An investigation of the mechanisms of piperaquine resistance in *Plasmodium falciparum* malaria

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Declaration

I hereby declare that this dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as specified in the text. It is not substantially the same as any work that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution. This dissertation does not exceed the prescribed 60,000 word limit for the Biology Degree Committee.

Abstract

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Antimalarial drug resistance is an unrelenting obstacle to malaria control programs. In Southeast Asia (SEA), parasites have developed some degree of resistance to nearly every malaria drug currently available, with the most recent emergence to artemisinin combination therapies (ACTs). ACTs are the recommended front-line treatments for *Plasmodium falciparum* malaria worldwide and decreased susceptibility of parasites to both artemisinin and one of the widely used partner drugs, piperaquine, have been reported in multiple locations in SEA. It is therefore necessary to have reliable methods for detecting and evaluating resistant phenotypes. The purpose of this study was to combine clinical data from Cambodia with findings from genomic studies to evaluate putative markers of piperaquine resistance. The study first developed high-throughput assays to reliably detect one of these markers, a copy number variation (CNV) in the *plasmepsin 2* and *plasmepsin 3* (*PM2-PM3*) genes, in parasites likely to be PPQ-resistant. In addition to assay development, this study used gene overexpression techniques and CRISPR-Cas9 gene editing to examine the functional role of molecular markers of piperaquine resistance, including the *PM2-3* CNV, and two gene candidates with nonsynonymous single nucleotide polymorphisms (SNPs): a putative exonuclease protein (*exo-E415G*) and a putative mitochondrial carrier protein (*mcp-N252D*). This research fills a knowledge gap in the lack of functional data for molecular markers of piperaquine resistance by examining the phenotypic relevance of the genotypes observed in contemporary isolates. To complement these functional studies, this doctoral work has also used a *P. falciparum* hypermutator parasite line to select for a piperaquine-resistant phenotype *in vitro*. Whole genome sequencing analysis (WGS) of the piperaquine-resistant lines obtained through these experiments has identified nonsynonymous SNPs in gene candidates that have been reported to play a role in antimalarial drug resistance, including SNPs in the chloroquine resistance transporter gene (*pfcrt*) and the multidrug resistant protein 1 (*pfmdr1*) transporter. SNPs in *pfcrt* have been reported to confer piperaquine resistance in the field and *in vitro* and our recent drug-pressure experiments provide additional evidence to support these findings. The WGS analysis also discovered novel SNPs in gene candidates not previously reported to modulate the piperaquine-resistant phenotype that will require further evaluation. Such work has enabled the possibility of examining whether genetic changes observed in patient isolates can also be investigated and observed *in vitro*. By combining functional molecular approaches with genomic analyses, this study provides new insights into the mechanisms of piperaquine resistance.

To my parents, grandparents, & Uncle Gene

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Abbreviations

ACT	Artemisinin Combination Therapy
AQ	amodiaquine
AS	artesunate
bp	base pair
BSD	blasticidin
CNV	copy number variation
CPDA	citrate-phosphate-dextrose-adenine
CQ	chloroquine
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DHA	dihydroartemisinin
DV	digestive vacuole
exo-E415G	exonuclease SNP, E415G
FNT	formate-nitrite transporter
gRNA	guide RNA
GWAS	genome-wide association study
Hb	hemoglobin
hDHFR	human dihydrofolate reductase
IC50	inhibitory concentration assay
iRBCs	parasite-infected red blood cells
K13	kelch13
ldh	lactate dehydrogenase
LUM	lumefantrine
mcp-N252D	mitochondrial carrier protein SNP, N252D
MQ	mefloquine
MRP2	multidrug resistance-associated protein 2
NHEJ	non-homologous end joining
PCR	polymerase chain reaction
PfCRT	chloroquine resistance transporter
PfMDR1	multidrug resistance protein 1
PM2	plasmepsin 2
PM3	plasmepsin 3
PPQ	piperaquine
PPQ-R	piperaquine-resistant
PPQ-S	piperaquine-sensitive
pRBCs	packed red blood cells
PSA	piperaquine survival assay
RBC	red blood cell
RSA	ring stage survival assay
SD	standard deviation
SEA	Southeast Asia
SNP	single nucleotide polymorphism
TACT	Triple Artemisinin Combination Therapy
TRAC	Tracking Resistance to Artemisinin Collaboration

uRBCs	uninfected red blood cells
WGS	whole genome sequencing
WWARN	Worldwide Antimalarial Research Network

Preface

How does a disease that we have known about for thousands of years–for which we have cures—still cause nearly half a million deaths each year? This question has driven the entirety of this thesis project. By focusing on one facet of the complex problem, the intricate biology of the malaria parasite, this doctoral dissertation attempts to provide insight into the role that drug resistance plays in the treatment of *Plasmodium falciparum* malaria. Though the goal of this work was never to answer the overarching question explicitly, it continues to hold the larger perspective and impact of this devastating disease at the forefront of this research.