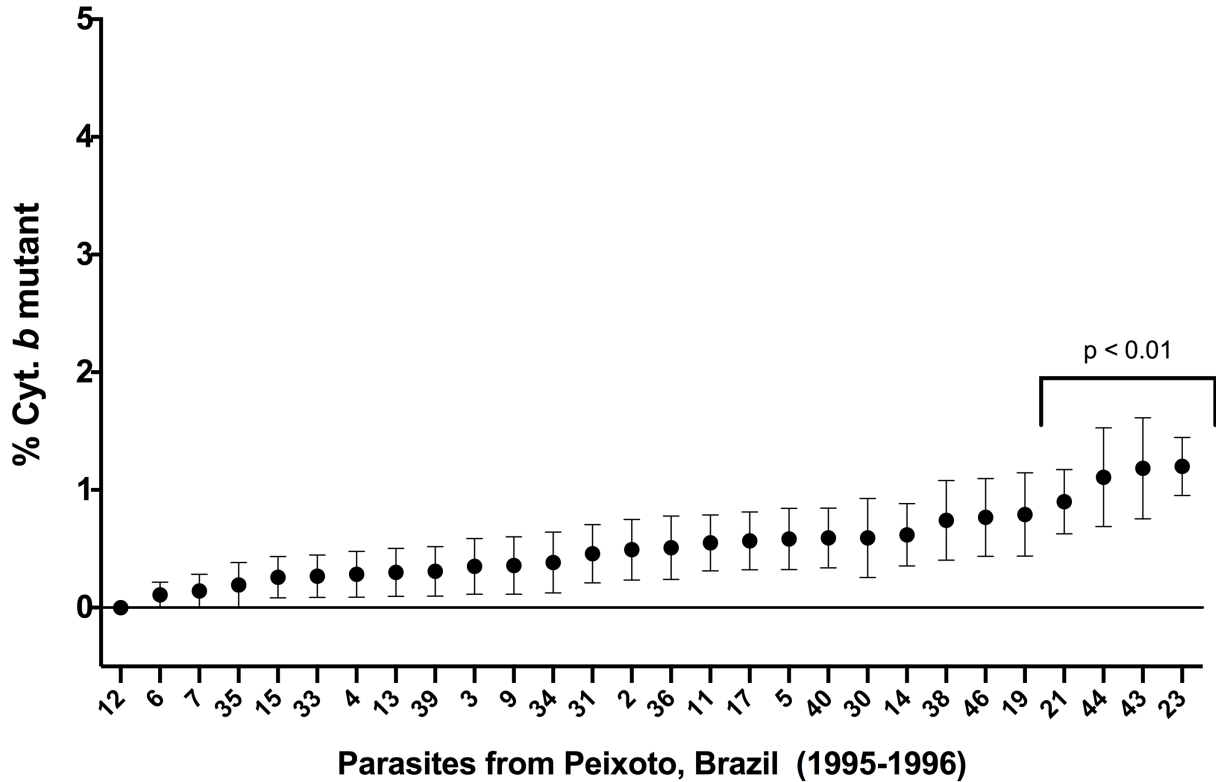


Figure 4.2. Pyrosequencing Detection of Cryptic Y268S Mutants in Brazil. Brazilian parasites ($n=28$) collected between 1995-1996, and though this population detected the most nonzero events, it had only four parasites with significant Y268S heteroplasmy with $p < 0.01$, a positive rate of 14%. All parasites were sequenced with 6 individual PCRs and pyrosequenced in duplicate ($n=12$).

Frequency of Y268S cyt. *b* in S.E. Asian *P. falciparum*

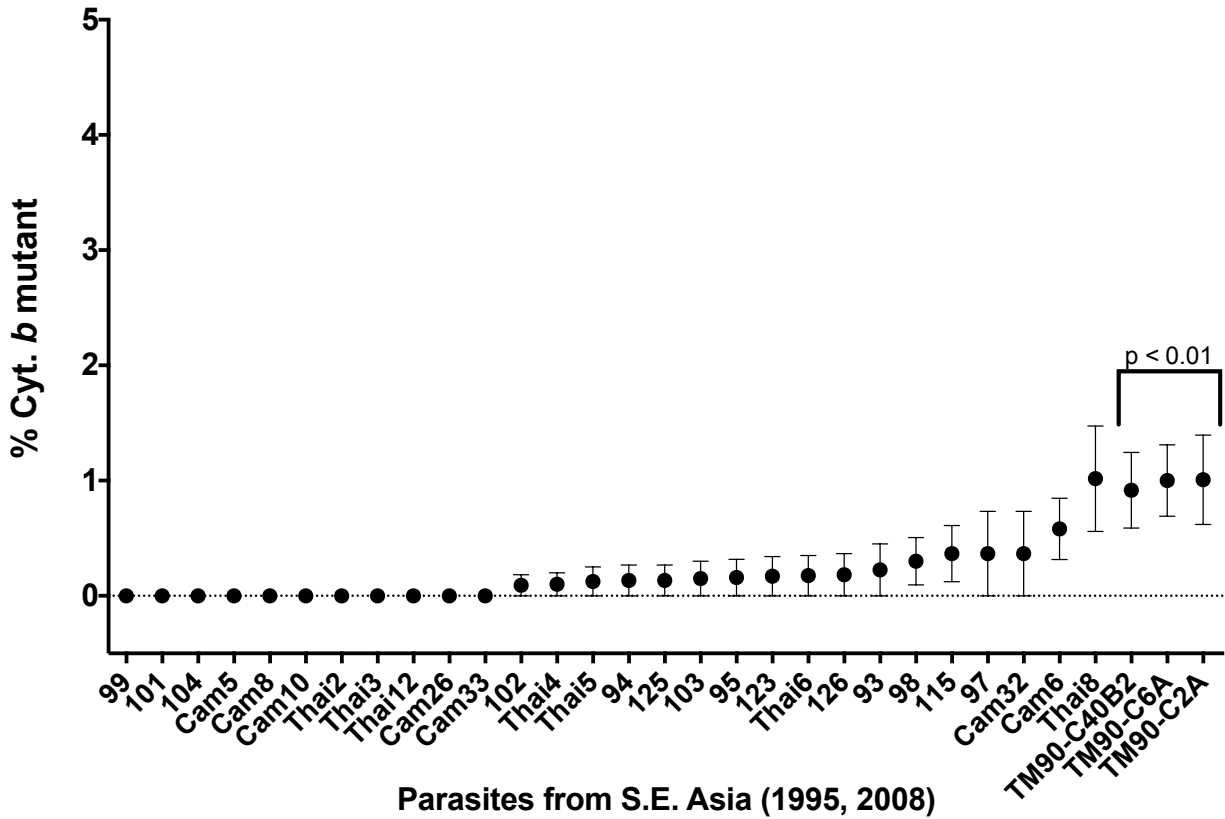


Figure 4.3. Pyrosequencing Detection of Cryptic Y268S Mutants in S.E. Asia. De-identified patient isolates from Bangkok Hospital for Tropical Diseases (1995) and Shoklo Malaria Research Unit in Thailand (2008), as well as patient isolates from Pailin Province in Cambodia (2008) were compiled to use to detect cryptic mtDNA Y268S heteroplasmy (n=31). The only parasites that reached significance with a p-value < 0.01 were the isolates in the Phase II studies at the Bangkok Hospital (n=3), for a positive rate of 10%. All parasites were sequenced with 6 individual PCRs and pyrosequenced in duplicate (n=12).

The MalariaGEN Pf3K Project is Used to Estimate the Scope of Global Mitochondrial Diversity

The MalariaGEN Pf3K Project is a collection of ~2600 parasites that have been collected from patients and deep-sequenced all over the world. In order to make this dataset useful for the purpose of mining for mitochondrial heteroplasmy, it had to be re-analyzed based on different criteria for SNP-calling and differential criteria for read quality and depth of coverage. First, all reads had to be high-quality (Phred >30), since a phred quality score is logarithmically tied to error probabilities; a phred score of 30 indicates the probability of an incorrect base call being 1:1000, or a base-call accuracy of 99.9%. Typically, a quality score of 20 or above is used in deep-sequencing, so to enable a lower MAF detection-threshold, this had to be modified to reflect more stringent base-calling criteria. In addition, any SNPs called had to be present equally on the forward and reverse strand, as sequencing errors are typically strand-specific and appear disproportionately. Following re-analysis of the raw reads, detection of heteroplasmic sites in the mtDNA was made possible with the maximum likelihood estimation model, where sites were identified as homoplasmic REF (WT), homoplasmic ALT (MUT), or heteroplasmic ALT (MUT) by defining homoplasmy as being AF=1, and a moderate heteroplasmy allele being the detection of an AF > 0.3 as represented in Figure 4.5.

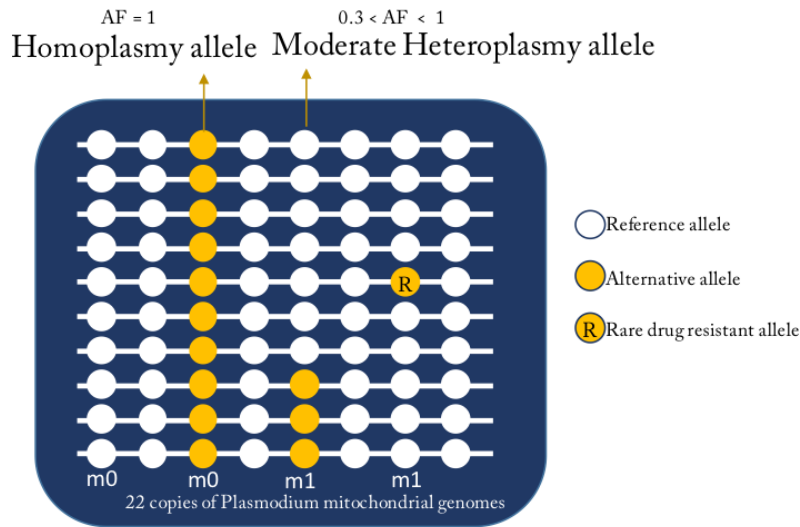
From the Pf3K project, we were able to estimate the mtDNA copy number of each parasite in the dataset by comparing the median chromosome 7 nuclear coverage depth to the median mtDNA coverage depth as described in Figure 4.6 below. Using the maximum likelihood model to estimate the Gaussian distribution, where the integral

area under the Gaussian distribution curve estimated the mtDNA copy number of the dataset to lie between 10 and 27 mtDNA copies.

The distribution of heteroplasmy could be observed in the context of a single SNP, as in Figure 4.7 shows a highly polymorphic SNP at position 4819 of the mtDNA genome. When taking homoplasmic SNPs at this locus into consideration, only 1.8% of the parasites surveyed constituted mutants. However, when the same dataset is surveyed with a $MAF > 0.3$, 15.2% of parasites were heteroplasmic at this locus, bringing the total mtDNA diversity up 10-fold. Once heteroplasmy is taken into account, overall diversity in the population at this locus increases from only 1.8% to 17%, making mitochondrial diversity vastly underestimated. The geographic distribution of SNP-calling remains the same, with predominantly parasites from African origins having the highest SNP prevalence at this locus.

When looking at mitochondrial diversity at the genome level, a similar trend emerges. In the top panel of Figure 4.7, the mitochondrial genome is plotted in terms of heteroplasmy (blue) and in terms of heteroplasmy between a MAF of $0.3 < 0.5$ (red), and for $MAF 0.5 < 1$ (yellow). When compared to homoplasmic alleles, heteroplasmy with a $MAF < 0.3$ contributed 5 times more diversity, showing that homoplasmic SNPs are only a subset of the total mtDNA diversity in these parasites. In the bottom panel, geographic differences between SNP prevalence can be seen, showing that regions have mitochondrial haplotypes specific for Cambodia and Malawi, characterized by both homoplasmic and heteroplasmic SNPs. In Cambodia, 30 homoplasmic and 597 heteroplasmic SNPs were characterized from a total of 570 parasites, when compared

with Malawi, which had 44 homoplasmic and 641 heteroplasmic SNPs in only 367 isolates, indicating that African parasites may be more diverse than those in Asia.



maximum likelihood estimation f_{m_1}, f_{m_0} can be calculated based on pre-classified potential heteroplasmy(m1) and homoplasmy (m0) data. The $\log(L(f_{m_1}) / L(f_{m_0}))$ was used for finding significant heteroplasmy site

Figure 4.4. Maximum Likelihood Estimation of Mitochondrial Heteroplasmy. Using the maximum likelihood estimation model, moderate heteroplasmic sites were classified as $MAF < 0.3$, where a homoplasmic site exists as $AF=1$. Heteroplasmic loci were located using this model, where outcomes may be homoplasmic REF (WT), homoplasmic ALT (MUT), or heteroplasmic ALT.

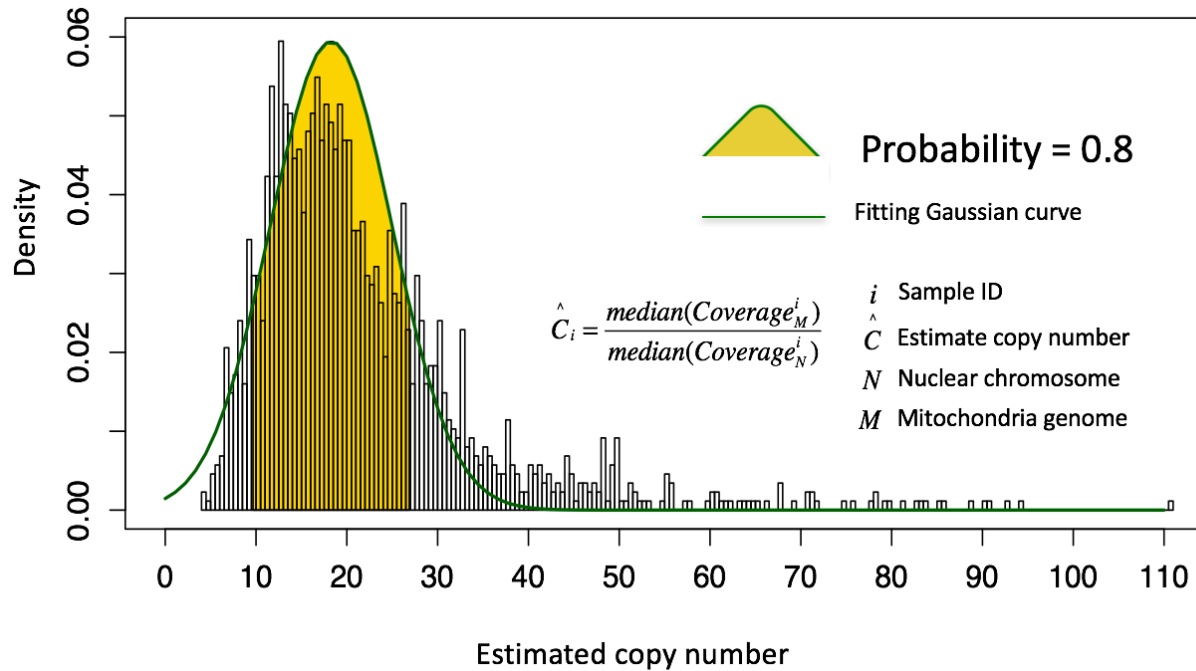


Figure 4.5. Estimating the mtDNA Copy Number in the MalariaGEN Pf3K Project. A histogram of estimated copy number for each sample used in the analysis (i) was plotted showing the overall copy number distribution of the Pf3K dataset. The green curve fit to the dataset indicates the background Gaussian distribution estimated by the maximum likelihood model. The yellow shadow represents the probability of 0.8 (integral area) under the Gaussian distribution curve, which estimates the mitochondrial copy number of the parasites sampled to be between 9.6 and 26.8 copies. Only parasites collected from patients with single infections were included for use in this analysis.

P. falciparum mitochondrial polymorphism is underestimated ~10 fold
at site *Pf_M76611* A4819C

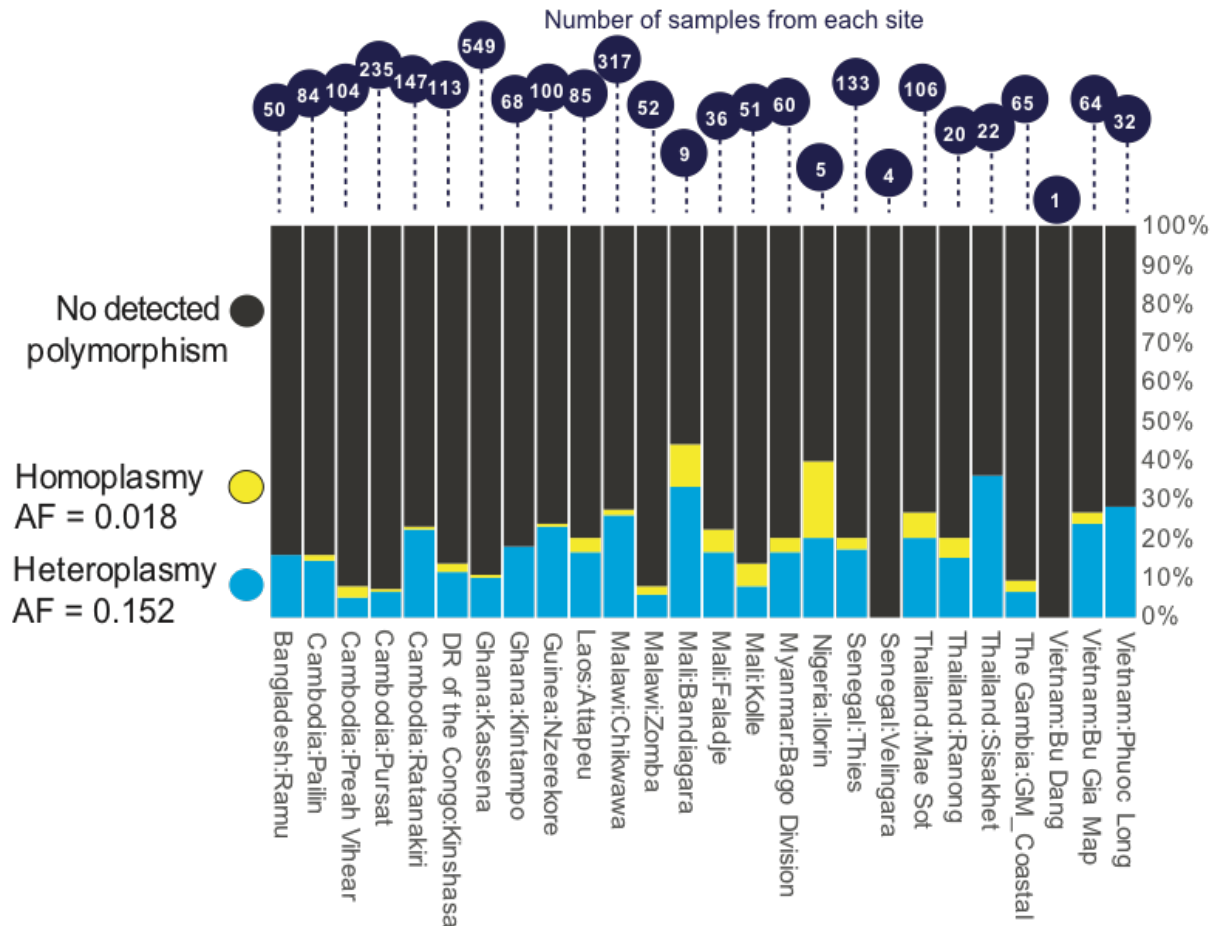


Figure 4.6. Homoplasmy and Heteroplasmy Contribute to Mitochondrial Diversity in a Single *Pf*-SNP. At the A4819 locus in the mitochondrial genome (*Pf_M76611*), homoplasmic SNPs (in yellow) can be detected in 1.8% of the ~2600 samples worldwide, with the SNP being called (A to C substitution) most prevalently in African samples. Using this enhanced method of detecting SNPs at a minor allele frequency of 30% or higher, the heteroplasmy detected (in blue) increases the amount of mitochondrial diversity ~10 fold (15.2% of parasites were heteroplasmic (A → C) at this locus. Taking heteroplasmy into account shows same overall distribution of SNPs geographically, but increases the prevalence of these SNPs. The navy circles indicate how many samples were represented from each geographic site.

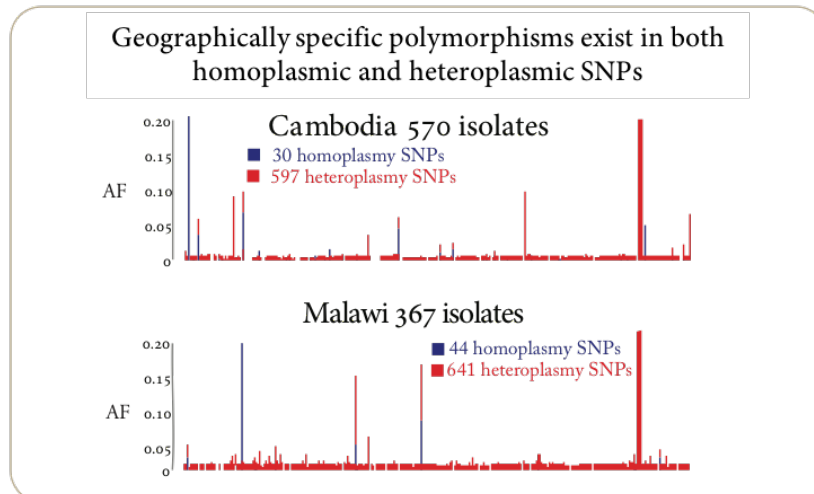
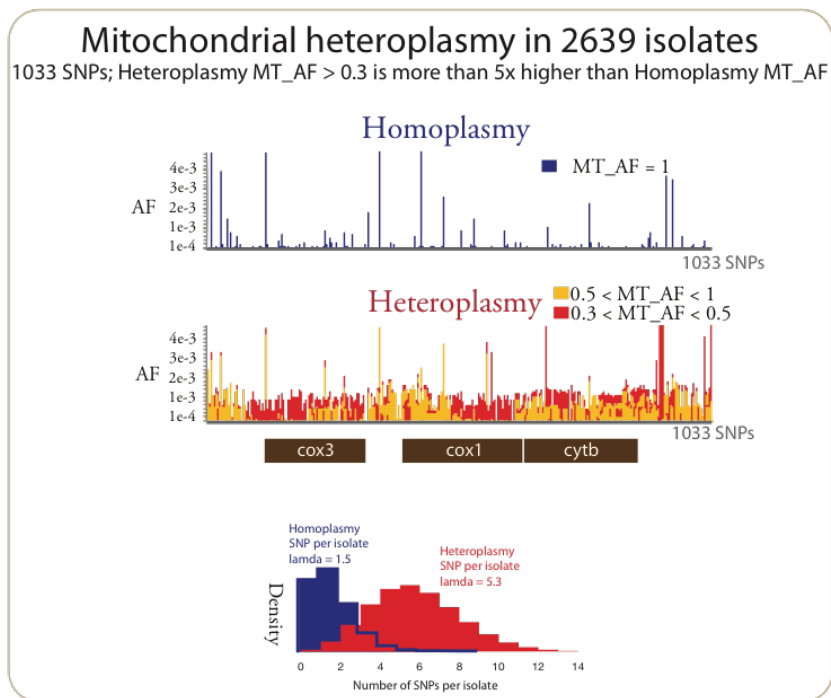


Figure 4.7. Both Homoplasmic and Heteroplasmic SNPs Vary by Region. 1033 SNPs were characterized in the ~2600 genome dataset, showing mitochondrial homoplasmy represents a small subset of overall diversity compared to heteroplasmy throughout the mitochondrial genome. Heteroplasmy detected at minor allele frequencies of between 30-50% are plotted in red for the top panel, and in yellow for frequencies between 50-100%. Homoplasmic SNPs are plotted in blue. In the lower panel, geographic differences exist between Asian and African SNPs, where some SNPs are common to both regions, and some are signatures of their region. In Cambodia, 570 parasites were sequenced that contained 30 homoplasmic SNPs, and 597 heteroplasmic SNPs, compared with Malawi which had 367 parasites sequenced and 44 homoplasmic SNPs were detected compared to 641 heteroplasmic SNPs.

Mitochondrial Deep-Sequencing of Patient Isolates/Drug Selections shows cryptic Y268S and common heteroplasmic loci

Combining high-coverage (>10,000x) mtDNA sequencing with the same stringent base-calling strategies described earlier allowed us to detect minor variants at lower detection thresholds than with the Pf3K Project, as low as 1% MAF, and would enable detection of low variants such as Y268S. The full set of SNP frequencies, mtDNA copy number estimations, and *dhodh* copy number estimations for all the parasites sequenced can be seen in Table 4.1. Sequencing of admission and recrudescence isolates from the Phase II studies in Thailand (Figure 4.8) confirmed the presence of cryptic Y268S heteroplasmy in all admission isolates sequenced, including TM90-C2A, TM90-C2B, TM90-C6A, and TM93-C1051. Interestingly, TM90-C6B was shown to have a majority K272R mutation, one that is typically only seen in conjunction with M133I mutations and confers additional resistance to the M133I mutation (alone EC₅₀ is ~68 nM, but combined with K272R jumps to over 1 μM (see Table 3.5) [192]. This K272R mutation was not present in Sanger sequencing of multiple PCR products prior to this. Interestingly, a K272R mutation was found in the MR4-TM93-C1088 parasite upon Sanger sequencing, where TM93-C1088 kept in our laboratory did not. Deep-sequencing of the MR4-C6B parasite did not have the K272R mutation, but was Y268S mutant, while the TM90-C6B in our laboratory is WT and known as the “atovaquone WT treatment-failure.”

Further, low level Y268S heteroplasmy was absent in the control parasites D6 and W2, who are either incapable of developing atovaquone resistance (D6) or favor alternative atovaquone resistance mutations like M133I (W2) [192, 196]. Cryptic Y268N

heteroplasmy was not detected in TM93-C1051, nor was cryptic M133I detected in W2. PL08-025, a patient isolate from Pailin Province, Cambodia in 2008 was found to have cryptic Y268S heteroplasmy as well. All of the TM90-C2A+10xATOV selections were 98-99% mutant, as well as ARC08-88-8A+10xATOV-1A and C2A+10xMEN-1A. The C2B+2xDSM1 parasites were all confirmed to be at various points of transition between WT and mutant Y268S because of DSM1 pressure, and the C2A+10xDSM1-2B showed ~30% Y268S selection, 8% Y268N, as well as ~34% R265G in the nuclear-encoded DHODH gene. In contrast, C2A+10xDSM1-3B parasite was completely R265G DHODH mutant. All parasites showed cryptic heteroplasmy in at least one mtDNA locus, typically at 1-2% frequencies, and interestingly, mtDNA mitotypes were not conserved between isolates and their clones in TM90-C2A and TM90-C2A-F6 or TM90-C2B and TM90-C2B-A3 (Table 4.1).

As far as estimated mtDNA copy number, parasites were estimated to have between 11 and 104 mtDNA copies, where highest copy numbers (> 50) were seen in the *in vitro* atovaquone or mefloquine-selected lines (which were maintained under drug pressure) or the extreme mtETC-resistant parasites TM92-C1086 (54 mtDNA copies, 99% Y268S mutant). When you compare that to TM90-C2B, which was 89% Y268S mutant and 15 mtDNA copies, real phenotypic differences could be caused by that observation alone. If that is viewed in terms of Y268S gene dose, that would be 51.48 Y268S copies per parasite in TM92-C1086, and only 13.35 Y268S copies in TM90-C2B. DHODH qPCR results correlated well with deep-sequencing coverage-based estimations of DHODH copy number, with the exception of 5 samples that were off by 1 copy (Table 4.1).

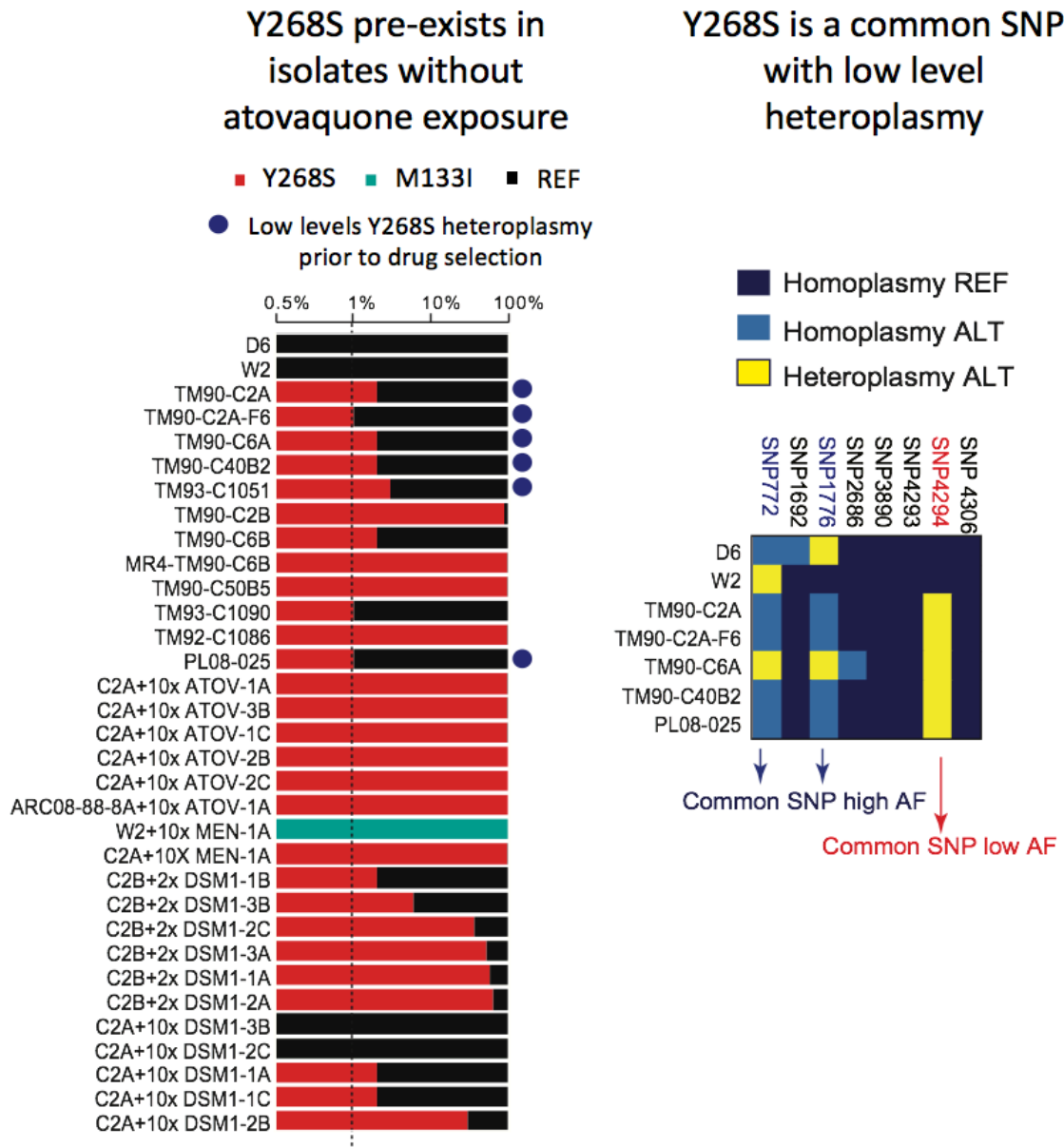


Figure 4.8. Y268S Status of Patient Isolates/Drug Selections Using Mitochondrial Deep-Sequencing. Using a MAF cutoff of 1%, parasites were plotted according to their Y268 frequencies of either REF (WT-black bars) or Y268S (red bars). Blue dots indicate parasites that have cryptic Y268S. Mitochondrial deep-sequencing confirms the cryptic Y268S status of admission isolates, unrelated parasites (PL08-025), and confirms absence of Y268S in lab strains W2 and D6. M133I (green bar) is seen in W2+10xMEN-1A selection. Drug selected parasites also indicate the gradual conversion of Y268S status to WT in the C2B+2DSM1 parasites, as well as the partial Y268S selection in C2A+10xDSM1-2B. Actual percentages of SNPs are reported in Table 4.1. The right panel shows cryptic Y268S parasites and control parasites plotted against SNPs reported in the dataset, with common SNP sites on the top axis. Navy represents homoplasmic REF (WT), yellow corresponds to heteroplasmic SNP, and blue represents homoplasmic SNP.

Table 4.1 Mitochondrial Deep-Sequencing of Patient Isolates/Drug Selections. Green boxes indicate high-frequency heteroplasmy, and orange boxes indicate low-level heteroplasmy. Estimated mtDNA copy number, DHODH copy number, and qPCR DHODH copy number are included for comparison. Mt coverage at Y268 is the sequencing read depth of the Y268 locus. Est # Mut copy is the estimated copy number of Y268S genotype in the parasite population, and calculated as previously described. The Est # WT copy was performed similarly. All mt SNPs are listed by their nucleotide position in the mt-genome, as well as the amino acid change they code for and nucleotide substitution. Two parasites were found to have DHODH mutations, and confirmed the prior sequencing results in Aim 2.

Parasite name	Est. mtDNA copy number	Est. WGS DHODH copy number	Est. qPCR DHODH copy number	Mito. seq. coverage at Y268	Y268S heteroplasmy	est # MUT copy	est # WT copy	SNP4294 Y268S SNP% A->C	SNP4293 Y268N SNP% T->A	SNP4306 K272R SNP% A->G	SNP3890 M133I SNP% G->C	SNP2686 SYN SNP% T->C	SNP1776 (ncr) SNP% T->C	SNP1723 (RNA2) SNP% T->G	SNP1692 (RNA2PR) SNP% G->A	SNP772 I239V SNP% T->C	DHODH mutation
W2	37	1	ND	25000	No	0	37	0	0	0	0	0	1	2	0	1	
D6	39	1	1	24000	No	0	39	0	0	0	0	0	1	1	98	100	
TM90-C2A-F6	37	1	1	26000	Yes	0.37	36.6	1	0	0	0	0	100	1	0	100	
TM90-C2A	24	1	1	10500	Yes	0.48	23.5	2	0	0	0	0	98	2	0	98	
TM90-C40B2	11	1	1	12500	Yes	0.22	10.8	2	0	0	0	0	99	2	0	99	
TM90-C6A	20	1	ND	12000	Yes	0.4	19.6	2	0	0	0	97	3	2	0	3	
1051	12	1	1	7000	Yes	0.36	11.6	3	0	0	0	0	99	2	0	99	
C2B-53A	40	1	1	3500	Yes	36.8	3.2	92	0	0	0	0	99	2	0	99	
TM90-C2B	15	1	1	12000	Yes	13.35	1.65	89	0	0	0	0	90	2	9	99	
TM90-C50B5	13	1	1	8500	Yes	12.74	0.26	98	0	0	0	0	99	2	0	99	
TM90-C6B	24	1	1	15500	Yes	0.48	23.5	2	0	99	0	98	2	2	0	2	
MR4-TM90-C6B	16	1	ND	7600	Yes	15.68	0.32	98	0	0	0	0	3	2	0	3	
TM93-C1090	26	1	1	14000	Yes	0.52	25.5	2	98	0	0	0	2	2	0	2	
TM92-C1086	52	1	1	17500	Yes	51.48	0.52	99	0	0	0	0	2	2	0	1	
C2A+10xATOV-1A	87	1	1	36500	Yes	86.13	0.87	99	0	0	0	0	100	2	0	100	
C2A+10xATOV-3A	74	1	1	29500	Yes	74	0	100	0	0	0	0	100	1	0	100	
C2A+10xATOV-1B	60	1	1	26000	Yes	59.4	0.6	99	0	0	0	0	99	1	0	100	
C2A+10xATOV-2B	17	1	1	8500	Yes	16.83	0.17	99	0	0	0	0	100	2	0	99	
C2A+10xATOV-3B	89	1	1	42500	Yes	89	0	100	0	0	0	0	99	2	0	99	
ARC08-10xATOV-1A	104	1	ND	36000	Yes	104	0	100	0	0	0	0	100	1	0	100	
C2A+10xMEN-1A	90	1	ND	36000	Yes	89.1	0.9	99	0	0	0	0	100	1	0	100	
W2+10xMEN-1A	90	2	ND	32000	No	0	90	0	0	0	99	0	1	2	0	1	
C2B+2xDSM1-1A	23	2	2	13000	Yes	13.11	9.89	57	0	0	0	0	99	2	0	100	
C2B+2xDSM1-2A	28	2	ND	13500	Yes	18.2	9.8	65	0	0	0	0	99	2	0	99	
C2B+2xDSM1-3A	44	2	ND	30000	Yes	22.88	21.1	52	0	0	0	0	100	2	0	100	
C2B+2xDSM1-1B	30	1	2	27000	Yes	0.6	29.4	2	0	0	0	0	100	1	0	100	
C2B+2xDSM1-3B	26	1	1.5	25000	Yes	1.56	24.4	6	0	0	0	0	99	2	0	99	
C2B+2xDSM1-2C	37	2	1	10500	Yes	13.69	23.3	37	0	0	0	0	100	1	0	100	
C2A+10xDSM1-1A	21	4	3	10500	Yes	0.42	20.6	2	0	0	0	0	99	2	0	99	
C2A+10xDSM1-2B	27	1	1	10200	Yes	8.1	18.9	30	8	0	0	10	60	2	3	68	34% R265G
C2A+10xDSM1-3B	43	1	1	25000	No	0	43	0	0	0	0	0	100	2	0	100	97% R265G
C2A+10xDSM1-1C	24	4	3	18000	Yes	0.48	23.5	2	0	0	0	0	100	2	0	100	
C2A+10xDSM1-2C	34	4	ND	30000	No	0	34	0	0	0	0	0	100	1	0	100	
ARC08-22-5G	20	1	ND	7854	Yes	0.2	19.8	1	0	0	0	0	2	2	0	1	
ARC08-88-5A	34	1	ND	20000	No	0	34	0	0	0	0	0	1	2	0	1	
PL08-025	34	1	ND	22500	Yes	0.34	33.7	1	0	0	0	0	100	2	0	100	

Discussion

Using a pre-existing global malaria genome dataset from the MalariaGEN Pf3K Project, we were able to demonstrate that mitochondrial heteroplasmy is a common event in the *Plasmodium falciparum* parasite, and that mitochondrial diversity is vastly underestimated between 5-10 fold both at individual loci and as a whole mt-genome (Figures 4.8 and 4.9). In addition, we've shown that geographic differences exist in the prevalence of both homoplasmic and heteroplasmic SNPs, with African parasites and Asian parasites showing evidence of their own distinct mitotypes (mitochondrial haplotypes) and this is in agreement with barcoding studies that show region-SNP specificity [163]. There was also a wide distribution of mitochondrial copy number among the 2600 mt-genomes (Figure 4.6) which is also in agreement with previous analyses, and we estimated the copy number to be between 10-27 copies, although the method for determining the copy number differed, as we were providing a range and not a single representative number [163]. Preston *et al.* plotted the mean autosomal coverage depth against the mean mitochondrial coverage depth and then fit a slope to the line, giving them an average mtDNA copy number to be 21.7. This is relatively consistent with our findings. Considering the parasites in this population were taken directly out of patients, and they have to be ring stages since later stages sequester, the relative mtDNA to nuclear coverage depth ratio should be a good representation of mtDNA copy number. Even in the instance of using later stage trophozoites and schizonts for genetic material as we did in the patient isolate/drug selections deep-sequencing, the mitochondria and nucleus replicate DNA at nearly equal rates, implying that the nuclear to mtDNA ratio is unchanged in any parasite stage [165]. As seen in

Table 4.1, the mtDNA copy number is seen as low as 11 (TM90-C40B2) and as high as 104 (ARC08-88+10xATOV-1A). All parasites with high mtDNA copy numbers (>50) were *in vitro* drug selected lines, with the exception of one, which is the extreme-mtETC resistant parasite TM92-C1086. Whether mtDNA copy number is involved in the resistance profile cannot be determined from this dataset, as drug susceptibility assays would have to have been performed on the day of gDNA extraction. This idea of high-copy number and its influence on parasite phenotype should be investigated further, as it could clarify the fluctuating atovaquone phenotype seen in parasites such as TM90-C2B, or even the high 63 μ M atovaquone EC₅₀ of ARC08-88-8A+10xATOV-1A, which had an estimated 104 mutant copies, and C2A+10xMEN-1A with 54 μ M atovaquone EC₅₀ with 89.1 mutant copies (Table 4.1). Further, the estimation of DHODH copy number was consistent with the majority of DHODH copy number estimations performed by qPCR. There are five samples that were not in agreement, and only differed by 1 copy. This is likely a discrepancy because the qPCR copy number determinations were performed on gDNA from immediately after parasite recovery, and the gDNA samples used in mitochondrial deep-sequencing were parasites remained in culture longer in the process of being thawed and subsequently expanded to obtain enough gDNA for deep-sequencing. For the 5 samples that had a 1-copy disagreement, a qPCR run of DHODH in the gDNA samples used for deep-sequencing would clarify further as to the accuracy of estimating DHODH copy number in this manner for 100% of cases.

The mtDNA-deep sequencing data revealed that the Y268S mutation is found in the admission isolates from the Phase II studies, and that Y268S is not present in

parasites such as D6 and W2 (Table 4.1). Interestingly, the Y268N mutation expected in TM93-C1051 was not found, but did have cryptic Y268S. Its recrudescence partner TM93-C1090 was a Y268N mutant as expected, leading to questions as to why we did not see cryptic Y268N in TM93-C1051. It is possible that the Y268N mutation occurs *de novo*, where Y268S is maintained at low levels but Y268N is generated spontaneously. However, considering that every single parasite sequenced in this dataset is heteroplasmic in at least one site, it seems more likely that the Y268N mutation is present at levels undetected at our MAF cutoff of 1%. This is also the case with the M133I mutation, where we cannot detect a cryptic presence in W2, yet the W2+10xMEN-1A developed the M133I mutation. However, we did not use the exact W2 parasite that was the parent for the W2+10xMEN-1A, and that could be the reason for lack of detection. The extent to which these mutant frequencies occur in heteroplasmy has not been evaluated, and some could be more common variants than others. Indeed, it is easy to speculate that the variants we are seeing are relatively common, as we were able to detect 3-4 parasites in Brazil, Africa, and S.E. Asia using Y268S pyrosequencing. The possibility of there being rarer or lower-frequency variants than the ones observed here warrant further investigation.

The mitochondrial genome replication of the linear, concatamerized 6-kb elements of *P. falciparum* was described as having highly-branched networks of replication and recombination intermediates, some rolling circle lariat intermediate species characteristic of the T₄ phage, and small circular form DNAs [165]. As unusual of a replication strategy as that may be, this same mt-genome and replication structure is described in plant species as well, and was first seen in the mitochondria of soybeans

and yeast. Indeed, the recombination activity of these plant genomes is now recognized as a critical and effective influence of plant mtDNA that dictates its structure, organization and evolution [225]. Prior to the understanding of the role of heteroplasmy in plants, there was also a long-standing argument that the plastid and mitochondrial genomes were homogeneous and lacked diversity. When heteroplasmy was first suggested, it was only attributed to deleteriously mutated mtDNA that caused phenotypic abnormalities, not a commonplace event. Now, heteroplasmy is viewed as the “normal situation” of plant mtDNA, and interestingly, plastid DNA heteroplasmy is also common [216]. The dynamics of heteroplasmy in plants has been intensively investigated, and much is known about the involvement of heteroplasmy in the evolution and maintenance of this unusual replication strategy. The main mt-genome is described as the genome that determines the plant phenotype, which in the case of *P. falciparum* is the 6 kb linear, tandemly repeating elements. The copy number estimation in *P. falciparum* is said to be 20-30, but in plants this number varies substantially even within clonal lines, and could vary by a factor of 10 [216]. It is of particular interest that there is a description of small circular forms of mtDNA in *P. falciparum*, and they were described as being products of recombination that interacted with the main linear mtDNA molecules and showed no evidence of self-replication, and represented less than 5% of the total mtDNA molecules [165]. The small circular forms of mtDNA described in *P. falciparum* have a striking resemblance to the description of sublimons in plants, and they are also present in low numbers compared to the main genome. These substoichiometric mtDNA circular molecules can be 10-1000 fold less abundant than the main mt-genome, and even so low that small fractions of cells contain them, and

also are not uniformly distributed within mitochondria. These sublimons are functionally silent and maintained as a substoichiometric population, and are capable of interacting with the main mt-genome, recombining and essentially becoming the main genome, functionally switching the contents of the main genome and sublimon. This phenomenon is called substoichiometric switching, and has been shown to be a mechanism used for rapid evolution and phenotype switching. This maintenance of main mt-genome and the substoichiometric sublimon genome is a dynamic process, and the unique recombination-based replication strategy rapidly expands advantage-conferring traits encoded in the sublimon. Figure 4.9 represents the mechanism of substoichiometric switching common in plants and is shown below.

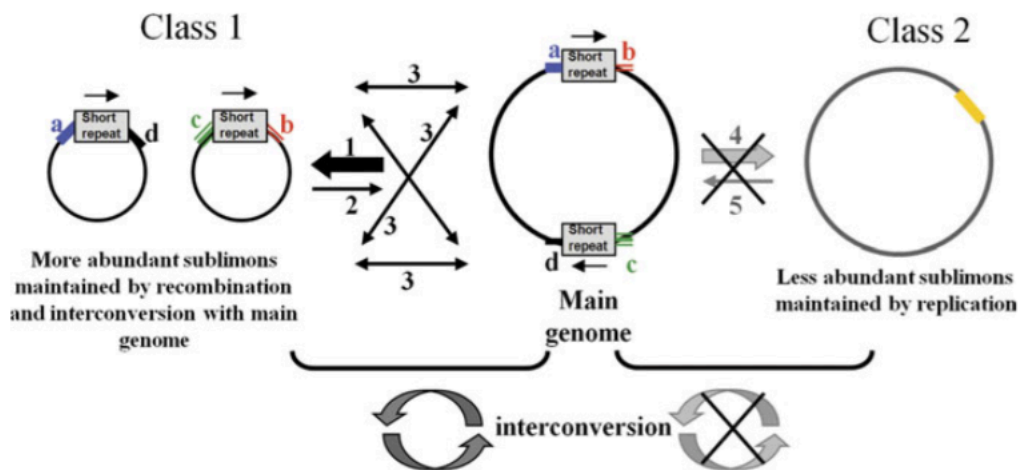


Figure 4.9. Substoichiometric shifting in plants with Class 1 and class 2 sublimons, from Woloszynska 2010, *J Exp Bot*, 61(3):657-671 [216]. The class 1 sublimon is the more abundant type, regularly produced by recombination using short repeats in the main genome (arrow 1). The sublimons and main genome can interconvert using either the process of reversible recombination (arrow 2) or non-reciprocal recombination (arrows 3). The class 2 sublimons are not as abundant as class 1, cannot be produced de novo and autonomously replicate, and have regions of non-homology with the main genome (yellow). Considering the small circular DNA molecules of *Pf* are products of recombination and interact with the main genome, they are presumed to be of Class 1.

The fact that we have shown the presence of heteroplasmy in *Plasmodium* genomes all over the world with mitotypes (mitochondrial haplotypes) that are geographically clustered suggests that heteroplasmy could be inherited in some way. This method of maintaining mitochondrial diversity could explain why some treatment failures look like wild-type parasites upon sequencing, as substoichiometric shifting can rapidly swap the main mtDNA and sublimon mtDNA. Interestingly, similar observations were made during the clinical trials in Thailand, where cryopreservation of a mutant parasite would be thawed and have a sensitive phenotype, and another vial frozen on the same day would maintain the resistant phenotype (Dennis Kyle, personal communication). This is a plausible explanation for our experience with TM90-C6B, which was sequenced and showed no mutations in mtDNA, yet a cryopreserve expanded years later for sequencing showed a predominant K272R mutation that could not have been missed the first time, as multiple PCRs and overlapping sequencing primers would have detected it since it was present at 99% frequency. Further, we have never put selection pressure on TM90-C6B, so this appearance of K272R was an example of spontaneous substoichiometric shifting, if the model is to be believed. In addition, we could not have mixed this parasite up and mislabeled it, considering we don't have any other parasites with this genotype. This substoichiometric shifting is known to happen spontaneously in both cultivated and wild plants [226, 227]. We have a similar scenario with the MR4-repository having different parasite genotypes than our own in the case of MR4-C6B and MR4-1088. MR4-1088 was both Y268S and K272R instead of just Y268S, and MR4-C6B was Y268S and should have been WT. These events are unlikely to be spontaneous mutation, and without the hypothesis of

substoichiometric shifting, there is nothing that comes to mind that could possibly explain these observations.

Indeed, all the seemingly bizarre selection observations make sense in the context of substoichiometric shifting, especially when it comes to the two C2A parasites that selected for a R265G mutation upon DSM1 selection, C2A+10xDSM1-3B, which selected for a R265G genotype (near 100%), and C2A+10xDSM1-2B. C2A+10xDSM1-2B is 30-34% Y268S and DHODH R265G, 8-10% Y268N and SNP1776, and 60% SNP1776. If these were grouped by their frequency in the flask, one could imagine the possibility of three different mitotypes (mitochondrial haplotypes) being present, which would mean that the R265G DHODH mutant parasites in the flask happened to have a Y268S/N mitotype. In that context, the Y268S/N mutations could be more of an accidental hitchhiker with their nucleus, which might explain why the parasite never made it to 100% R265G like C2A+10xDSM1-3B, since 3B didn't have to fight selection with a dysfunctional enzyme. There was no evidence of any partially-selected mitotypes in C2A+10xDSM1-3B, and it had a 97% base-call for R265G, so the population was nearly pure. We have provided evidence that these mechanisms are incompatible in Aim 2, so the notion that by chance a Y268S-mtDNA-dominant parasite would be the parasite to develop the R265G mutation and fail to thrive in the flask would be evidence of cytoplasmic incompatibility in action.

Further, the concept of the sublimon explains why some researchers have failed to detect cryptic mutants in sensitive parasite admission samples, and others have [217, 228]. As stated earlier, sublimon populations are maintained at low levels, even as low as 1 sublimon copy per 100 cells, and nothing aside from very high coverage ultra-deep

sequencing would catch it, if at all. Indeed, the only two instances of seeing low-level cryptic mutants prior to atovaquone exposure is our own data, and another group that used a very high coverage WGS approach much like our own [228]. In their work, Talundzic *et al.* detected a cryptic I258M mutation at a 0.1% MAF, but dismissed the finding in favor of the conventional *de novo* mutation theory because 0.1% was within the threshold that could be considered error, despite high-quality, 45,000x sequencing coverage. The main argument for the *de novo* development of atovaquone resistance is that mutants cannot be detected prior to atovaquone treatment, yet we have parasites that have high enough copy presence to detect the low-level mutants, at 2% MAF. Indeed, we do not see Y268N at our 1% MAF cutoff, yet it could be argued that TM90-C2A does possess Y268N at very low levels, as it was partially selected in the C2A+10xDSM1-2B parasite. Much higher-resolution deep sequencing (ultra-deep sequencing) or sequencing of whole mtDNA molecules (3rd generation sequencing) will be required to sort out what truly is lurking at low levels in mtDNA.

This model would also provide clarification for what we see with the loss of the TM92-C1086 Y268S mutant populations. The application of drug selective-pressure would immediately select a parasite with a Y268S sublimon, have rapid sublimon-mtDNA recombination and mtDNA recombination-based expansion of the sublimon's mitotype leading to survival, while all other parasites rapidly succumb to atovaquone pressure. With the wt-mtDNA now being the sublimon, it is maintained at substoichiometric levels. If spontaneous substoichiometric shifting were to occur again in one parasite that converts back to wild-type dominant mtDNA, it would look much like a fitness study, seeing gradual conversion of wt-dominant parasites in the flask because

of increased fitness in normal growth conditions. However, TM90-C2A nor TM90-C2B match exact mtDNA mitotypes with their clones TM90-C2A-F6 and TM90-C2B-A3, suggesting that substoichiometric shifting of highly similar mitotypes must be a common occurrence, not just the work of drug-selective forces.

In conclusion, there are three main pieces of direct evidence that this model applies to *Plasmodium falciparum*. First is its mtDNA replication strategy, which is the same as many plants, who are well known to have their main linear mt-genome replicate with a branching, recombination-based mechanism [165]. Second is the observation that the mtDNA in *P. falciparum* has small circular forms that were shown to directly interact with the linear, highly branched forms. Lastly, our parasites typically maintain either majority wt or majority mutant states in their mtDNA, which is important in the context of sublimon heteroplasmy in plants, as subtelomeric shifting does not result in homoplasmy, but instead results in a low-maintenance number of sublimon mtDNA and its main mt-genome. For the few that appear homoplasmic at sites that were once heteroplasmic in our mitochondrial deep-sequencing dataset, it is likely that they are simply below the 1% MAF cutoff used, as coverage limits the depth one can go in confident base-calling [229]. Looking at the SNP-calling in our mtDNA collection as a whole, every single parasite was low-level heteroplasmic in at least one site. Continuing to develop methods to look at low-level variants and fully investigate this new branch of mitochondrial DNA research is warranted. Mitochondrial heteroplasmy may be contributing to parasite fitness in nuanced ways that have yet to be investigated, such as the potential to investigate mitonuclear interactions in the parasite, which would

undoubtedly affect parasite metabolism in important ways, including the possibility of cytoplasmic incompatibility [173].

CHAPTER FIVE: SUMMARY

Although the mechanism of action and resistance to atovaquone was first suggested in 1999, the publication rate of this compound has not tapered off [114]. Atovaquone has fueled 20 years of debate in the malaria community, from its mechanism of action, to mechanism of resistance, and most recently the potential transmission of resistance. But before we can decide whether or not atovaquone resistance can be transmitted, it seems that first we must know how resistance is actually generated and maintained, which is something that is vastly understudied. There was a single paper on the mitochondrial replication of *Plasmodium falciparum*, and since there has been 20 years of silence. All we know about mitochondrial replication and inheritance come from shockingly little information, and where the malaria community trailed off, botanists continued on, to move past the 'low-diversity mitochondria' phase and into the current line of thinking that instead portrays plant mitochondria as having diverse, dynamic mitochondrial genomes that are orchestrated by the presence of mitochondrial heteroplasmy and carried out by their mitochondrial recombination-based replication strategy. Since *P. falciparum* has the same mtDNA structure and replication strategy, we might consider the possibility that the case is not closed.

This dissertation sought to understand the phenotypic diversity among atovaquone-resistant parasites. In Aim 1, we sequenced candidate genes looking for potential clues, but no explanations were found, and created a pyrosequencing assay that detected cryptic mutants in atovaquone-sensitive parasites. With the pyrosequencing assay, we recognized that atovaquone-resistant phenotypes are unstable even with a stable genotype, as with TM90-C2B. We then noticed that the extreme mtETC-resistant phenotype is genotypically unstable, rapidly reverting to WT in less than two months. These observations led to Aim 2, which involved several drug selection experiments in cryptic Y268S mutant genetic backgrounds. Using clonal parasites and atovaquone pressure, we found similar phenotypic variation to that found in patient isolates, and most importantly, we recapitulated the Y268S clinical resistance genotype in *Plasmodium falciparum*, which has never been successful in 20 years of research. Many people speculated that the reason for non-relevant genotypes being selected by atovaquone *in vitro* had to do with complex immunological interactions within the host to generate the *de novo* resistance. In reality, it was as simple as picking the right genetic background to select for atovaquone resistance. TM90-C2A, TM90-C40B2, and wild parasite ARC08-88 all had cryptic Y268S heteroplasmy according to pyrosequencing, and they all successfully generated Y268S resistance. Interestingly, we also found a way to de-select for resistance, by exposing TM90-C2B to low levels of DSM1, as higher doses proved to be lethal in the Y268S background because of likely incompatible resistance consequences. All parasites on continuous DSM1 pressure gradually reverted their Y268S atovaquone-resistance conferring mutation in favor of copy number amplification of DHODH. Then, when exposing the sensitive TM90-C2A to

DSM1, most parasites responded with copy number amplifications, with the exception of two parasites that mutated their DHODH enzyme instead to an R265G. One of these parasites, TM90-C2A+10xDSM1-2B was a ~30% mixture of R265G/Y268S/Y268N mutations and the other was completely R265G mutant, which suggests that the Y268 mutations combined with R265G struggled to thrive. Finally, we were able to show that menoctone is also capable of generating the Y268S and the M133I mutations, in TM90-C2A and W2, respectively. All of this evidence points to the selection of pre-existing mitochondrial mutants that are present at low-levels, which led to Aim 3, where we sought to make sense of the low-level heteroplasmy we were seeing. We pyrosequenced parasites from geographically diverse locations and found cryptic Y268S heteroplasmy, and found massive, previously uncharacterized heteroplasmic diversity in 2400 parasites from around the world. Finally, we used a high-mitochondrial DNA coverage approach to look for cryptic variants at low levels in our *in vitro* cultured parasite lines which gave us the first true evidence of how heteroplasmic diversity is maintained in *Plasmodium falciparum*. Deep-sequencing of the mtDNA led to the observation of distinct, low-level mitochondrial haplotypes that are frequently interchanged in our parasite lines over time, which is made possible by the sublimon, known in the plant world as the small, circular mtDNA molecule by which mtDNA diversity is maintained.

The implications of this work are that we know very little about mitochondrial diversity maintenance, and while I hope I've convinced you that this diversity exists, the work to prove the mechanism of substoichiometric shifting would be a logical step in a future direction. In order to do this, 3rd generation sequencing of intact mtDNA

molecules at very high coverage would essentially allow you to sequence every single mtDNA mitotype present without shearing DNA, and you could enrich for molecules of 6-kb size in search of the sublimon's mtDNA contents. This would be enhanced with a method to reliably isolate intact mtDNA in sufficient quantities.

Other future experiments would include trying to make a decisive correlation between parasite phenotype and mtDNA copy number, as gene dose is likely to influence the extent of phenotypic resistance to atovaquone. Pairing high-coverage mitochondrial sequencing with phenotypic drug assays would solidify this matter, or the development of a highly-sensitive qPCR method to estimate mtDNA copy number could also work.

Future work with the TM90-10xDSM-3B needs revisiting, as it was one of the parasites that did not get phenotyped; I selected one from each treatment flask to evaluate, and did not know its unique R265G DHODH mutant status until deep-sequencing was carried out. We do not know what its resistance profile is and the results of a true DHODH mutant that is uncharacterized deserves attention. Along that same line, careful observation of the seemingly confused TM90-C2A+10xDSM1-2B parasite in conditions of prolonged drug pressure would be interesting to see if the parasite can/will retain a combined Y268S/R265G mutant status, it would be my guess that this parasite is struggling because of cytoplasmic incompatibility, which of course is an unintended consequence stemming from mitochondrial heteroplasmy. Observations in plants have shown heteroplasmy-induced substoichiometric shifts causing male sterility [226, 227]. The implications of heteroplasmy in an organism are not always fitness-conferring results, so mitonuclear interactions would be a very interesting subject

to study, especially in the transmission subtext. The question of whether transmission can occur in atovaquone resistance would also be a question of whether sublimons can be transmitted, as low-level mutants would be inherited if sublimons are inherited. The possibility of the main-genome being transmitted by mosquitos has already been confirmed in our lab, which are in direct contrast to the most recent paper by Goodman *et al.* 2016 that claims atovaquone resistance is not transmitted [113]. I hope that all of this work is the beginning of a new branch of research that looks into the impact of mitochondrial diversity in *Plasmodium falciparum*.

CHAPTER SIX: LIST OF REFERENCES

1. Silva JC, Egan A, Friedman R, Munro JB, Carlton JM, Hughes AL: **Genome sequences reveal divergence times of malaria parasite lineages.** *Parasitology* 2010, **138**(13):1737-1749.
2. Whitfield J: **Portrait of a serial killer: a roundup of the history and biology of the malaria parasite.** *Nature* 2002.
3. Ingram VM: **Abnormal human haemoglobins. III. The chemical difference between normal and sickle cell haemoglobins.** *Biochimica et biophysica acta* 1959, **36**:402-411.
4. Krause MA, Diakite SAS, Lopera-Mesa TM, Amaratunga C, Arie T, Traore K, Doumbia S, Konate D, Keefer JR, Diakite M *et al*: **α -Thalassemia Impairs the Cytoadherence of Plasmodium falciparum-Infected Erythrocytes.** *PLoS ONE* 2012, **7**(5).
5. Miller LH, Mason SJ, Clyde DF, McGinniss MH: **The resistance factor to Plasmodium vivax in blacks. The Duffy-blood-group genotype, FyFy.** *The New England journal of medicine* 1976, **295**(6):302-304.
6. Garnham P: **Malaria parasites and other haemosporidia.** *Malaria Parasites and Other Haemosporidia* 1966.
7. Perkins SL, Schall JJ: **A molecular phylogeny of malarial parasites recovered from cytochrome b gene sequences.** *Journal of Parasitology* 2002, **100**(3).
8. Ta TH, Hisam S, Lanza M, Jiram AI, Ismail N, Rubio JMM: **First case of a naturally acquired human infection with Plasmodium cynomolgi.** *Malaria journal* 2014, **13**:68.
9. World Health O: **World Malaria Report 2015.** In: *World Health Organization*. 2015.
10. World Health O: **World Malaria Report 2014.** *World Health Organization* 2014.
11. Cox FEG: **History of the discovery of the malaria parasites and their vectors.** *Parasites & Vectors* 2010, **3**(1):5.
12. Valkiūnas G, Anwar AM, Atkinson CT, Greiner EC, Paperna I, Peirce MA: **What distinguishes malaria parasites from other pigmented haemosporidians?** *Trends in Parasitology* 2005, **21**(8):357-358.
13. Carlton JM, Perkins SL, Deitsch KW: **Malaria Parasites: Comparative Genomics, Evolution and Molecular Biology.** *Caister Academic Press* 2013.
14. Bousema T, Okell L, Felger I, Drakeley C: **Asymptomatic malaria infections: detectability, transmissibility and public health relevance.** *Nature Reviews Microbiology* 2014, **12**(12):833-840.
15. **Anopheles Mosquitoes** [<http://www.cdc.gov/malaria/about/biology/mosquitoes/>]

16. Kiszewski A, Mellinger A, Spielman A, Malaney P, Sachs S, Sachs J: **A global index representing the stability of malaria transmission.** *The American journal of tropical medicine and hygiene* 2004, **70**(5):486-498.
17. Sinka ME, Bangs MJ, Manguin S, Rubio-Palis Y, Chareonviriyaphap T, Coetzee M, Mbogo CM, Hemingway J, Patil AP, Temperley WH *et al*: **A global map of dominant malaria vectors.** *Parasites & Vectors* 2012, **5**(1):69.
18. Ménard R: **Gliding motility and cell invasion by Apicomplexa: insights from the Plasmodium sporozoite.** *Cellular Microbiology* 2001, **3**(2):63-73.
19. Ejigiri I, Sinnis P: **Plasmodium sporozoite-host interactions from the dermis to the hepatocyte.** *Current opinion in microbiology* 2009, **12**(4):401-407.
20. Arnot DE, Ronander E, Bengtsson DC: **The progression of the intra-erythrocytic cell cycle of Plasmodium falciparum and the role of the centriolar plaques in asynchronous mitotic division during schizogony.** *International journal for parasitology* 2011, **41**(1):71-80.
21. Maier AG, Cooke BM, Cowman AF, Tilley L: **Malaria parasite proteins that remodel the host erythrocyte.** *Nature Reviews Microbiology* 2009, **7**(5):341-354.
22. Moura PA, Dame JB, Fidock DA: **Role of Plasmodium falciparum Digestive Vacuole Plasmepsins in the Specificity and Antimalarial Mode of Action of Cysteine and Aspartic Protease Inhibitors.** *Antimicrobial Agents and Chemotherapy* 2009, **53**(12):4968-4978.
23. Tilley L, Dixon M, Kirk K: **The Plasmodium falciparum-infected red blood cell.** *The International Journal of Biochemistry & Cell Biology* 2011, **43**(6).
24. Scherf A, Lopez-Rubio J, Riviere L: **Antigenic Variation in Plasmodium falciparum.** *Annual review of microbiology* 2008, **62**(1):445-470.
25. Kwiatkowski D, Nowak M: **Periodic and chaotic host-parasite interactions in human malaria.** *Proceedings of the National Academy of Sciences* 1991, **88**(12):5111-5113.
26. Mideo N, Reece SE, Smith AL, Metcalf JCE: **The Cinderella syndrome: why do malaria-infected cells burst at midnight?** *Trends in Parasitology* 2012, **29**(1).
27. World Health O: **Guidelines for the Treatment of Malaria, 3rd Edition.** *World Health Organization* 2015.
28. Gatton ML, Martin LB, Cheng Q: **Evolution of resistance to sulfadoxine-pyrimethamine in Plasmodium falciparum.** *Antimicrob Agents Chemother* 2004, **48**(6):2116-2123.
29. Meremikwu MM, Donegan S, Sinclair D, Esu E, Oringanje C: **Intermittent preventive treatment for malaria in children living in areas with seasonal transmission.** *The Cochrane database of systematic reviews* 2012, **2**:Cd003756.
30. **Choosing a Drug to Prevent Malaria**
[\[http://www.cdc.gov/malaria/travelers/drugs.html\]](http://www.cdc.gov/malaria/travelers/drugs.html)
31. **Tables of malaria vaccine projects globally**
[\[http://www.who.int/immunization/research/development/Rainbow_tables/en/\]](http://www.who.int/immunization/research/development/Rainbow_tables/en/)
32. Nussenzweig R, Vanderberg J, Most H: **Protective immunity produced by the injection of x-irradiated sporozoites of Plasmodium berghei. IV. Dose**

- response, specificity and humoral immunity.** *Military medicine* 1969, **134**(10):1176-1182.
33. Nussenzweig RS, Vanderberg J, Most H, Orton C: **Protective immunity produced by the injection of x-irradiated sporozoites of plasmodium berghei.** *Nature* 1967, **216**(5111):160-162.
 34. Arama C, Troye-Blomberg M: **The path of malaria vaccine development: challenges and perspectives.** *Journal of Internal Medicine* 2014, **275**(5):456-466.
 35. Hoffman SL, Vekemans J, Richie TL, Duffy PE: **The march toward malaria vaccines.** *Vaccine* 2015.
 36. Seder RA, Chang L-J, Enama ME, Zephir KL, Sarwar UN, Gordon IJ, Holman LA, James ER, Billingsley PF, Gunasekera A *et al*: **Protection Against Malaria by Intravenous Immunization with a Nonreplicating Sporozoite Vaccine.** *Science* 2013, **341**(6152):1359-1365.
 37. Friesen J, Matuschewski K: **Comparative efficacy of pre-erythrocytic whole organism vaccine strategies against the malaria parasite.** *Vaccine* 2011, **29**(40):7002-7008.
 38. Esen M, Kremsner PG, Schleucher R, Gässler M, Imoukhuede E, Imbault N, Leroy O, Jepsen S, Knudsen B, Schumm M *et al*: **Safety and immunogenicity of GMZ2 — a MSP3–GLURP fusion protein malaria vaccine candidate.** *Vaccine* 2009, **27**(49):6862-6868.
 39. Genton B, Betuela I, Felger I, Al-Yaman F, Anders RF, Saul A, Rare L, Baisor M, Lorry K, Brown GV *et al*: **A recombinant blood-stage malaria vaccine reduces Plasmodium falciparum density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea.** *The Journal of infectious diseases* 2002, **185**(6):820-827.
 40. Jepsen MPG, Jogdand PS, Singh SK, Esen M, Christiansen M, Issifou S, Hounkpatin AB, Ateba-Ngoa U, Kremsner PG, Dziegiel MH *et al*: **The Malaria Vaccine Candidate GMZ2 Elicits Functional Antibodies in Individuals From Malaria Endemic and Non-Endemic Areas.** *Journal of Infectious Diseases* 2013.
 41. Ogutu BR, Apollo OJ, McKinney D, Okoth W, Siangla J, Dubovsky F, Tucker K, Waitumbi JN, Diggs C, Wittes J *et al*: **Blood Stage Malaria Vaccine Eliciting High Antigen-Specific Antibody Concentrations Confers No Protection to Young Children in Western Kenya.** *PLoS ONE* 2009, **4**(3).
 42. Palacpac NQ, Ntege E, Yeka A, Balikagala B, Suzuki N, Shirai H, Yagi M, Ito K, Fukushima W, Hirota Y *et al*: **Phase 1b Randomized Trial and Follow-Up Study in Uganda of the Blood-Stage Malaria Vaccine Candidate BK-SE36.** *PLoS ONE* 2013, **8**(5).
 43. Sagara I, Dicko A, Ellis RD, Fay MP, Diawara SI, Assadou MH, Sissoko MS, Kone M, Diallo AI, Saye R *et al*: **A randomized controlled phase 2 trial of the blood stage AMA1-C1/Alhydrogel malaria vaccine in children in Mali.** *Vaccine* 2009, **27**(23).
 44. Sahly EHM, Patel SM, Atmar RL, Lanford TA, Dube T, Thompson D, Sim BKL, Long C, Keitel WA: **Safety and immunogenicity of a recombinant nonglycosylated erythrocyte binding antigen 175 Region II malaria vaccine**

- in healthy adults living in an area where malaria is not endemic. *Clinical and vaccine immunology : CVI* 2010, **17**(10):1552-1559.
45. Majori G: **Short History of Malaria and Its Eradication in Italy With Short Notes on the Fight Against the Infection in the Mediterranean Basin.** *Mediterranean Journal of Hematology and Infectious Diseases* 2012, **4**(1).
 46. Williams LL: **Malaria Eradication in the United States.** *American Journal of Public Health and the Nations Health* 1963, **53**(1):17-21.
 47. **Indoor Residual Spraying: An Operational Manual for Indoor Residual Spraying (IRS) for Malaria Transmission Control and Elimination** [<http://www.who.int/malaria/publications/atoz/9789241508940/en/>]
 48. Raghavendra K, Barik TK, Reddy NBP, Sharma P, Dash AP: **Malaria vector control: from past to future.** *Parasitology Research* 2011, **108**(4):757-779.
 49. D'Alessandro U, Olaleye BO, McGuire W, Thomson MC, Langerock P, Bennett S, Greenwood BM: **A comparison of the efficacy of insecticide-treated and untreated bed nets in preventing malaria in Gambian children.** *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1995, **89**(6):596-598.
 50. Sreehari U, Raghavendra K, Rizvi MM, Dash AP: **Wash resistance and efficacy of three long-lasting insecticidal nets assessed from bioassays on Anopheles culicifacies and Anopheles stephensi.** *Tropical medicine & international health : TM & IH* 2009, **14**(5):597-602.
 51. **WHO Recommended Long-lasting Insecticidal Nets** [[http://scholar.google.com/scholar?q=WHO Recommended Long-lasting Insecticidal Nets&btnG=&hl=en&num=20&as_sdt=0%2C22](http://scholar.google.com/scholar?q=WHO+Recommended+Long-lasting+Insecticidal+Nets&btnG=&hl=en&num=20&as_sdt=0%2C22)]
 52. Singh SK, Singh S: **A Brief History of Quinoline as Antimalarial Agents.** *Int J Pharm Sci Rev Res* 2014, **25**(1).
 53. Kremsner PG, Winkler S, Brandts C, Neifer S, Bienzle U, Graninger W: **Clindamycin in combination with chloroquine or quinine is an effective therapy for uncomplicated Plasmodium falciparum malaria in children from Gabon.** *The Journal of infectious diseases* 1994, **169**(2):467-470.
 54. Thaithong S, Beale GH, Chutmongkonkul M: **Susceptibility of Plasmodium falciparum to five drugs: an in vitro study of isolates mainly from Thailand.** *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1983, **77**(2):228-231.
 55. Wongsrichanalai C, Webster HK, Wimonwattawatee T, Sookto P, Chuanak N, Thimasarn K, Wernsdorfer WH: **Emergence of multidrug-resistant Plasmodium falciparum in Thailand: in vitro tracking.** *The American journal of tropical medicine and hygiene* 1992, **47**(1):112-116.
 56. Wongsrichanalai C, Webster HK, Wimonwattawatee T, Sookto P, Chuanak N, Timasarn K, Wernsdorfer WH: **In vitro sensitivity of Plasmodium falciparum isolates in Thailand to quinine and chloroquine, 1984-1990.** *The Southeast Asian journal of tropical medicine and public health* 1992, **23**(3):533-536.
 57. da Silva AF, Benchimol JL: **Malaria and quinine resistance: a medical and scientific issue between Brazil and Germany (1907-19).** *Medical history* 2014, **58**(1):1-26.
 58. Nkrumah LJ, Riegelhaupt PM, Moura P, Johnson DJ, Patel J, Hayton K, Ferdig MT, Wellems TE, Akabas MH, Fidock DA: **Probing the multifactorial basis of**

- Plasmodium falciparum quinine resistance: evidence for a strain-specific contribution of the sodium-proton exchanger PfNHE.** *Mol Biochem Parasitol* 2009, **165**(2):122-131.
59. Cowman AF, Karcz S, Galatis D, Culvenor JG: **A P-glycoprotein homologue of Plasmodium falciparum is localized on the digestive vacuole.** *The Journal of cell biology* 1991, **113**(5):1033-1042.
60. Duraisingh MT, Cowman AF: **Contribution of the pfmdr1 gene to antimalarial drug-resistance.** *Acta tropica* 2005, **94**(3):181-190.
61. Mu J, Ferdig MT, Feng X, Joy DA, Duan J, Furuya T, Subramanian G, Aravind L, Cooper RA, Wootton JC *et al*: **Multiple transporters associated with malaria parasite responses to chloroquine and quinine.** *Mol Microbiol* 2003, **49**(4):977-989.
62. Trenholme CM, Williams RL, Desjardins RE, Frischer H, Carson PE, Rieckmann KH, Canfield CJ: **Mefloquine (WR 142,490) in the treatment of human malaria.** *Science* 0400, **190**(4216).
63. Humphreys GS, Merinopoulos I, Ahmed J, Whitty CJ, Mutabingwa TK, Sutherland CJ, Hallett RL: **Amodiaquine and artemether-lumefantrine select distinct alleles of the Plasmodium falciparum mdr1 gene in Tanzanian children treated for uncomplicated malaria.** *Antimicrob Agents Chemother* 2007, **51**(3):991-997.
64. Fitch CD: **Ferriprotoporphyrin IX, phospholipids, and the antimalarial actions of quinoline drugs.** *Life sciences* 2004, **74**(16):1957-1972.
65. Duraisingh MT, Jones P, Sambou I, von Seidlein L, Pinder M, Warhurst DC: **The tyrosine-86 allele of the pfmdr1 gene of Plasmodium falciparum is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin.** *Molecular and biochemical parasitology* 2000, **108**(1):13-23.
66. Price RN, Uhlemann A-CC, Brockman A, McGready R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ *et al*: **Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number.** *Lancet (London, England)* 2004, **364**(9432):438-447.
67. Anderson TJ, Nair S, Qin H, Singlam S, Brockman A, Paiphun L, Nosten F: **Are transporter genes other than the chloroquine resistance locus (pfcr) and multidrug resistance gene (pfmdr) associated with antimalarial drug resistance?** *Antimicrobial agents and chemotherapy* 2005, **49**(6):2180-2188.
68. Uhlemann A-CC, Ramharter M, Lell B, Kreamsner PG, Krishna S: **Amplification of Plasmodium falciparum multidrug resistance gene 1 in isolates from Gabon.** *The Journal of infectious diseases* 2005, **192**(10):1830-1835.
69. Uhlemann AC, Krishna S: **Antimalarial multi-drug resistance in Asia: mechanisms and assessment.** *Current topics in microbiology and immunology* 2005, **295**:39-53.
70. White NJ: **Antimalarial drug resistance.** *The Journal of clinical investigation* 2004, **113**(8):1084-1092.
71. Woodrow CJ, Krishna S: **Antimalarial drugs: recent advances in molecular determinants of resistance and their clinical significance.** *Cellular and molecular life sciences : CMLS* 2006, **63**(14):1586-1596.

72. Schlitzer M: **Malaria Chemotherapeutics Part I: History of Antimalarial Drug Development, Currently Used Therapeutics, and Drugs in Clinical Development.** *ChemMedChem* 2007, **2**(7).
73. Ginsburg H: **Should chloroquine be laid to rest?** *Acta Tropica* 2005, **96**(1):16-23.
74. Payne D: **Did medicated salt hasten the spread of chloroquine resistance in *Plasmodium falciparum*?** *Parasitology Today* 1988, **4**(4):112-115.
75. Kublin JG, Cortese JF, Njunju EM: **Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi.** *Journal of Infectious ...* 2003.
76. Taylor WRJ, White NJ: **Antimalarial drug toxicity.** *Drug safety* 2004.
77. Valderramos SG, Fidock DA: **Transporters involved in resistance to antimalarial drugs.** *Trends in pharmacological sciences* 2006, **27**(11):594-601.
78. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LM, Sidhu AB, Naude B, Deitsch KW *et al*: **Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance.** *Mol Cell* 2000, **6**(4):861-871.
79. Guantai E, Chibale K: **Chloroquine resistance: proposed mechanisms and countermeasures.** *Current drug delivery* 2010, **7**(4):312-323.
80. Martin RE, Kirk K: **The malaria parasite's chloroquine resistance transporter is a member of the drug/metabolite transporter superfamily.** *Molecular biology and evolution* 2004, **21**(10):1938-1949.
81. Ecker A, Lehane AM, Clain J, Fidock DA: **PfCRT and its role in antimalarial drug resistance.** *Trends Parasitol* 2012, **28**(11):504-514.
82. Bray PG, Martin RE, Tilley L, Ward SA, Kirk K, Fidock DA: **Defining the role of PfCRT in *Plasmodium falciparum* chloroquine resistance.** *Molecular microbiology* 2005, **56**(2):323-333.
83. Johnson DJ, Fidock DA, Mungthin M, Lakshmanan V, Sidhu AB, Bray PG, Ward SA: **Evidence for a central role for PfCRT in conferring *Plasmodium falciparum* resistance to diverse antimalarial agents.** *Molecular cell* 2004, **15**(6):867-877.
84. Sidhu AB, Verdier-Pinard D, Fidock DA: **Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by pfCRT mutations.** *Science* 2002, **298**(5591):210-213.
85. Sidhu AB, Valderramos SG, Fidock DA: **pfmdr1 mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*.** *Molecular microbiology* 2005, **57**(4):913-926.
86. Mita T, Kaneko A, Hombhanje F, Hwaihwanje I, Takahashi N, Osawa H, Tsukahara T, Masta A, Lum JK, Kobayakawa T *et al*: **Role of pfmdr1 mutations on chloroquine resistance in *Plasmodium falciparum* isolates with pfCRT K76T from Papua New Guinea.** *Acta tropica* 2006, **98**(2):137-144.
87. Gupta B, Xu S, Wang Z, Sun L, Miao J, Cui L, Yang Z: ***Plasmodium falciparum* multidrug resistance protein 1 (pfmrp1) gene and its association with in vitro drug susceptibility of parasite isolates from north-east Myanmar.** *J Antimicrob Chemother* 2014, **69**(8):2110-2117.

88. Raj DK, Mu J, Jiang H, Kabat J, Singh S, Sullivan M, Fay MP, McCutchan TF, Su XZ: **Disruption of a Plasmodium falciparum multidrug resistance-associated protein (PfMRP) alters its fitness and transport of antimalarial drugs and glutathione.** *J Biol Chem* 2009, **284**(12):7687-7696.
89. Conrad MD, LeClair N, Arinaitwe E, Wanzira H, Kakuru A, Bigira V, Muhindo M, Kanya MR, Tappero JW, Greenhouse B *et al*: **Comparative impacts over 5 years of artemisinin-based combination therapies on Plasmodium falciparum polymorphisms that modulate drug sensitivity in Ugandan children.** *J Infect Dis* 2014, **210**(3):344-353.
90. Naisbitt DJ, Williams DP, O'Neill PM, Maggs JL, Willock DJ, Pirmohamed M, Park BK: **Metabolism-dependent neutrophil cytotoxicity of amodiaquine: A comparison with pyronaridine and related antimalarial drugs.** *Chem Res Toxicol* 1998, **11**(12):1586-1595.
91. Kitchen LW, Vaughn DW, Skillman DR: **Role of US military research programs in the development of US Food and Drug Administration--approved antimalarial drugs.** *Clin Infect Dis* 2006, **43**(1):67-71.
92. Alving AS, Carson PE, Flanagan CL, Ickes CE: **Enzymatic deficiency in primaquine-sensitive erythrocytes.** *Science* 1956, **124**(3220):484-485.
93. Pang LW, Limsomwong N, Boudreau EF, Singharaj P: **Doxycycline prophylaxis for falciparum malaria.** *Lancet (London, England)* 1987, **2**(8565):970.
94. Kopetzki D, Levesque F, Seeberger PH: **A continuous-flow process for the synthesis of artemisinin.** *Chemistry (Weinheim an der Bergstrasse, Germany)* 2013, **19**(17):5450-5456.
95. Zhu C, Cook SP: **A concise synthesis of (+)-artemisinin.** *Journal of the American Chemical Society* 2012, **134**(33):13577-13579.
96. Petras JM, Kyle DE, Gettayacamin M, Young GD, Bauman RA, Webster HK, Corcoran KD, Peggins JO, Vane MA, Brewer TG: **Arteether: risks of two-week administration in Macaca mulatta.** *Am J Trop Med Hyg* 1997, **56**(4):390-396.
97. Witkowski B, Khim N, Chim P, Kim S, Ke S, Kloeung N, Chy S, Duong S, Leang R, Ringwald P *et al*: **Reduced artemisinin susceptibility of Plasmodium falciparum ring stages in western Cambodia.** *Antimicrob Agents Chemother* 2013, **57**(2):914-923.
98. Tucker MS, Mutka T, Sparks K, Patel J, Kyle DE: **Phenotypic and genotypic analysis of in vitro-selected artemisinin-resistant progeny of Plasmodium falciparum.** *Antimicrob Agents Chemother* 2012, **56**(1):302-314.
99. Chen N, Chavchich M, Peters JM, Kyle DE, Gatton ML, Cheng Q: **Deamplification of pfmdr1-containing amplicon on chromosome 5 in Plasmodium falciparum is associated with reduced resistance to artelinic acid in vitro.** *Antimicrob Agents Chemother* 2010, **54**(8):3395-3401.
100. Cui L, Wang Z, Miao J, Miao M, Chandra R, Jiang H, Su XZ, Cui L: **Mechanisms of in vitro resistance to dihydroartemisinin in Plasmodium falciparum.** *Mol Microbiol* 2012, **86**(1):111-128.
101. Takala-Harrison S, Clark TG, Jacob CG, Cummings MP, Miotto O, Dondorp AM, Fukuda MM, Nosten F, Noedl H, Imwong M *et al*: **Genetic loci associated with**

- delayed clearance of Plasmodium falciparum following artemisinin treatment in Southeast Asia.** *Proc Natl Acad Sci U S A* 2013, **110**(1):240-245.
102. Cheeseman IH, Miller BA, Nair S, Nkhoma S, Tan A, Tan JC, Al Saai S, Phyo AP, Moo CL, Lwin KM *et al*: **A major genome region underlying artemisinin resistance in malaria.** *Science* 2012, **336**(6077):79-82.
 103. Ariev F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, Kim S, Duru V, Bouchier C, Ma L *et al*: **A molecular marker of artemisinin-resistant Plasmodium falciparum malaria.** *Nature* 2014, **505**(7481):50-55.
 104. Straimer J, Gnadig NF, Witkowski B, Amaratunga C, Duru V, Ramadani AP, Dacheux M, Khim N, Zhang L, Lam S *et al*: **Drug resistance. K13-propeller mutations confer artemisinin resistance in Plasmodium falciparum clinical isolates.** *Science* 2015, **347**(6220):428-431.
 105. Taylor SM, Parobek CM, DeConti DK, Kayentao K, Coulibaly SO, Greenwood BM, Tagbor H, Williams J, Bojang K, Njie F *et al*: **Absence of putative artemisinin resistance mutations among Plasmodium falciparum in Sub-Saharan Africa: a molecular epidemiologic study.** *J Infect Dis* 2015, **211**(5):680-688.
 106. Meshnick SR, Taylor TE, Kamchonwongpaisan S: **Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy.** *Microbiological reviews* 1996, **60**(2):301-315.
 107. Mok S, Ashley EA, Ferreira PE, Zhu L, Lin Z, Yeo T, Chotivanich K, Imwong M, Pukrittayakamee S, Dhorda M *et al*: **Drug resistance. Population transcriptomics of human malaria parasites reveals the mechanism of artemisinin resistance.** *Science* 2015, **347**(6220):431-435.
 108. Porter TH, Folkers K: **Antimetabolites of coenzyme Q. Their potential application as antimalarials.** *Angew Chem Int Ed Engl* 1974, **13**(9):559-569.
 109. Boehm P, Cooper K, Hudson AT, Elphick JP, McHardy N: **In vitro activity of 2-alkyl-3-hydroxy-1,4-naphthoquinones against Theileria parva.** *J Med Chem* 1981, **24**(3):295-299.
 110. Hudson AT, Dickins M, Ginger CD, Gutteridge WE, Holdich T, Hutchinson DB, Pudney M, Randall AW, Latter VS: **566C80: a potent broad spectrum anti-infective agent with activity against malaria and opportunistic infections in AIDS patients.** *Drugs Exp Clin Res* 1991, **17**(9):427-435.
 111. Hudson AT, Randall AW, Fry M, Ginger CD, Hill B, Latter VS, McHardy N, Williams RB: **Novel anti-malarial hydroxynaphthoquinones with potent broad spectrum anti-protozoal activity.** *Parasitology* 1985, **90**(Pt 1):45-55.
 112. Nixon GL, Moss DM, Shone AE, Laloo DG, Fisher N, O'Neill PM, Ward SA, Biagini GA: **Antimalarial pharmacology and therapeutics of atovaquone.** *J Antimicrob Chemother* 2013, **68**(5):977-985.
 113. Fry M, Pudney M: **Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-4-(4'-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone (566C80).** *Biochem Pharmacol* 1992, **43**(7):1545-1553.
 114. Srivastava IK, Morrissey JM, Darrouzet E, Daldal F, Vaidya AB: **Resistance mutations reveal the atovaquone-binding domain of cytochrome b in malaria parasites.** *Mol Microbiol* 1999, **33**(4):704-711.

115. Srivastava IK, Rottenberg H, Vaidya AB: **Atovaquone, a broad spectrum antiparasitic drug, collapses mitochondrial membrane potential in a malarial parasite.** *J Biol Chem* 1997, **272**(7):3961-3966.
116. Ke H, Lewis IA, Morrisey JM, McLean KJ, Ganesan SM, Painter HJ, Mather MW, Jacobs-Lorena M, Llinás M, Vaidya AB: **Genetic Investigation of Tricarboxylic Acid Metabolism During the Plasmodium falciparum Lifecycle.** *Cell Rep* 2015, **11**(1):164-174.
117. Painter HJ, Morrisey JM, Mather MW, Vaidya AB: **Specific role of mitochondrial electron transport in blood-stage Plasmodium falciparum.** *Nature* 2007, **446**(7131):88-91.
118. LaRocque RC, Rao SR, Lee J, Ansdell V, Yates JA, Schwartz BS, Knouse M, Cahill J, Hagmann S, Vinetz J *et al*: **Global TravEpiNet: a national consortium of clinics providing care to international travelers--analysis of demographic characteristics, travel destinations, and pretravel healthcare of high-risk US international travelers, 2009-2011.** *Clin Infect Dis* 2012, **54**(4):455-462.
119. 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, **54**(1):62-66.
120. Peterson DS, Walliker D, Wellem TE: **Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria.** *Proc Natl Acad Sci U S A* 1988, **85**(23):9114-9118.
121. 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, **85**(23):9109-9113.
122. Bacon DJ, Tang D, Salas C, Roncal N, Lucas C, Gerena L, Tapia L, Llanos-Cuentas AA, Garcia C, Solari 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, **4**(8):e6762.
123. Basco LK, Eldin de Pecoulas P, Wilson CM, Le Bras J, Mazabraud A: **Point mutations in the dihydrofolate reductase-thymidylate synthase gene and pyrimethamine and cycloguanil resistance in Plasmodium falciparum.** *Mol Biochem Parasitol* 1995, **69**(1):135-138.
124. Nzila-Mounda A, Mberu EK, Sibley CH, Plowe CV, Winstanley PA, Watkins WM: **Kenyan Plasmodium falciparum field isolates: correlation between pyrimethamine and chlorcycloguanil activity in vitro and point mutations in the dihydrofolate reductase domain.** *Antimicrob Agents Chemother* 1998, **42**(1):164-169.
125. Crowther AF, Levi AA: **Proguanil—the isolation of a metabolite with high antimalarial activity.** *British Journal of Pharmacology and Chemotherapy* 1953, **8**(1):93-97.

126. Canfield CJ, Pudney M, Gutteridge WE: **Interactions of atovaquone with other antimalarial drugs against Plasmodium falciparum in vitro.** *Exp Parasitol* 1995, **80**(3):373-381.
127. Srivastava IK, Vaidya AB: **A mechanism for the synergistic antimalarial action of atovaquone and proguanil.** *Antimicrob Agents Chemother* 1999, **43**(6):1334-1339.
128. Fivelman QL, Adagu IS, Warhurst DC: **Modified fixed-ratio isobologram method for studying in vitro interactions between atovaquone and proguanil or dihydroartemisinin against drug-resistant strains of Plasmodium falciparum.** *Antimicrob Agents Chemother* 2004, **48**(11):4097-4102.
129. Hernandez T, Myatt AV, Coatney GR, Jeffery GM: **Studies in human malaria. XXXIV. Acquired resistance to pyrimethamine (daraprim) by the Chesson strain of plasmodium vivax.** *Am J Trop Med Hyg* 1953, **2**(5):797-804.
130. Roper C, Pearce R, Nair S, Sharp B, Nosten F, Anderson T: **Intercontinental spread of pyrimethamine-resistant malaria.** *Science* 2004, **305**(5687):1124.
131. Lumb V, Das MK, Singh N, Dev V, Khan W, Sharma YD: **Multiple origins of Plasmodium falciparum dihydropteroate synthetase mutant alleles associated with sulfadoxine resistance in India.** *Antimicrob Agents Chemother* 2011, **55**(6):2813-2817.
132. Alifrangis M, Nag S, Schousboe ML, Ishengoma D, Lusingu J, Pota H, Kavishe RA, Pearce R, Ord R, Lynch C *et al*: **Independent origin of plasmodium falciparum antifolate super-resistance, Uganda, Tanzania, and Ethiopia.** *Emerging infectious diseases* 2014, **20**(8):1280-1286.
133. Jain V, Basak S, Bhandari S, Bharti PK, Thomas T, Singh MP, Singh N: **Burden of complicated malaria in a densely forested Bastar region of Chhattisgarh State (Central India).** *PLoS ONE*, **9**(12):e115266.
134. Yuthavong Y, Tarnchompoo B, Vilaivan T, Chitnumsub P, Kamchonwongpaisan S, Charman SA, McLennan DN, White KL, Vivas L, Bongard E *et al*: **Malarial dihydrofolate reductase as a paradigm for drug development against a resistance-compromised target.** *Proc Natl Acad Sci U S A* 2012, **109**(42):16823-16828.
135. Jimenez-Diaz MB, Ebert D, Salinas Y, Pradhan A, Lehane AM, Myrand-Lapierre ME, O'Loughlin KG, Shackelford DM, Justino de Almeida M, Carrillo AK *et al*: **(+)-SJ733, a clinical candidate for malaria that acts through ATP4 to induce rapid host-mediated clearance of Plasmodium.** *Proc Natl Acad Sci U S A* 2014, **111**(50):E5455-5462.
136. Spillman NJ, Allen RJ, McNamara CW, Yeung BK, Winzeler EA, Diagana TT, Kirk K: **Na(+) regulation in the malaria parasite Plasmodium falciparum involves the cation ATPase PfATP4 and is a target of the spiroindolone antimalarials.** *Cell host & microbe* 2013, **13**(2):227-237.
137. Lehane AM, Ridgway MC, Baker E, Kirk K: **Diverse chemotypes disrupt ion homeostasis in the Malaria parasite.** *Mol Microbiol* 2014, **94**(2):327-339.
138. White NJ, Pukrittayakamee S, Phyo AP, Rueangweerayut R, Nosten F, Jittamala P, Jeeyapant A, Jain JP, Lefevre G, Li R *et al*: **Spiroindolone KAE609 for falciparum and vivax malaria.** *N Engl J Med* 2014, **371**(5):403-410.

139. Meister S, Plouffe DM, Kuhlen KL, Bonamy GM, Wu T, Barnes SW, E. BS, Borboa R, Bright AT, Che J *et al*: **Imaging of Plasmodium liver stages to drive next-generation antimalarial drug discovery**. *Science* 2011, **334**(6061):1372-1377.
140. Biot C, Nosten F, Fraise L, Ter-Minassian D, Khalife J, Dive D: **The antimalarial ferroquine: from bench to clinic**. *Parasite (Paris, France)* 2011, **18**(3):207-214.
141. Wiesner J, Jomaa H: **Isoprenoid biosynthesis of the apicoplast as drug target**. *Current drug targets* 2007, **8**(1):3-13.
142. Jomaa H, Wiesner J, Sanderbrand S, Altincicek B, Weidemeyer C, Hintz M, Turbachova I, Eberl M, Zeidler J, Lichtenthaler HK *et al*: **Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs**. *Science* 1999, **285**(5433):1573-1576.
143. Wiesner J, Henschker D, Hutchinson DB, Beck E, Jomaa H: **In vitro and in vivo synergy of fosmidomycin, a novel antimalarial drug, with clindamycin**. *Antimicrob Agents Chemother* 2002, **46**(9):2889-2894.
144. Lell B, Ruangweeraayut R, Wiesner J, Missinou MA, Schindler A, Baranek T, Hintz M, Hutchinson D, Jomaa H, Kreamsner PG: **Fosmidomycin, a novel chemotherapeutic agent for malaria**. *Antimicrob Agents Chemother* 2003, **47**(2):735-738.
145. Borrmann S, Adegnikaa AA, Matsiegui PB, Issifou S, Schindler A, Mawili-Mboumba DP, Baranek T, Wiesner J, Jomaa H, Kreamsner PG: **Fosmidomycin-clindamycin for Plasmodium falciparum Infections in African children**. *J Infect Dis* 2004, **189**(5):901-908.
146. Wells TN, Hooft van Huijsduijnen R, Van Voorhis WC: **Malaria medicines: a glass half full?** *Nat Rev Drug Discov* 2015, **14**(6):424-442.
147. Ehrhardt K, Davioud-Charvet E, Ke H, Vaidya AB, Lanzer M, Deponte M: **The antimalarial activities of methylene blue and the 1,4-naphthoquinone 3-[4-(trifluoromethyl)benzyl]-menadione are not due to inhibition of the mitochondrial electron transport chain**. *Antimicrob Agents Chemother* 2013, **57**(5):2114-2120.
148. R. BS, Hitchings GH: **Trimethoprim, a sulphonamide potentiator**. *British journal of clinical pharmacology* 1968, **33**(1):72-90.
149. Laurens MB, Mungwira RG, Nyirenda OM, Divala TH, Kanjala M, Muwalo F, Mkandawire FA, Tsirizani L, Nyangulu W, Mwinjiwa E *et al*: **TSCQ study: a randomized, controlled, open-label trial of daily trimethoprim-sulfamethoxazole or weekly chloroquine among adults on antiretroviral therapy in Malawi: study protocol for a randomized controlled trial**. *Trials* 2016, **17**(1):322.
150. Walsh DS, Looareesuwan S, Wilairatana P, Heppner DG, Jr., Tang DB, Brewer TG, Chokejindachai W, Viriyavejakul P, Kyle DE, Milhous WK *et al*: **Randomized dose-ranging study of the safety and efficacy of WR 238605 (Tafenoquine) in the prevention of relapse of Plasmodium vivax malaria in Thailand**. *J Infect Dis* 1999, **180**(4):1282-1287.
151. Llanos-Cuentas A, Lacerda MV, Rueangweeraayut R, Krudsood S, Gupta SK, Kochar SK, Arthur P, Chuenchom N, Mohrle JJ, Duparc S *et al*: **Tafenoquine**

- plus chloroquine for the treatment and relapse prevention of Plasmodium vivax malaria (DETECTIVE): a multicentre, double-blind, randomised, phase 2b dose-selection study.** *Lancet* 2014, **383**(9922):1049-1058.
152. Price RN, Nosten F: **Single-dose radical cure of Plasmodium vivax: a step closer.** *Lancet* 2014, **383**(9922):1020-1021.
 153. MacRae JI, Dixon MW, Dearnley MK, Chua HH, Chambers JM, Kenny S, Bottova I, Tilley L, McConville MJ: **Mitochondrial metabolism of sexual and asexual blood stages of the malaria parasite Plasmodium falciparum.** *BMC biology* 2013, **11**:67.
 154. Lunt SY, Vander Heiden MG: **Aerobic glycolysis: meeting the metabolic requirements of cell proliferation.** *Annu Rev Cell Dev Biol* 2011(27):441-464.
 155. Krungkrai J: **The multiple roles of the mitochondrion of the malarial parasite.** *Parasitology* 2004, **129**(5):511-524.
 156. van Dooren GG, Marti M, Tonkin CJ, Stimmler LM, Cowman AF, McFadden GI: **Development of the endoplasmic reticulum, mitochondrion and apicoplast during the asexual life cycle of Plasmodium falciparum.** *Mol Microbiol* 2005, **57**(2):405-419.
 157. Strauss M, Hofhaus G Fau - Schroder RR, Schroder Rr Fau - Kuhlbrandt W, Kuhlbrandt W: **Dimer ribbons of ATP synthase shape the inner mitochondrial membrane.** *EMBO J* 2008, **27**(7):1154-1160.
 158. Joseph JT, Aldritt SM, Unnasch T, Puijalon O, Wirth DF: **Characterization of a conserved extrachromosomal element isolated from the avian malarial parasite Plasmodium gallinaceum.** *Mol Cell Biol* 1989, **9**(9):3621-3629.
 159. Creasey AM, C. R-CL, Moore DJ, Williamson DH, Wilson RJ, Walliker D, Carter R: **Uniparental inheritance of the mitochondrial gene cytochrome b in Plasmodium falciparum.** *Curr Genet* 1993, **23**(4):360-364.
 160. Feagin JE: **The 6-kb element of Plasmodium falciparum encodes mitochondrial cytochrome genes.** *Mol Biochem Parasitol* 1992, **52**(1):145-148.
 161. Hikosaka K, Watanabe Y, Kobayashi F, Waki S, Kita K, Tanabe K: **Highly conserved gene arrangement of the mitochondrial genomes of 23 Plasmodium species.** *Parasitol Int* 2011, **60**(2):175-180.
 162. Vaidya AB, Arasu P: **Tandemly arranged gene clusters of malarial parasites that are highly conserved and transcribed.** *Mol Biochem Parasitol* 1987, **22**(2-3):249-257.
 163. Preston MD, Campino S, Assefa SA, Echeverry DF, Ocholla H, Amambua-Ngwa A, Stewart LB, Conway DJ, Borrmann S, Michon P *et al*: **A barcode of organellar genome polymorphisms identifies the geographic origin of Plasmodium falciparum strains.** *Nat Commun* 2014, **5**:4052.
 164. van Dooren GG, M. SL, McFadden GI: **Metabolic maps and functions of the Plasmodium mitochondrion.** *FEMS Microbiol Rev* 2006, **30**(4):596-630.
 165. Preiser PR, Wilson RJ, Moore PW, McCreedy S, Hajibagheri MA, Blight KJ, Strath M, Williamson DH: **Recombination associated with replication of malarial mitochondrial DNA.** *EMBO J* 1996, **15**(3):684-693.
 166. Breton S, Stewart DT: **Atypical mitochondrial inheritance patterns in eukaryotes.** *Genome* 2015, **58**(10):423-431.

167. Greiner S, Sobanski J, Bock R: **Why are most organelle genomes transmitted maternally?** *BioEssays* 2015, **37**(1):80-94.
168. Jarvi SI, Farias ME, Lapointe DA, Belcaid M, Atkinson CT: **Next-generation sequencing reveals cryptic mtDNA diversity of Plasmodium relictum in the Hawaiian Islands.** *Parasitology* 2013, **140**(14):1741-1750.
169. Giuliani C, Barbieri C, Li M, Bucci L, Monti D, Passarino G, Luiselli D, Franceschi C, Stoneking M, Garagnani P: **Transmission from centenarians to their offspring of mtDNA heteroplasmy revealed by ultra-deep sequencing.** *Aging* 2014, **6**(6):454-467.
170. Messenger LA, Llewellyn MS, Bhattacharyya T, Franzén O, Lewis MD, Ramírez JD, Carrasco HJ, Andersson B, Miles MA: **Multiple Mitochondrial Introgression Events and Heteroplasmy in Trypanosoma cruzi Revealed by Maxicircle MLST and Next Generation Sequencing.** *PLoS Negl Trop Dis* 2012, **6**(4):e1584.
171. Stewart JB, Chinnery PF: **The dynamics of mitochondrial DNA heteroplasmy: implications for human health and disease.** *Nat Rev Genet* 2015, **16**(9):530-542.
172. Payne BAI, Wilson IJ, Yu-Wai-Man P, Coxhead J, Deehan D, Horvath R, Taylor RW, Samuels DC, Santibanez-Koref M, Chinnery PF: **Universal heteroplasmy of human mitochondrial DNA.** *Human Molecular Genetics* 2013, **22**(2):384-390.
173. Wolff JN, Ladoukakis ED, Enríquez JA, Dowling DK: **Mitonuclear interactions: evolutionary consequences over multiple biological scales.** *Philosophical Transactions of the Royal Society B: Biological Sciences* 2014, **369**(1646):20130443.
174. Ishii H, Fountaine J, Chung WH, Kansako M, Nishimura K, Takahashi K, Oshima M: **Characterisation of Qol-resistant field isolates of Botrytis cinerea from citrus and strawberry.** *Pest Manag Sci* 2009, **65**(8):916-922.
175. Vaidya AB, J. M, Plowe CV, Kaslow DC, Wellems TE: **Unidirectional dominance of cytoplasmic inheritance in two genetic crosses of Plasmodium falciparum.** *Mol Cell Biol* 1993, **13**(12):7349-7357.
176. Vaidya AB, Mather MW: **Mitochondrial evolution and functions in malaria parasites.** *Annu Rev Microbiol* 2009, **63**:249-267.
177. Ke H, Morrissey JM, Ganesan SM, Painter HJ, Mather MW, Vaidya AB: **Variation among Plasmodium falciparum strains in their reliance on mitochondrial electron transport chain function.** *Eukaryot Cell* 2011, **10**(8):1053-1061.
178. Balabaskaran Nina P, Morrissey JM, Ganesan SM, Ke H, Pershing AM, Mather MW, Vaidya AB: **ATP synthase complex of Plasmodium falciparum: dimeric assembly in mitochondrial membranes and resistance to genetic disruption.** *J Biol Chem* 2011, **286**(48):41312-41322.
179. Sturm A, Mollard V, Cozijnsen A, Goodman CD, McFadden GI: **Mitochondrial ATP synthase is dispensable in blood-stage Plasmodium berghei rodent malaria but essential in the mosquito phase.** *Proc Natl Acad Sci U S A* 2015, **112**(33):10216-10233.
180. Ginsburg H: **Mitochondrial Electron Flow.** In.; 2016.

181. Fisher N, Bray PG, Ward SA, Biagini GA: **The malaria parasite type II NADH:quinone oxidoreductase: an alternative enzyme for an alternative lifestyle.** *Trends Parasitol* 2007, **23**(7):305-310.
182. Pidathala C, Amewu R, Pacorel B, Nixon GL, Gibbons P, Hong WD, Leung SC, Berry NG, Sharma R, Stocks PA *et al*: **Identification, design and biological evaluation of bisaryl quinolones targeting Plasmodium falciparum type II NADH:quinone oxidoreductase (PfNDH2).** *J Med Chem* 2012, **55**(5):1831-1843.
183. Vallieres C, Fisher N, Antoine T, Al-Helal M, Stocks P, Berry NG, Lawrenson AS, Ward SA, O'Neill PM, Biagini GA *et al*: **HDQ, a potent inhibitor of Plasmodium falciparum proliferation, binds to the quinone reduction site of the cytochrome bc1 complex.** *Antimicrob Agents Chemother* 2012, **56**(7):3739-3747.
184. Phillips MA, Rathod PK: **Plasmodium dihydroorotate dehydrogenase: a promising target for novel anti-malarial chemotherapy.** *Infectious disorders drug targets* 2010, **10**(3):226-239.
185. Nixon GL, Pidathala C, Shone AE, Antoine T, Fisher N, O'Neill PM, Ward SA, Biagini GA: **Targeting the mitochondrial electron transport chain of Plasmodium falciparum: new strategies towards the development of improved antimalarials for the elimination era.** *Future medicinal chemistry* 2013, **5**(13):1573-1591.
186. Biagini GA: **Mitochondrial electron transport chain of Plasmodium falciparum.** In: *Encyclopedia of Malaria*. Edited by Hommel M, Kremsner, P.G.: Springer-Verlag Berlin Heidelberg; 2014.
187. Schnell JV, Siddiqui WA, Geiman QM: **Biosynthesis of coenzymes Q by malarial parasites. 2. Coenzyme Q synthesis in blood cultures of monkeys infected with malarial parasites (Plasmodium falciparum and P. knowlesi).** *J Med Chem* 1971, **14**(11):1026-1029.
188. Vaidya AB: **Mitochondrial and plastid functions as antimalarial drug targets.** *Curr Drug Targets Infect Disord* 2004, **4**(1).
189. Kessler JJ, Lange BB, Merbitz-Zahradnik T, Zwicker K, Hill P, Meunier B, Palsdottir H, Hunte C, Meshnick S, Trumpower BL: **Molecular basis for atovaquone binding to the cytochrome bc1 complex.** *J Biol Chem* 2003, **278**(33):31312-31318.
190. Biagini GA, Fisher N, Shone AE, Mubarak MA, Srivastava A, Hill A, Antoine T, Warman AJ, Davies J, Pidathala C *et al*: **Generation of quinolone antimalarials targeting the Plasmodium falciparum mitochondrial respiratory chain for the treatment and prophylaxis of malaria.** *Proc Natl Acad Sci U S A* 2012, **109**(21):8298-8303.
191. Seymour KK, Yeo AE, Rieckmann KH, Christopherson RI: **dCTP levels are maintained in Plasmodium falciparum subjected to pyrimidine deficiency or excess.** *Annals of tropical medicine and parasitology* 1997, **91**(6):603-609.
192. Korsinczky M, Chen N, Kotecka B, Saul A, Rieckmann K, Cheng Q: **Mutations in Plasmodium falciparum cytochrome b that are associated with atovaquone resistance are located at a putative drug-binding site.** *Antimicrob Agents Chemother* 2000, **44**(8):2100-2108.

193. Fisher N, R. AM, Antoine T, Al-Helal M, Warman AJ, Johnson DJ, Lawrenson AS, Ranson H, O'Neill PM, Ward SA *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, **287**(13):9731-9741.
194. Bopp SE, Manary MJ, Bright AT, Johnston GL, Dharia NV, Luna FL, McCormack S, Plouffe D, McNamara CW, Walker JR *et al*: **Mitotic evolution of Plasmodium falciparum shows a stable core genome but recombination in antigen families.** *PLoS Genet* 2013, **9**(2):e1003293.
195. Schwobel B, Alifrangis M, Salanti A, Jelinek T: **Different mutation patterns of atovaquone resistance to Plasmodium falciparum in vitro and in vivo: rapid detection of codon 268 polymorphisms in the cytochrome b as potential in vivo resistance marker.** *Malar J* 2003, **2**:5.
196. Rathod PK, McErlean T, Lee PC: **Variations in frequencies of drug resistance in Plasmodium falciparum.** *Proc Natl Acad Sci U S A* 1997, **94**(17):9389-9393.
197. Bueno JM, Herreros E, Angulo-Barturen I, Ferrer S, Fiandor JM, Gamo FJ, Gargallo-Viola D, Derimanov G: **Exploration of 4(1H)-pyridones as a novel family of potent antimalarial inhibitors of the plasmodial cytochrome bc1.** *Future medicinal chemistry* 2012, **4**(18):2311-2323.
198. Capper MJ, O'Neill PM, Fisher N, Strange RW, Moss D, Ward SA, Berry NG, Lawrenson AS, Hasnain SS, Biagini GA *et al*: **Antimalarial 4(1H)-pyridones bind to the Qi site of cytochrome bc1.** *Proc Natl Acad Sci U S A* 2015, **112**(3):755-760.
199. Nilsen A, LaCrue AN, White KL, Forquer IP, Cross RM, Marfurt J, Mather MW, Delves MJ, Shackelford DM, Saenz FE *et al*: **Quinolone-3-diarylethers: a new class of antimalarial drug.** *Sci Transl Med* 2013, **5**(177):177ra137.
200. Phillips MA, Gujjar R, Malmquist NA, White J, El Mazouni F, Baldwin J, Rathod PK: **Triazolopyrimidine-based dihydroorotate dehydrogenase inhibitors with potent and selective activity against the malaria parasite Plasmodium falciparum.** *J Med Chem* 2008, **51**(12):3649-3653.
201. Ross LS, Gamo FJ, Lafuente-Monasterio MJ, Singh OM, Rowland P, Wiegand RC, Wirth DF: **In vitro resistance selections for Plasmodium falciparum dihydroorotate dehydrogenase inhibitors give mutants with multiple point mutations in the drug-binding site and altered growth.** *J Biol Chem* 2014, **289**(26):17980-17995.
202. Guler JL, White J, 3rd, Phillips MA, Rathod PK: **Atovaquone tolerance in Plasmodium falciparum parasites selected for high-level resistance to a dihydroorotate dehydrogenase inhibitor.** *Antimicrob Agents Chemother* 2015, **59**(1):686-689.
203. Bueno JM, Manzano P, Garcia MC, Chicharro J, Puente M, Lorenzo M, Garcia A, Ferrer S, Gomez RM, Fraile MT *et al*: **Potent antimalarial 4-pyridones with improved physico-chemical properties.** *Bioorg Med Chem Lett* 2011, **21**(18):5214-5218.
204. Yeates CL, Batchelor JF, Capon EC, Cheesman NJ, Fry M, Hudson AT, Pudney M, Trimming H, Woolven J, Bueno JM *et al*: **Synthesis and structure-activity**

- relationships of 4-pyridones as potential antimalarials.** *J Med Chem* 2008, **51**(9):2845-2852.
205. Winter RW, Kelly JX, Smilkstein MJ, Dodean R, Bagby GC, Rathbun RK, Levin JI, Hinrichs D, Riscoe MK: **Evaluation and lead optimization of anti-malarial acridones.** *Exp Parasitol* 2006, **114**(1):47-56.
206. Raether W, Enders B, Hofmann J, Schwannecke U, Seidenath H, Hanel H, Uphoff M: **Antimalarial activity of new floxacrine-related acridinedione derivatives: studies on blood schizontocidal action of potential candidates against *P. berghei* in mice and *P. falciparum* in vivo and in vitro.** *Parasitol Res* 1989, **75**(8):619-626.
207. Cross RM, Maignan JR, Mutka TS, Luong L, Sargent J, Kyle DE, Manetsch R: **Optimization of 1,2,3,4-tetrahydroacridin-9(10H)-ones as antimalarials utilizing structure-activity and structure-property relationships.** *J Med Chem* 2011, **54**(13):4399-4426.
208. Biagini GA, Fisher N, Berry N, Stocks PA, Meunier B, Williams DP, Bonar-Law R, Bray PG, Owen A, O'Neill PM *et al*: **Acridinediones: selective and potent inhibitors of the malaria parasite mitochondrial bc1 complex.** *Mol Pharmacol* 2008, **73**(5):1347-1355.
209. Cross RM, Namelikonda NK, Mutka TS, Luong L, Kyle DE, Manetsch R: **Synthesis, antimalarial activity, and structure-activity relationship of 7-(2-phenoxyethoxy)-4(1H)-quinolones.** *J Med Chem* 2011, **54**(24):8321-8327.
210. Cross RM, Monastyrskiy A, Mutka TS, Burrows JN, Kyle DE, Manetsch R: **Endochin optimization: structure-activity and structure-property relationship studies of 3-substituted 2-methyl-4(1H)-quinolones with antimalarial activity.** *J Med Chem* 2010, **53**(19):7076-7094.
211. Winter R, Kelly JX, Smilkstein MJ, Hinrichs D, Koop DR, Riscoe MK: **Optimization of endochin-like quinolones for antimalarial activity.** *Exp Parasitol* 2011, **127**(2):545-551.
212. Edstein MD, Looareesuwan S, Viravan C, Kyle DE: **Pharmacokinetics of proguanil in malaria patients treated with proguanil plus atovaquone.** *Southeast Asian J Trop Med Public Health* 1996, **27**(2):216-220.
213. Sutherland CJ, Laundry M, Price N, Burke M, Fivelman QL, Pasvol G, Klein JL, Chiodini PL: **Mutations in the *Plasmodium falciparum* cytochrome b gene are associated with delayed parasite recrudescence in malaria patients treated with atovaquone-proguanil.** *Malar J* 2008, **7**:240.
214. Trager W, Jensen JB: **Human Malaria Parasites in Continuous Culture.** *Journal of Parasitology* 2005, **91**(3):484-486.
215. Desjardins RE, Canfield CJ, Haynes JD, Chulay JD: **Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique.** *Antimicrob Agents Chemother* 1979, **16**(6):710-718.
216. Woloszynska M: **Heteroplasmy and stoichiometric complexity of plant mitochondrial genomes--though this be madness, yet there's method in't.** *J Exp Bot* 2010, **61**(3):657-671.
217. Musset L, Le Bras J, Clain J: **Parallel evolution of adaptive mutations in *Plasmodium falciparum* mitochondrial DNA during atovaquone-proguanil treatment.** *Mol Biol Evol* 2007, **24**(8):1582-1585.

218. Guler JL, Freeman DL, Ahyong V, Patrapuvich R, White J, Gujjar R, Phillips MA, DeRisi J, Rathod PK: **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.
219. Chavchich M, Gerena L, Peters J, Chen N, Cheng Q, Kyle DE: **Role of *pfmdr1* amplification and expression in induction of resistance to artemisinin derivatives in *Plasmodium falciparum*.** *Antimicrob Agents Chemother* 2010, **54**(6):2455-2464.
220. Pfaffl MW: **A new mathematical model for relative quantification in real-time RT-PCR.** *Nucleic Acids Research* 2001, **29**(9):e45-e45.
221. 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, **40**(4):914-919.
222. Vaidya AB, Mather MW: **Atovaquone resistance in malaria parasites.** *Drug Resist Updat* 2000, **3**(5):283-287.
223. Zalis MG, Pang L, Silveira MS, Milhous WK, Wirth DF: **Characterization of *Plasmodium falciparum* isolated from the Amazon region of Brazil: evidence for quinine resistance.** *Am J Trop Med Hyg* 1998, **58**(5):630-637.
224. Basco LK, Ringwald P: **Molecular epidemiology of malaria in Yaounde, Cameroon IV. Evolution of pyrimethamine resistance between 1994 and 1998.** *Am J Trop Med Hyg* 1999, **61**(5):802-806.
225. Kanazawa A, Shimamoto Y: **Soybean Recombination Sites are Present as Dispersed Segments in Arabidopsis and Liverwort Mitochondrial DNA.** *Plant Molecular Biology Reporter* 1999, **17**(1):19-29.
226. Arrieta-Montiel M, Lyznik A, Woloszynska M, Janska H, Tohme J, Mackenzie S: **Tracing evolutionary and developmental implications of mitochondrial stoichiometric shifting in the common bean.** *Genetics* 2001, **158**(2):851-864.
227. Janska H, Sarria R, Woloszynska M, Arrieta-Montiel M, Mackenzie SA: **Stoichiometric shifts in the common bean mitochondrial genome leading to male sterility and spontaneous reversion to fertility.** *Plant Cell* 1998, **10**(7):1163-1180.
228. Talundzic E, Plucinski MM, Biliya S, Silva-Flannery LM, Arguin PM, Halsey ES, Barnwell JW, Vannberg F, Udhayakumar V: **Advanced Molecular Detection of Malarone Resistance.** *Antimicrob Agents Chemother* 2016, **60**(6):3821-3823.
229. Li M, Stoneking M: **A new approach for detecting low-level mutations in next-generation sequence data.** *Genome Biology* 2012, **13**(5):1-15.

APPENDIX A: PUBLICATION COPYRIGHT



Sasha Siegel <svsiegel@gmail.com>

ID: 213884 Permission authorization for WHO copyrighted material

1 message

permissions@who.int <permissions@who.int>
To: svsiegel@gmail.com
Cc: permissions@who.int

Thu, Nov 10, 2016 at 3:43 PM

Dear Dr Siegel

Thank you for your request for permission to reprint and reproduce certain WHO copyrighted material.

On behalf of the World Health Organization, we are pleased to authorize your request to reproduce the WHO materials as detailed in the form below, subject to the terms and conditions of the non-exclusive licence below.

If you have questions regarding this authorization, please contact permissions@who.int.

We thank you for your interest in WHO published materials.

Kind regards,
WHO Permissions team

WORLD HEALTH ORGANIZATION (WHO)

Non-exclusive licence to use selected WHO published materials

You submitted a request, through WHO's online platform, for permission to reprint and reproduce certain WHO copyrighted material (the "Licensed Materials"). This is a legal agreement (the "Agreement") between you and WHO, granting you a licence to use the Licensed Materials subject to the terms and conditions herein.

Read this Agreement in its entirety before using the Licensed Materials.

By using the Licensed Materials, you enter into, and agree to be bound by, this Agreement.

This licence is granted only for original materials belonging to WHO. If any part of the WHO published materials you wish to reproduce are credited by WHO to a source other than WHO, those materials are not covered by this Agreement and are not part of the Licensed Materials. You are responsible for determining if this is the case, and if so, you are responsible for obtaining any necessary permission from the source of those third-party materials prior to their use.

If you enter into this Agreement on behalf of an organization, by using the Licensed Materials you confirm (represent and warrant) that you are authorized by your organization to enter into this Agreement on the organization's behalf. In such a case, the terms "you" and "your" in this Agreement refer to, and this Agreement applies to, the organization.

World Health Organization, World Malaria Report, 2014.

**NATURE PUBLISHING GROUP LICENSE
TERMS AND CONDITIONS**

Nov 10, 2016

This Agreement between Sasha V Siegel ("You") and Nature Publishing Group ("Nature Publishing Group") consists of your license details and the terms and conditions provided by Nature Publishing Group and Copyright Clearance Center.

License Number	3985470943477
License date	Nov 10, 2016
Licensed Content Publisher	Nature Publishing Group
Licensed Content Publication	Nature Reviews Microbiology
Licensed Content Title	Asymptomatic malaria infections: detectability, transmissibility and public health relevance
Licensed Content Author	Teun Bousema, Lucy Okell, Ingrid Felger, Chris Drakeley
Licensed Content Date	Oct 20, 2014
Licensed Content Volume Number	12
Licensed Content Issue Number	12
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 1
Author of this NPG article	no
Your reference number	
Title of your thesis / dissertation	Mitochondrial Heteroplasmy Contributes to the Dynamic Atovaquone Resistance Response in Plasmodium falciparum
Expected completion date	Dec 2016
Estimated size (number of pages)	175
Requestor Location	Sasha V Siegel 3720 Spectrum Blvd Suite 304 TAMPA, FL 33612

CC0 1.0 Universal (CC0 1.0) Public Domain Dedication

This is a human-readable summary of the [Legal Code \(read the full text\)](#).

[Disclaimer](#)

No Copyright



The person who associated a work with this deed has **dedicated** the work to the public domain by waiving all of his or her rights to the work worldwide under copyright law, including all related and neighboring rights, to the extent allowed by law.

You can copy, modify, distribute and perform the work, even for commercial purposes, all without asking permission. See **Other Information** below.



Other Information

- In no way are the patent or trademark rights of any person affected by CC0, nor are the rights that other persons may have in the work or in how the work is used, such as **publicity or privacy** rights.
- Unless expressly stated otherwise, the person who associated a work with this deed makes no warranties about the work, and disclaims liability for all uses of the work, to the fullest extent permitted by applicable law.
- When using or citing the work, you should not imply **endorsement** by the author or the affirmer.

Sinka *et al.*, Parasites & Vectors 2012, 5(1):69.



RightsLink®

Creative Commons Attribution License (CC BY)

This article is available under the terms of the [Creative Commons Attribution License \(CC BY\)](#). You may distribute and copy the article, create extracts, abstracts, and other revised versions, adaptations or derivative works of or from an article (such as a translation), to include in a collective work (such as an anthology), to text or data mine the article, including for commercial purposes without permission from Elsevier. The original work must always be appropriately credited.

Permission is not required for this type of reuse.

CLOSE WINDOW

Copyright © 2016 [Copyright Clearance Center, Inc.](#) All Rights Reserved.
Comments? We would like to hear from you. E-mail us at customercare@copyright.com

Mideo *et al.* 2012, *Trends in Parasitology*, 29(1).

**JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS**

Nov 10, 2016

This Agreement between Sasha V Siegel ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	3985490094191
License date	Nov 10, 2016
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	Journal of Internal Medicine
Licensed Content Title	The path of malaria vaccine development: challenges and perspectives
Licensed Content Author	C. Arama, M. Troye-Blomberg
Licensed Content Date	Apr 18, 2014
Licensed Content Pages	11
Type of use	Dissertation/Thesis
Requestor type	University/Academic
Format	Print and electronic
Portion	Figure/table
Number of figures/tables	1
Original Wiley figure/table number(s)	Figure 2
Will you be translating?	No
Title of your thesis / dissertation	Mitochondrial Heteroplasmy Contributes to the Dynamic Atovaquone Resistance Response in Plasmodium falciparum
Expected completion date	Dec 2016
Expected size (number of pages)	175
Requestor Location	Sasha V Siegel 3720 Spectrum Blvd Suite 304 TAMPA, FL 33612 United States Attn: Sasha V Siegel

**JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS**

Nov 10, 2016

This Agreement between Sasha V Siegel ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	3985541011751
License date	Nov 10, 2016
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	ChemMedChem
Licensed Content Title	Malaria Chemotherapeutics Part I: History of Antimalarial Drug Development, Currently Used Therapeutics, and Drugs in Clinical Development
Licensed Content Author	Martin Schlitzer
Licensed Content Date	May 25, 2007
Licensed Content Pages	43
Type of use	Dissertation/Thesis
Requestor type	University/Academic
Format	Print and electronic
Portion	Figure/table
Number of figures/tables	1
Original Wiley figure/table number(s)	Figure 33
Will you be translating?	No
Title of your thesis / dissertation	Mitochondrial Heteroplasmy Contributes to the Dynamic Atovaquone Resistance Response in Plasmodium falciparum
Expected completion date	Dec 2016
Expected size (number of pages)	175
Requestor Location	Sasha V Siegel 3720 Spectrum Blvd Suite 304 TAMPA, FL 33612 United States Attn: Sasha V Siegel
Publisher Tax ID	EU826007151

NATURE PUBLISHING GROUP LICENSE TERMS AND CONDITIONS

Nov 10, 2016

This Agreement between Sasha V Siegel ("You") and Nature Publishing Group ("Nature Publishing Group") consists of your license details and the terms and conditions provided by Nature Publishing Group and Copyright Clearance Center.

License Number	3985490348550
License date	Nov 10, 2016
Licensed Content Publisher	Nature Publishing Group
Licensed Content Publication	Nature Reviews Drug Discovery
Licensed Content Title	Malaria medicines: a glass half full?
Licensed Content Author	Timothy N. C. Wells, Rob Hooft van Huijsduijnen, Wesley C. Van Voorhis
Licensed Content Date	May 22, 2015
Licensed Content Volume Number	14
Licensed Content Issue Number	6
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 3
Author of this NPG article	no
Your reference number	
Title of your thesis / dissertation	Mitochondrial Heteroplasmy Contributes to the Dynamic Atovaquone Resistance Response in Plasmodium falciparum
Expected completion date	Dec 2016
Estimated size (number of pages)	175



Sasha Siegel <svsiegel@gmail.com>

Using a scheme from MPMP

2 messages

Hagai Ginsburg <hagai.ginsburg@gmail.com>
To: svsiegel@gmail.com

Thu, Nov 10, 2016 at 4:29 PM

Dear Sasha,

You are hereby authorized to use the scheme of mitochondrial electron flow from MPMP.

Please give credit.

Cheers and greetings to Dennis,

Hagai

[Are you using MPMP, the website on the functional genomics of *P. falciparum*?](#)
[Try it MPMP.huji.ac.il and you will never regret!](#)

Ginsburg 2016. Malaria Metabolic Pathways, Mitochondrial Electron Flow.

**SPRINGER LICENSE
TERMS AND CONDITIONS**

Nov 10, 2016

This Agreement between Sasha V Siegel ("You") and Springer ("Springer") consists of your license details and the terms and conditions provided by Springer and Copyright Clearance Center.

License Number	3985510545501
License date	Nov 10, 2016
Licensed Content Publisher	Springer
Licensed Content Publication	Springer eBook
Licensed Content Title	Mitochondrial Electron Transport Chain of Plasmodium falciparum
Licensed Content Author	Nicholas Fisher
Licensed Content Date	Jan 1, 2014
Type of Use	Thesis/Dissertation
Portion	Figures/tables/illustrations
Number of figures/tables/illustrations	1
Author of this Springer article	No
Order reference number	
Original figure numbers	Figure 2
Title of your thesis / dissertation	Mitochondrial Heteroplasmy Contributes to the Dynamic Atovaquone Resistance Response in Plasmodium falciparum
Expected completion date	Dec 2016
Estimated size(pages)	175
Requestor Location	Sasha V Siegel 3720 Spectrum Blvd Suite 304 TAMPA, FL 33612 United States Attn: Sasha V Siegel
Billing Type	Invoice
Billing Address	Sasha V Siegel 3720 Spectrum Blvd Suite 304