

PHARMACOLOGY AND PHARMACODYNAMICS
OF SELECTED ANTIMALARIALS AGAINST *P.*
falciparum GAMETOCYTES

Thesis is submitted in accordance with the requirements
of the University of Liverpool for the degree of Doctor
of Philosophy

By

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DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Ahmed Mohammed Saif

This thesis is a product of my own work which has been carried out during my PhD study in the Department of Parasitology, Liverpool School of Tropical Medicine, University of Liverpool, between January 2013 and December 2016. All the experiments presented in the result chapters were performed by me under the supervision of my supervisors, Professor Steve Ward, Professor Giancarlo Biagini. The thesis was written by me with their guidance.

Dedication

To my Parents, my wife and my daughters

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Table of Contents

Dedication	II
Acknowledgments	III
Publications, Presentations and Awards	IV
Table of Contents	V
List of Figures	IX
List of Tables	XI
Abbreviations	XII
Abstract	XVI
1 General introduction	1
1.1 Overview:.....	2
1.2 Malaria as a disease:.....	3
1.2.1 Malaria History:	3
1.2.2 Malaria Pathogenesis:	5
1.2.3 Malaria Epidemiology:.....	7
1.2.4 Malaria Control and prevention:	9
1.3 Malaria parasite biology:.....	10
1.3.1 Human malaria parasite life cycle:.....	10
1.3.2 Malaria Physiology and bioenergetics process:	12
1.3.3 <i>P. falciparum</i> sexual blood stages (gametocytes) Structure:	14
1.3.4 <i>P. falciparum</i> gametocytes morphology development:	16
1.4 Gametocytes metabolism:	17
1.5 Malaria Chemotherapy:.....	20
1.5.1 Antimalarial drugs:.....	22
1.6 Combination therapy of malaria treatment:	35
1.6.1 The non-Artemisinin-based combination therapy:.....	35
1.6.2 The Artemisinin-based Combination therapy:	35
1.7 Drug metabolism:.....	39
1.7.1 Artemisinin metabolism:.....	41
1.7.2 8-aminoquinoline metabolism (PMQ and TF):.....	42
1.8 Gametocytocidal activity assays development:	45
1.8.1 Florescence indicator of metabolic activity:	45
1.8.2 Florescence imaging:	46
1.8.3 Chemiluminescence:	48
1.8.4 Bioluminescence assay:	50
1.9 Thesis objectives:	51
2 Experimental Methods:	53
2.1 Introduction:.....	54

2.2	Culture system for <i>P. falciparum</i> maintenance:.....	54
2.2.1	<i>P. falciparum</i> parasite strains:.....	55
2.2.2	Preparation of uninfected red blood cells:	56
2.2.3	Cryopreservation Procedure of parasite cultures:	57
2.2.4	Parasite Cultures Retrieve (Thawing):.....	57
2.2.5	Routine monitoring of asexual stages parasitaemia:.....	58
2.2.6	Synchronisation of parasite cultures:	59
2.2.7	Culture procedure:.....	59
2.2.8	Sub-culturing Procedure of asexual stages:	60
2.3	<i>P. falciparum</i> Gametocytes (Sexual Stage) Culture:	61
2.3.1	<i>P. falciparum</i> gametocytes drug treatments:.....	64
2.3.2	Luciferase assay of gametocytocidal activity:	64
2.3.3	Data analysis:	66
2.4	Modelling Drug activity against Gametocytes.....	66
2.4.1	Identification of kill rates and pharmacodynamic parameters of drugs .	66
2.4.2	PK-PD modelling and predictions of clinical drug activity	68
3	Screening of known and potential gamitocytocidal drugs and PK/PD validation of methylene blue	69
3.1	Introduction:.....	70
3.2	Methods and materials:	73
3.2.1	Reagents:	73
3.2.2	Test compounds:	73
3.2.3	Compounds chemical structures:	74
	Figure 3.1: Chemical structures of antimalarial drugs used in chapter 3.....	74
3.2.4	Methods:.....	74
3.3	Results:.....	75
3.3.1	<i>In-vitro</i> anti-gametocytocidal activity screening of a panel of 13 known & potential antimalarial drugs and clinical candidates at 10 μ M concentration:.....	75
3.3.2	Dose-response of gametocytocidals activity profile during the gametocytes development (Early & late stages):.....	77
3.3.3	Identification of kill rates and pharmacodynamic parameters of drugs:	82
3.3.4	PK-PD modelling and predictions of clinical drug activity:.....	85
3.4	Discussion:	89
3.4.1	Panel of known antimalarials activity as gametocytocidal:	89
3.4.2	Activity of recent potential anti-malarial compounds against gametocytes:.....	94
3.4.3	PK-PD Modelling of MB towards Gametocytes Clearance:	95

3.4.4	Conclusion:	97
4	<i>In-vitro</i> pharmacodynamics of antimalarial endoperoxides against <i>P. falciparum</i> gametocytes and PK/PD validation of DHA	99
4.1	Introduction:	100
4.2	Material & Methods:	103
4.2.1	Reagents:	103
4.2.2	Test Compounds:	103
4.2.3	Compounds chemical structures:	104
4.2.4	Methods:.....	104
4.3	Results:.....	105
4.3.1	In-vitro gametocytocidal activity of selected endoperoxide compounds at a single concentration (5 μ M) against specific gametocyte life-cycle stages: .	105
4.3.2	Assessment of the dose-response relationship of selected endoperoxide against early and late stage gametocytes:.....	107
4.3.3	Determination of kill rates and characterisation of key pharmacodynamic properties of endoperoxides against late stage gametocytes:.....	111
4.3.4	PK-PD modelling and predictions of clinical drug activity:	114
4.4	Discussion:	117
4.4.1	Stage-specific kinetics and dynamics of Endoperoxides:	117
4.4.2	Dose-Response Endoperoxide Activity Profiles:.....	118
4.4.3	PK-PD Modelling of DHA and Gametocytes Clearance Predictions: .	120
4.4.4	Conclusion:	122
5	Gametocytocidal activity of primaquine and Its CYP2D6 metabolites	124
5.1	Introduction:	125
5.2	Material and Methods:	128
5.2.1	Reagents:	128
5.2.2	Test compounds:	129
5.2.3	Compounds chemical structures:	130
5.2.4	Experimental Methods:	131
5.3	Results:.....	139
5.3.1	CYP2D6 enzyme kinetic inhibition:	139
5.3.2	Hydrogen peroxide production through CYP drug metabolites reaction:.....	145
5.3.3	Gametocytocidal activity of PMQ compounds vs CYP 2D6-treated compounds:	148
5.3.4	Gametocytocidal activity of PMQ compounds vs HLM-treated compounds:	150
5.4	Discussion:	153

5.4.1	PMQ and its phenolic metabolites can inhibit the metabolism of a CYP2D6 enzyme substrate and generate H ₂ O ₂ :.....	153
5.4.2	Gametocytocidal activity of PMQ is CYP2D6-dependant metabolism:	156
5.4.3	Conclusion:	157
6	Tafenoquine and novel 8-aminoquinoline analogues: Gametocytocidal activity, haemolytic toxicity in the role of CYP2D6-dependent drug metabolism	159
6.1	Introduction:	160
6.2	Material and Methodes:.....	162
6.2.1	Reagents:	162
6.2.2	Test Compounds:	162
6.2.3	Compounds chemical Structures:.....	163
6.2.4	Methods:.....	164
6.3	Results:.....	165
6.3.1	TF autoflorescence spectrum:	165
6.3.2	CYP2D6 enzyme kinetic inhibition:	166
6.3.3	CYP metabolism dependent hydrogen peroxide production:	170
6.3.4	Hemolysis in red blood cell G6PD deficient:	173
6.3.5	The role of CYP2D6 specific metabolism and gametocytocidal activity:.....	174
6.3.6	The role of microsomal metabolism on gametocytocidal activity:	176
6.4	Discussion:	179
6.4.1	The interaction between selected 8-aminoquinolines and CYP2D6:...	179
6.4.2	Gametocytocidal activity of TF is not associated with CYP2D6-dependent metabolism:.....	181
6.4.3	Conclusion:	183
7	Conclusion, Limitations and Future Perspectives	185
7.1	Summary and conclusion:	186
7.2	Study limitations and future perspectives:	191
	REFERENCES	194

List of Figures

Figure 1.1: Malaria distribution globally and confirmed malarial cases.	8
Figure 1.2: Human malarial parasite life cycle of <i>P. falciparum</i> .	11
Figure 1.3: The gametocyte development stages (I-V) in <i>P. falciparum</i> .	17
Figure 1.4: The demonstration of TCA cycle in sexual gametocytes of malaria parasite <i>P. falciparum</i> .	18
Figure 1.5: Artemisinin and its semisynthetic derivatives chemical structures.	23
Figure 1.6: Proposed of cell-death and proceedings of cell survival after treatment with antimalarial artemisinins in <i>P. falciparum</i> .	24
Figure 1.7: Chemical structures of quinolone-based antimalarial drugs.	25
Figure 1.8: Chemical structure of antifolate drugs and their mode of action.	29
Figure 1.9: Diagram of putative ATQ mechanism of action.	31
Figure 1.10: Metabolism of artemisinin and its semi-synthetic derivatives.	42
Figure 2.1: Gametocytes development of stage composition of the cultures used in the experiment.	61
Figure 2.2: <i>In-vitro</i> production of <i>P. falciparum</i> gametocytogenesis in culture flask.	63
Figure 2.3: The 96- well white microplate use for tumescence reading of the luciferase assay.	64
Figure 2.4: Luciferase kinetic intensities steady state characterization.	65
Figure 3.1: Chemical structures of antimalarial drugs used in chapter 3.	74
Figure 3.2: Bar chart illustrate the inhibition profile of antimalarial at 10 μ M against early and late gametocyte stages.	77
Figure 3.3: Early and late gametocytes stages showing different profile activities.	79
Figure 3.4: Early and late gametocytes stages showing different profiles activity with spiroindolones.	80
Figure 3.5: Early and late gametocytes stages showing different profiles activity with MPS1.	80
Figure 3.6: Time-dependent killing assay of MB at different concentration against late stages gametocytes over discrete time intervals.	83
Figure 3.7: Model of pharmacodynamics of MB to determine the kill rate per h.	84
Figure 3.8: Simulation of PK exposure in a population of 1000 people given a 30% variation on all reported PK parameters of MB.	87
Figure 3.9: PK-PD relationship of MB and its effect upon late stages gametocyte for three day-dosing regimens (every 12 h).	88
Figure 4.1: Chemical structure of endoperoxides.	104
Figure 4.2: Bar chart illustrates the inhibition profile of antimalarial peroxides against early and late gametocyte stages at 5 μ M concentration.	106
Figure 4.3: Typical curves showing the dose response of (A) artemisinin, and the fully synthetic peroxides (B) OZ439 and (C) TDD-E209 against early (black) and late (red) gametocyte stages.	108
Figure 4.4: Typical curves showing the exposure-effect relationship of the semi-synthetic derivatives of artemisinin (A) DHA, (B) Artesunate and (C) Artemether against early (black) and late (red) gametocyte stages.	109
Figure 4.5: Time-dependent killing assay of DHA at different concentrations against late stages gametocytes over discrete time intervals.	112
Figure 4.6: Dynamic concentration-kill rate response of DHA.	113
Figure 4.7: Simulation of PK exposure in a population of 1000 people given a 30% variation on all reported PK parameters.	115
Figure 4.8: PK-PD relationship of DHA and its effect upon gametocytes.	116

Figure 5.1: The proposed mechanism of action of PMQ bio-activation via CYP 2D6 metabolism and antimalarial efficacy.	126
Figure 5.2: Chemical structure of primaquin and selected PMQ metabolites.	130
Figure 5.3: CYP2D6 steady-state kinetics and inhibition of tracer metabolism vs. PMQ concentration.	140
Figure 5.4: CYP2D6 steady-state kinetics of PMQ metabolites 5OH PMQ and PQQI.	142
Figure 5.5: CYP2D6 steady-state kinetics of PMQ metabolites 5,6-OH PMQ and 6-OH PQQI.	143
Figure 5.6: CYP2D6 steady-state kinetics of carboxy and 5-hydroxycarboxyPMQ compounds.	144
Figure 5.7: Catalase-mediated oxygen release from PMQ and its metabolites compounds before and after CYP2D6 metabolism prior to normalization (adjustment).	146
Figure 5.8: Normalized catalase-mediated oxygen release post CYP2D6 metabolism of PMQ and its metabolites.	147
Figure 5.9: %Viability of <i>P. falciparum</i> gametocytes after exposed to PMQ metabolites compounds in the context of CYP 2D6 metabolism.	149
Figure 5.10: %Viability of <i>P. falciparum</i> gametocytes after exposed to PMQ metabolites compounds in the context of HLM metabolism.	151
Figure 6.1: Chemical structure of PMQ, TF and newly synthetic 8-aminoquinoline (SL-6-41, SL-6-46 and SL-6-56).	163
Figure 6.2: Spectrum auto florescence excitation and emission detected at high concentration of TF.	165
Figure 6.3: The ability of TF to compete for CYP2D6 via competition with the metabolism of a fluorescent probe substrate.	167
Figure 6.4: The ability of three 8-minoquinoline PMQ analogues to compete for CYP2D6 via competition with the metabolism of a fluorescent probe substrate.	169
Figure 6.5: Catalase-mediated oxygen release before and after CYP2D6 metabolism of TF compared to PMQ prior to normalization (adjustment).	171
Figure 6.6: Normalized catalase-mediated oxygen release post CYP2D6 metabolism of TF compare to PMQ.	172
Figure 6.7: <i>In-vitro</i> hemolysis induced by PMQ and TF.	173
Figure 6.8: The effect of CYP2D6-dependent metabolism on the viability gametocytes after exposure to TF vs PMQ.	175
Figure 6.9: The effect of CYP2D6-dependent metabolism on the viability of gametocytes after exposure to novel synthetic 8-aminoquinoline analogues.	175
Figure 6.10: The effect of HLM-dependent metabolism on the viability gametocytes after exposure to TF vs PMQ.	177
Figure 6.11: The effect of HLM-dependent metabolism on the viability gametocytes after exposure to new synthetic 8-aminoquinoline analogues.	178

List of Tables

Table 1.1: Antimalarial drug classes, their target locations, parasite stages and their possible combinations.	21
Table 1.2: Antimalarial drugs that have been described to exert limited improvement on gametocytes clearance/mosquito infectivity.	33
Table 1.3: Antimalarial drugs that have been described to exert significant reductions in gametocytes clearance/mosquito infectivity.	34
Table 2.1: <i>In-vitro</i> gametocytogenesis of <i>P. falciparum</i> culture under various conditions.	62
Table 3.1: Known and new potential antimalarial drugs used in this chapter.	73
Table 3.2: Gametocytocidal activity of 13 known and potential candidate antimalarials.	76
Table 3.3: IC ₅₀ values for the gametocytocidal activity of 7 current and candidate antimalarial drugs.	78
Table 3.4: The kill rate measurement achieved by each concentration of Methylene blue.	83
Table 3.5: PK parameters of MB for adult patients as reported in the literature.	86
Table 4.1: Endoperoxides antimalarial drugs that are used in this chapter.	103
Table 4.2: Gametocytocidal activity of 6 current and candidate endoperoxide drugs.	107
Table 4.3: The measured DHA dose dependent kill rates:	112
Table 4.4: PK parameters of DHA for adult patients as reported in the literature.	114
Table 5.1: PMQ and its metabolites used in this chapter.	129
Table 5.2: Summary of CYP2D6 reaction procedure.	132
Table 5.3: Summary of the CYP-reaction constituents used in the late stage gametocyte assay.	136
Table 5.4: The summary of statistical significances between (-) CYP and (+) CYP activity against late gametocytes (Welch's <i>t</i> -test, two tail).	149
Table 5.5: Summary of statistical significances between (-) HLM and (+) HLM activity against late gametocytes (Welch's <i>t</i> -test, two tail).	152
Table 6.1: TF and the newly synthetic 8-aminoquinoline compounds studied.	162
Table 6.2: The summary of statistical analysis between (-) CYP and (+) CYP incubations with TF, primaquone and the 8-aminoquinoline analogues in terms of effects on viability of late gametocytes (Welch's <i>t</i> -test, two tail).	176
Table 6.3: The summary of statistical significances between (-) HLM and (+) HLM of TF, primaquone and 8-aminoquinoline analogues against late gametocytes (Welch's <i>t</i> -test, two tail).	178

Abbreviations

ACTs	Atemisinin-based combination therapies
AQ	Amodiaquine
ATP	Adenosine triphosphate
ATQ	Atovaquone
AUC	Area under the curve
BL	Bioluminescence signal
<i>C</i>	Concentration
CL	Central Clearance
cm ²	Centimeter squared
<i>C_{max}</i>	Peak concentration
CO ₂	Carbon dioxide
<i>C_p</i>	plasma concentration
CQ	CQ
CYP	Cytochrome P450
dH ₂ O	Distilled water
DHA	Dihydroartemisinin
DHFR	Dihydrofolate reductase
DHODH	Dihydroorotate dehydrogenase
DHPS	Dihydropteroate synthase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dTMP	deoxythymidylate
e.g.	For example
<i>EC₅₀</i>	The concentration required to achieve 50% of <i>E_{max}</i>
<i>E_{max}</i>	The maximum possible kill rate
<i>E_{min}</i>	the background effect achieved in negative controls
EM	Extensive metabolizers
ETC	Mitochondrial electron transport chains
FAD	Flavin adenine dinucleotide
g	gram
G6PD	Glycerol-6-phosphate dehydrogenase
GSH	Glutathione
GTP	Guanosine triphosphate
h	hour
h ⁻¹	Per hour
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxides
Hb	Hemoglobin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV	Human immunodeficiency virus
HLM	Human liver microsomes
i.e.	In another word
IC ₅₀	Half-maximal inhibitory concentration
IC ₉₉	99% inhibitory concentration
IM	inter-mediate metabolizers
iRBC	Infected red blood cell
$K\ h^{-1}$	Kill rate per hour
K_a	The rate of absorption
K_{CP}	The rate for transit from central to peripheral compartments
K_e	The rate of elimination
K_{PC}	The rate for transit from peripheral to central compartment
L	Litre
M	Molar
MB	Methylene Blue
MDR	Multidrug resistance
MeOH	Methanol
mg	miligram
min	minute
ml	milliliter
mM	Millimolar
mm	millimeter
MoA	Mode of action
MPS1	Mitotic Kinase Monopolar Spindle 1
MQ	mefloquine
MQO	Malate:quinone oxidoreductase
MS	Mass spectrometry
mtETC	Mitochondrial electron transport chain
MW	Molecular weight
n	Number of experiments replicates
N ₂	Nitrogen
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	reduced Nicotinamide adenine dinucleotide phosphate
ND	Not determined
NAG	N-acetyl-glucosamine
nmol/L	Nanomol per liter
nM	Nanomolar
NPP	New permeation pathway
O ₂	Oxygen
°C	Degrees Celsius

OH	Hydroxy
PBS	Phosphate buffer saline
PD	Pharmacodynamic
PfCRT	<i>P. falciparum</i> CQ resistance transporter
PfEMP 1	<i>P. falciparum</i> erythrocyte protein 1
PM	poor metabolizers
PfMDR 1	<i>P. falciparum</i> multidrug resistance transporter 1
PfNDH2	<i>P. falciparum</i> type II NADH:quinone oxidoreductase
PG	Proguanil
pH	Power of hydronium
PK	Pharmacokinetic
PK-PD	Pharmacokinetic-Pharmacodynamic
PMQ	Primaquine
PPQ	Piperaquine
Q	Ubiquinone
Q	The inter-compartmental clearance
Q_0	Ubiquinone oxidation site
QH_2	Ubiquinol
Q_i	Ubiquinone reduction site
RBC	Red blood cell
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per min
RPMI	Roswell Park Memorial Institute
s	Second
SD	Standard deviation
SEM	Standard error of the mean
SP	Sulfadoxine-pyrimethamine
t	time
TCA	Tricarboxylic acid
TF	Tafenoquine
T_{max}	Time to reach maximum concentration
UK	United Kingdom
UM	Ultra-rapid metabolizers
UMP	Uridine monophosphate
USA	United State of America
UTP	Uridine triphosphate
V_C	Central Volume distribution
V_d	volume of distribution
w/w	Weight per weight
WHO	World Health Organization
X_l	The drug mass in the gut

X_{2a}	The mass of drug in blood
X_3	The mass of drug in the peripheral compartment
μg	microgram
μl	Microliter
μm	Micrometer
μM	Micromolar
%	Percentage
/	per
μ	Mean
1 st	First
2 nd	Second
3 rd	Third

Abstract

Malaria is a vector-borne disease that is still responsible for high human morbidity and mortality. Of the five *Plasmodium* species that can cause malaria in humans, *Plasmodium falciparum* is regarded the most virulent species. The most fundamental component of sustained control and eradication efforts is the development of effective drugs for malaria treatment and prophylaxis. *Plasmodium falciparum*'s sexual stages (gametocytes) are not associated with malarial pathogenesis or the clinical symptoms, but they are responsible for the transmission of the disease from human hosts to mosquitos. As such, the development of gametocytocidal interventions that targets the transmission stage to break the disease's lifecycle forms the basis of efforts towards malaria elimination and eradication. However, despite the importance of this developmental stage, the biology and pharmacology of gametocytes are still very poorly understood. This thesis has set out to gain a better understanding of the identity of gametocyte-active antimalarials and a deeper understanding of the mechanisms underpinning the activity. Using a newly generated luciferase-reporting transgenic line, pharmacodynamic gametocyte studies could be performed to help characterise the activity of selected known reference antimalarials, new potential gametocyte inhibitors in pre-clinical development as well as newly developed fully synthetic compounds designed against the sexual stages. This novel assay revealed that the efficacy of active tested compounds is highly stage-specific. Of all the tested reference antimalarial drugs, MB and DHA were the most potent antimalarial across all gametocyte stages and importantly they were active at clinically relevant levels. These observations were progressed further, developing a time- dependent killing assay that was performed with different concentrations of targeted drug over discrete time intervals to determine the drug's kill rate. These parameters were then used to simulate the PK/PD relationship of the drug in order to estimate gametocyte clearance profiles during the human treatment period (Chapter 3 and 4). A main focus of the thesis was conducted to better understand the mechanism of drug activity of the 8-aminoquinolines against gametocytes. The ability of a series of 8-aminoquinolines (primaquine as the parent drug, synthesised metabolites in chapter 5, three novel analogues and tafenoquine in (chapter 6) to interact with CYP2D6 was tested by measuring their ability to specifically inhibit the metabolism of fluorescently-tagged tracer substrate by recombinant human CYP 2D6. Reaction products from the CYP metabolites and HLM were then used to test firstly their ability to kill gametocytes, and then to establish their ability to generate hydrogen peroxides and finally measure their haemolytic toxicity. At 10 μ M, primaquine CYP metabolites showed activity against the gametocytes that was higher than that of the parent drugs, with the exception of tafenoquine which, interestingly, demonstrated good activity and haemolytic toxicity as a parent drug. These analyses are presented and discussed in the context of strategies that aim at the discovery and development of new transmission-reducing antimalarial drugs.

Chapter 1

General introduction

1.1 Overview:

Malaria is one of the main tropical diseases of major importance in several parts of the world. It remains the main source of morbidity and mortality in the tropical zones (Bruce-Chwatt, 1987, Murray et al., 2012, Guerin et al., 2002). Malaria is transmitted via infected female *Anopheles* mosquitos and is caused by the protozoan parasite species known as *Plasmodium*. Part of the *Plasmodium* life cycle occurs in vertebrates (birds, animal, humans) whilst the transmission cycle occurs in mosquitos. Human beings are infected by various *Plasmodium* species including *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* and lastly the primate parasite *Plasmodium knowlesi* that has recently been shown to infect humans (Cox-Singh and Singh, 2008, Cox-Singh et al., 2008, White, 2008a).

In 2016, malaria continues to be one of the lethal infectious diseases in spite of the huge efforts to eliminate/eradication the disease during last decades. The disappointment results of malarial vaccine trials, the development of drug-resistant *Plasmodium* strains, insecticide resistance and the enormous global changes regarding population increase and the environment make the challenges towards disease elimination/eradication extremely complicated.

This chapter reviews current knowledge regarding malaria disease, parasite biology, malaria chemotherapy, drug metabolism, and whole cell gametocytocidal activity assays.

1.2 Malaria as a disease:

1.2.1 Malaria History:

Malaria has been in existence for over 4000 years. The initial description of malaria is found in the ancient medical writings by the Chinese called *Nei Ching* around 2700 BC. The disease was documented by the Greeks in the 4th century BC where Hippocrates wrote about Malaria symptoms (Cox, 2010). A Sanskrit medical article documented malaria symptoms and linked the disease to an insect bite as the cause. In the 2nd century BC, the initial medication for treating malaria was discovered by the Chinese. Specifically, the use of *Artemisia annua* or the Qinghao plant for treating malaria was documented in a medical article called 52 Remedies. However, it was only until 340 CE that the description of the antifever properties of the plant was described in medical text. The Qinghao plant's active ingredient is artemisinin that was isolated in 1971 by scientists from China. Presently, drugs with artemisinin compounds are utilised in treating malaria in areas where individuals have developed resistance to the chloroquine (CQ). In the 17th century, another medication was documented by Jesuit missionaries from Spain. The medication came from the bark of a tree in Peru. This was after the bark cured the wife of the Viceroy of Peru who had been infected with malaria. The Peruvian bark is used in deriving quinine for treating malaria.

On November 6, 1880, an army surgeon known as Alphonse Laveran while treating a patient suffering from malaria in Algeria discovered a parasite in the patient's blood during examination. The surgeon came to the conclusion that malaria was caused by a single parasite that he named as *Oscillaria malariae* (Cox, 2010). Furthermore, Laveran discovered part of the malaria's lifecycle after observing how the male

gametocyte exflagellated in the blood. Based on this, he concluded that the parasite was a protozoan. Laveran was unable to detect the parasite in soil, water, or air, and deduced that the parasite lived inside a mosquito. This was confirmed in 1884 by Angello Celli and Ettore Marchiafava who named it Plasmodium. In 1886, it was determined by Camillo Golgi that malaria presented itself in multiple forms. This conclusion was arrived at after observations of the fever trend in malaria patients. Particularly, one form of malaria was characterised by fever on each second day, while in another form the fever presented on each third day. Golgi connected the malarial fever to the rupturing of red blood cells because of the release of mature merozoites accumulated in red blood cells (Cox, 2010).

In 1890, Raimondo Filetti, Giovanni Batista, and Grassi named initial species of plasmodium particularly *Plasmodium malariae* and *Plasmodium vivax*. In 1897, *Oscillaria malariae* was renamed *Plasmodium falciparum* by William Welch. In 1897, the initial proof that mosquitoes transferred plasmodia to humans was done by Ronald Ross after observing how birds bitten by infected mosquitoes developed malaria. In 1898, the occurrence of the sexual cycle of the plasmodium genus in the Anopheles mosquito was demonstrated by Guisepe Bastianelli, Amico Bignami, Giovanni Battista Grassi, Camillo Golgi, Ettore Marchiafava, and Angelo Cell. These investigators gathered mosquitoes and allowed them to bite healthy individuals who went on to develop the disease. This resulted in the discovery of how the disease is transmitted in people. In 1922, *Plasmodium ovale* was described and named by John William Stephens. In 1931, *Plasmodium knowlesi* was discovered in a monkey by Biraj Mohan das Gupta and Robert Knowles. The initial malaria case due to *P. knowlesi* was reported in 1965. In 1948, Cyril Garnham and Henry Scott finished the lifecycle of Plasmodium by demonstrating that a stage of division in the liver preceded

the development of the parasite in the blood. The long duration between being infected and the appearance of the parasites in blood was confirmed by the demonstration of the existence of a dormant phase in the liver by Wojciech Krotoski.

1.2.2 Malaria Pathogenesis:

Initially, patients are asymptomatic after being bitten by the infected anopheles mosquito during the incubation of the parasite in the liver. After the parasite starts intraerythrocytic development cycles, patients start manifesting splenomegaly, a fever cycle lasting for 48 h, and flu-like symptoms. While developing in the erythrocyte, the parasite remodels the infected red blood cell to ensure survival within the human host (Haase and de Koning-Ward, 2010). The red blood cell does not have the endogenous cellular mechanism required for protein trafficking and thus the parasite is involved in coordinating the transport and expression of proteins to the plasma membrane of the infected red blood cell for survival of the parasite. These cellular changes enable host and parasite interactions that eventually contribute to the clinical manifestation of malaria (Goldberg and Cowman, 2010). Infected red blood cells are capable of adhering to other cells via particular host-cell receptor and parasite ligand interactions that result in the sequestration of the parasite (Rowe et al., 2009). The parasites sequester in vascular beds of various tissues and organs such as subcutaneous tissue, placenta, liver, kidney, lungs, and the brain, which prevent removal by the spleen (Buffet et al., 2011). Consequently, only the ring stage infected red blood cells circulate in the blood stream. Sequestration and severe disease is caused by particular parasite proteins. Specifically, a parasite ligand that contributes to sequestration is the Erythrocyte Membrane Protein 1 (PfEMP1) surface protein. PfEMP1 is responsible for the adhesion of the infected red blood cells to various host cells (Pasternak and Dzikowski, 2009). Approximately 60 *var* genes encoded PfEMP1 protein that is

exported to the surface of the infected red blood cell. The expression of a single PfEMP1 at a time occurs on the surface of the infected red blood cell in the intraerythrocytic development cycle (Buffet et al., 2011). Although the expression of PfEMP1 is homogenous on one infected red blood cell, its expression across a population is heterogenous and might affect pathogenesis and tissue distribution (Warimwe et al., 2012). PfEMP1 variants contain particular host-cell receptors for determining which types of cells they might bind (Claessens et al., 2012). Although all mature infected red blood cells can undergo sequestration, not all infections result in severe disease (Haldar et al., 2007).

Sequestration together with related systemic and local cytokine release play a vital role in severe malaria (Taylor et al., 2013, Manning et al., 2012). Patients with severe malaria have hyperparasitemia and severe systemic complications including organ failure, shock, thrombocytopenia, and severe anaemia. Major complications of infection by *P. falciparum* include cerebral malaria. Cerebral malaria is a fatal complication of malaria that leads to severe impairment of the neurological function (Ponsford et al., 2012). When infected red blood cells sequester in the brain's microvasculature, they cause the blockage and the passage of the red blood cells thereby disrupting gas exchange, which causes inflation, haemorrhaging, and localised acidosis that impair the patient's behaviour due to increased neurological damage that often culminates in coma, seizure, and death (Postels and Birbeck, 2013). In cerebral malaria, infected red blood cells bind CD36 and ICAM-1 scavenger receptors found on the surface of endothelial cells that line the brain's microvasculature (Turner et al., 2013). Cerebral malaria accounts for the majority of malaria deaths especially in young children after initial exposure to the malaria parasite (Haldar et al., 2007). The mortality rate of patients with cerebral malaria is between 15% and 20%.

1.2.3 Malaria Epidemiology:

An estimated 200 species of *Plasmodium* parasites infect mammals, birds, rodents, and reptiles. Human beings are infected by various *Plasmodium* species including *P.knowlesi*, *P.ovale*, *P. malariae*, *P.vivax*, and *P. falciparum*. According to WHO report in 2015, about 214 million malaria estimated cases in the world with the full of malaria deaths being approximately 438,000 people. This report represents a reduction in the malaria reported cases and deaths since 2000 by 37% and 60%, respectively. Approximately 90% of the of disease deaths occur in Africa and over two-third of this cases taking place in children under five years old (WHO, 2015). The vast majority of the mortality and morbidity of malaria is attributed to *P. falciparum* and is found worldwide, but mainly in Africa in tropical and subtropical areas (Snow et al., 2005).

P. vivax is geographically widespread and exist mostly in Latin America, Asia, and in some parts of Africa (Gunn, 2012). It is responsible for a many of disease cases and also a source of severe malaria disease and death (WHO, 2012, Kochar et al., 2005). Interestingly, *P. vivax* is linked to erythrocyte expressing Duffy blood group antigens (Fy^a and Fy^b) and not found in people of West Africa because they mainly do not express those antigens on erythrocytes (negative duffy blood group) (Gunn, 2012). *P. vivax* as well as *P. ovale* have dormant liver stages (hypnozoites) that can activate and invade the blood (relapse) several months or years after the infecting mosquito bite (Gunn, 2012). In addition, *P. vivax* parasites as well as *P. ovale* parasites are only able to develop in reticulocyte (Gunn, 2012)

P. ovale is distributed generally in Africa, particularly West Africa and Western Pacific islands (Collins and Jeffery, 2005). This parasite species is similar to *P. vivax* physiologically and morphologically as they are only able to develop in reticulocyte

and can be dormant in liver stages (hypnozoites). However, *P. ovale* can infect people with negative duffy blood group antigens which is the case in Africa (Gunn, 2012). This contributes to the high prevalence of *P. ovale* in Africa more than *P. vivax* (Collins and Jeffery, 2005).

P. malariae is distributed all over world and is the only the human parasites which has specific cycle with three-day cycle (Collins and Jeffery, 2007). It is common in human and chimpanzee and the dormant infection is able to continue in the body for years if untreated (Hayakawa et al., 2009).

P. knowlesi is a primate malarial disease that is mostly found in Southeast Asia and causes the disease in long-tailed and pig-tailed macaques (reservoir hosts). Recently, *P. knowlesi* was shown to be an important cause of zoonotic malaria disease in that region, especially in Malaysia (Cox-Singh and Singh, 2008, Cox-Singh et al., 2008).

Figure 1.1 shows the world distribution of malaria in 2014.

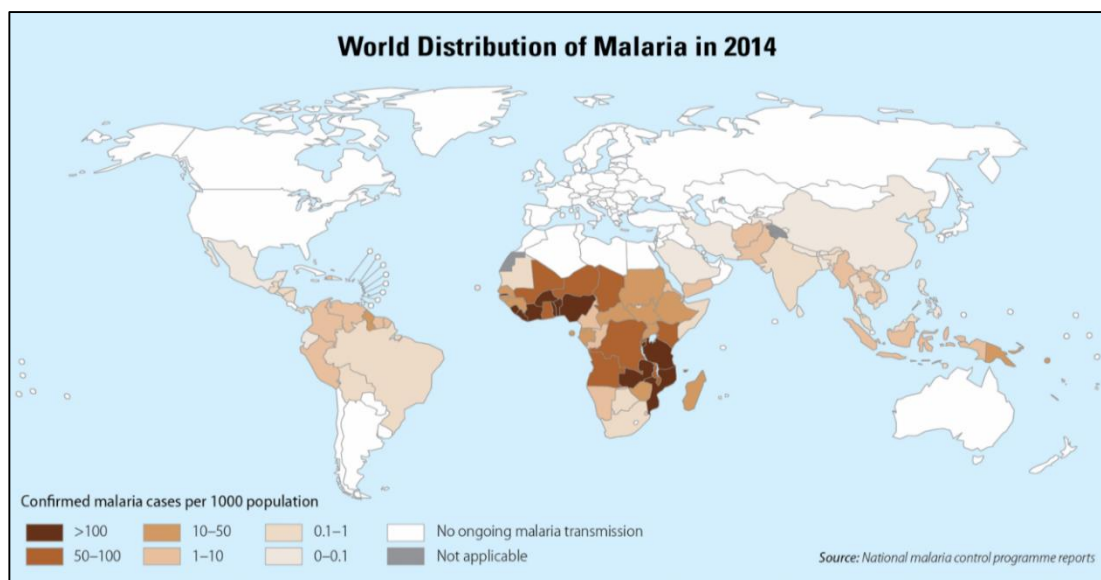


Figure 1.1: Malaria distribution globally and confirmed malarial cases.

Global map shows malaria confirmed malarial cases per 1000 population countries in 2014. Dark brown colour indicates countries with malaria cases >100. Brown indicates countries with malaria cases 50-100. Light brown indicates countries with malaria cases 10-50. Pink indicates countries with malaria cases 1-10. Light Pink indicates countries with malaria cases 0-1. White colour indicates countries that are not affected by malaria. Grey colour is not applicable to any countries (Adapted from WHO,2014).

1.2.4 Malaria Control and prevention:

The control and prevention of malaria involves a multi-faceted approach that include transmission interruption, treatment, vector control, and vaccines (Chambers, 2012). Vector control techniques through insecticide spraying and bed net are aimed at reducing the transmission of the disease by preventing bites from mosquitoes. Although these techniques have some effectiveness, bed nets have to be replaced on a regular basis and mosquitoes might develop insecticide resistance (Liu, 2015). Transmission interruption involves using lasting insecticide-treated nets. The standard treatment for malaria is artemisinin-based combination therapies (ACTs). ACTs augment malaria elimination and control efforts by reducing disease transmission and the spread of drug resistance at the population level (Abdul-Ghani and Beier, 2014). Specifically, ACTs significantly reduce the duration of gametocyte carriage after treatment and minimise the transmission potential in addition to rapid action in clearing asexual stages of the parasite (Abdul-Ghani and Beier, 2014). However, resistance to anti-malarial is rapidly developing in endemic regions (Abdul-Ghani et al., 2014). Currently, there is lack of an approved vaccine for malaria. However, a vaccine being developed called, RTS, S that offers protection against the disease during the pre-erythrocytic parasite moderately protects against malaria (Crompton et al., 2010). Phase II clinical trials indicate that approximately 30% to 50% of malaria-naïve individuals that got the RTS, S immunisation were protected following a malaria infection challenge (Guinovart et al., 2009). In addition, vaccine initiatives aimed at blocking malaria transmission by infecting people with genetically or irradiated attenuated sporozoites have been challenging because the vaccine offered protection only to a small population of people (Hoffman et al., 2010). The development of vaccine against malaria is hindered by deficient knowledge of the interactions between

the intrerythrocytic forms and the host, particularly how the polymorphic parasite proteins found on the infected red blood cells cause the disease (Crompton et al., 2010).

1.3 Malaria parasite biology:

1.3.1 Human malaria parasite life cycle:

The malaria parasite has a complex life cycle that requires two hosts (Figure 1.2). In the human host, asexual replication of the parasite occurs, while sexual reproduction takes place in the anopheles' mosquito. Malaria infection starts when an infected female anopheles mosquito feeds on human blood and injects sporozoites into the blood stream. After this, the sporozoites travel quickly and reach the liver where they mature within the hepatocytes for nearly 14 days followed by asexual multiplication to form merozoites (Hansen et al., 2014). The merozoites exit the liver through the rupturing of hepatic tissues and enter into the bloodstream. In the blood stream, the merozoites invade the red blood cells. When inside the red blood cells, the parasite starts the intraerythrocytic development cycle that enables it to mature from a ring stage parasite to trophozoites and schizonts (Delves et al., 2012). Some of the merozoites are transformed into male and female gametocytes that circulate in the bloodstream. When an infected human is bit by a second mosquito, the gametocytes travel to the midgut of the mosquito where sexual development takes place. Fertilization of the gametocytes occurs to form zygotes that then develop into ookinetes, which then transform into oocyst inside the mosquito's midgut (Miller et al., 2013b). The parasite is released to the salivary gland as sporozoites that cause infections in new human hosts. This completes the transmission cycle of the malaria parasite. The figure below demonstrates the malaria lifecycle.

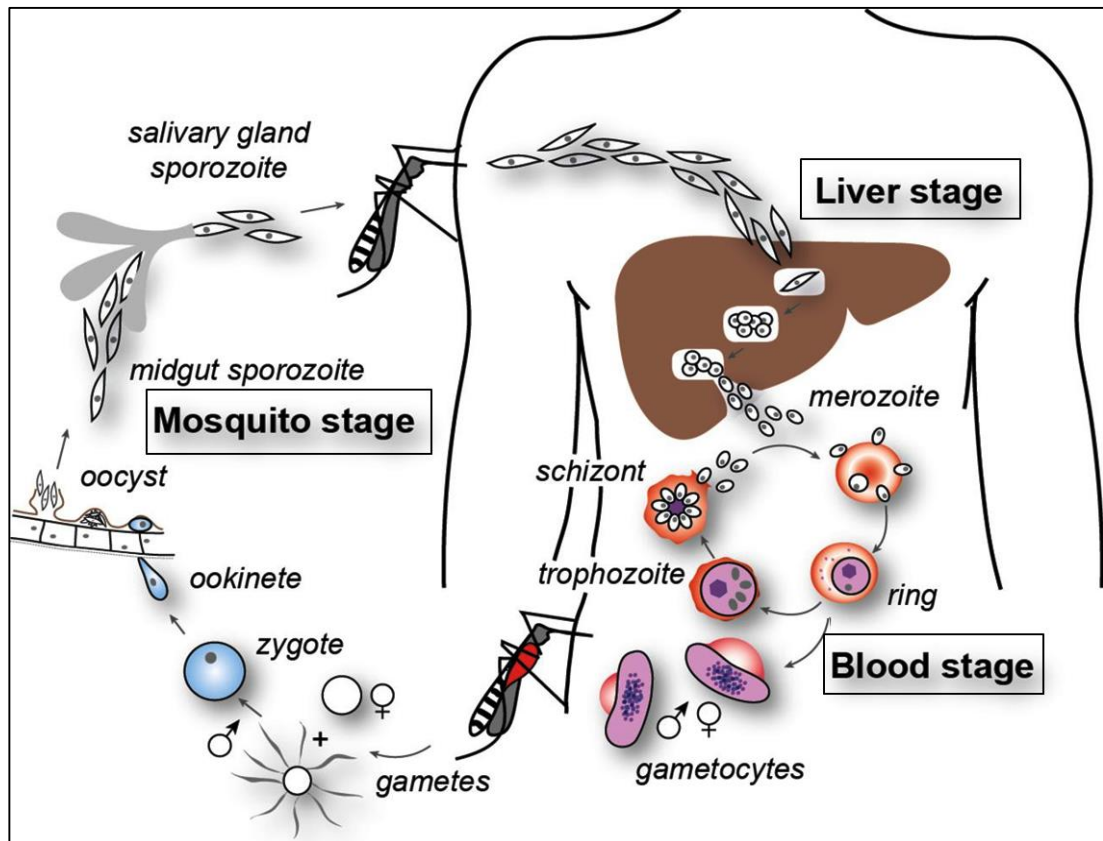


Figure 1.2: Human malarial parasite life cycle of *P. falciparum*.

The infected female *Anopheles* mosquito bites a human and inoculates sporozoite. This sporozoite form invade hepatocytes in liver, and develop and rupture hepatocytes to produce liver stage (merozoite) as an exoerythrocytic and then released into the blood stream. During the presence of merozoites in blood stream, they invade erythrocytes. When inside the red blood cells (blood stage), the parasite starts the intraerythrocytic development cycle that enables it to mature from a ring stage parasite to trophozoites and schizonts. The schizonts rupture and release merozoites which reinvade new erythrocytes. Some of the merozoites are transformed into male and female gametocytes that circulate in the bloodstream. In Mosquito stage, when an infected human is bitten by a second mosquito, the gametocytes travel to the midgut of the mosquito where sexual development takes place. Fertilization of the gametocytes occurs to form zygotes that then develop into ookinetes, which then transform into oocyst inside the mosquito's midgut. Finally, sporozoites that migrate to the salivary glands. Figure adapted from Cowman et al., 2012 (Cowman et al., 2012).

1.3.2 Malaria Physiology and bioenergetics process:

The nanomotor ATP synthase is critical in the bioenergetics process of most organisms. Specifically, the ATP synthase is involved in the generation of ATP in the mitochondria. Plasmodium parasites have only one mitochondrion. The size and morphology of the mitochondria vary between sexual and asexual stages of plasmodium parasites. During the early trophozoite and ring phases of the sexual stage, the mitochondrion is a discrete, small and single organelle. Prior to transitioning between a mature trophozoite and schizont, the mitochondrion transforms into an elongated, branched and wider structure. When in the schizogony stage, the mitochondrion is highly branched prior to the onset of cytokinesis. When cytokinesis begins, the mitochondrion with branches undergoes division by fission into various organelles with divided apicoplasts moving into newly created merozoites (Fisher et al., 2014). In the sexual stage, the mitochondria of the gametocyte develop unique cristae. Gametocytes can have varied mitochondrial features, including distinctive and branched, elongated and single, or rounded and multiple (Fisher et al., 2014). Female gametocytes have a higher number of cristate mitochondria than males at phases III, IV, and V. The genome of the mitochondrion is 6kb linear mitochondrial DNA (mtDNA) that is the smallest among eukaryotes. Hikosaka et al., 2011 noted that the mitochondrial genome of the plasmodium parasite is much conserved between the species with over 90% similarity between *plasmodium* species that infect human beings (Hikosaka et al., 2011). The mtDNA exists as various copies with nearly 30 in *P. falciparum*. The female gamete is responsible for transmitting the mtDNA when it mates with the male gametocyte in the gut of the mosquito.

The physiology of the mitochondrion of Plasmodium is complex. The Mitochondrion acts as the main cellular energy source as ATP in higher eukaryotes. The contribution

and the function of the TCA (Tri-carboxylic acid) cycle in the bioenergetics of the malaria parasite have generated debate. In high level eukaryotes, the TCA cycle takes place under specific aerobic conditions through various chemical reactions for generating energy through the consumption of acetyl-CoA. The required enzymes for the TCA cycle that undergo active synthesis during the asexual phases are encoded by the plasmodium genome. Conversely the non-existence of a mitochondrial pyruvate dehydrogenase is an indication that the TCA cycle in plasmodium parasites differs from other eukaryotes (Foth et al., 2005). Following entry into the parasite, the glucose molecule undergoes metabolism into pyruvate molecules via the glycolysis pathway. In high level eukaryotes, the pyruvate molecule is transported into the mitochondrion where conversion into acetyl-CoA occurs for integration with the TCA cycle. The malaria parasite does not have the pyruvate dehydrogenase in its mitochondrion and thus it lacks the ability of generating acetyl-COA from pyruvate that undergoes fermentation into lactate, which yields two ATP molecules for every molecule of glucose that is consumed (Foth et al., 2005). Thus, the acetyl-CoA is only generated from phosphoenolpyruvate in the apicoplast for synthesising amino sugars in the endoplasmic reticulum (Fisher et al., 2014). Plasmodium species rely on an oxygen acquisition system and functional respiratory chain for survival and growth. The electron transport chain in the sexual and asexual stages of the *P. falciparum* has been detected (Uyemura et al., 2004). The respiratory chain of the plasmodium contains five dehydrogenases including glycerol-3-phosphate dehydrogenase (G3PDH), dihydroorotate dehydrogenase (DHODH), and malate: quinone oxidoreductase (MQO), type II NADH dehydrogenase (*Pf*NDH2), and succinate: quinone oxidoreductase (SDH). Other components of the electron transport chain include F₁F₀-ATP synthase (complex v), bc₁ complex (complex III), and cytochrome c oxidase

(complex IV). The respiratory chain is the main source of the potential generation of the mitochondrial membrane that is critical to the survival of the parasite. Plasmodium parasites are sensitive to electron transport chains (ETC) inhibitors.

1.3.3 *P. falciparum* sexual blood stages (gametocytes) Structure:

Gametocytogenesis is the necessary process for the formation of gametocytes for transmitting plasmodium. Past studies have indicated that all merozoites from one schizont can become either sexual or asexual where those merozoites committed to sexual differentiation to form exclusively female or male gametocytes (Ikadai et al., 2013). After the *P. falciparum* is committed to gametocytogenesis, it starts the pre-stage 1 development. Particularly, sexual schizogony occurs in the committed parasite to produce sexually committed merozoites. When these merozoites are released into the red blood cell of the host, they end up invading erythrocytes that lead to a sexually committed rings being formed. In a period of between 24 and 30 h, the committed ring parasites undergo differentiation to form a molecularly and morphologically recognised phase 1 gametocyte (Silvestrini et al., 2012). After the formation of the phase 1 gametocyte, post-phase I development follows where the gametocyte undergoes further maturation to form a mature male or female gametocyte.

Studies have been conducted to examine the biology of gametocytogenesis in terms of molecular basis for this process. Gametocytogenesis is triggered by both host and environmental factors that promote transcriptional changes through epigenetic changes in the blood-stage parasite (Guttery et al., 2015). Commitment to sexual development is influenced by AP2 genes (Painter et al., 2011, Hughes et al., 2010). ApiAP2 proteins have been shown to regulate various processes the cycle of a parasite including development inside hepatocytes (Iwanaga et al., 2012), maturation of

sporozoite (Yuda et al., 2010), and formation of ookinete (Yuda et al., 2009). AP2-G, a transcription factor has been demonstrated in studies of *P. falciparum* and *P. berghei* to activate a transcriptional flow that initiates gametocytogenesis and sexual commitment (Kafsack et al., 2014, Sinha et al., 2014). Utilising particular strains with loss of gametocyte production, a single mutation found in the *ap2-g* locus has been identified where deleting or disrupting *ap2-g* stopped sexual conversion and *ap2-g* expression in schizonts was associated with the up-regulation of various genes involved in the development of gametocytes (Sinha et al., 2014, Kafsack et al., 2014). Such studies show that commitment to gametocytes is regulated by AP2-G. Another gene responsible for sexual commitment in *P. falciparum* is the ATP-binding cassette transporter ABCG2 (Tran et al., 2014). In the study, disrupting the gene led to a threefold increase in the production of gametocyte in comparison with parental wild type.

A transcriptional analysis of gametocyte parasite lines with defects demonstrated that the gametocyte development 1(*pfgdv1*) gene encoded a nuclear protein necessary for sexual development (Eksi et al., 2012). Furthermore, the study showed that early genes in gametocytogenesis specifically *pfgc* was considerably down-regulated without the presence of *pfgdv1* (Eksi et al., 2012), which shows that this protein is important in sexual commitment during gametocytogenesis. The application of transposon-mediated mutagenesis has been critical in screening for non-producers of gametocytes to establish the genes necessary for the development of the gametocyte (Ikadai et al., 2013). This approach led to the identification of 16 genes most of which were not implicated in the gametocytogenesis process in the past. Some of the non-producers of gametocytes lacked the ability to form initial stage gametocytes, while others had the ability to do this but could not achieve this in later stages of gametocyte

development. The complementation of 5 of the 16 genes, including *pfhip* (*P. falciparum* interacting protein; PF3D7_0527500/PFE1370w), *pfmaf1*, *pfgeco* (gametocyte erythrocyte cytosolic protein PF3D7_1253000/PFL2550w), *pfsf3a3* (splicing factor 3A subunit 3; PF3D7_0924700/PFI1215w), and PF3D7-0532600/PFE1615c indicated that all but *pfgeco* restored the ability to form gametocytes (Josling and Llinas, 2015). The implication of this study is that these genes play a particular function in some phases of the development of gametocytes.

1.3.4 *P. falciparum* gametocytes morphology development:

The development of the gametocytes of the *P. falciparum* occurs in five phases as shown in Figure 1.3. These phases focus on the changes in appearance occurring after a red blood cell has been invaded by a sexually committed merozoite. After this, the gametocyte undergoes growth and elongation to increasingly occupy most of the red cell of the host. In phase 1, the gametocytes cannot be easily differentiated from asexual trophozoites. However, the gametocytes have a roundish shape with a pointed end. A unique pigment pattern might also be visible. Between phases II and V, the gametocytes can be easily differentiated in Giemsa-Stained blood films. In phase II, subpellicular microtubules start to form with few microtubules that give gametocytes pointed ends and elongated shape (Baker, 2010). In phase III, further elongation occurs in the gametocyte with ends becoming rounded. In female gametocytes, Golgi bodies and mitochondria proliferate. Beginning from phase IV, sexual dimorphism is clear where the gametocyte has an elongated shape with pointed ends. While the female gametocyte has a comparatively small nucleus and concentrated pigment pattern, the male gametocyte has a larger nucleus and more diffuse pigment pattern (Baker, 2010). In male gametocytes, the occurrence of electron dense osmiophilic bodies at the

cytoplasm's periphery is significant lower. Female gametocytes have higher densities or ribosomes than male gametocytes that accounts for the former's blue appearance and latter's pink appearance in Giemsa stained-blood films (Baker, 2010). In phase V, the gametocytes attain maturity and might be crescent-shaped and have rounded ends.

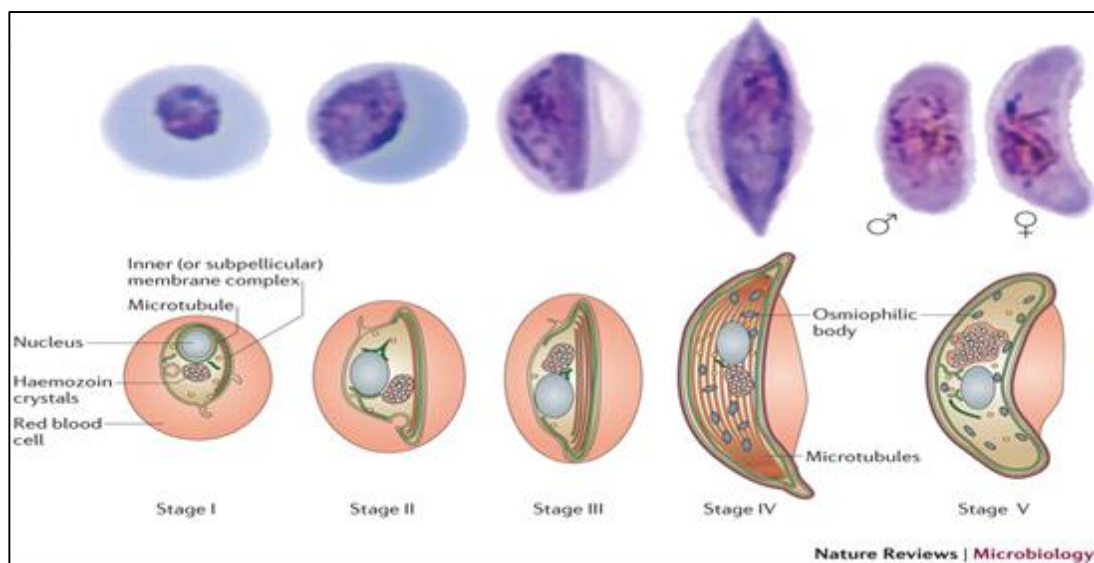


Figure 1.3: The gametocyte development stages (I-V) in *P. falciparum*.
Adapted from (Josling and Llinas, 2015).

1.4 Gametocytes metabolism:

Gametocytes rely on glycolysis and glucose uptake for ATP synthesis and survival (MacRae et al., 2013). Using the glycolytic pathway, gametocytes are able to convert glucose to lactate. All the enzymes involved in the glycolytic pathway are encoded by the plasmodium genome (Bozdech et al., 2003). Trends towards a decrease in gene expression responsible for Hb catabolism, protein biosynthesis, and glycolysis have been observed in the late gametocyte development stages (Baker, 2010). TCA cycle also plays an important role in gametocyte metabolism. MacRae et al. (2013) examined the role of TCA cycle in gametocyte development and observed that

gametocytes catabolise the host's glutamine and glucose in mitochondria via the TCA cycle. The carbon skeletons generated from glucose drive this flux. The authors concluded that gametocytes demonstrated an increased level of TCA catabolism of pyruvate, glycolytic flux, and glucose utilisation (MacRae et al., 2013). It has been suggested that the switch to a more efficient energy generation method via the TCA cycle by gametocytes might be necessary for sustaining the development of gametocytes under hypoglycaemic conditions that are common among patients suffering from severe malaria (Daily et al., 2007). Furthermore, the increased utilisation of the TCA cycle in gametocytes might be an indication of the increased energy requirements in female gametes that are preparing for the stages after fertilisation when accessing glucose in the vector might be restricted (Talman et al., 2004). Figure 1.4 below demonstrates the TCA cycle in malaria metabolism.

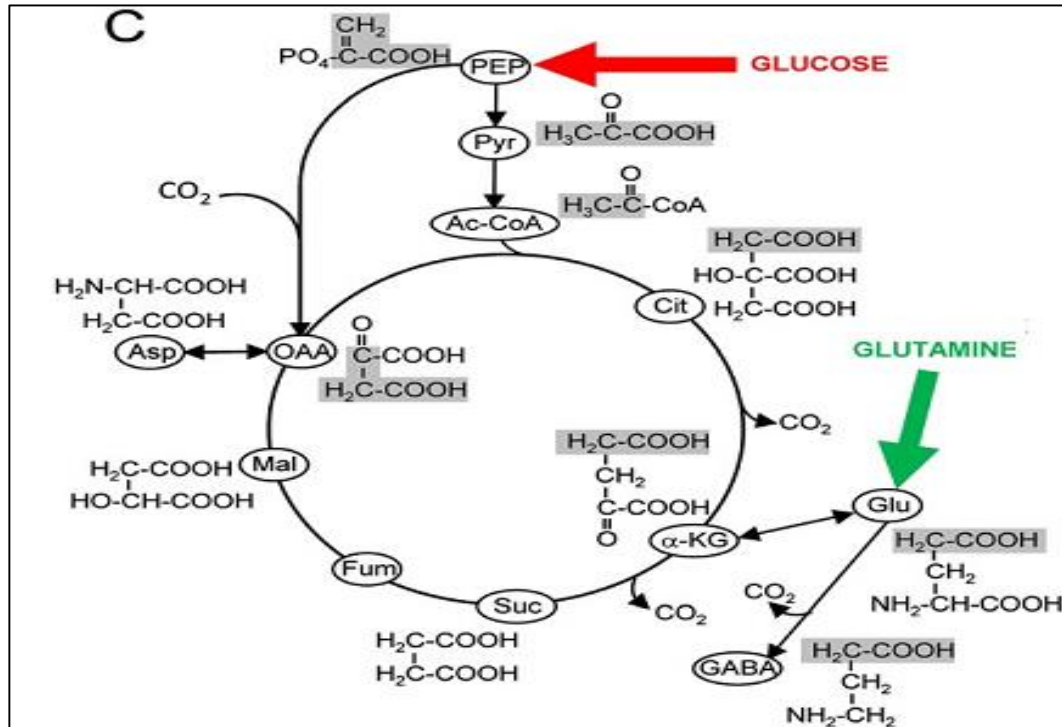


Figure 1.4: The demonstration of TCA cycle in sexual gametocytes of malaria parasite *P. falciparum*.

Adapted from (MacRae et al., 2013).

In addition, gametocytes need fatty acids during their development. *Plasmodium* has the ability of generating some fatty acids de novo in its apicoplast (Lamour et al., 2014); however, most of the parasite scavenges most of the fatty acids from the host serum (Mi-ichi et al., 2006). Transcriptomic analysis demonstrates the upregulation of the five of the six categories of the type II fatty acid pathway during the development of gametocytes (Young et al., 2005). It has also been demonstrated that the lipid transporter gABC2 is critical in neutral lipid accumulation within the parasite and this is expressed primarily in female gametocytes (Tran et al., 2014). Thus, this suggests that lipid metabolism is vital in gametocyte development.

1.5 Malaria Chemotherapy:

In the absence of an effective malaria vaccine, antimalarial chemotherapy has been the sole treatment solution against malaria for decades. Malaria treatment basically involves parasite killing in the blood stream (treatment) and liver (radical cure and/or prophylaxis) while giving supportive treatment to human host (Winstanley, 2000). Five main groups of antimalarial drugs are available with regards to their biological activity and chemical structure (Table 1.1) (Antoine, 2012). These groups include the following: Endo peroxide compounds (artemisinin-type), antifolates, quinoline-containing, antibacterial agents and (5) hydroxynaphthoquinones. All of these compounds have different mechanism of action within different subcellular organelles which interfere with their metabolic pathways processes that lead finally to parasite death (Table 1.1) (Greenwood et al., 2008, Biagini et al., 2003).

Treatment failure has been demonstrated to all antimalarial drugs, the underlying causes include the generation of parasite drug resistance, and non-optimal pharmacokinetic/ pharmacodynamics (White, 2004). The usage of antimalarial drugs combinations between two or three compounds with different target and different modes of action have been introduced in an attempt to reduce the emergence of resistance and improve the drug action and efficacy (Table 1.1) (Kremsner and Krishna, 2004, White, 2004).

Table 1.1: Antimalarial drug classes, their target locations, parasite stages and their possible combinations.

Drug groups	Target location				Parasite stage					Combination
	C	M	DV	A	BS	LS	GS	HS	SS	
Quinoline-Containing Drugs										
Quinine	•		•	•	•		•			+ Sulfadoxine-pyrimethamine (SP); + Tetracycline; + Clindamycin; + Doxycycline + Sulfadoxine-pyrimethamine (SP) + Sulfadoxine-pyrimethamine (SP) + DHA + Sulfadoxine-pyrimethamine (Fansimef®) + Artemether (Coartem®, Riamet®)
CQ	•		•	•	•		•			
AQ	•		•	•	•		•			
PPQ	•		•	•	•					
PMQ	•		•	•		•	•	•		
TF	•		•	•		•	•			
Meflequine (MQ)	•		•	•	•		•			
Halofantrine	•		•	•	•					
Lumefantrine	•		•	•	•					
Antifolates										
Pyrimethamine	•		•	•	•	•			•	+ Sulfadoxine (SP) + Pyrimethamine (SP) + ATQ (Malarone®) + Dapsone + Artesunate (Dacart®); + Dapsone (LapDap®) + Artesunate + ChloroPG (Dacart®); + ChloroPG (LapDap®)
Sulfadoxine	•		•	•	•					
Proguanil (PG)	•		•	•	•	•			•	
ChloroPG	•		•	•	•					
Dapsone	•		•	•	•					
Hydroxynaphthaquinones										
ATQ	•	•	•	•	•				•	+ PG (Malarone®)
Antibiotics										
Doxycycline	•		•	•	•					+ Quinine
Clindamycin	•		•	•	•					+ Quinine
Tetracycline	•		•	•	•	•				+ Quinine
Endoperoxide compounds										
Artemisinin	•	•?	•	•	•		•?			+ Lumefantrine (Coartem®, Riamet®) + Dapsone + ChloroPG (Dacart®); + amadioquine (ASAQ); + MQ (ASMQ); + Sulfadoxine-pyrimethamine (SP) + PPQ
Artemether	•	•?	•	•	•		•?			
Artesunate	•	•?	•	•	•		•?			
DHA	•	•?	•	•	•		•?			

Abbreviations: C, cytosol; M, mitochondrion; DV, digestive vacuole; A, apicoplast; BS, blood stage; LS, liver stage; GS, gametocytes stage; HS, hypnozoites stage and SS, sporozoites stage. (Adapted from Antoine, 2013).

1.5.1 Antimalarial drugs:

Table 1.2 and Table 1.3 summarise the antimalarial drugs used against transmission stages of *plasmodium*.

1.5.1.1 Endoperoxide Compounds:

Artemisinin (ART) is derived from the *Artemisia annua* plant (Chinese wormwood) and its active compounds called Qinghaosu which has been used as herbal cure in traditional Chinese medicine for fever treatment (White, 2008b). ART has a high potency and considered a safe drug with a low toxicity against all types of malaria parasites even the parasite strains which are resistance to conventional antimalarial drugs (Li et al., 1994, Krishna et al., 2008). Semi-synthetic ART, e.g. DHA, artesunate and artemether, are first generation derivatives that have been synthesized (Figure 1.5) with improved solubility and increased drug efficacy leading to high demand in cultivation to obtain more extract of *Artemisia annua* plant (O'Neill and Posner, 2004). These derivatives were used previously as monotherapy treatments and then were used in combination with other antimalarial drugs, known as Artemisinin-based combination therapy (ACTs) (White, 2008b).

Endoperoxide compounds are deemed one of the most important antimalarial drugs. It is considered to have originated from the ancient Chinese curative techniques (Butterworth et al., 2013). The rationality and the drug design are considered to be centred on artemisinin (Duez et al., 2015). This is due to the fact that the drug's mechanism of action remains somewhat unresolved (O'Neill et al., 2010b, Mercer et al., 2011). The antimalarial drug has a specific way of working where it creates an intrusion with the catabolic pathway related to the plasmodial Hb. Moreover, it also creates a big hindrance towards hemepolymerization (Butterworth et al., 2013). The

proposed mode of action of this class in asexual stage of *P. falciparum* is shown in Figure 1.6 (Tilley et al., 2016). The antimalarial drug ensures there is complete and effective inhibition of the multiplication of the plasmodium pathogens (Duez et al., 2015). Here, drug does not allow the sexual stages concerning the pathogen multiplication to take place, a condition that has helped to control the spread of malaria. Lucantoni et al. (2015) assert that the drug helps to mitigate the overall gametocyte carriage, thus, helping to reduce the overall spread of malaria (Lucantoni et al., 2015).

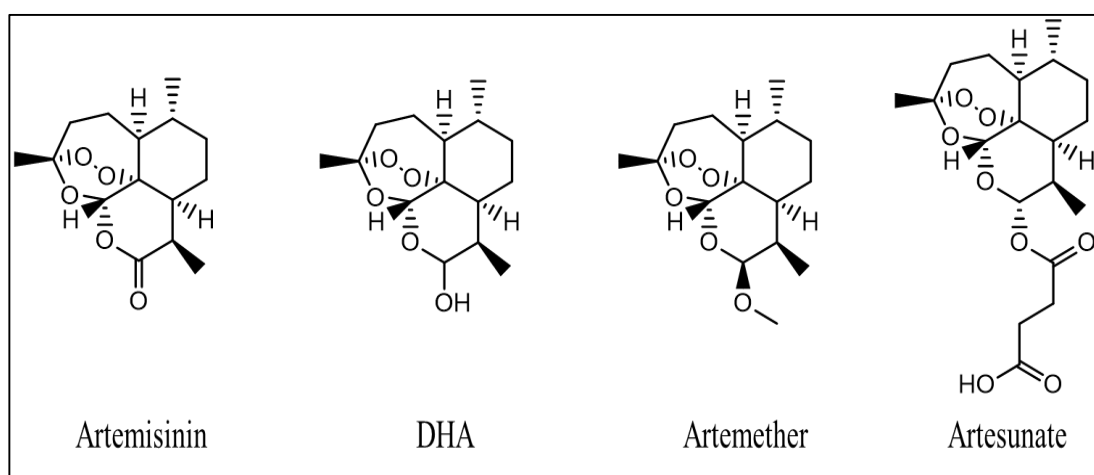


Figure 1.5: Artemisinin and its semisynthetic derivatives chemical structures.

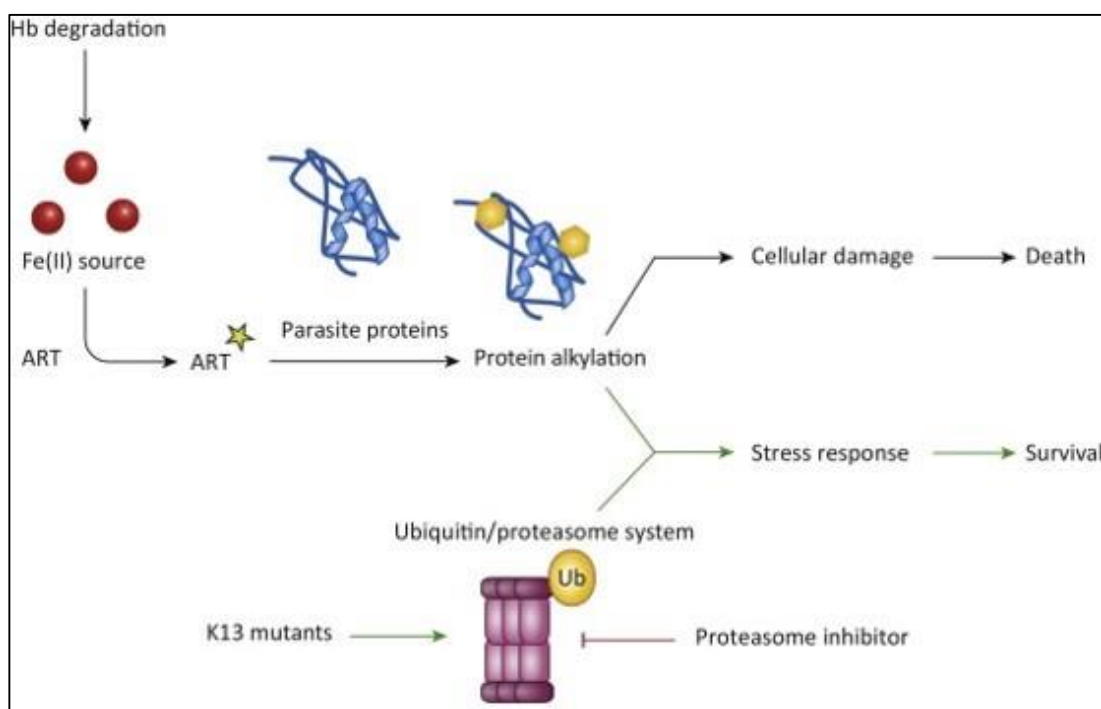


Figure 1.6: Proposed of cell-death and proceedings of cell survival after treatment with antimalarial artemisinins in *P. falciparum*.

Antimalarial artemisinins are activated via a reduced iron source where mainly heme released from Hb digestion as well as biosynthesis. The nucleophile-harboring cellular components interacts with the activated drug and resulting to cellular damage and eventually cell death. *P. falciparum* are assumed to mount a stress response that engages the unfolded protein response (UPR), including the ubiquitin (proteasome system). It is proposed that the chance of K13 mutants stress response is increased, leading to parasite survival. The stress response is proposed to decrease in the presence proteasome inhibitors and consequently promoting parasite death (Tilley et al., 2016).

1.5.1.2 Antimalarial quinoline-based class:

Quinoline-based antimalarial drugs consist of roughly the common antimalarial drug classes. Chemically, quinolines are aromatic nitrogen compounds characterised by a central solid-ring structure, basically this ring forms benzene merged to pyridine at two adjacent carbon atoms (Figure 1.7, A). Nevertheless, the core molecule of quinoline can link to different functional groups that characterised by solubility improvement and drug efficacy. The quinolin-based drugs can be classified them into three different chemical classes as follows: the first class is 4-aminoquinolines such as CQ, AQ, PPQ. They are characterised by weak bases, deprotonated, and hydrophilic

at neutral pH. Second class is aryl-amino alcohols such as quinine, MQ, lumefantrine. This class also characterised by weak bases and lipid soluble at pH 7.0 (Olliaro, 2001, O'Neill et al., 2012). Finally, 8-aminoquinoline include the, PMQ (Figure 1.7).

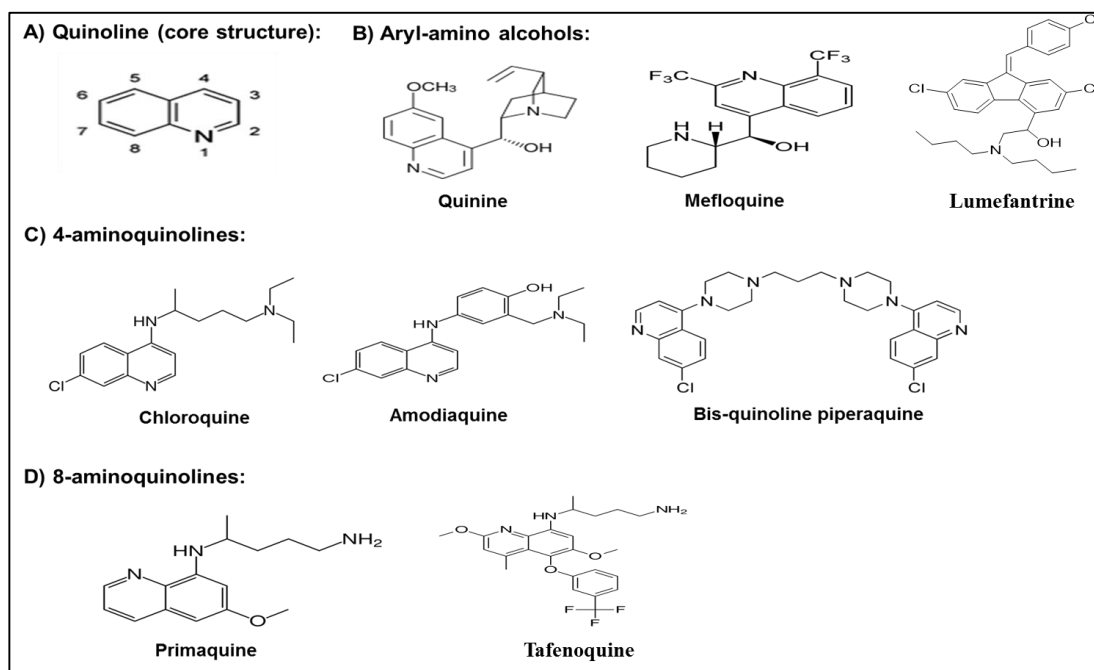


Figure 1.7: Chemical structures of quinolone-based antimalarial drugs.

(A) quinoline (core structure). (B) Aryl-amino alcohols drugs: quinine, mefloquine and lumefantrine. (C) 4-aminoquinolines drugs: chloroquine, amodiaquine and bis-quinoline piperazine. (D) 8-aminoquinoline drug: primaquine and tafenoquine.

Historically, the treatment value of cinchona (fever bark tree) was recognised before understanding the nature of malaria parasite (Achan et al., 2011). In 19th century, the active ingredient, quinine, was successfully isolated and synthesised to solve the main obstacle related to limit action of its supplies (Seeman, 2007, Foley and Tilley, 1998, Woodward and Doering, 1944). Understanding the quinine chemical structure has led to the identification quinoline as the crucial pharmacophore which resulted to the synthesis and development of quinoline class as seen in Figure 1.7, A.

It is proper with drug of choice in malaria disease prophylaxis and treatment for many years, 4-aminoquinolines CQ (Muller and Hyde, 2010). The achievement of this drug

relies on its clinical activity, economic-effective, limited toxicity and simplicity to use and synthesise. (Hyde, 2007, Muller and Hyde, 2010, Biagini et al., 2003). CQ has saved millions of peoples since it was introduced in 1940s, however its use has restricted to some areas due to reduction of its efficacy in the last 20 years, mainly because of the development and appearance of widespread of resistance in many regions where *P. falciparum* is ubiquitous (Muller and Hyde, 2010, Wellems and Plowe, 2001, O'Neill et al., 2012, Biagini et al., 2003, Payne, 1987). Thus, the essential need for other synthetic 4-aminoquinoline became the priority. This resulted in development and synthesised AQ and PPQ which have demonstrated activity against *P. falciparum* CQ-resistant strains (O'Neill et al., 2012, O'Neill et al., 1998, Biagini et al., 2003). Moreover, as a response to drug resistance, research has led to synthesis new generation of quinine called aryl-amino alcohols, including quinoline methanol: MQ, lumefantrine and halofantrine. In southeast Asia, for example, quinine is used in combination with tetracycline or doxycycline (antibiotics) as a results of emergence of quinine resistance in that regions (Ejaz et al., 2007).

8-Aminoquinolines including PMQ have been used to be effective against malaria hypnozoites (liver stages) and for prophylaxis. In most cases, such condition is manifested in the liver, thus, becoming the main target whenever the antimalarial drug is used (Abdul-Ghani and Beier, 2014). However, it is worth noting the drug cannot be used by patients suffering from the G6PD deficiency (Graves et al., 2015b). This is due to the fact that 8-Aminoquinoline has very great potential of making the patients experience haemolysis, which can be fatal (Abdul-Ghani and Beier, 2014). 8-Aminoquinolines are also effective at blocking malaria transmission of the parasite from an already infected person to the final vector, mosquito (Graves et al., 2015b).

The actual mechanism of action of quinoline-based compounds has not been completely explained but it is accepted that a vital step in its mode of action, with the exception of PMQ (used primarily to act against hypnozoite and sexual stages infection), is a binding of bi-product of Hb digestion in parasite food vacuole (ferriprotoporphyrin IX, or heme) to drug, although other targets may also be involved in some cases (Fitch, 2004, Muller and Hyde, 2010, Bray et al., 2005b, O'Neill et al., 2012, Slater and Cerami, 1992). The unclear mode of action of this class of drug as well as the lack of sufficient knowledge of parasite physiology and biochemistry leave the mechanism of CQ resistance still ambiguous.

Many evidence to support the essential role of a heme-dependent mode of action for both 4-aminoquinoline and aryl-amino alcohol (Mungthin et al., 1998). The ability of CQ to accumulate in the parasite's food vacuole as a weak base allows it to raise the osmolality of the vesicles and pH, thus leading to swelling and membrane disruption (Krogstad and Schlesinger, 1987). CQ converts to deprotonate once it accumulates inside the parasite's digestive vacuole and trapped in it (Olliaro, 2001). Therefore, the putative mode of action of CQ is to block the polymerization of heme (toxic) generated from Hb digestion into hemozoin, and thereafter monomeric heme accumulates to levels in the digestive vacuole that kill the parasite (Slater and Cerami, 1992).

1.5.1.3 Antifolate:

Anti-folate drugs are a category of chemotherapeutic agents utilised in treating infections caused by bacteria and protozoa such as malaria. Antimalarial anti-folate drugs include dapson, chlorocycloguanil, pyrimethamine and sulphadoxine. Folate derivatives are critical for the replication of DNA and protein synthesis and, hence, cell survival (Heinberg and Kirkman, 2015). In the treatment of malaria, antifolates work by disrupting the balance of metabolites in the folate pathway (Yuthavong,

2014). Malaria parasites acquire folate cofactors by scavenging them from the host or through de novo synthesis (Heinberg and Kirkman, 2015). Human beings depend only on diet as the external supply of folate cofactors as these are not produced by their bodies. Medications that target the folate pathway are vital in fighting against pathogens. The action of antifolates involves inhibiting the main enzymes in the folate cofactor synthesis as well as in the phases after the cofactors have been salvaged from an exogenous source (Yuthavong, 2014). The main enzymes targeted by antifolate drugs include dihydrofolate synthase (DHFS) and dihydropteroate synthase (DHPS) (Muller and Hyde, 2013) as seen in Figure 1.8. Therefore, anti-folates mechanism of action is characterised by the inhibition of DNA synthesis.

Antifolates are considered as very effective antimalarial drugs, which create an interference with the metabolism of the folate (Beavogui et al., 2010). According to Chatterjee (2013), some of the drugs classified under this category include the therapeutic and causal prophylactic. These drugs can have some synergetic properties when used in combination. However, over the recent past, the antifolate drugs have proved to be highly susceptible towards some malarial causing parasites and they have become ineffective (Beavogui et al., 2010). The drug is very effective in creating resistance towards the transmission of the malarial parasites (Mharakurwa et al., 2011). For instance, the ingestion of the drug is considered highly effective in reducing the rate of mutation of the '*Plasmodium falciparum* dihydrofolatereductase (DHFR) (Chatterjee, 2013). Therefore, this reduces the rate of the transmission of the malaria parasites. Apart from that, the antifolates have also been shown to be highly effective in trying to mitigate carriage of the gametocytes, thus, reducing the spread of malaria disease (Mharakurwa et al., 2011).

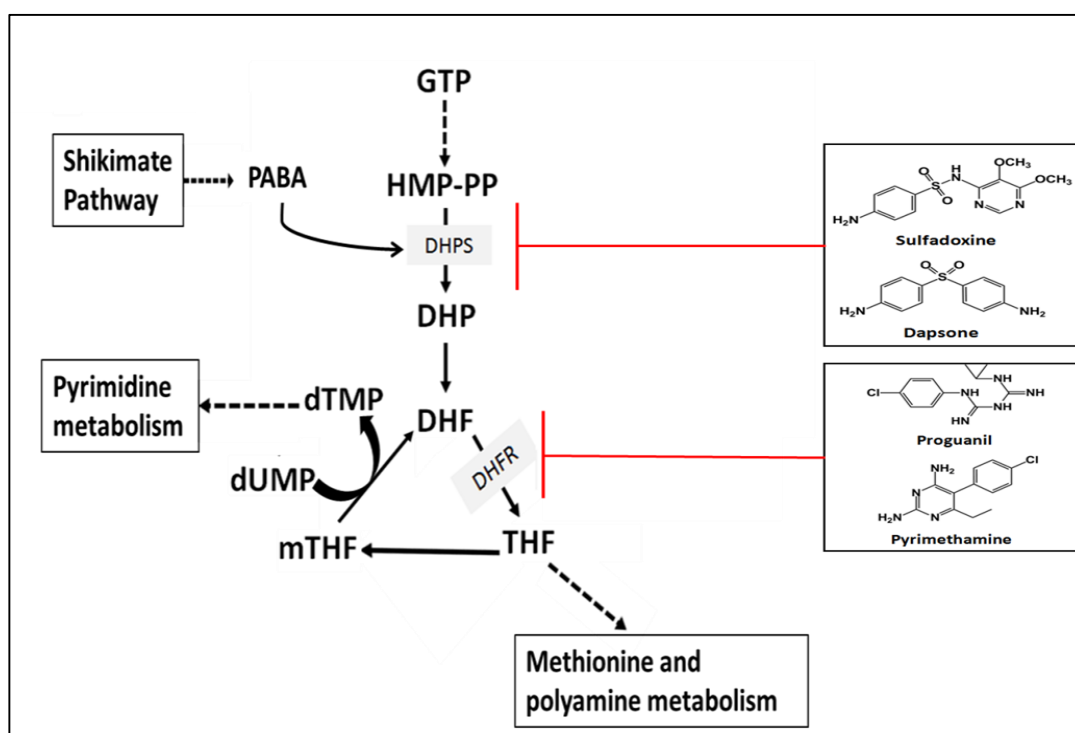


Figure 1.8: Chemical structure of antifolate drugs and their mode of action.

The biochemical folate pathway in *P. falciparum* and site targeting the antifolate drugs. Abbreviations: DHF, dihydrofolate; DHP, dihydropteroate; PABA, para-Aminobenzoic acid; THF, tetrahydrofolate; DHFR, dihydrofolate reductase; DHPS, dihydropteroate synthase; GTP, guanosine triphosphate; mTHF, methyltetrahydrofolate; dUMP, deoxy-uridine monophosphate; HMP-PP, hydroxymethyl-pteridine-PP; dTMP, thymidine monophosphate.

1.5.1.4 ATQ (Hydroxynaphthoquinone):

ATQ is a hydroxynaphthoquinone (2-[trans-4-(4'-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthoquinone) developed 20 years ago to selectively compete for coenzyme ubiquinone in electron transport chain of *P. falciparum* mitochondria (Nixon et al., 2013). It shows activity against malaria parasites (Hudson et al., 1991) and interestingly, it is more active against parasite mitochondria (1000 fold) than mammalian mitochondria (Fry and Pudney, 1992), specifically targeting the binding site of ubiquinone oxidation in bc_1 complex as seen in Figure 1.9 (Syafuruddin et al., 1999, Birth et al., 2014).

Throughout parasite blood stage, a vital function of malarial mitochondrion is to deliver orotate for the purpose of pyrimidine biosynthesis through dihydroorotate dehydrogenase (DHODH) activity. The cytochrome *bc₁* complex, however, is inhibited by ATQ, leading to interruption of metabolites concentrations in pyrimidine biosynthesis (Seymour et al., 1997, Hammond et al., 1985). Transgenic parasites ubiquinone-independent yeast DHODH proved the resistant phenotype of ATQ (Painter et al., 2007). ATQ can kill the intra-erythrocytic parasites stage relatively slower than other antimalarial drugs including CQ and artemisinin (Biagini et al., 2012, White, 1997, Sanz et al., 2012). This leads to possible acting of this drug and other mitochondrial drugs only on late trophozoite with no action on early ring parasites (Biagini et al., 2012). Remarkably, ATQ has shown activity against liver stage, and as a result used as prophylactic treatment, even though no activity response has been shown against relapsing malaria *Plasmodium vivax* hypnozoites (Lalloo and Hill, 2008, Dembele et al., 2011).

The combination of ATQ and PG (Malarone®) exhibited synergistic activity in clinical work and *in-vitro* (Looareesuwan et al., 1999a, Canfield et al., 1995, Looareesuwan et al., 1996). This combination is used largely as chemoprophylaxis for people used to travel endemic regions rather than for patients (Kessl et al., 2007, Looareesuwan et al., 1999b, Looareesuwan et al., 1999a).

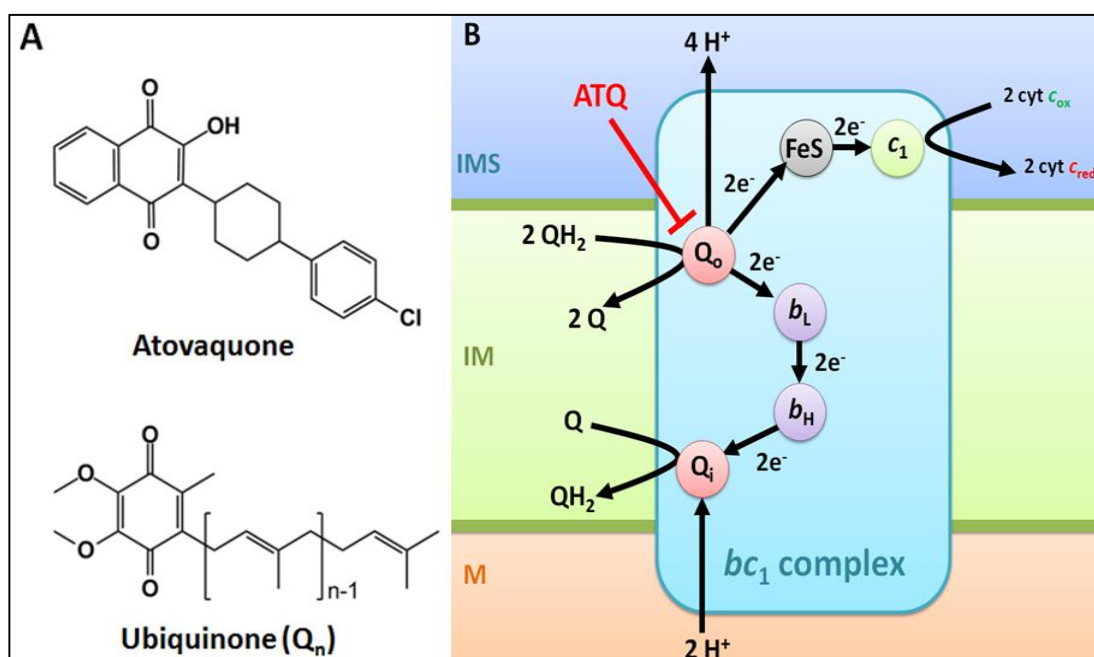


Figure 1.9: Diagram of putative ATQ mechanism of action.

(A) Chemical structure of ATQ and ubiquinone. (B) illustrates cytochrome *bc*₁ complex reaction pathway and its inhibition by ATQ. Abbreviations: ATQ, Atovaquone; Q, ubiquinone; QH₂, ubiquinol; b_L, heme b_L binding site; b_H, heme b_H binding site; FeS, iron-sulfur cluster; cyt c, cytochrome c; c₁, cytochrome c binding site. (Adapted from Antoine, 2013).

1.5.1.5 Methylene blue:

MB is not a registered antimalarial drug but its antimalarial properties have long been known and the drug and related analogues are currently in clinical development. Methylene is known to kill asexual *Plasmodium* parasites and more recently it has been shown to kill sexual gametocyte stages (Pascual et al., 2011). Although the mode of action of MB is not completely understood, it is thought to inhibit the antioxidant glutathione reductase activity (Buchholz et al., 2008, Adjalley et al., 2011a). However, MB shows significant activity against *Plasmodium* parasites in which the antioxidant enzyme is missing (Pastrana-Mena et al., 2010).

According to Delves et al. (2013), methylene can be used to suppress the multiplication of the malaria parasites by making sure they lack sufficient oxygen

necessary for their multiplication. This is due to the fact that methylene can be used to regulate the level of methemoglobin level in the patient's blood (Delves et al., 2013a). However, one has to be careful in order to ensure the normal level of methemoglobin is maintained in the long run. Methylene is used to affect the production of the gametocytes *in vivo*. Thus, the move helps to reduce the increment in the rate of the transmission of the disease causing agents (Pascual et al., 2011). This is due to the fact that methylene is considered to be an inhibiting factor towards the proper biological functioning of the parasites, which, in turn, interferes with their multiplication (Pascual et al., 2011).

Table 1.2: Antimalarial drugs that have been described to exert limited improvement on gametocytes clearance/mosquito infectivity.

Drug	Effect on gametocytes	Gametocyte infectivity to mosquitoes	Reference
Quinine	Limited gametocytocidal effect on young gametocytes	Significant decline in oocyst numbers in <i>A. dirus</i> only at relatively high concentrations (over 600ng/ml)	(Chotivanich et al., 2006)
CQ	Partially inhibits gametocytogenesis High likelihood (by 4.4 times) of gametocytemia in drug resistant more than drug-sensitive parasites after treatment	No noticeable impact on sporogony in <i>A. stephensi</i> mosquitoes Improved infectivity to <i>A. arabiensis</i> mosquitoes	(Chutmongkonkul et al., 1992) (Hogh et al., 1998)
Sulfadoxine-pyrimethamine (SP)	Peak in gametocytemia 12 days after a single dose treatment Peak in gametocytemia by day 7 following a single dose treatment Peak in gametocytemia 7 days after a single dose treatment Gametocytogenesis in mutant NF-135 strain but gametocytocidal impact on wild-type NF-54 strain	No infectivity to <i>A. arabiensis</i> mosquitoes Infectivity of gametocytes from parasites with resistance-related mutations to <i>A. albiminus</i> mosquitoes Low infectivity to wild <i>A. gambiae</i> mosquitoes Reduced maturity of gametocyte and infectivity to <i>A. stephensi</i> mosquitoes	(Govere et al., 2003) (Mendez et al., 2007) (Beavogui et al., 2010) (Kone et al., 2010)
Lumefantrine	Reduced gametocytocidal activity on late-stage gametocytes Dose-dependent inhibition on early and late stage gametocyte development in vitro	Noticeable inhibition of gametocyte infectivity to mosquitoes and decreases oocyst numbers Dose-dependent reduction in oocyst numbers in <i>A. stephensi</i>	(Adjalley et al., 2011b) (van Pelt-Koops et al., 2012)
Halofantrine	Partially inhibits gametocytogenesis	Lack of effect on sporogonic development in <i>A. stephensi</i>	(Chutmongkonkul et al., 1992)
Pyronaridine	Gametocytocidal activity restricted to stage I to II gametocytes	Noticeably inhibits gametocyte infectivity to mosquitoes and reduces oocyst numbers	(Adjalley et al., 2011b)
PPQ	Gametocytocidal activity restricted to stages I to II gametocytes	Lack of effect on the decrease of oocyst numbers in mosquitoes or blocking transmission	(Adjalley et al., 2011b)
Monodesethyl-AQ	Rapid gametocytocidal activity limited to stages I to II gametocytes	Lack of effect on the decrease in oocyst numbers in mosquitoes or blocking transmission	(Adjalley et al., 2011b)

Table 1.3: Antimalarial drugs that have been described to exert significant reductions in gametocytes clearance/mosquito infectivity.

Drug	Effect on gametocytes	Gametocyte infectivity to mosquitoes	Reference
Artemisinin	Significantly reduces gametocyte density between day 7 and 14 after treatment	Reduces infectivity to <i>A. dirus</i> mosquitoes between 7 and 14 after treatment	(Chen et al., 1994)
	Reduces likelihood of gametocyte carriage 4 weeks after treatment on a six-dose regimen	Decreases infectivity of gametocyte carriers of <i>A. gambiae</i> mosquitoes on day 7	(Sutherland et al., 2005b)
Artemether-lumefantrine artesunate	Gametocyte carriage for a mean period of 5.5 days	Reduces infectivity to <i>A. gambiae</i> on day 7 following treatment	(Sawa et al., 2013)
		Reduces infectivity to <i>A. dirus</i> , oocyst numbers, and infection rate	(Chotivanich et al., 2006)
DHA	Partially active against mature gametocytes	Reduces oocyst numbers	(Adjalley et al., 2011b)
DHA-pyranoridine	Gametocyte carriage for a period of 15.3 days	Higher infectivity(two-fold) to <i>A. gambiae</i> on day 7 following treatment in comparison with artemether-lumefantrine	(Sawa et al., 2013)
PMQ	Eliminates gametocytemia after a single-dose treatment	Prevents infection to <i>A. gambiae</i> mosquitoes	(Burgess and Bray, 1961)
	Active against all gametocytes stages but marked against stages (I-IV)	Reduces oocyst numbers in <i>A. dirus</i>	(Chotivanich et al., 2006) (Adjalley et al., 2011b)
TF	Active against all gametocyte stages but marked against stages I to IV		(Adjalley et al., 2011b)

1.6 Combination therapy of malaria treatment:

1.6.1 The non-Artemisinin-based combination therapy:

WHO recommends the utilisation of non-artemisinin based combination therapy where artemisinin-based combination therapy is unavailable and where the component drugs are well tolerated and efficacious. Non-ACT drugs that have been used include sulphadoxine-pyrimethamine + AQ (SP +AQ). Studies have evaluated the efficacy of non-ACT drugs in the treatment of uncomplicated malaria. In a study in Mali, it was found that SP +AQ was more efficacious than SP alone in treatment uncomplicated falciparum malaria (Maiga et al., 2015). Similar results were found in a study in Malawi (Bell et al., 2008). In Equatorial Guinea, the efficacy of SP + AQ was found to be 96.5% compared to 97.5% for artesunate + sulphadoxine-pyrimethamine (Charle et al., 2009). However, there is increased parasite resistance to sulphadoxine and pyrimethamine (SP). Despite this, SP combinations are still utilised in endemic areas as part of intermittent preventive treatment in pregnant women and children or seasonal chemoprophylaxis in children (Heinberg and Kirkman, 2015).

1.6.2 The Artemisinin-based Combination therapy:

The World Health Organisation recommends artemisinin-based combination therapy as the first line treatment for severe and uncomplicated falciparum malaria. To enhance acceptability and adherence, ACTs are formulated in fixed dose combinations (Visser et al., 2014). The artesunate derivative in combination treatments are active against all the phases of the asexual malaria parasite and result in considerably shorter parasite clearance time than other antimalarials (Visser et al., 2014). Furthermore, they demonstrate effects against gametocytes and thus minimise the risk of the perpetuation of the parasite's lifecycle in patients after treatment, which is vital in optimising the

control of malaria in endemic regions (Douglas et al., 2013). ACTs are administered over a period of 3 days. The rationale is that the administration of two blood schizontocidal drugs with varied targets and mechanisms of action is highly effective than using only a single drug. Thus, in the pre-existence of polymorphisms that confer resistance or the emergence of de novo mutations to one of the medications, the resistant parasite is killed by the other drug that is still effective (Visser et al., 2014). Furthermore, artemisinin derivatives are administered in combination due to their short half-life. Thus, recrudescence might occur if administered as a single therapy for a short time. The current ACTs for malaria include dihydroartemisinin-PPQ, artemether-lumefantrine, artesunate-AQ, and artesunate-MQ, and artesunate-sulfadoxine-pyrimethamine.

Various studies have evaluated the efficacy of artemisinin-based combination therapies. Smithuis et al. (2010) performed a randomised trial that compared the effectiveness of the four ACTs and loose tablet combination of MQ and artesunate, and assessed the addition of a single dose of PMQ. The results indicated that recrudescence of *P. falciparum* infections were found in 14 patients on artesunate-AQ. This rate was significantly higher than that of DHA-PPQ, loose-artesunate-MQ, fixed dose artesunate-MQ, and artemether-lumefantrine where recrudescence was found in only two patients for each drug combination. *P. falciparum* gametocyte carriage was substantially reduced after a single dose of PMQ (0.75mg/kg) was added. The researchers concluded that artesunate-AQ ought not to be utilised in Myanmar due to the effectiveness of other ACTs. Artesunate-MQ offered the greatest malaria suppression after treatment. Furthermore, adding a single dose of PMQ significantly reduces the potential for malaria transmission (Smithuis et al., 2010a).

Yeka et al. (2016) examined the effectiveness of artemether-lumefantrine versus artesunate-AQ in treating uncomplicated malaria in Uganda. The results demonstrated that the risk of recurrent parasitemia was lower in the artesunate-AQ group than in the artemether-lumefantrine cohort. Recrudescence occurred following treatment with artemether-lumefantrine. The researchers concluded that artemether-lumefantrine treatment is followed by more recurrences than treatment with artesunate-AQ (Yeka et al., 2016). In a related randomised study, Ndounga et al. (2015) compared the efficacy of artemether-lumefantrine versus artesunate-AQ in treating acute uncomplicated malaria among Congolese children aged below 10 years. The efficacy of the drugs expressed as percentage of adequate parasitological and clinical response was 96.4% for artemether –lumefantrine and 97% for artesunate-AQ. In both cohorts, the adverse events included diarrhoea, abdominal pain, vomiting, dizziness, headache, jaundice, and nausea. The frequency of adverse events was higher in the cohort on artesunate-AQ than in the artemether-lumefantrine group. It was concluded that these ACTs were effective in treating malaria among children (Ndounga et al., 2015).

Gbotosho et al. (2011) evaluated the efficacy of artemether-lumefantrine and artesunate-AQ during the 5 years of adoption as the initial line of treatment for malaria in Nigeria (Gbotosho et al., 2011). The results showed that in comparison with artemether-lumefantrine, AQ-artesunate led to a significant reduction in the number of children with parasitemia and fever 1 day following treatment. The number of children with parasitemia on day 2 and gametocytemia on presentation and carriage reduced considerably over the years. The overall efficacy of the drugs was 96.5% and it did not change over 5 years. The researchers concluded that artemether-lumefantrine and artesunate-AQ were efficacious treatment for malaria 5 years after being adopted in Nigeria. In a related study, Oguiche et al. (2014) examined the efficacy of artesunate-

AQ and artemether-lumefantrine in a sample of 747 children aged below five years old (Oguche et al., 2014). The results indicated that the number of children with parasitemia 1 day following treatment was significantly lower in artesunate-AQ in comparison with artemether-lumefantrine. However, a similarity in parasite clearance time was observed in both treatments. The overall efficacy of both treatment regimens was 96.3%. The parasitological cure rates on the 28th day were 98.3% for artesunate-AQ and 96.9% for artemether-lumefantrine, suggesting that both treatments are efficacious in treating uncomplicated malaria in children in young.

Although studies demonstrate the efficacy of ACTs in treating malaria, the malaria parasite has developed resistance to these drugs. *P. falciparum* parasite with lowered in-vivo susceptibility to derivatives of artemisinin was found in western Cambodia (Noedl et al., 2008, Dondorp et al., 2009). This is a threat to the control and the elimination of malaria across the globe. Artemisinin resistance involves slow parasite clearance (Phyo et al., 2012). Clearance of *P. falciparum* is attained in 2 days in a majority of patients (95%) (Phyo et al., 2012). However, artemisinin resistance infection is still positive on the slide for 3 or more days where the failure of treatment is more frequent in such infections following artemisinin-based combination treatment in Thailand (Carrara et al., 2013) and Cambodia (Rogers et al., 2009, Denis et al., 2006, Saunders et al., 2014). The main causal determinant of artemisinin resistance in Southeast Asia has been identified as mutations that transform the main aminoacid sequence in the propeller region of the kelch motif containing gene called K13, which acts via upregulation of unfolded protein response pathways (Tun et al., 2015). Studies have documented several K-13 propeller mutations in southeast Asia being linked to delays in parasite clearance following treatment with artemisinin (Ariey et al., 2014, Ashley et al., 2014a, Thriemer et al., 2014, Takala-Harrison et al., 2015) and

lowered in-vitro responses. Studies have yet to identify frequently occurring mutations in the propeller region linked to normal parasite clearance rates (Ariey et al., 2014, Ashley et al., 2014a). Evidence indicates that excluding artemisinin resistance areas, K13 propeller mutations are not present at considerable frequencies (Mohon et al., 2014, Kamau et al., 2015, Conrad et al., 2014, Torrentino-Madamet et al., 2014, Taylor et al., 2015) and the total K-13 propeller mutation prevalence is less than 5% in surveys from various transmission contexts (Tun et al., 2015).

1.7 Drug metabolism:

Cytochrome P450 (CYP) is a family of enzymes that catalyses the oxidative biotransformation of majority of drugs (Zanger and Schwab, 2013). Out of the 57 functional CYPs in humans, only about a dozen enzymes that belong to CYP1, 2, and 3 families are responsible for drug metabolism (Zanger and Schwab, 2013). Polymorphisms in gene-encoding for drug transporters and metabolising enzymes are related to individual differences in responses to drugs (Shah, 2005). Genetic polymorphisms in CYP450 genes have an influence on the safety and efficacy of drugs because of their effect on enzyme activity and/ or expression (Bains, 2013). Nearly 40 % of drug metabolism that is dependent on cytochrome P450 is attributed to polymorphic enzymes (Marwa et al., 2014). Polymorphism takes place in all the members of the CYP2C sub-family including CYP2C19, CYP2C18, CYP2C9, and CYP2C8. The genes for these sub-families are localised on chromosome 10q24 (Marwa et al., 2014). The function of CYP genes in drug metabolism differs considerably. CYP2C plays a critical role in the metabolism for nearly 20% of clinical drugs (Goldstein, 2001). CYP2C8 constitutes 7% of the liver's whole CYP content (Totah and Rettie, 2005) and is responsible in the metabolism of clinical drugs (Daily

and Aquilante, 2009). The CYP3A sub-family is the most abundant CYP in the small intestine and liver (Marwa et al., 2014). CYP3A4 participates in the metabolism of nearly 50% of clinical drugs (Marwa et al., 2014). CYP2B6 gene accounts for between 2% and 10% of the total content of CYP (Wang and Tompkins, 2008). The most prevalent single nucleotide polymorphism within the CYP3A family is CYP3A4*1B (Marwa et al., 2014).

Variations in the expression and function of CYP450 lead to four clinical phenotypes including ultra-rapid metabolizers(UM), extensive metabolizers(EM), inter-mediate metabolizers(IM), and poor metabolizers(PM) (Zanger and Schwab, 2013). Poor metabolizers have two allele copies that minimise the expression of a specific CYP450. Thus, they lack a specific enzyme activity and inefficiently metabolize drugs in comparison with UM, IM, and EM (Zanger et al., 2008). Intermediate metabolizers are heterozygous for a single copy of a functional allele and null allele of a specific CYP450, which leads to minimal reduction in the activity of the enzyme (Bains, 2013). Ultra-rapid metabolizers have more than two active gene copies that results in increased expression of protein levels and rapid substrate metabolism (Bains, 2013). Finally, extensive metabolizers are homozygous for two functional alleles that causes rapid metabolism of drugs and higher drug concentrations than PM and IM (Bains, 2013).

The frequencies of variant alleles that encode CYP families differ among individuals based on ethnic background and race (Seripa et al., 2010). The CYP2C8*2 allele is rare in Caucasians and Asians but prevalent in Africans, while CYP2C8*3 is prevalent in Caucasians but rare in Asians or Africans. The CYP2C8*3 has been linked with a noticed reduction in the metabolism of AQ among Caucasians (Daily and Aquilante, 2009). The CYP2B6*6 allele is more prevalent in individuals of African descent than

Caucasians (Klein et al., 2005). This allele is linked with increased plasma concentrations of anti-malarial drugs such as artemisinin (Kerb et al., 2009). The CYP3A4*1B allele is linked to poor metabolism of quinine (Rodriguez-Antona et al., 2005), and artemether lumefantrine (Staehli Hodel et al., 2013). Evidence also indicates that various Cytochrome P450 genes are involved in the metabolism of antimalarial drugs. CYP2B6 and CYP2A6 are involved in the metabolism of artemisinins (Piedade and Gil, 2011), while CYP2C8 is critical in the metabolism of CQ (Projean et al., 2003), and AQ (Mehlotra et al., 2009), and CYP2C19 is involved in the metabolism of artemether (Marwa et al., 2014). CYP2D6 is important in metabolizing CQ (Projean et al., 2003) and PMQ (section 1.7.2), while CYP3A4 is involved in the metabolism of lumefantrine (Piedade and Gil, 2011), quinine, PMQ (Mehlotra et al., 2009), CQ (Kim et al., 2003), and artemisinins (Mehlotra et al., 2009). Finally, the activity of CYP3A5 influences the metabolism of lumefantrine and CQ (Mehlotra et al., 2009), and artemether (White et al., 1999). Therefore, different CYP 450 isoenzymes are vital in the metabolism of antimalarial drugs and influence treatment outcomes.

1.7.1 Artemisinin metabolism:

The co-administration of various drugs in artemisinin-based combination therapy increases the risk of drug-drug interactions (Ericsson et al., 2014). Such interactions are due to the changes in the enzymatic activity of CYP450 particularly via inhibitory effects. Various studies have shown that artemisinin inhibits the activity of various CYP450 enzymes. For instance, it has been shown that DHA and artemisinin inhibit the action of CYP1A2 (He et al., 2007, Bapiro et al., 2001). The inhibitory impact on CYP1A2 has been established in healthy individuals with an artemisinin exerting 66%

inhibition of this enzyme (Bapiro et al., 2005, Asimus et al., 2007). Such in vivo findings indicate high and medium risk for drug-drug interactions with artemisinin and dihydroartemisinin, respectively, on CYP1A2 (Ericsson et al., 2014). Studies have also indicated that artemisinin compounds inhibit the activity of CYP2B6 (Therese et al., 2012, Xing et al., 2012). Bapiro et al. (2001) found that the enzymatic activity of microsomal and recombinant CYP2C19 is inhibited by DHA and artemisinin. In their study, Ericsson et al. (2014) found that artemisinin inhibited the activities of CYP3A4, CYP2C19, CYP2B6, and CYP1A2. The inhibitory activity of artemisins on these iso-enzymes leads to drug-drug interactions that increase the risk of adverse effects.

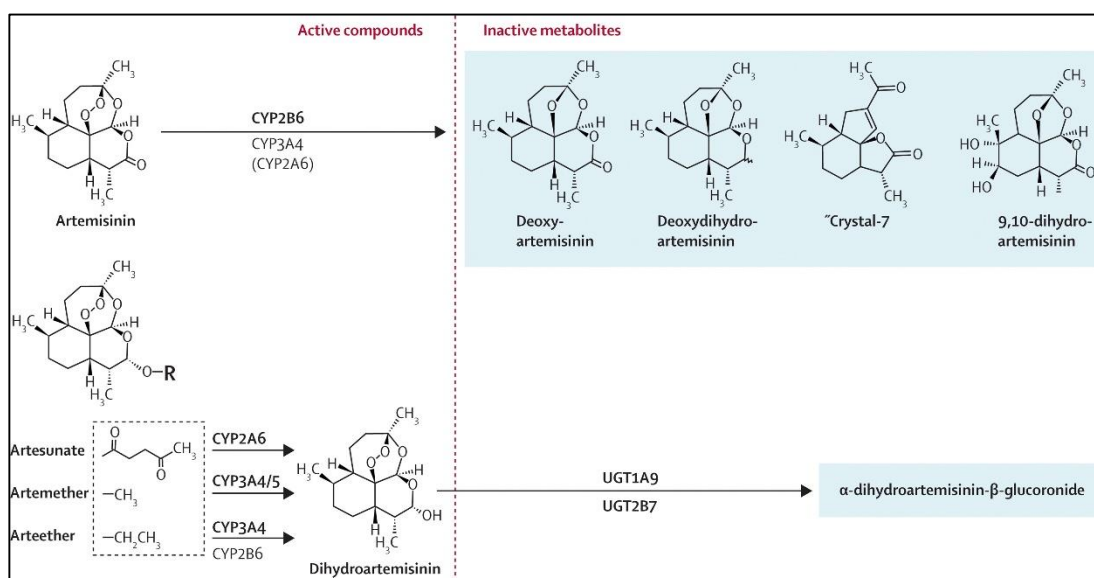


Figure 1.10: Metabolism of artemisinin and its semi-synthetic derivatives.

Primary pathways in bold, minor contribution in parenthesis. This figure adapted from Kerb and his colleagues (Kerb et al., 2009).

1.7.2 8-aminoquinoline metabolism (PMQ and TF):

PMQ and TF are 8- aminoquinolines used for anti-hypnozoite activity against malaria caused by *P. vivax* (Krotoski et al., 1980, Potter et al., 2015b). The administration of PMQ at low doses is recommended for blocking malaria transmission because of the drug's activity against gametocytes. Recent studies have shown that the metabolism

of 8-aminoquinolines is dependent on CYP2D6 in the liver stage anti-malarial activity in human and mouse studies (Potter et al., 2015b). Specifically, CYP2D6 converts the PMQ molecule into oxidized metabolites that cause the anti-hypnozoite activity of the drug (Marcsisin et al., 2016). Pybus et al. (2012) showed that the metabolism of PMQ is primarily occurs through the MAO-A, and CYP2D6 pathways. In this study, Pybus et al. (2012) demonstrated that CYP3A4, CYP2C19, MAO-A, and CYP2D6 metabolised PMQ. However, CYP2D6 demonstrated the highest catalytic efficiency toward PMQ of all the MAOs and CYPs tested in the study (Pybus et al., 2012b). Thus, CYP2D6 plays a vital role in the metabolism of 8-aminoquinolines. Pybus et al. (2013) showed that PMQ was active only in mice with the capability of metabolizing substrates of CYP2D6. Deleting the mouse enzyme that is closest to human CYP2D6 in the mice led to a complete blockage of the liver stage antimalarial activity in vivo (Pybus et al., 2013). Other studies have shown that stereo-selectivity is critical in the metabolism of 8-aminoquinolines by CYP2D6 both in vivo and in-vitro (Tekwani et al., 2015, Fasinu et al., 2014).

1.7.2.1 CYP2D6 metabolism effects on 8-aminoquinoline efficacy:

The efficacy of 8-aminoquinolines depends on CYP2D6 metabolism. Bennett et al. (2013) reported that therapy failures of PMQ in *Plasmodium vivax* challenge trial were directly attributed to CYP2D6 polymorphisms of the null or intermediate phenotype (Bennett et al., 2013a). Consistently, Pybus et al. (2013) showed how the efficacy of PMQ is dependent on CYP2D6 metabolism in a model involving a malaria mouse. In their study, Pybus et al. (2013) found that PMQ failed to exhibit any activity at a dose of 20mg/kg when it was administered to CYP2D6 knockout mice, which was challenged with *P. berghei*. The investigators attempted to associate particular metabolite to this impact by pre-incubating the human CYP2D6 inhibitor paroxetine

with recombinant CYP2D6 followed by monitoring of the production of the PMQ metabolite. Results from the *in vitro* pre-incubation of CYP2D6 with paroxetine showed a noticeable decline in phenolic metabolite production (Pybus et al., 2013). Similarly, Potter et al. (2015) found that phenolic metabolite levels of PMQ were highest in mice with the capability of metabolizing CYP2D6 substrates. Consistently, Vuong et al. (2015) found the same results for TF metabolism in mice (Vuong et al., 2015). St. Jean et al. (2016) suggested that the reduced metabolism of CYP2D6 was not linked to relapse among intermediate metabolizers following the administration of TF for treating *P. vivax* in a phase 2b DETECTIVE trial of the drug (St Jean et al., 2016). According to Marcsisin et al. (2016), PMQ hydroxylation via CYP2D6 results in unstable metabolites such as 5-hydroxyPMQ with the capability of redox cycling and the production of oxidative stress. The redox recycling of such metabolites and the oxidative stress from the metabolism of PMQ explains the efficacy of PMQ. However, the reliance of CYP2D6 metabolism for the efficacy of 8-aminoquinoline is a problem because of the high polymorphism of CYP2D6 in humans. The activity of CYP2D6 in humans is highly changeable with over 74 CYP2D6 alleles having been reported so far (Potter et al., 2015b). The phenotypic and genetic differences in CYP2D6 contribute to significant differences in drug metabolism and efficacy among patients.

1.7.2.2 CYP2D6 metabolism and 8-aminoquinoline toxicity in G6PD patients:

Therapy with 8-aminoquinolines such as PMQ has risks especially for individuals with a glucose-6-phosphate dehydrogenase (G6PD) deficiency who have a diminished capacity for mitigating cellular damage due to oxidative damage (Marcsisin et al., 2016). Treating G6PD-deficient individuals with PMQ increases the risk of haemolysis. The characteristics of haemolysis include mild jaundice, intra-vascular

haemolysis with dark urine, and severe anaemia (Ashley et al., 2014b). The severity of G6PD deficiency and the dose of PMQ administered to an individual determine the clinical signs of hemolysis (Ashley et al., 2014b). The mechanism through which PMQ exactly causes haemolytic effects and if or how CYP2D6 metabolism is involved in such effects is yet to be established. However, the toxicity of 8-aminoquinolines in G6PD deficient individuals is thought to be caused by CYP2D6-dependent phenolic metabolites such as 5-hydroxyPMQ (Marcsisin et al., 2016). Studies show that the production of 5-hydroxy-PMQ is dependent on CYP2D6 (Potter et al., 2015b, Pybus et al., 2012b), and that 5-hydroxy-PMQ is unstable in environments where oxygen is not carefully excluded and this has been shown to cause haemolysis both in vivo and in vitro (Marcsisin et al., 2016). Therefore, taking PMQ among G6PD deficient individuals increases haemolysis.

1.8 Gametocytocidal activity assays development:

1.8.1 Florescence indicator of metabolic activity:

Bolscher et al. (2015) developed high throughput assays to screen for compounds against gametocytes. The researchers cultured *P. falciparum* NF54 parasites followed by treating them with 50mM N-acetyl-d-glucosamine from day 4 to 7 to kill asexual parasites. A discontinuous 63% Percoll gradient centrifugation was used in isolating early and late stage gametocytes at day 7 and day 11, respectively. The researchers adapted a current gametocyte parasitic lactate dehydrogenase assay and used it in 384-well plates together with a new homogenous immunoassay for monitoring the transition of female gametocytes into gametes. The researchers screened 48 antimalarials using these assays to test the effect on sporogony in the anopheles' mosquito as a way of quantifying the transmission-blocking properties of anti-malaria

drugs in terms of their impact on gametogenesis. The results indicated that these screening assays revealed unique phase-particular kinetics and the dynamics of the drug effects. Peroxide demonstrated the most potent transmission –blocking effects with IC₅₀ values that were 20 to 40 fold higher than the IC₅₀ against the asexual phases that cause clinical symptoms of malaria (Bolscher et al., 2015).

Tanaka et al. (2013) developed an assay based on fluorescent indicator of metabolic activity. Gametocytogenesis was induced by culturing *P. falciparum* 3D7 following by selecting and enriching stage III to V gametocytes with 50mM NAG and Percoll density gradient centrifugation, respectively. Malaria gametocytes were plated in a 1,536-well plate using the Multidrop Combi followed by incubation for 72 h. The AlamarBlue dye was utilised for measuring cell viability. After the addition of the AlamarBlue to the assay plates, the researchers measure fluorescence intensity at 4, 8, 10, and 24 h time points. The assay was used in evaluating 1,280 malaria compounds. The results indicated that the miniaturised assay led to a significant reduction in the total number of reagents and gametocytes necessary for screening of large compound collections. The assay had a signal-to-basal ratio of 3.2 fold, and Z'-factor value of 0.68. Furthermore, using the assay to evaluate 1,280 anti-malarial compounds demonstrated that two compounds Cyclohexyl-amine and Antabuse had 54 and 7.8-fold potency towards gametocytes compared to their cell cytotoxicity effect in the SH-SY5Y cell line (Tanaka et al., 2013).

1.8.2 Florescence imaging:

Duffy and Avery (2013) used two anti-gametocytes high throughput screening assays based on confocal fluorescence microscopy that utilised both a viability marker (MitoTracker Red CM-H₂XRos) and a gametocyte particular protein (*pfs16*-Luc-GFP) in measuring anti-gametocytocidal activity. Using this assay, the researchers obtained

IC₅₀ values for 36 existing malarial compounds targeting the late and early stage gametocytes as well as the asexual stage. Screening of the MMV malaria box was undertaken to determine active compounds using IC₅₀ assessment. The results indicated that 7% of the ‘‘drug like’’ and 21% of the ‘‘probe-like’’ compounds from the MMV malaria box showed comparable activity against both late stage gametocytes and the asexual phases of the parasite. Thus, the two assays selectively identified compounds that targeted gametocytes and were suitable for screening large compounds (Duffy and Avery, 2013).

Lucantoni et al. (2015) developed a predictive phenotypic high content imaging assay for identifying malaria compounds targeting mature gametocytes. Strains of *P. falciparum* including 3D7-PFL1675c: GFP and 3D7A were cultured in-vitro. On day 3, trophozoite parasites were isolated followed by the addition of fresh red blood cells. The residual asexual parasites were removed with the addition of 50mM N-acetyl glucosamine. On the fourth day, the researchers isolated the 3D7-PFL1675C: GFP gametocytes using magnetic purification, followed by incubation to obtain mature stage V gametocytes on day 14. 3D7A gametocytes utilised in the non-transgenic assay were diluted with fresh blood and incubated to achieve maturation at day 12. This was followed by incubation with compound in 384- well plates for 48 h then exposure to Acridine Orange fluorescent dye and Xanthurenic acid, and readout acquisition following 2 h of incubation. Cultures that showed an activation of 95% or above on day 12 were used in the assay. This HTS assay involved using non-transgenic parasite by staining gametocytes with a fluorescent dye. The resulting Acridine Orange Gamete assay was used in evaluating the activity of existing anti-malarial drugs. The results showed that PMQ, ATQ, artemisone, cycloheximide, and thiostrepton reduced the number of gametes more than the total numbers of the

parasite. Furthermore, the assay was used in determining the activity of the compounds in the malaria venture box against mature gametocytes where standard membrane feeding assay tests were performed. The results of luminescence signals indicated that compounds MMV005830, MMV007591, and MMV006172 reduced the intensity of oocytes by over 85% at 10 μ M. The researchers concluded that the assay was robust and could be used in identifying quality hits with the likelihood to confirm the transmission blocking activity of malaria compounds (Lucantoni et al., 2015).

1.8.3 Chemiluminescence:

Lucantoni et al. (2013) developed a luciferase-based high throughput screening assay to assess the chemical compound activity on stages I to III of gametocytes. Gametocytogenesis was induced by magnetically purifying trophozoite culture using a MACS column. The trophozoite culture was incubated for 24 h. Further magnetic purification was conducted to differentiate early stage gametocytes from asexual schizonts and trophozoites as well as late stage gametocytes. After this, the parasites were plated without further addition of red blood cells in 384-well white plates using a multidrop reagent dispenser and incubated for 72 h. The researchers measured luminescence activity on the 4th day of gametocytogenesis using a luminescence reporter gene assay system. The HTS assay was developed using recombinant *P. falciparum* line expressing green fluorescent protein luciferase. The assay was aimed at evaluating the initial phase gametocytocidal activity of the MMV Malaria Box that contains 400 malarial compounds targeting the asexual blood stage activity of the malaria parasite. This collection was screened against stage I to III gametocytes and produced 64 compounds that were active against gametocytes with IC_{50s} below

2.5 μ M. Based on this, it was concluded that this assay is appropriate in screening large compound libraries (Lucantoni et al., 2013).

Lucantoni et al. (2016) developed and validated a high throughput luciferase based assay to identify compounds that are active against gametocytes in stages IV and V. The researchers cultured the *P. falciparum* NF54 gene for the production of gametocytes. Gametocyte production involved the isolation of the parasite at the trophozoite phase using a magnetic column followed by the addition of fresh red blood cells. On day 0 of gametocytogenesis, magnetic purification was done followed by the addition of NGA for clearing asexual parasites. Clearing of asexual parasites using NAG was done daily until the 8th day where final magnetic purification was done on the gametocyte culture. The researchers utilised a luciferase reporter gene assay system where the luciferase signal was evaluated in 384-well plate after incubation for 72 h. The researchers validated the assay by testing a panel of 39 antimalarial drugs and clinical candidates. The assay was also used in screening three chemical libraries (MMV Malaria Box, ERS_01, and GDB_04). The results showed that artemisinin and its derivatives inhibited late-stage gametocytes with IC₅₀ values ranging between 5 and 91nM. TF had weak gametocytocidal activity against late stage gametocytes with IC₅₀s of 2.03 \pm 0.49 and 2.50 \pm 0.004 μ M. However, PMQ did not have any effect on late stage gametocytes. MQ and halofantrine had limited potency on late stage gametocytes with values between 2.31 μ M and 3.39 μ M. Thiostrepton moderately inhibited late-stage gametocytes with IC₅₀ 1.00 \pm 0.07 and 2.31 \pm 0.13 μ M. PPQ, naphthoquine, AQ, hydroxyCQ and CQ did not inhibit late-stage gametocytes. High concentration of chlorPG inhibited late-stage gametocytes. MB was potent against late stage gametocytes with IC₅₀ value of 38 \pm 14nM, while cycloheximide was moderately potent against stage IV and V gametocytes with IC₅₀ of 2.31 \pm 0.13 μ M, and

pentamidine and pyronaridine weakly inhibited with IC₅₀ values above 3 μM. The most active compounds from the MMV Malaria box with activity against stage IV and V gametocytes included MMV006172 with IC₅₀ value of 1.364 μM, MMVO19918 with IC₅₀ values ranging between 0.32 μM and 0.89 μM, and MMV667491 with IC₅₀ value of 1.06 μM. Overall, this assay demonstrated the compounds that are active against stage IV and V gametocytes (Lucantoni et al., 2016b).

1.8.4 Bioluminescence assay:

Cevenini et al. (2014) developed a dual-colour (red and green) bioluminescence assay for gametocytes. The researchers cultured *P. falciparum* 3D7A line followed by transfection of the ring stage parasites. Transgenic lines with integrated luciferase cassettes in the pfelo 1 locus were produced by equipping the luciferase cassettes with pfelo 1 homology genes for Zinc Finger Nuclease-mediated genome editing. The researchers treated Stage II gametocytes with 50mM NAG for eliminating asexual stages. Stage IV and V gametocytes were also treated with NAG to allow maturation. The luciferase assay expressing green and red emitting luciferases were used in the study. Luciferase assays were performed to compare luciferase activities on stage III gametocytes following percoll purification. The assays were conducted on six transgenic lines of the parasites in 96-well plates. Single-cell bioluminescence imaging was done on gametocytes treated with CQ and epoxomicin as well as on a control sample. The results indicated that the single-cell bioluminescence imaging assisted in visualizing live gametocytes in various stages. A weak Bioluminescence signal (BL) was seen in CQ-treated gametocytes, while a BL signal was not detectable in gametocytes treated with epoxomin, and strong BL signals were seen in the control parasites. Dual luciferase assays were also on CBR and CBG99 expressing parasites at stage V and II of development in a 96-well plate. The findings of the dual luciferase

assay showed that epoxomin efficiently killed both stages V and II gametocytes. It was concluded that this assay that requires incubation for only 48 h and utilises a luminogenic substrate leads to a considerable reduction in the time and cost of assays (Cevenini et al., 2014).

D'Alessandro et al. (2016) developed an assay for determining the activity of anti-malarial compounds against early stage (I-III) and late stage (IV-V) gametocytes. The assay in the study was a high throughput screening assay with *P. falciparum* that gametocytes expressing a potent luciferase activity. The gametocyte lactate dehydrogenase assay and the luciferase assays were evaluated by screening antimalarial drugs to determine the IC₅₀ Values of the compounds against gametocytes. The results demonstrated that gametocyte lactate dehydrogenase and luciferase assays are vital in identifying anti-malarial compounds that are potent against gametocytes both in their late and early stages of development (D'Alessandro et al., 2016a).

1.9 Thesis objectives:

If we are to achieve a sustainable malaria elimination strategy, it is vital that malaria transmission is targeted within an integrated approach. To that end, targeting the malaria parasite gametocytes remains an attractive and tractable strategy. The historical difficulty in culturing gametocyte stages and assaying gametocyte viability has resulted in a fragmented understanding of the gametocidal activity of the existing antimalarial armamentarium. This thesis has therefore set out to gain a better understanding of the identity of gametocyte-active antimalarials and a deeper understanding of the mechanisms underpinning activity. Specific objectives include:

1. To screen different known antimalarials and some selected newly developed compounds against *P. falciparum* gametocyte stages and validate their stage-specific activity. This is followed by the development of time-dependant killing assays and subsequent Pharmacokinetic-Pharmacodynamic (PK-PD) simulations. This objective has been achieved successfully as described in Chapter 3.
2. To assess the susceptibility profile to endoperoxides of *P. falciparum* gametocytes stages in both static and time-dependent assays and subsequent PK-PD simulations. This objective has been accomplished successfully and described in Chapter 4.
3. To determine the ability of PMQ and its metabolites to interact with recombinant human Cytochrome (CYP 2D6). To test PMQ and PMQ-metabolites following metabolism with CYP2D6 and HLM metabolites to test activity against late stages gametocytes. This work is described in chapters 5.
4. To determine the ability of TF to interact with CYP2D6 and test the activity of TF as a parent drug, TF-metabolism with CYP2D6 and HLM metabolites for activity against late stage gametocytes. In addition, three novel 8-AQ structural analogues also investigated their gametocytocidal efficacy prior to and after CYP2D6 and HLM metabolites activation. This work is described in details in chapter 6.

Chapter 2

Experimental Methods:

2.1 Introduction:

The aim of the experimental methods chapter is to provide the information on *P. falciparum* culture system and the developmental methods of sexual (gametocytes) cultures. In addition, a description of the selected method of gametocitocodal viability assays (luciferase based) which have been utilized to generate the data of the thesis and the general steps of PK/PD methods are provided. More specific details and of methods and modifications to the general methods are provided within the experimental chapters.

2.2 Culture system for *P. falciparum* maintenance:

The parasite isolate of *Plasmodium falciparum* culturing in vitro was adjusted from (Trager and Jensen, 1976) and (Jensen and Trager, 1977). The culture was accomplished using standard aseptic techniques in an Envair class II laminar flow safety cabinet. The biocidal cleaner (Biocleanse Concentrate, TEKNON, UK) was used to clean the laminar flow cabinet first and then with 70% ethanol in order to minimize the contamination. Consumables, for example: 75 cm² and 25 cm² Nunc™ polystyrene tissue culture flasks (Fisher Scientific, UK), 2.5 ml bijoux bottles and 15 ml and 50 ml centrifuge tubes. Solutions used for parasite culture were prepared with distilled water (dH₂O) and sterilized by filter through a sterile bottle top filter unit with a 0.22 µm membrane (Fisher Scientific, UK). All pipette tips (10 µl, 100 µl, 200 µl and 1000 µl) prior to use were sterilized by autoclaving (121 °C, 15 psi for 30 min). Protective gloves were used and regularly covered with 70% ethanol when working in culture room to reduce contamination. Used RPMI-1640 complete culture medium, uninfected red blood cell (RBC), culture flasks, used pipettes and supernatants were

disinfected in biocidal cleaner (Biocleanse Concentrate, TEKNON, UK) first and then wasted in the specified trash.

2.2.1 *P. falciparum* parasite strains:

The *P. falciparum* strains used in this Project was the newly generated luciferase-reporting transgenic line 3D7elo1-pfs16-CBG99. This CBG99 were generously provided by Prof Pietro Alano lab (INBB, Istituto Nazionale di Biostrutture e Biosistemi, 00136 Rome, Italy) to (Cevenini et al., 2014). The 3D7 strain was cloned from the isolate NF54 obtained from one of an airport staff in Amsterdam (Cowman et al., 1991).

2.2.1.1 Culture medium:

In all the experiments, RPMI-1640 (Roswell Park Memorial Institute) culture medium containing L-glutamine and sodium bicarbonate (NaHCO_3) were used and purchased from Sigma (Sigma, UK). Complete culture medium was prepared by adding 12.5 ml of pre-sterilized 1 M HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid), 50 ml of pooled human AB+ serum and 200 μl of 50 mg/ml gentamicin (Sigma, UK) to a 500 ml bottle of RPMI-1640 culture medium. For the purpose of checking for contamination, the medium was incubated at 37 °C overnight prior to use. The increase in turbidity and changing colour of medium from red/orange to yellow indicates the occurrence of contamination. The complete culture medium was used for up to one week and unused medium was discarded.

2.2.1.2 HEPES:

HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid) was obtained from VWR International Ltd (UK). The preparation of 1 M stock of HEPES (MW = 238.03 g) was as follows: 238.03 g of HEPES was dissolved in 700 ml of distilled water and

the pH was adjusted using 5 M NaOH to 7.4 after which the solution was made up to 1 L using distilled water. The 1 M stock of HEPES was then sterilized through a sterile bottle top filter unit with a 0.22 μm membrane (Fisher Scientific, UK), labelled, and stored at 4 $^{\circ}\text{C}$.

2.2.1.3 Gentamicin:

A stock of 50 mg/ml Gentamicin was purchased from Sigma (Sigma, UK) and aliquoted into 2.5 ml bijoux bottles, labelled and stored at 4 $^{\circ}\text{C}$.

2.2.1.4 Serum:

Ward 7Y and the Gastroenterology Unit, Royal Liverpool Hospital, Liverpool, UK, kindly supplied human AB+ serum.

2.2.1.5 Gas Phase:

The gas used in this study was composed of 3% O_2 , 4% CO_2 and 93% N_2 (British Oxygen Special Gases, UK). The flasks were gassed aseptically in the class II laminar flow safety cabinet. They were gassed separately by a pre-sterilized cotton plugged pipette fitted to the terminal acrylic filter for approximately 30 seconds per 25 cm^2 flasks and 1 min per 75 cm^2 flasks.

2.2.2 Preparation of uninfected red blood cells:

The North West Regional Blood Transfusion Service, Liverpool, UK, kindly supplied human O+ blood which is uninfected red blood cells (RBC). The whole blood was supplied in citrate-phosphate-dextrose bags and had been tested for HIV and HBV antibodies. The blood was aseptically aliquoted into sterile 50 ml centrifuge tubes and stored at 4 $^{\circ}\text{C}$ for only two weeks. A 25 ml aliquot of whole blood was centrifuged aseptically at 3000 rpm for 5 min and then buffy coat layer and serum were carefully removed using a pre-sterilized 10 ml pipette. The remaining packed RBC was washed

three times in RPMI-1640 incomplete culture medium supplemented with 25 mM HEPES (pH 7.4) and 20 μ M gentamicin and was collected by centrifugation at 3000 rpm for 5 min. The washed packed RBC's were labelled and stored at 4 °C for no more than one week and discarded the unused.

2.2.3 Cryopreservation Procedure of parasite cultures:

Firstly, the cryoprotectant solution was prepared as follows: 1.9 g of sodium chloride (Sigma Chemical Co, UK) was dissolved in 200 ml of distilled water to get 0.95% (w/v) physiological saline. To make 4.2% (w/v) sorbitol in physiological saline, 8.4 g of sorbitol (Sigma Chemical Co, UK) was then dissolved in the prepared saline. Subsequently, 70 ml of glycerol (Sigma Chemical Co, UK) was added to the solution. Then, the cryoprotectant solution was sterilized through a sterile bottle top filter unit with a 0.22 μ m membrane (Fisher Scientific, UK), labelled, and stored at 4 °C.

In order to freeze the parasite culture, a high parasitaemia (greater than 5%), mainly at ring stage, were transferred aseptically into sterile 50 ml centrifuge tubes and centrifuged at 2000 rpm for 5 min. The supernatant was discarded and an equal volume of cryoprotectant solution was added to the pellet and allowed to equilibrate for 5 min at room temperature. The suspension was placed into screw-capped cryotubes (Nunc, UK), labelled and then moved into liquid nitrogen tank (British Oxygen Special Gases, UK).

2.2.4 Parasite Cultures Retrieve (Thawing):

Cryotubes were retrieved as follows: cryotubes were taken from the liquid nitrogen storage tank and allowed to thaw at room temperature or quickly thawed at 37 °C. Prior to pouring the defrosted contents of cultures into a 15 ml centrifuge tube, 70% ethanol was used to wipe the rim of the vial to minimize any chances of contamination.

An equal volume of ice cold 3.5% (w/v) sodium chloride (Sigma Chemical Co, UK) was then added to 15 ml centrifuge tube and centrifuged at 2000 rpm for 5 min. The supernatant was removed and the cell pellet was washed once in an equal volume of RPMI-1640 complete culture medium, and centrifuged as before. The supernatant was removed and the cell pellet was re-suspended in 15 ml of RPMI-1640 complete culture medium which was then made up to the desired hematocrit with washed RBC. Cell pellet suspension was then transferred to a sterile 25 cm² culture flask, labelled, gassed and placed in an incubator at 37 °C for 48 h.

2.2.5 Routine monitoring of asexual stages parasitaemia:

In order to monitor the asexual stage, the parasitemia were checked daily by making a thin blood film from every culture flask. The parasite cultures should be healthy, well synchronised and within the required parasitaemia (typically, 5-10%). Thin blood films were then fixed in absolute 100% methanol for 5-10 seconds (Fisher Scientific, UK) and placed into a 10% Giemsa stain solution (VWR International Ltd, UK) buffered at pH 7.2 for 15-20 min. The films were washed carefully and thoroughly under running tap water. After that, they were dried and examined under oil immersion at x1000 magnification on a light microscope (Zeiss, Germany). The parasitaemia was calculated by counting the cells in approximately 5-10 fields of the blood film and expressing infected cells as a percentage of the total number of cells film as shown below:

$$Paeasitemia(\%) = \left(\frac{\text{No. of infected RBC's}}{\text{Total number of RBC's}} \right) \times 100$$

2.2.6 Synchronisation of parasite cultures:

A sugar-based alcohol (Sorbitol, Sigma, UK), has a steadying effect on the RBC plasma membrane (Meryman and Hornblow.M, 1968) and changes the permeability of the later stages of asexual parasites (Lambros and Vanderberg, 1979). These alterations of permeability of the late stage parasites and its host allow sorbitol to enter through the new permeability pathway (NPP). The parasites swell as a result of osmotic effect and ultimately lyse and die. This process allows selection of the young ring forms which are unaffected, thereby synchronising the culture.

In order to prepare 5% sorbitol (which is used for the purpose of synch.), 25 g of sorbitol was dissolved in 500 ml of distilled water. The solution was then sterilized through a sterile bottle top filter unit with a 0.22 µm membrane (Fisher Scientific, UK), labelled and stored at 4⁰C. The synchronisation procedure was as follows: cultures with a high proportion of ring stage parasites were transferred aseptically to pre-sterilised centrifuge tubes and centrifuged at 2000 rpm for 5 min. at room temperature. The supernatant was discarded, and 5 ml of 5% (w/v) sorbitol was added to the pellet. The suspension was left in the laminar flow safety cabinet to stand at room temperature for approximately 20 min, with occasional shaking of the tube, and then centrifuged and the supernatant was discarded. The cell pellet was washed twice in 10 ml RPMI-1640 complete culture medium and centrifuged as before. The remaining cell pellet was re-suspended in 50 ml RPMI-1640 complete culture medium for the continuous culture for a minimum of 48 h prior to use in the different experiments.

2.2.7 Culture procedure:

To maintain the parasite in continuous culture in 75 cm² flasks (Nunc, UK), a modification of the method of (Trager and Jensen, 1976) and (Jensen and Trager,

1978, Jensen and Trager, 1977) was used. The hematocrit in the culture flasks was usually 2% unless otherwise stated. Cultures were started from parasitised cells retrieved from cryopreserved stocks or by seeding RBC's in RPMI-1640 complete culture medium suspension with parasitized red cells from another flask. The culture flask was then gassed and placed in an incubator at 37°C. The culture medium was changed every 48 hrs when the parasitaemia was less than 1% and every 24 hrs when higher than 2%. The procedure for this was as follows: the culture suspension was transferred aseptically to a 50 ml centrifuge tube and centrifuged at 2000 rpm for 5 min at room temperature. The spent medium was carefully removed and discarded. Pre-warmed RPMI-1640 complete culture medium was then added to make up the volume to 15 ml and 50 ml in 25 cm² and 75 cm² culture flasks, respectively. The culture flasks were then gassed and placed in an incubator at 37°C. The parasites were sub-cultured when the target parasitaemia had been reached (usually at ~10% parasitaemia).

2.2.8 Sub-culturing Procedure of asexual stages:

The main reason of sub-culturing the parasites is to produce a large volume of parasite pellets for the experiments. The procedure was as follows: the culture suspension was centrifuged at 2000 rpm for 5 min at room temperature and then the supernatant was discarded. A specific volume of cell pellet was added to a new flask. Fresh uninfected erythrocytes and RPMI-1640 complete culture medium were added to make the required hematocrit (usually 2%) and parasitaemia depending on size of the flask used. The culture flask was then gassed and incubated at 37 °C. The rest of the parasitised cells were used either in an experiment, cryopreserved or thoroughly decontaminated and discarded.

2.3 *P. falciparum* Gametocytes (Sexual Stage) Culture:

P. falciparum gametocyte cultures were prepared as described previously (D'Alessandro et al., 2016a) with some modification and optimization. Briefly, asexual stage of good gametocytes producing lines of *P. falciparum* were cultured in O-positive RBCs at 5% hematocrit, in RPMI 1640 complete culture medium supplemented with 5ml of 4mM hypoxanthin. Parasites culture were initiated at 2% in mix stages of ring and trophozoites (~5% hematocrit) and grown until they reached high parasitemia. To trigger gametocytogenesis, the haematocrit was reduced by adding more medium in the flask to increase the stress factor.

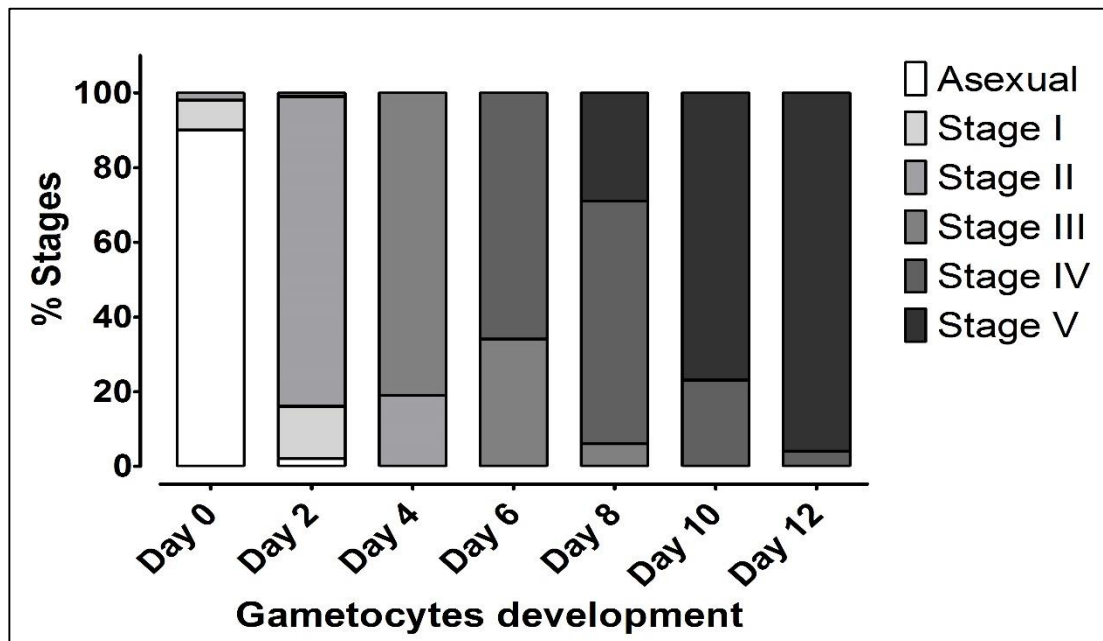


Figure 2.1: Gametocytes development of stage composition of the cultures used in the experiment.

Giemsa-stained thin smears were used every day to monitor the development of the cultures until the stage I and II gametocytes are clearly recognised and the asexual stage parasites seem unhealthy. Gametocytes were grown in the presence of 50 mM N-acetylglucosamine (NAG) for four days to clear residual asexual parasites and

obtain a practically pure gametocyte culture. Development of the gametocytes culture was monitored by Giemsa-stained thin smears as shown in Figure 2.1.

All previous steps were optimized in various conditions to influence gametocyte production. Mixed stage asexual parasites able to produce more gametocytes than the sorbitol synchronous parasites. Furthermore, the vertical position flasks and 5% haematocrit showed more gametocytes production than lower haematocrit and horizontal position flasks, indicating that the production of gametocytes in stress conditions can induce more gametocytes than normal (routine) steps of asexual culture. Serum medium composition moderately increase in gametocytes production more than AlbuMax and NAG clear residual asexual parasites significantly more than sorbitol addition to the culture.

Table 2.1: *In-vitro* gametocytogenesis of *P. falciparum* culture under various conditions.

Conditions		Gametocyaemia ^a
Asexual parasite synchrony to induce gametocytes		
Sorbitol sync.		2.3 ± 0.9 % (<i>n</i> = 3)
Mixed stages (ring, trophozoite, schizont)		4.3 ± 1.02 % (<i>n</i> = 5)
Flask position (Vertical / horizontal) and haematocrit percentage		
T25 cm ² / vertical	5% haematocrit	4.4 ± 1.0 % (<i>n</i> = 3)
T25 cm ² / horizontal	5% haematocrit	3.0 ± 1.4 % (<i>n</i> = 3)
T25 cm ² / vertical	2 % haematocrit	2.9 ± 0.9 % (<i>n</i> = 2)
T25 cm ² / horizontal	2 % haematocrit	2.3 ± 1.1 % (<i>n</i> = 2)
Medium composition (asexual, stationary gametocyte cultures T25 cm²)		
Asexual culture	Gametocyte culture	
AlbuMAX	AlbuMAX	3.2 ± 1.0 % (<i>n</i> = 2)
Serum	Serum	4.2 ± 1 (<i>n</i> = 3)
Asexual parasite elimination		
Fresh NAG addition		4.5 ± 0.4 % (<i>n</i> = 6)
Sorbitol synchronisation		2.4 ± 0.3 % (<i>n</i> = 2)
Different strains of <i>P. falciparum</i> parasites in better conditions		
3D7elo1- pfs16-CBG99		4.3 ± 0.49 % (<i>n</i> = 4)
3D7		0.9 ± 0.3 % (<i>n</i> = 3)
HB3		0.26 ± 0.3 % (<i>n</i> = 2)

^a Day 12 from gametocytes induction

The number of biological experiments is given by (*n*), with each biological experiment performed two or more times.

The kinetics of alteration from asexual stage to gametocytes followed the maturation to stage IV-V gametocytes were monitored as clearly seen in Figure 2.2. The number of parasitaemia increased up to maximum parasitaemia on days 0 (prior to NAG treatment) then double medium (DM) were add to reduce the haematocrit. Some sexual forms were first detected on day 0, and gametocytaemia reaching the maximum with average of 4 % on day 6-14.

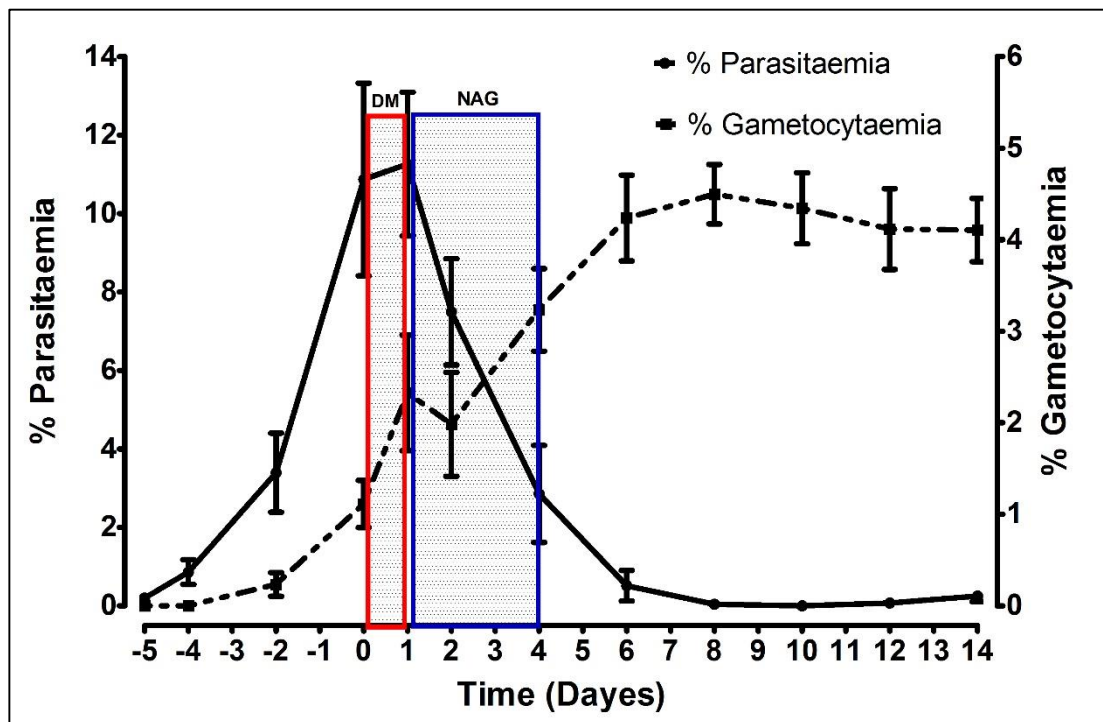


Figure 2.2: *In-vitro* production of *P. falciparum* gametocytogenesis in culture flask. Kinetics of asexual stages (parasitaemia) in solid line and sexual stages (gametocytaemia) in dashed line during gametocytogenesis. The number of parasitaemia increased up to maximum parasitaemia on days 0 (prior to NAG treatment) then double medium (DM) were add to reduce the haematocrit. Sexual forms were first detected on day 0, with gametocytaemia reaching an average of 4 % on day 6-14. Data are from three independent experiments each experiment performed in triplicate and data represent mean \pm SEM.

2.3.1 *P. falciparum* gametocytes drug treatments:

The assay was performed on gametocytes at two different stages. For early (stage II-III) gametocytes, induced cultures were treated for 48 h with 50 mM N-acetylglucosamine (NAG) to eliminate asexual stages before drug treatment. Late (IV-V) stage gametocytes had been NAG-treated for 96 h and then allowed to mature. Drug treatments were performed at a final hematocrit of 1% in 100 μ L final volume in 96-well culture plates. The gametocytemias were routinely ranging between 2% and 3% 3D7elo1- pfs16-CBG99 during the assay. Drugs were dissolved in dimethyl sulfoxide (DMSO) or any relevant solvent and control samples were treated with solvent at the highest concentration present in treated samples $\leq 0.1\%$. The incubation period of drug exposure is depending on the assay and is described in relevant chapter results section.

2.3.2 Luciferase assay of gametocytocidal activity:

All samples were tested in triplicate at least three times. Luciferase assay after drug-activity experiments were performed after transferring samples to 96-well white microplates as describe previously (Cevenini et al., 2014). The measurement of samples in the 96- well white microplate was as shown in figure 2.3 below to reduce the interfering of luminesce lights between the samples.

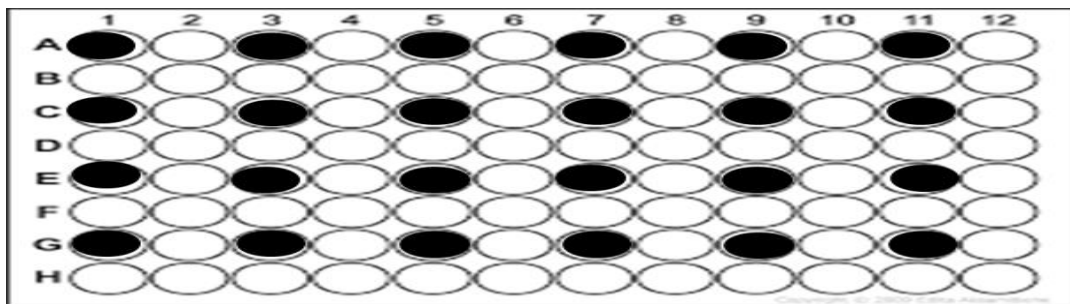


Figure 2.3: The 96- well white microplate use for tumescence reading of the luciferase assay.

The black filled in well-plate represent the transferred gametocyte-treated samples to reduce any interference of luminesce between the samples.

The optimal D-luciferin substrate in citrate buffer 0.1 M, pH 5.2 was 1 mM D-luciferin (final concentration). Substrate was added directly to the samples at a 1:1 ratio, and plates were read, in plate reader, Varioskan[®] Flash plate reader (Thermo Electron Corporation) at the stable kinetics enzyme at least between 8-18 min after addition (Figure 2.4).

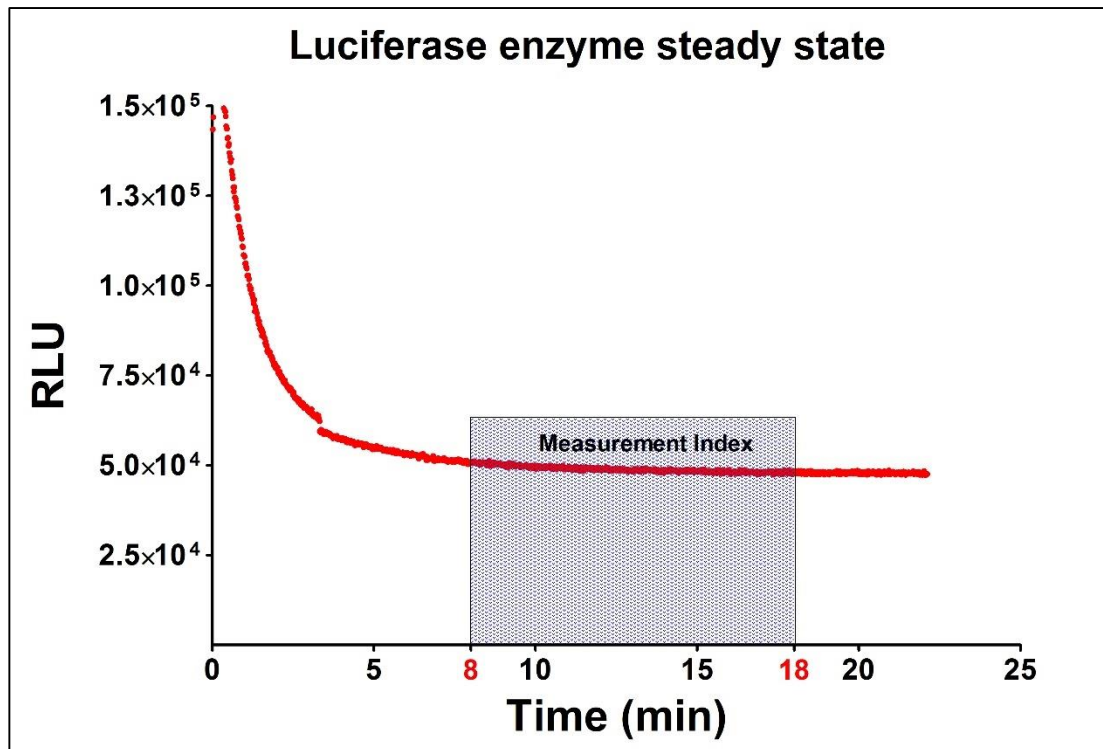


Figure 2.4: Luciferase kinetic intensities steady state characterization.

Bioluminescence intensity and kinetic profile obtained from CBG99 expressing gametocytes (2% haematocrit; 2-4% gametocytemia/well) using D-luciferin substrate in citrate buffer 0.1 M, pH 5.2. The measurement index pattern box shows the selected start and end reading time of test plates (8 to 18 mins) at the convenience steady state of enzyme activity.

2.3.3 Data analysis:

The results from luminescence plate reader, Varioskan ® Flash plate reader (Thermo Electron Corporation) were expressed as the percentage viability compared with untreated controls according to the following:

The average well reading for each drug concentration, the positive control wells (uninfected RBC's) and the negative control wells (10µM of MB) were calculated. Then, the negative control value subtracted from all the other averages. After that, the percentage of gametocytes viability was calculated to uninfected RBC control well (100% viability)

$$\% Viability = 100 \times \frac{\mu \text{ of treated sample}}{\mu_{c+}}$$

where μ is the mean of luminescence reading LR and (c+) uninfected RBCs. To express the results of the dose-response experiments, the percentage of viability calculated using Prism 5 for windows (© 1992-2010 Graphpad software, Inc.) through nonlinear regression analysis using the log (agonist) vs. response (four parameter) built-in equation.

2.4 Modelling Drug activity against Gametocytes

2.4.1 Identification of kill rates and pharmacodynamic parameters of drugs

The dynamics of gametocytes kill exerted by different drugs were systematically characterised by analysing the effects of each drug upon gametocyte counts at a range of clinically relevant concentrations. The kill rate exerted by each concentration was

calculated using Prism 5 for windows (© 1992-2010 Graphpad software, Inc.) through nonlinear regression using the “plateau followed by one phase decay” built-in equation as shown below in Equation 1.

$$Gametocyte\ count = (G_0 - Plateau) * e^{(-k \cdot t)} + Plateau \quad \dots Eq. 1$$

Where (G_0) refers to the initial number of gametocytes in culture, ($Plateau$) is the theoretical minimum level of gametocytes that can be achieved at the last time point, (k) is the kill rate per h and (t) is the time in h. When lag time is observed before activity the program would account for it using an IF statement which calculates the time at which activity is initiated.

After measuring the kill rate (k) achieved by each concentration of the drug, the overall activity of the drug is then assessed using a sigmoidal E_{max} model which calculates the EC_{50} for each drug as well as its maximal kill rate (E_{max}). The kill rates were fitted using Prism 5 by applying the log(agonist) vs. response (three parameter) built-in equation in Prism as shown below in equation 2:

$$Kill\ rate\ (hr^{-1}) = E_{min} + \frac{(E_{max} - E_{min})}{1 + 10^{LOG(EC_{50} - C)}} \quad \dots\dots Eq. 2$$

Where (E_{min}) is the background effect achieved in negative controls, (E_{max}) the maximum possible kill rate that could be achieved at the highest concentrations, (EC_{50}) is the concentration required to achieve 50% of E_{max} and (C) is concentration in Molar (M).

Using equation 2 above the main pharmacodynamic properties (E_{max} and EC_{50}) of each drug could be identified for use in further PK-PD predictions to identify the activity of each drug.

2.4.2 PK-PD modelling and predictions of clinical drug activity

Prediction of clinical activity of drugs against gametocytes were generated using Monte-Carlo simulations using the program Pmetrics® (Neely et al., 2012a) where the PK parameters of each drug were collected from the literature as shown in the relevant sections discussing each drug and the concentration-time profile modelled using either one or two compartments PK model.

To convert drug mass in blood to concentration in molar, the following equation is used below:

$$\text{Drug conc. (M)} = \frac{X_2/V_d}{Mwt}$$

Where (V_d) is the volume of distribution in litres, (X_2) the mass of drug in blood and Mwt is the molecular weight of the drug.

The overall kill profile of gametocytes is then defined by linking the dynamic concentration to the sigmoidal pharmacodynamic profile which has been characterized in the previous section in equation (2).

For Monte-Carlo simulations, we assumed 1000 subjects where the pharmacokinetic parameters were set at a variability of 30% to account for natural variation in pharmacokinetic profiles in different humans. Final results were plotted showing the median profile for gametocyte kill in addition to the 5% and 95% percentiles to show the range given pharmacokinetic variability.

Chapter 3

Screening of known and potential gamitocycidal drugs

and PK/PD validation of methylene blue

3.1 Introduction:

Malaria control and elimination cannot be achieved exclusively through vector control or treatment of malaria patients, but will require the development of strategies to block the transmission process between the human hosts and mosquitoes (vector). This goal can be accomplished by targeting the gametocyte stages in the human circulation and hepatic sporozoite and oocysts in mosquito midgut (Burrows et al., 2011a, Burrows et al., 2011b). Therefore, the most appropriate target that could block the host-vector transmission process is the gametocyte in the human host, which would result in interrupting the transmission and breaking the malaria cycle. To date, only a few known antimalarials have therapeutic activity against late gametocytes such as artemisinins and 8-aminoquinolines. However, toxicity and resistance emergence are serious obstacles that could impede the application of those classes of antimalarial drugs in the field (Lucantoni and Avery, 2012). As a result, the development of new compounds as gametocytocidals has become urgent.

In this chapter, screening of some known antimalarial compounds against gametocyte stages and a selection of newly developed anti-malarial compounds were used to investigate their potential gametocytocidal activity at clinically relevant concentration levels. To achieve the main aim, gametocyte bioactivity assays are key to determine the activity of transmission-blocking compounds (Lucantoni and Avery, 2012, Reader et al., 2015).

The process of *in-vitro* production of *P. falciparum* gametocytes in synchronised stage-specific high yields for screening assays is challenging. Recently, several methods have been published on the development gametocyte cultures (Fivelman et al., 2007, Lucantoni et al., 2013, Cevenini et al., 2014, D'Alessandro et al., 2016b,

Delves et al., 2016), however malaria isolates showed significant variability in their ability to generate gametocytes *in vitro* (Graves et al., 1984).

The screening of known antimalarials; e.g. 4-aminoquinoline, 8-aminoquinoline, aminoalcohol, naphthoquinone, antifolates, thiazine dye (methylene blue), and the new potential gametocytocidal compounds such as spiroindolones, Mitotic Kinase Monopolar Spindle 1 (MPS1) inhibitors and aurora kinase inhibitor have been investigated to measure their activity against gametocyte stages. In the present study, a newly generated luciferase-reporting transgenic line 3D7elo1- pfs16-CBG99, derived from well generated gametocytes 3D7 lab strain, was used to introduce gametocytogenesis developmental stages *in vitro*. A highly sensitive luciferase-based gametocyte assay method was used in this study. This assay uses a newly generated luciferase-reporting transgenic line, and a non-lysing D-luciferin substrate formulation (Cevenini et al., 2014, D'Alessandro et al., 2016a) to monitor gametocyte viability during gametocytogenesis development and determine the stage-specific sensitivity and the kill rate of the antimalarials.

Spiroindolones represent a new antimalarial class that targets a P-type Na⁺-ATPase enzyme (PfATP4) by inhibiting this plasma membrane protein of the parasite and then disrupting its sodium haemostasis leading to parasite death (Spillman et al., 2013, Flannery et al., 2013, Turner, 2016). This novel mode of action gives us a better understanding of the pathways involved in parasite biology and haemostasis and has resulted in the discovery of new antimalarial targets to face the emerging challenges of drug resistance.

Although there is no Mitotic Kinase Monopolar Spindle 1 (MPS1) in *P. falciparum*, suspected MPS1 inhibitors (azaindoles, potential kinase) AWZ compounds screened in house have shown reasonable activity against *P. falciparum* asexual stages. The

activity of those kinase inhibitors has high level of structural similarities to the 1H-pyrrolo [3, 2-c] pyridine scaffold (Naud et al., 2013).

Pharmacodynamic dose-response curves and IC_{50} values have been established for the compounds showing significant activity during gametocyte stages. Thereafter, the kill rate of the most potent known antimalarial compound was determined by conducting a time-dependent killing assay, building a pharmacodynamic E_{max} sigmodal model and studying its pharmacokinetic/pharmacodynamic (PK/PD) properties in eliminating gametocytes when administered to patients at clinical levels.

3.2 Methods and materials:

3.2.1 Reagents:

P. falciparum 3D7elo1-pfs16-CBG99 were generously provided by Prof Pietro Alano lab (INBB, Istituto Nazionale di Biostrutture e Biosistemi, 00136 Rome, Italy). 4-aminoquinolines, 8-aminoquinolines, antifolates, MB were purchased from sigma (DHA, artesunate and artemether) were purchased from sigma (Dorset, UK). Lumefantrine was purchased from TCI. The spiroindolones (SJ-7 & SJ-10) were kindly provided from Amy Matheny, St. Jude Children's Research hospital (Memphis, TN USA) with no chemical structure provide. MPS1 inhibitors were provided in house from our medicinal chemistry team. Aurora kinase inhibitor from AstraZeneca. The chemical structures of these compounds are shown in Figure 3.1.

3.2.2 Test compounds:

Table 3.1: Known and new potential antimalarial drugs used in this chapter.

<i>Class</i>	<i>Compound</i>	<i>Molecular weight g/mol</i>	<i>Solvent</i>
4-Aminoquinoline	CQ	319.872	50% MeOH
	PPQ	535.517	
8-Aminoquinoline	PMQ	259.347	75% MeOH
	TF	581.589	
Aminoalcohols	Lumefantrine	528.939	DMSO
naphthoquinones	ATQ	366.837	50% MeOH
Antifolates	Pyrimethamine	248.71	DMSO
Others (thiazine dye)	Methylene blue	319.85	DMSO
Dihydroisoquinolines (Spiroindolones)	SJ000573359-7	457.38	DMSO
	SJ000571311-10	457.38	
MPS1 inhibitor	AWZ5019	401.48	DMSO
	AWZ5023	376.43	
Aurora kinase inhibitor	AZD3646	523.54	DMSO

3.2.3 Compounds chemical structures:

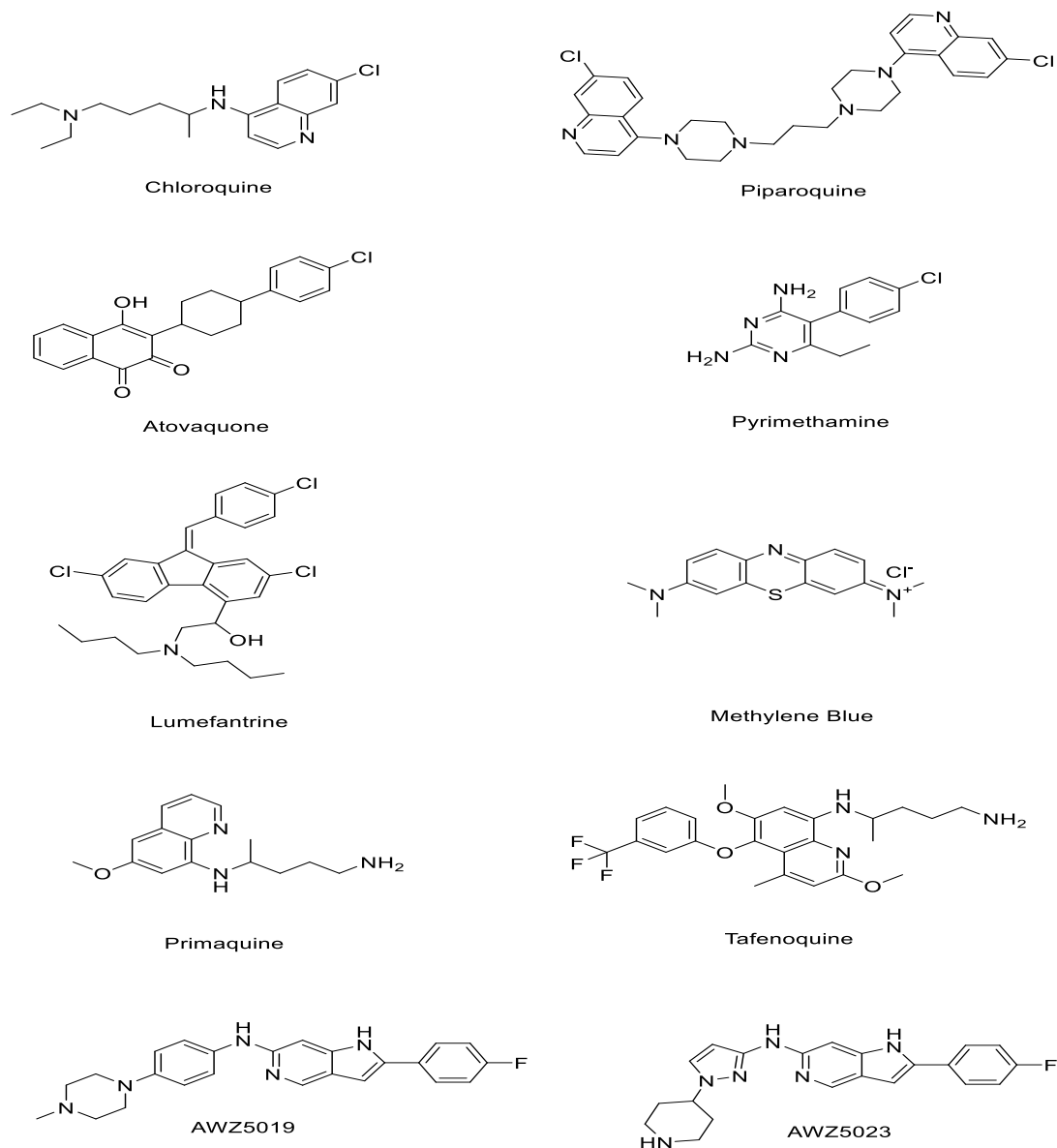


Figure 3.1: Chemical structures of antimalarial drugs used in chapter 3.

3.2.4 Methods:

P. falciparum gametocytes culture and gametocytocidal assay were performed as described in methodology chapter 2, section 2.3 and then PK-PD model were performed as described in chapter 2, section 2.4.

3.3 Results:

3.3.1 *In-vitro* anti-gametocytocidal activity screening of a panel of 13 known & potential antimalarial drugs and clinical candidates at 10 μ M concentration:

To begin the gametocyte luciferase assay, a newly generated luciferase-reporting transgenic line (3D7elo1- pfs16-CBG99) was used to determine the activity of the selected known and potential antimalarial compounds on gametocyte viability at two different stages. The main findings show the distinct stage-specific effects different compounds. MB has been used as a positive drug control in this study. MB exhibits complete inhibition of gametocytes at all stages (Figure 3.2 and Table 3.2).

AWZ compounds (MPS1 inhibitor) used in this chapter resulted in elimination of gametocytes at all stages by about 100%. Two Spiroindolone compounds have also been tested against gametocyte stages. SJ000573359-7 and SJ000571311-10 showed significant activity against early stages by 92% and 86%, respectively. Similarly, both SJ compounds exhibited significant activity against late stages gametocytes by 74% and 70% respectively.

At the concentration tested (10 μ M), lumefantrine resulted in > 50% inhibition of gametocyte viability during early stages. However, the inhibition of late stage gametocytes by the same compound was less pronounced with only 26% inhibition observed. Among the 4-Aminoquinoline tested, CQ & PPQ at 10 μ M did not show significant activity during all stages, however the early stages still showed a more pronounced response in comparison to late-stage gametocytes.

Although PMQ did not exhibit significant inhibitory activity against gametocytes, all tested 8-aminoquinoline compounds showed interesting results as they were more active against late stages in comparison to early stages. TF showed remarkable activity

in late stages (95.7 % inhibition) and in early stages, the drug resulted in inhibiting viability by 85.6%.

ATQ resulted in the inhibition of 21 % of the immature gametocytes stages, whereas its inhibitory capacity against mature late stages was only 10.2% inhibition. The antifolate pyrimethamine did not show any activity against gametocytes at the test concentration (10 μ M). Lastly, aurora kinase inhibitor, AZD3646 exhibited ~50% inhibition of early stage gametocytes, but late stages did not display any significant inhibition by this compound (Figure 3.2, Table 3.2).

The results above have led us to examine the compounds that showed 50 % or more inhibition at 10uM in the luciferase-based assay at a wide range of concentrations which includes clinically relevant blood exposures.

Table 3.2: Gametocytocidal activity of 13 known and potential candidate antimalarials.

Class	Compound	%inhibition \pm SEM	
		Early@ 10 μ M	Late@ 10 μ M
4-Aminoquinoline	CQ	33.4 \pm 3.8	11.4 \pm 2.3
	PPQ	9.2 \pm 2.4	2.5 \pm 1.5
8-Aminoquinoline	PMQ	7.2 \pm 4.1	15.6 \pm 6.4
	TF	85.5 \pm 4.3	95.7 \pm 1.1
Aminoalcohols	Lumefantrine	52.5 \pm 1.8	25.8 \pm 1.2
naphthoquinones	ATQ	21.2 \pm 3.7	10.2 \pm 4.4
Antifolates	Pyrimethamine	4.01 \pm 1.9	2.2 \pm 2.1
Others (thiazine dye)	Methylene blue	97.1 \pm 2.1	98.3 \pm 1.7
Dihydroisoquinolines (Spiroindolones)	SJ000573359-7	92.5 \pm 3.1	74.2 \pm 3.2
	SJ000571311-10	86.7 \pm 2.3	70.5 \pm 3.2
(MPS1) inhibitors	AWZ5019	99.8 \pm 2.1	100.2 \pm 7.5
	AWZ5023	97.5 \pm 7.1	96.8 \pm 9.4
Aurora kinase inhibitor	AZD3646	53.3 \pm 3.7	8.2 \pm 4.6

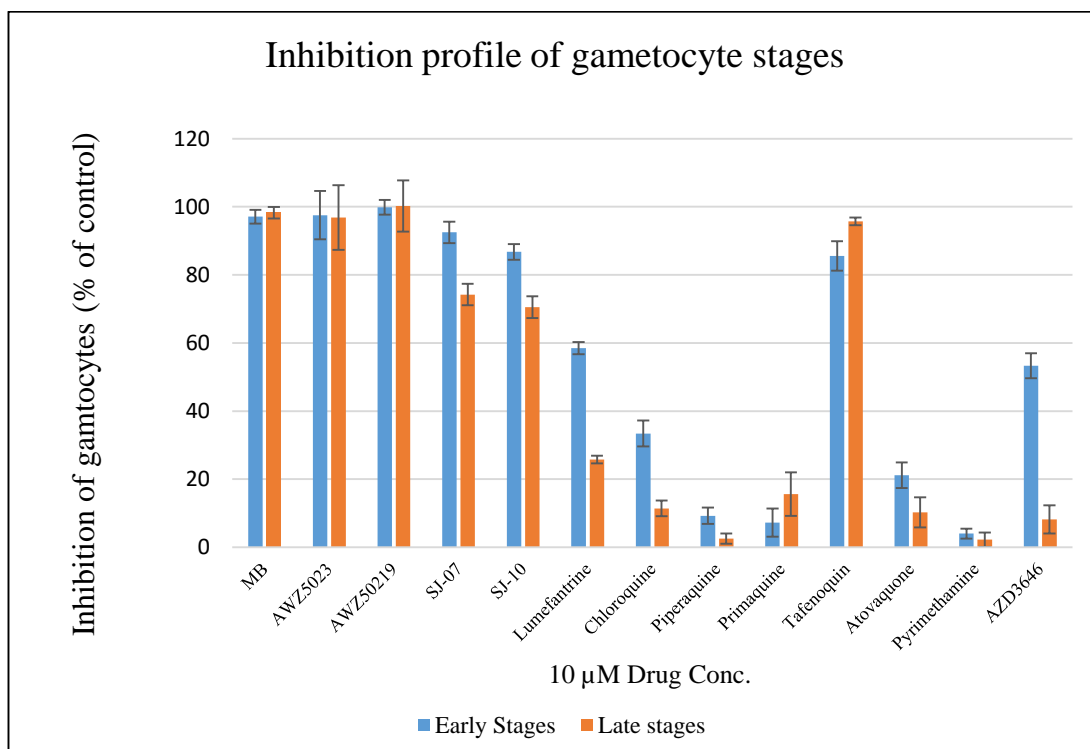


Figure 3.2: Bar chart illustrate the inhibition profile of antimalarial at 10μM against early and late gametocyte stages.

3.3.2 Dose-response of gametocytocidals activity profile during the gametocytes development (Early & late stages):

Amongst the 13 compounds tested at 10uM, 7 showed promising response (>50%) against gametocyte stages (Table 3.2 and Figure 3.2). Therefore, the activity of these potential gametocytocidal drugs was assessed throughout the process of gametocytogenesis by measuring the inhibitory effects at a wide range of concentrations and determined the IC_{50} and maximal kill values at 2 time points: days 2-4 (stage II-III) and days 10-12 (IV-V) during the development of gametocytes after 48 h-long exposures to drugs (section 2.3).

The selected compounds included a number of known antimalarials: MB, lumefantrine and TF. Additionally, less known compounds such as AWZ compounds (MPS1) inhibitors (AWZ5019 & AWZ5023) as well as spiroindolone compounds

(SJ000573359-7 & SJ000571311-10) have also shown >50% activity against gametocytes.

The mean of the $IC_{50} \pm SEM$ values were determined by in-vitro gametocyte viability assay for both early and late stages gametocytes. The relative concentration-response curves of tested compounds in this chapter were illustrated in Figure 3.3, Figure 3.4 and Figure 3.5.

Table 3.3 shows the mean of the $IC_{50} \pm SEM$ values of all tested compounds which was determined by dose-response of gametocytes viability assay for both early and late gametocyte stages. In general, the determined IC_{50} levels against early stage gametocytes (II-III) were significantly lower than IC_{50} levels calculated against late stage gametocytes (indicating a significantly higher potency in eliminating gametocytes during early stages in comparison to late stages). TF, however, showed more active against late stages in comparison to early gametocytes where the IC_{50} of early gametocytes was $6.45 \pm 0.4 \mu M$ and in late stages was $4.8 \pm 0.2 \mu M$.

Table 3.3: IC_{50} values for the gametocytocidal activity of 7 current and candidate antimalarial drugs.

Class	Compound	IC_{50} (nM) \pm SEM	
		Early	Late
Thiazine dye	Methylene blue	$38.05^* \pm 2.7$	622.1 ± 8.8
8-Aminoquinoline	TF	$6450^* \pm 413$	4800 ± 201
Aminoalcohols	Lumefantrine	$187.4^* \pm 6.6$	579 ± 7.3
Dihydroisoquinolines (Spiroindolones)	SJ000573359-7	$467.2^* \pm 3.7$	2361 ± 31
	SJ000571311-10	$586.5^* \pm 2.6$	3150 ± 7.1
(MPS1) inhibitors	AWZ5019	$669.7^* \pm 6.1$	3530 ± 21.1
	AWZ5023	$575.3^* \pm 8.6$	1351 ± 5.5

* indicates $P < 0.05$ (Student's t-test, one-tailed, $n=3$ separate experiments, each in triplicates).

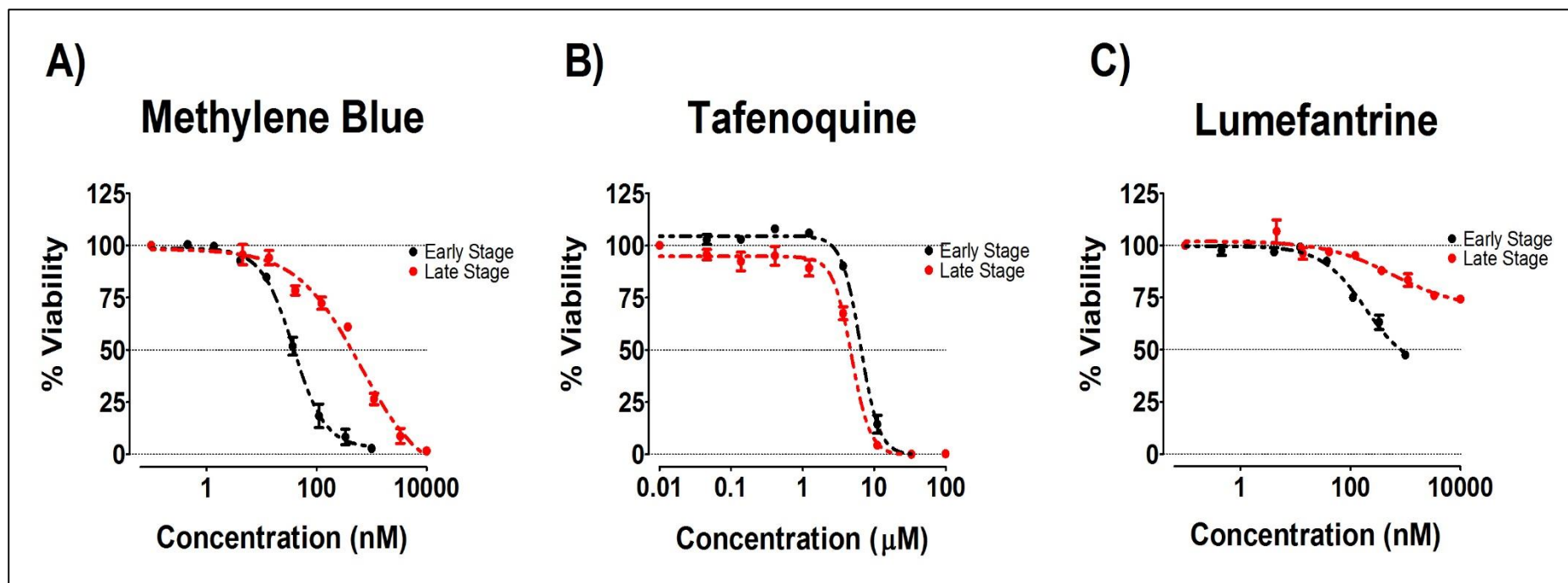


Figure 3.3: Early and late gametocytes stages showing different profile activities.

Typical curves show the dose response of (A) MB, (B) TF and (C) Lumefantrine compounds against gametocyte stages. IC_{50} values were calculated through nonlinear regression using the “log (inhibitor) vs. response -- Variable slope (four parameters)” model in Prism 5. The error bars represent the standard error of the mean (SEM) of three independent experiments each in triplicates (N= 3).

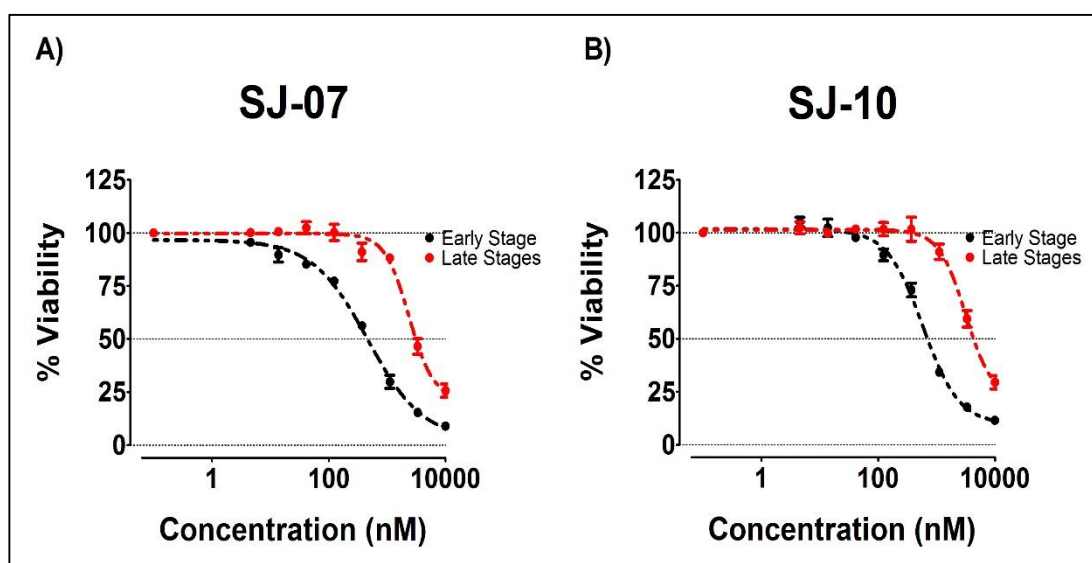


Figure 3.4: Early and late gametocytes stages showing different profiles activity with spiroindolones.

Typical curves show the dose response of spiroindolones (A) SJ000573359-7 or SJ-07 and (B) SJ000571311-10 or SJ-10 compounds against gametocyte stages. IC_{50} values were calculated through nonlinear regression using the “log (inhibitor) vs. response -- Variable slope (four parameters)” model in Prism 5. The error bars represent the standard error of the mean (SEM) of tree independent experiments each in triplicates ($n= 3$).

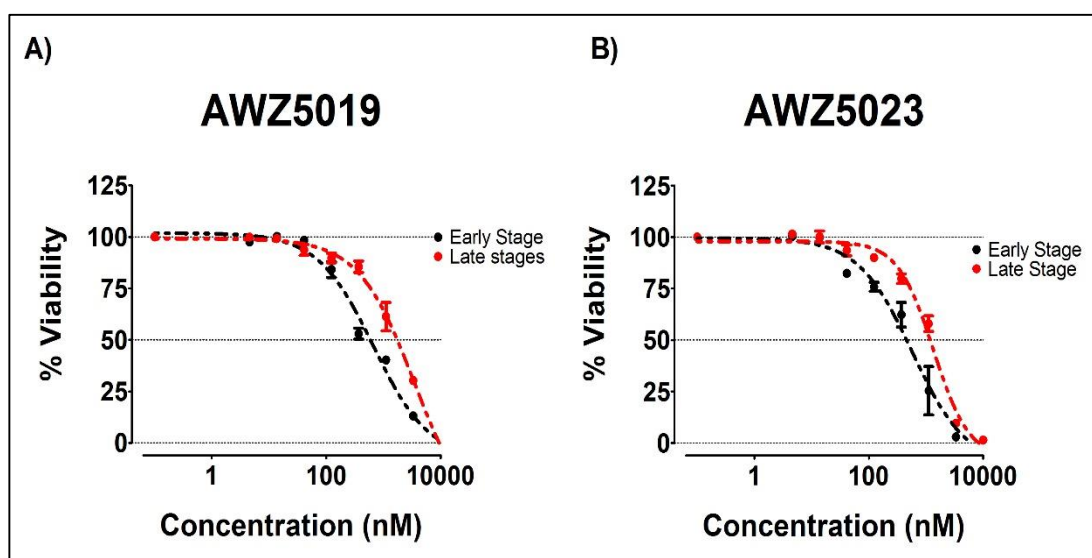


Figure 3.5: Early and late gametocytes stages showing different profiles activity with MPS1.

Dose response curves of (suspected) Mitotic Kinase Monopolar Spindle 1 (MPS1) inhibitors (A) AWZ5019 and (B) AWZ5023 compounds against gametocyte stages. IC_{50} values were calculated by nonlinear regression using the “log (inhibitor) vs. response -- Variable slope (four parameters)” model in Prism 5. The error bars represent the standard error of the mean (SEM) of tree independent experiments each in triplicates ($n= 3$).

The IC_{50} of MB increased significantly ($P < 0.05$) from early stage to late stages by ~18 folds from 38.05 ± 2.7 nM to 622.1 ± 8.8 nM, respectively (Table 3.3 and Figure 3.3A). Lumefantrine demonstrated moderate activity against gametocytes in both stages by reducing the parasite viability to 53.5% and 75.9 %, and the IC_{50} values were 3 fold higher in late stages in comparison to early stages (187.4 ± 6.6 to 579 ± 7.3 nM) as seen in Table 3.3 and Figure 3.3C.

Spiroindolone compounds used in this study demonstrate a significant response to kill gametocytes and showed stage-specific inhibitory responses using our luciferase viability assay. SJ-07 & SJ-10 exhibited higher potency against early gametocytes in comparison to late gametocytes (> 5-fold increase in IC_{50}). The IC_{50} values of SJ-07 is 467.2 ± 3.7 nM in early stages and 2361 ± 31 nM in late stages, whereas SJ-10 IC_{50} values in early and late stages gametocytes were 568.5 ± 2.6 nM; 3150 ± 7.1 nM respectively indicating that SJ-07 is significantly more potent than SJ-10 at inhibiting gametocytes during all stages (Table 3.3 and Figure 3.4).

Similarly, AWZ compounds have also demonstrated significant activity against gametocytes and have also shown a stage-specific inhibitory response with IC_{50} values 3 to 5 times higher in late stages than in early stage gametocytes. The IC_{50} values of AWZ5019 in early and late gametocytes were 669.7 ± 6.1 and 3530 ± 21.1 nM respectively. Additionally, AWZ5023 is more potent against early and late gametocytes than AWZ5019 with IC_{50} of 575.3 ± 8.6 nM and 1351 ± 5.5 nM ($P < 0.05$) as shown in Table 3.3 and Figure 3.5.

Figure 3.3, Figure 3.4 and Figure 3.5 demonstrate the discrepancy in activity against early and late stage gametocytes for all drugs.

3.3.3 Identification of kill rates and pharmacodynamic parameters of drugs:

Of all tested known and potential antimalarial drugs, MB was selected to perform time-dependent killing assays, as it showed the highest potency against all gametocyte stages, and because it has been used for malaria therapy in patients. The IC_{50} level for MB against late stage gametocytes was also clinically relevant: $(622.1 \pm 8.8 \text{ nM})$, indicating a possibility for the drug to significantly reduce gametocytes in patients when administered at standard doses. The determination of kill rates for MB was done as described in chapter 2 section 2.4.1.

The dynamics of gametocyte kill exerted by different drugs was systematically characterised by analysing the effects of MB upon gametocyte counts at a range of clinically relevant concentrations (10, 8, 4, 2, 1, 0.5, 0.25, 0.1, 0.05 and 0.001 μM) over discrete time intervals (0, 6, 12, 24, 48, 72, 96 h). The kill rate exerted by each concentration was calculated using Prism 5 for windows through nonlinear regression using the “plateau followed by one phase decay” built-in equation as shown below in Equation 1 (Figure 3.6).

$$\text{Gametocyte count} = (G_0 - \text{Plateau}) * e^{(-k \cdot t)} + \text{Plateau} \quad \dots \text{Eq. 1}$$

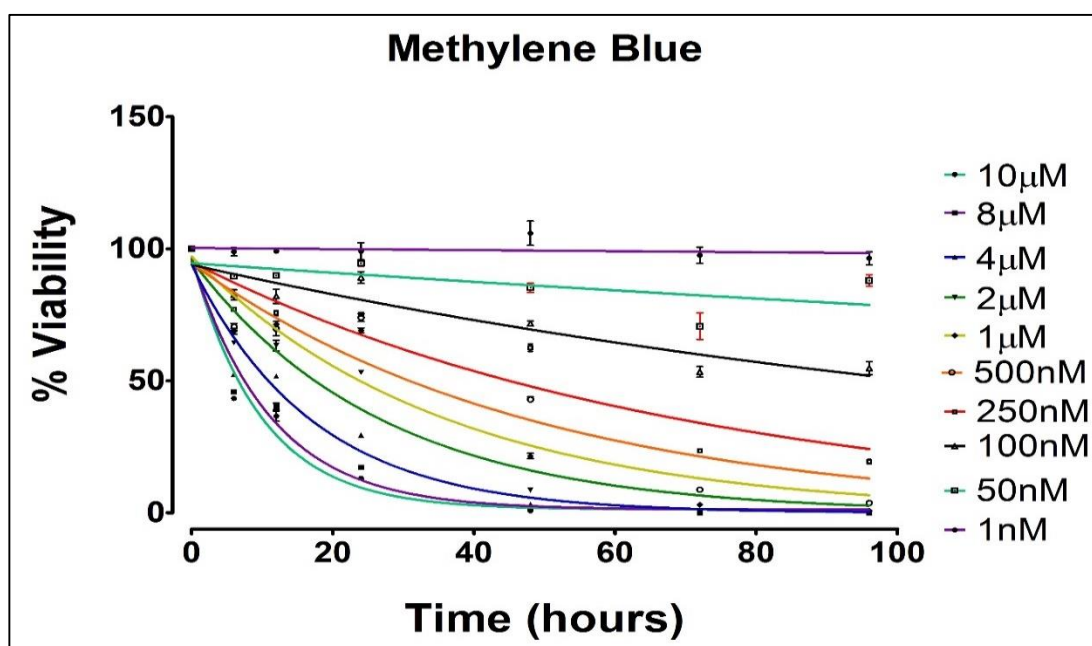


Figure 3.6: Time-dependent killing assay of MB at different concentration against late stages gametocytes over discrete time intervals.

Data show mean +/- SEM from three independent experiments performed in triplicate.

Table 3.4: The kill rate measurement achieved by each concentration of Methylene blue.

MB conc.	Kill rate ($K h^{-1}$)	SEM	N
10 µM	0.101017	0.007560	3
8 µM	0.1019	0.008613	3
4 µM	0.08903	0.008214	3
2 µM	0.0581	0.007143	3
1 µM	0.03712	0.006749	3
500 nM	0.02784	0.007657	3
250 nM	0.02063	0.009032	3
100 nM	0.01425	0.007433	3
50 nM	0.006944	0.008742	3
1 nM	0.002473	0.02483	3
0	0.0002024	0.002584	3

As can be clearly seen in Figure 3.6, there was no delay or (lag time) on the effect of gametocyte kill starting from the first drug exposure. After measuring the kill rate (k) achieved by each concentration of MB (Table 3.4), the overall activity of the drug is then assessed using a sigmoidal E_{max} model which calculates the EC_{50} for MB as well as its maximal kill rate (E_{max}). The kill rates were fitted using Prism 5 by applying the log (agonist) vs. response (three parameter) built-in equation in Prism as shown below in equation 2:

$$\text{Kill rate (hr}^{-1}\text{)} = E_{min} + \frac{(E_{max}-E_{min})}{1+10^{\text{LOG}(EC_{50}-C)}} \quad \dots \text{Eq. 2}$$

The EC_{50} of MB for the E_{max} model as well as the E_{max} were determined. MB EC_{50} equal to 3.22 μM with 95% confident interval CI (2.132, 4.853) μM and the E_{max} value which represents the maximal kill rate is 0.11 h^{-1} (Figure 3.7).

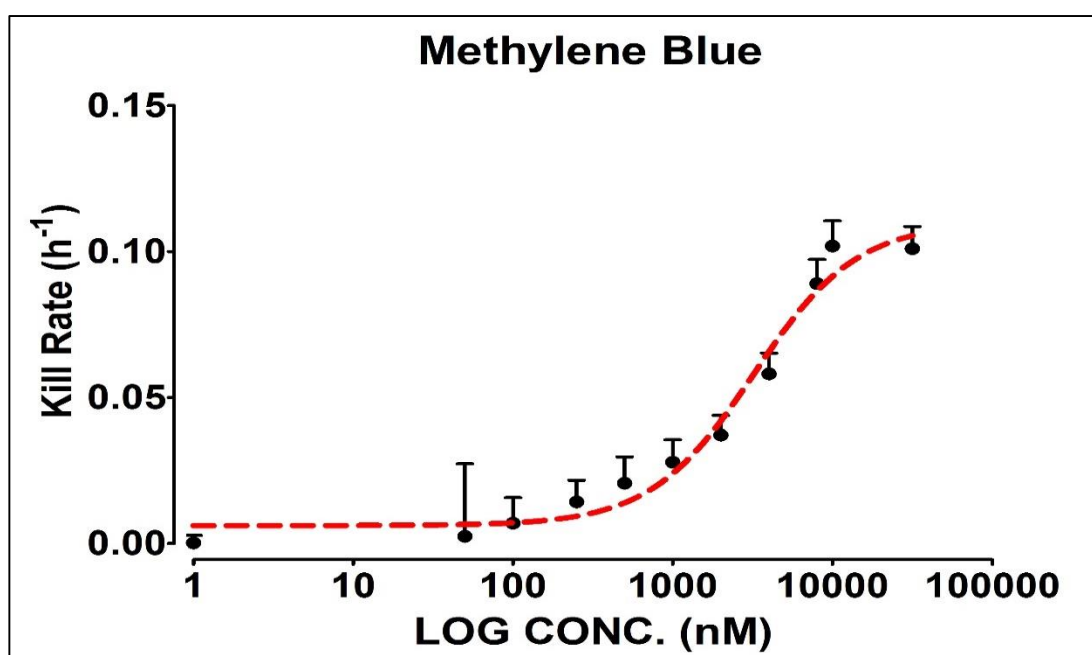


Figure 3.7: Model of pharmacodynamics of MB to determine the kill rate per h.

The figure shows relationship between MB concentration (X axis) and the kill rate exerted at each concentration (Y axis). The red line shows the sigmoidal fit of the data which is used to calculate an EC_{50} and an E_{max} value. Data show mean \pm SEM from three independent experiments performed in triplicate.

3.3.4 PK-PD modelling and predictions of clinical drug activity:

3.3.4.1 Pharmacokinetic profile of MB in human:

The clinical activity prediction of MB against late gametocyte stages were generated using Monte-Carlo simulations using the program Pmetrics® (Neely et al., 2012b). The PK parameters of MB were collected as reported in clinical PK studies in the literature as shown in Table 3.5 using a 2 compartment PK model defined by the following two differential equations:

$$\frac{dX_1}{dt} = -k_a \cdot X_1 \quad \dots\dots \text{Eq. 3}$$

$$\frac{dX_2}{dt} = k_a \cdot X_1 - (k_e + k_{cp}) \cdot X_2 + k_{pc} \cdot X_3 \quad \dots\dots \text{Eq. 4}$$

$$\frac{dX_3}{dt} = k_{cp} \cdot X_2 - k_{pc} \cdot X_3 \quad \dots\dots \text{Eq.5}$$

Where (X_1) represents drug mass in the gut in grams, K_a the rate of absorption, (X_2) the mass of drug in blood, (X_3) the mass of drug in the peripheral compartment, K_e the rate of elimination, K_{CP} rate constants for transit from central to peripheral compartments, K_{PC} the rate peripheral to central compartment, t sand (t) the time in h. The drug mass of MB in blood was converted to concentration in molar using the following equation:

$$\text{Drug conc. } (M) = \frac{X_2/V_d}{Mwt} \quad \dots\dots \text{Eq.6}$$

Table 3.5: PK parameters of MB for adult patients as reported in the literature.

Parameter	Predictable value	References
Dose (mg)	500mg given twice daily for 3 days	(Walter-Sack et al., 2009)
Central Volume distribution/F (V _c) [L]	33.4	
Peripheral Volume of distribution/F (V _p) [L]*	209.7	
Inter-compartmental Clearance (Q/F) [L/hr] **	20.3	
Central Clearance/F (CL) (L/h)	8.4	
Absorption rate (k _a) (h ⁻¹)	0.72	

$$* Q = K_{CP} \cdot V_c$$

$$** V_p = \frac{Q}{K_{PC}}$$

The PK exposure simulation in a population of 1000 people with 30% variation of PK parameters was used for the determination of PK profile in a typical human population. Solid black line in Figure 3.8 shows the median PK exposure whereas dashed red lines show the 5% and 95% of the population from the same simulation. Peak concentration (C_{max}) and time to reach maximum concentration (T_{max}) can be determined from the data of the PK profile as follows: median C_{max} of MB is 18.1 μM and T_{max} value of 2.0 h. The threshold of MB activity shown in Figure 3.8 starting from IC_{50} level as dashed in blue. The dug concentration can reach the IC_{50} levels with a safe dosing level (500mg twice daily for 3 days).

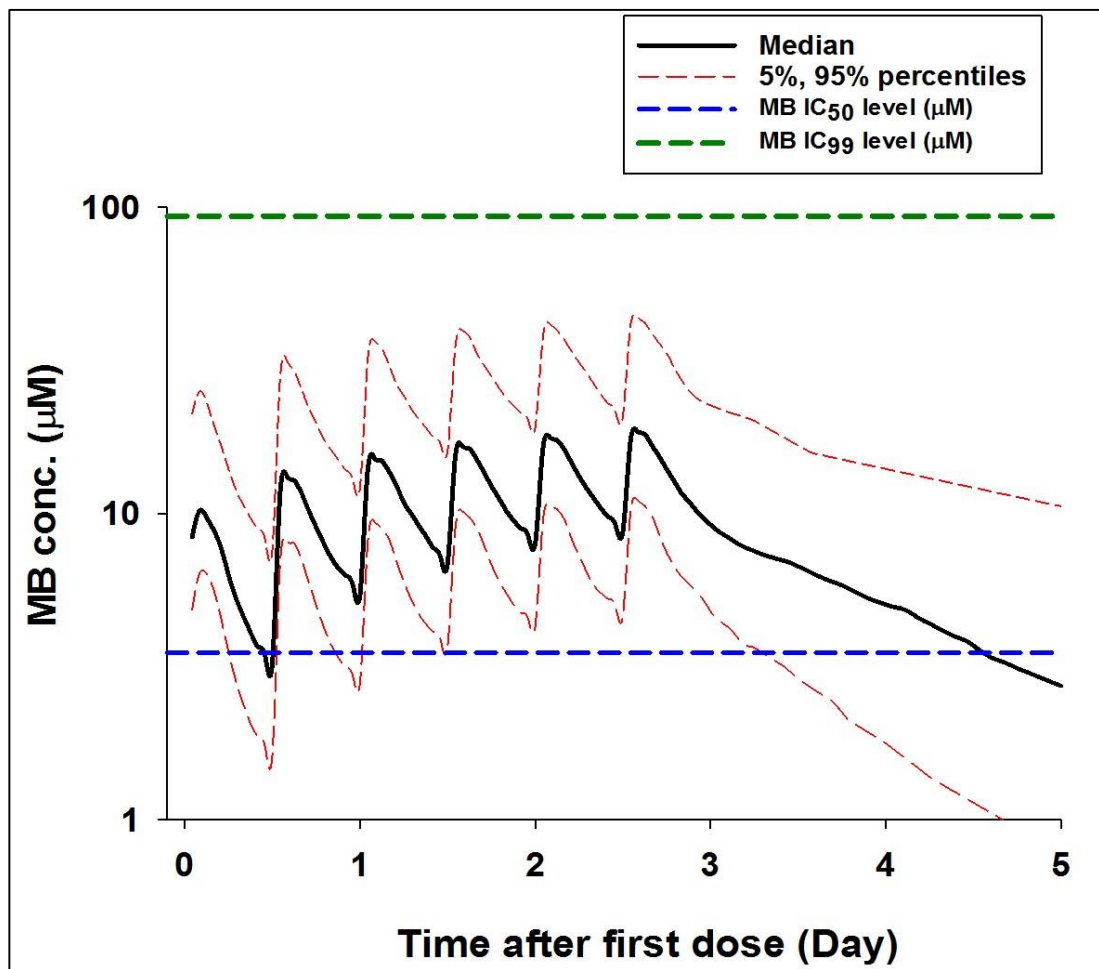


Figure 3.8: Simulation of PK exposure in a population of 1000 people given a 30% variation on all reported PK parameters of MB. Solid black line shows the median PK exposure whereas dashed red lines show the 5% and 95% from the same simulation. IC_{50} and IC_{99} levels of MB are shown in the graph as dashed blue and green lines, respectively.

3.3.4.2 The prediction model of MB effect on late gametocytes:

The prediction model of MB kill profile on late gametocytes clearance is then defined by linking the dynamic concentration (calculated using equations 3-5 for PK profile) to the sigmoidal pharmacodynamic profile (Figure 3.7) which has been characterized in the previous section in equation (2). The final results plotted for the prediction model showing the median profile for gametocyte kill in addition to the 5% and 95% percentiles to show the range given pharmacokinetic variability. The prediction graph of PK-PD relationship of MB shows dramatic levels of gametocyte reduction, achieving complete depletion within less than 3 days (Figure 3.9).

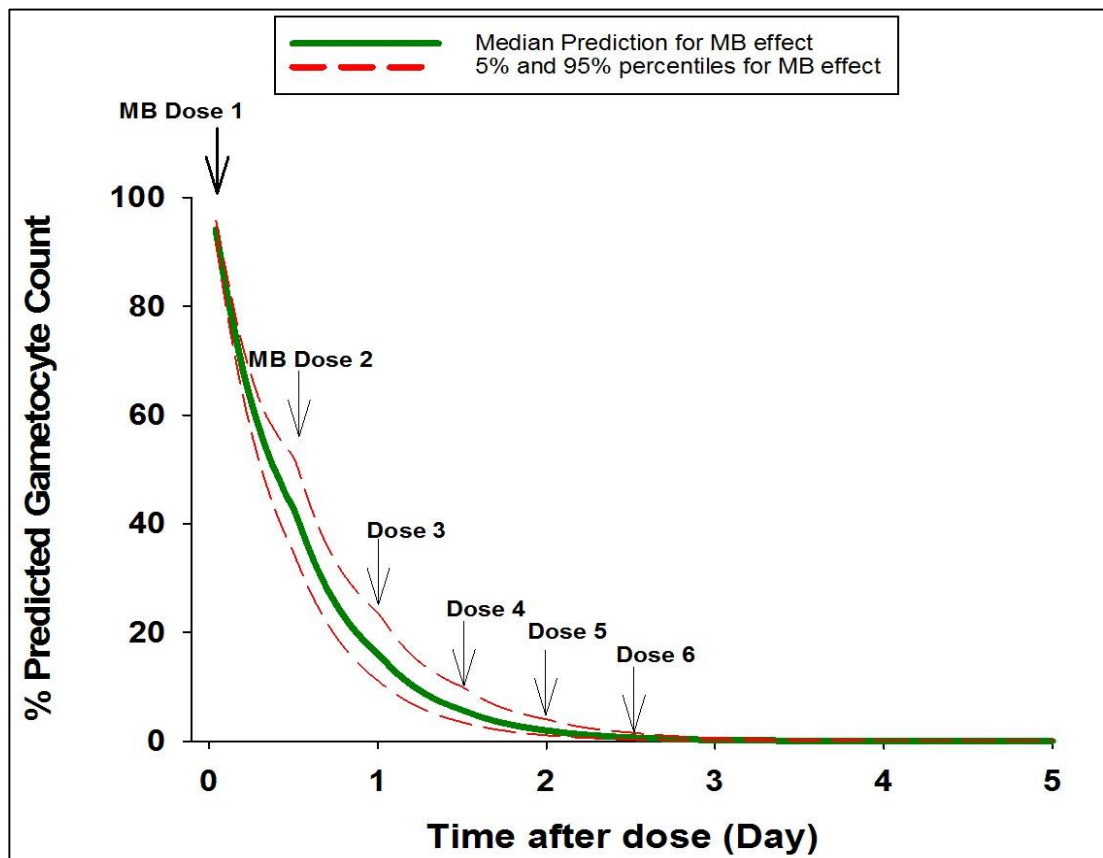


Figure 3.9: PK-PD relationship of MB and its effect upon late stages gametocyte for three day-dosing regimens (every 12 h).

The figure shows a simulation of MB effect upon gametocytes with a standard dose of 500mg twice a day for 3 days given the PK and PD properties reported earlier in this chapter. The green line shows the median effect upon gametocyte in a population of 1000 people while the dashed red lines show the 5% and 95% percentiles of the same population.

3.4 Discussion:

The sustainability of malaria control needs combination of the treatment schemes targeting pathogenic malaria form (asexual) and transmission gametocyte form (sexual). The efforts to find gametocytocidal compounds are disrupted by the limited understanding of the fundamental biology of gametocytogenesis. During Gametocyte development, the parasite morphologically differentiates and tends to be less metabolically active during this process (Peatey et al., 2011). Also, a replication processes does not occur in the gametocyte genome in all stages as gametocyte arrested in phase G₀ of cell cycle (Raabe et al., 2009). The action nucleic acid synthesis is more likely limited to RNA synthesis and this is genetically proven by the haploid of gametocytes. After day 6 of gametocytogenesis the RNA synthesis is terminated as reported in previous studies, leading to the lack of Hb digestion or protein synthesis in late stage gametocytes (Canning and Sinden, 1975, Sinden et al., 1978).

Here, the aim was to determine the pharmacodynamic response (*in-vitro*) of selected known antimalarials and a panel of new inhibitor compounds against *P. falciparum* gametocyte-specific stages. In addition, a pharmacodynamic sigmoidal model of MB was developed to measure the gametocytes kill rate to use it for the PK-PD simulation methods to predict the efficacy of MB as gametocytocidal in malaria patients.

3.4.1 Panel of known antimalarials activity as gametocytocidal:

The selected reference antimalarial compounds that were tested using our luciferase-based assay showed significant agreement with other recently published existing assays. Our results using MB, showed that this compound displayed a high potency against all gametocyte stages. Interestingly, we observed that the gametocytocidal activity of MB against early stage gametocytes is more potent than that against late

stages, with IC₅₀ concentrations of approximately 38 nM and 622 nM, respectively. MB is used mostly in humans for methemoglobinemia, where the ferrous ion (Fe²⁺) undergoes an oxidation process and is converted to ferric (Fe³⁺). MB treats this by acting as an electron donor which reduces the oxidation. In the parasite, the actual mode of action of MB is still ambiguous (Vennerstrom et al., 1995, Schirmer et al., 2003). One hypothesis is that MB interferes with the oxidation-reduction cycling of glutathione reductase (Adjalley et al., 2011b). However, this hypothesis was not supported in one study which demonstrated that MB retains significant activity against *Plasmodium* parasites in which the antioxidant enzyme (glutathione reductase) is missing (Pastrana-Mena et al., 2010).

MB is considered chemically as a weak base and can accumulate in acidic organelles such as the digestive vacuole (Goodman et al., 2011). Other studies suggest that MB interferes with the formation of hemozoin in *Plasmodium* food vacuole (Schirmer et al., 2003). The asexual *P. falciparum* parasite is assumed to share its major metabolic activities with early stage gametocytes with respect to Hb degradation (Sinden, 1982). Although this could interpret the high sensitivity of MB to kill the early stage gametocytes, the mechanism of action would be similar to CQ (4-aminoquinoline) as it is thought to be causative agent supporting the significant activity of 4-aminoquinoline drug class. However, the mode of resistance of 4-aminoquinoline, defined by PfMDR1 and PfCRT (Sa et al., 2009, Valderramos et al., 2010), doesn't convey cross-resistance to MB (Vennerstrom et al., 1995, Pascual et al., 2011).

In mature gametocytes, the male and females are in cell cycle arrest and are effectively quiescent in blood circulation and ready for uptake by mosquitos during a blood meal (Sinden and Smalley, 1979). Consequently, once the gametocyte becomes entirely mature and infectious, its metabolic activity is concentrated (reduced) to the main

housekeeping metabolic processes such as redox activity and generation of ATP (Tanaka and Williamson, 2011, Lelievre et al., 2012). Hb digestion will also stop according to Hanssen and colleagues in their previous study to measure the Hb content by soft X-ray microscopic analysis (Hanssen et al., 2012). This is supported by the idea of the different mechanisms of action underlying MB activity against late gametocytes, as the drug still exhibit significant activity in late stage gametocytes by $IC_{50} > 17$ fold lower than that in early stages (Table 3.3).

Lumefantrine is amino alcohol antimalarial used as partner drug in Coartem, the most commonly used ACT (Wells et al., 2009). Lumefantrine displayed moderate gametocytocidal activity against early stages of gametocyte development by inhibiting 52 % of gametocyte viability. This is consistent with a recent study using GFP-luciferase reporters as viability assay (Adjalley et al., 2011b). In mature late gametocytes, however less gametocytocidal activity was exhibited by lumefantrine compared to the early stages with about 25 % inhibition of the gametocytes viability at 10 μ M (Figure 3.3C).

The significant inhibition of gametocytes viability with lumefantrine was at concentration (10 μ M) approximately equal to the average peak plasma concentration in malaria patients which was described in previous studies to be about 11 μ M and half-life of 4-5 days (Eastman and Fidock, 2009, Djimde and Lefevre, 2009). Interestingly, lumefantrine has been observed to significantly inhibit *P. falciparum* oocyst numbers in mosquitoes fed on drug-treated gametocytes (Adjalley et al., 2011a) and to also moderately inhibit the oocyst production in a rodent malaria model (Delves and Sinden, 2010). These data indicate that the decrease in the number of oocysts by lumefantrine may not only inhibit exflagellation of male gametocytes, but lumefantrine may also have an influence upon female gametocytes, gamete formation,

and the development of oocysts in mosquitos through a hitherto unknown mechanisms of action.

PMQ is an 8-aminoquinoline that is widely used to treat *P. vivax* and *P. ovale* malaria infection and is also used to prevent relapse cases as it has efficacy against hypnozoites or liver stages (Bousema and Drakeley, 2011). A single dose of PMQ given to malaria patients with ACT can significantly reduce the gametocyte carriage period (Smithuis et al., 2010a, Bousema et al., 2010). Here, PMQ does not display significant inhibitory activity against gametocytes at 10 μ M. However, PMQ is rapidly metabolised to different reactive intermediates which are supposed to be responsible for antimalarial activity as well as erythrocytes (haemolysis) toxicity (Idowu et al., 1995, Vale et al., 2009). Therefore, the Data are consistent with the hypothesis that the parent compound does not possess inhibitory activity and that inhibitory activity is only displayed by the metabolites of PMQ. The inhibitory activity of PMQ metabolites is explored in more detail in Chapter 5.

The gametocytocidal activity of TF was also examined. TF is 8-aminoquinoline compound that is a promising clinical candidate with potentially less toxicity and possessing a longer half-life compare to primaquine, at 14 days and 5 h respectively (Mihaly et al., 1984b, Ward et al., 1985, Brueckner et al., 1998). Our results showed gametocytocidal activity for TF in all gametocytes stages compared to PMQ. Also, it is noted that the potency of TF against late stage gametocytes is significantly more pronounced than its activity against early stages. TF and its metabolic activity against plasmodium transmission stages will be further investigated and described in Chapter 6.

The 4-aminoquinoline CQ, (former first line malaria therapy), and PPQ are reported to exert gametocytocidal activity only against early stage gametocytes, as their mode

of action allows the drug to inhibit the hemozoin formation in parasite digestive vacuole (Butcher, 1997, Peatey et al., 2009, Buchholz et al., 2011, D'Alessandro et al., 2016b). The luciferase-based assay used in our study is consistent with these reports and shows a moderate, albeit not significant, activity against early stages gametocytes with only 33 % inhibition of the viability and no activity against mature late stages. PPQ, a bisquinoline which is used as a partner drug with the first line malaria drug ACT, failed to demonstrate significant activity upon early and late stage gametocytes. The luciferase viability assay used in our experiment has been used in the study of D'Alessandro and her colleagues as they have shown excellent gametocytocidal activity of CQ and PPQ against early gametocytes in contrast to what we have previously described in this chapter (D'Alessandro et al., 2016b). The incubation period of drug exposure used in their work was 72 h compared to 48 h incubation in our results. This could interfere with the activity of drug in the final readings. Consequently, we should mention that the immature stage gametocytes are assumed to share metabolic activity (Hb digestion) with asexual stages (Sinden, 1982) which is the targeted mode of action of 4-aminoquinolines. Another study according to Hanssen and his colleagues confirms that early gametocytes still generate and maintain the Hb digestion (main metabolite pathway) by 50% whereas in later stage III digestion appears to be completed in mature stages (Hanssen et al., 2012).

Enzymes involved in nucleotide synthesis pathways, such as dihydrofolate reductase are not active after gametocytogenesis induction, where DNA replication is absent. This is supported by the failure of the antifolate drug, pyrimethamine, to exert gametocytocidal activity against all gametocytes stages in our data and in the recently published studies in the literature using luciferase-based assay and pLDH (D'Alessandro et al., 2016b).

ATQ is naphthoquinone antimalarial that targets the mitochondrial respiratory chain by inhibiting cytochrome *bc*₁. This pathway plays a significant role in cell proliferation (Fleck et al., 1996). In this study, ATQ did not display gametocytocidal activity against any gametocyte stages. These results are in agreement with recent studies that describe a reduction in the mitochondrial metabolite activity of sexual stage gametocytes (Adjalley et al., 2011a, D'Alessandro et al., 2016b).

3.4.2 Activity of recent potential anti-malarial compounds against gametocytes:

A number of potential antimalarial compounds have been tested for the first time against *P. falciparum* gametocyte stages. Those include two spiroindolones, two MPS1 inhibitors and one aurora kinase inhibitor, and it is worth mentioning that all of them were tested against asexual stages (in house) and showed significant activity as antimalarials against asexual stages. Although membrane transport inhibitors are generally less effective to treat infectious diseases, spiroindolone, a recent compound in a new generation of antimalarial compounds (Rottmann et al., 2010, White et al., 2014) which targets Na⁺-efflux ATPase (PfATP4) in parasite plasma membrane and disrupts Na⁺ haemostasis (Spillman et al., 2013), has confirmed the susceptibility of ion transport mechanism of parasites to chemical drug attack.

SJ000573359-7 and SJ000571311-10 have already been investigated in house against asexual stages and demonstrated significant activity with *IC*₅₀ values at nanomolar ranges: 1.5 and 10.2 nM respectively. In our results, both compounds showed significant activity at 10 μM being more active against early stages compare to late gametocytes. The shift in *IC*₅₀ values from asexual stages to sexual stages in more than 100 fold, however the sensitivity of these compounds against transmission stages indicate the consistency of PfATP4 being essential in sexual stages. As those compounds have been tested for the first time against gametocytes and displayed

gametocytocidal activity, a previous study showed that the spiroindolone compound (KAE609) also inhibits gametocytes development as well as oocyst formation (van Pelt-Koops et al., 2012), indicating the potential of this class to be gametocytocidal. The MPS1 inhibitors (azaindoles, potential kinase) AWZ compounds were investigated against gametocyte stages for the first time, and they have displayed good activity in asexual stages (in house). Interestingly, there is no MPS1 in *P. falciparum*, but the suspected MPS1 inhibitors demonstrate significant activity as antimalarial class in all intraerythrocytic stages. The activity of kinase inhibitors shares high levels of structural similarity to the 1H-pyrrolo [3, 2-c] pyridine scaffold (Naud et al., 2013). A preclinical aurora kinase inhibitor (AZD3646) has also been tested against gametocyte stages. It displayed moderate activity against early stages gametocyte (at high concentration, 10 μ M) and no effect against late stages. The aurora kinase contains serine/threonine kinases which is important for regulation of the cell cycle in mitotic processes (Bischoff et al., 1998, Giet and Prigent, 1999, Bavetsias and Linardopoulos, 2015). As a result, gametocytes are not able to respond to this kind of inhibitors as no replication processes occur in gametocyte genome in all stages as gametocytogenesis arrested in phase G₀ of cell cycle (Raabe et al., 2009).

3.4.3 PK-PD Modelling of MB towards Gametocytes Clearance:

In this chapter, our data revealed that MB demonstrated high potency as a gametocytocidal compound in all stages *in-vitro*. MB has also been able to reduce the mosquito infection prevalence dramatically by reducing the oocyst numbers (Adjalley et al., 2011a). In 1891, the antimalarial properties of MB that has been reported by the chemist Paul Ehrlich as the first synthetic drug used in clinical treatment (Guttman and Ehrlich, 1891, Schirmer et al., 2011). Recently, the interest of using MB in

combination with other antimalarial agents has been increasing to assess its advantage in reducing gametocyte carriage (Coulibaly et al., 2009, Bountogo et al., 2010).

Therefore, we have studied the pharmacodynamic profile of MB upon gametocyte clearance by studying its activity against gametocytes across a wide range of clinically relevant concentrations. A simulation method of the PK-PD model, which could provide a good understanding of drug efficacy on mature stages gametocyte has been developed in this study. MB was selected from all of the tested compounds because of its significant activity against all gametocyte stages; MB is also already clinically registered compound with known and published PK parameters.

The pharmacodynamics of gametocyte kill were systematically characterised by analysis of the effect of MB drug upon gametocyte counts at a range of clinically relevant concentrations over discrete time intervals. For the desirable PD model, PD parameters derived from the time-killing dependent assay were conducted to define gametocyte kill rate and calculate E_{max} and IC_{50} . The dynamic measurement shows a significant reduction of gametocyte viability starting from the first time point of 6 h. This reflects the high potency of MB against *P. falciparum* gametocytes even in late stages. PK parameters of MB were collected from the literature to identify the concentration-time profile modelled using a two-compartment PK model (Walter-Sack et al., 2009). During the gametocytogenesis, the gametocytes tend to be sequestered somewhere in the body (usually bone marrow and spleen) and once they mature to late stages, they are released to the bloodstream and become ready to be infectious to mosquitoes (Smalley and Sinden, 1977, Smalley et al., 1981, Rogers et al., 2000). Here, we are targeting the drug plasma concentration (C_P) in blood circulation, as gametocytes exist in the peripheral bloodstream, by using a two-compartment PK model.

Using aqueous formulation of oral MB, the drug can readily and safely achieve blood levels that are higher than MB's IC50 levels identified *in-vitro* (Walter-Sack et al., 2009). Linking the activity of MB as constructed in the PD model (Figure 3.7) to its simulated PK profile (Figure 3.8) results in a PK-PD model that shows the potential activity of this drug against gametocytes (Figure 3.9). The PK-PD simulation predicts a complete elimination of gametocytes from the blood stream in less than 3 days when administered twice daily at a dose of 500mg using the aqueous oral formulation.

Due to the limited clinical studies of measuring the effect of MB on transmission stage, the agreement of whether MB could have the ability to help reducing the gametocyte carriage or not is still debatable. One recent study from Burkina Faso detected strong clinical efficacy of MB-AQ when trialled in children with *P. falciparum* in uncomplicated malaria (Zoungrana et al., 2008) and another study suggested moderate effect of MB as monotherapy in reducing gametocyte carriages (Bountogo et al., 2010).

3.4.4 Conclusion:

In this chapter, we investigated some reference antimalarial drugs and new potential antimalarials against intraerythrocytic transmission stages and measured their activity as gametocytocidal compounds. All active compounds against gametocytes demonstrate stage-specific activity during gametocytogenesis development. Briefly, MB, spiroindolones and MPS1 inhibitors showed significant gametocytocidal activity at all stages. TF is the only 8-aminoquinoline that demonstrates gametocytocidal activity during all gametocyte stages, however TF was more potent against late gametocytes in comparison to early gametocytes which is the contrary from all other compounds as they have activity in early stages more than late stages. Lumefantrine displayed moderate gametocytocidal activity upon early stages by inhibiting 52 % of

gametocytes viability and about 25 % inhibition of the mature late stages. This suggests that lumefantrine as a partner drug of choice with artemisinin (Coartem), the most commonly used ACT (Wells et al., 2009), would display moderate gametocytocidal activity. 4-Aminoquinoline tested compounds, ATQ and antifolate (pyrimethamine) failed to display good activity against gametocytes, apart from CQ which exhibited about 50 % inhibition against early gametocyte stages.

A time-dependent killing assay has been developed and conducted to measure the exposure-effect relationship of MB on *P. falciparum* gametocytes. Thereafter, the PK/PD relationship of MB in a clinical context has been established to estimate the rate of clinical gametocyte clearance with the clinical treatment dose and shown to result in a 100% reduction of gametocytes from the blood stream in less than 3 days.

Chapter 4

***In-vitro* pharmacodynamics of antimalarial endoperoxides
against *P. falciparum* gametocytes and PK/PD validation of
DHA**

4.1 Introduction:

There have been major strategic alterations in international efforts to control malaria, and eradicate the disease. This major shift has revealed significant knowledge gaps. From this perspective, the identification of the stage specificity of new potent antimalarial drugs is being prioritised. Effective malarial disease elimination programmes must aim at reducing gametocyte carriers at the critical disease transmission stages.

Endoperoxide compounds, such as artemisinin and its derivatives, are considered to be the most important antimalarial drugs and the first line of defence against malaria, currently including all drug resistant malarial parasite strains (Winzeler and Manary, 2014). Due to the effectiveness of this class of drug, the following question arises: Does this drug class help to decrease the prevalence of malaria by targeting the transmission stages in the human body? A suggested answer is that all artemisinin combination therapies (ACTs) have reasonable effects, with some variation, on gametocyte carriage (Bousema et al., 2006, Group, 2016). However, it remains unclear as to whether the difference in gametocytocidal activity is directly related to the endoperoxides themselves or to the combination of antimalarial agents used in combination chemotherapy (Group, 2016).

Chemically, artemisinin is a tetracyclic 1,2,4-trioxane comprising an endoperoxide bridge but without the nitrogen ring system that exists in most antimalarial compounds (Meshnick et al., 1996, Van Geldre et al., 1997). The key pharmacophore of the artemisinin endoperoxide bridge was identified and is believed to be entirely responsible for the drug's mode of action (Klayman, 1985, Avery et al., 1993, Avery et al., 1996). To avoid the issue of solubility and enhance the pharmacological

properties of the parent drug, artemisinin, semi-synthetic artemisinin derivatives have been synthesised based on a similar backbone and alterations at the C₁₀ position, producing either ether or ester derivatives, then most notable of which are the clinically deployed drugs artesunate and artemether. The active metabolites of these semi-synthetics being dihydroartemisinin (DHA) (Figure 4.1).

Due to the emergence of drug and parasite resistance against most antimalarials, now including artemisinins (Jambou et al., 2005), extensive studies have been conducted to synthesise and generate novel classes of endoperoxides, such as OZ439 (trioxlane), RKA-182 and the recently developed tetraoxane, TDD-E209, based on the important peroxide bridge structure (O'Neill et al., 2010a, Charman et al., 2011). In 2011, RKA-182, a '1,2,4,5-tetraoxane' developed by the Liverpool group, was scheduled for preclinical development in phase I trials (Marti et al., 2011). However, some safety concerns (Copple et al., 2012) and the very short predicted half-life in man prompted the search for an alternative. In response to this challenge, TDD-E209 was developed (Wells et al., 2015).

In this chapter, we investigate the potential activity of three different classes of endoperoxides—the semi-synthetic artemisinins (plus the parent), the trioxolanes and the tetraoxanes—during gametocytogenesis at the malarial transmission stage. A highly sensitive luciferase-based gametocyte assay method was used in this study. This assay uses a newly generated luciferase-reporting transgenic line, and a non-lysing D-luciferin substrate formulation (D'Alessandro et al., 2016a). The method was selected to assess the susceptibility profile of endoperoxides in terms of their structure activity and the ability of the assay to allow the measurement of gametocyte stage-specific sensitivity and development as well as the kill rate.

Pharmacodynamic response curves and IC_{50} values were established using a series of endoperoxide compounds: artemisinin, semi-synthetics DHA, artesunate and artemether, and fully-synthetic ozonide OZ439 and the tetraoxane TDD-E209. In addition, the kill rate of the most potent endoperoxides was determined using a time-dependant killing assay using the variables of exposure time and drug concentration. Finally, a predictive pharmacokinetic/pharmacodynamic (PK-PD) model was developed using gametocyte counts in order to facilitate a broader understanding of the DHA bioactivity at the transmission stage in humans.

4.2 Material & Methods:

4.2.1 Reagents:

P. falciparum 3D7elo1-pfs16-CBG99 were generously provided by Prof Pietro Alano lab (INBB, Istituto Nazionale di Biostrutture e Biosistemi, 00136 Rome, Italy). Artemisinin and its semi-synthetic derivatives (DHA, artesunate and artemether) were purchased from TCI. The fully synthetic endo-peroxides (OZ439 and TDD-E209) were kindly provided in house from our medicinal chemistry team. The chemical structures of these compounds are shown in Figure 4.1.

4.2.2 Test Compounds:

Table 4.1: Endoperoxides antimalarial drugs that are used in this chapter.

Class	Compound	Molecular weight g/mol	Solvent
Endoperoxides	Artemisinin	282.332	DMSO
	OZ439	469.622	
	TDD-E209	501.63	
	DHA	284.35	
	Artesunate	384.421	
	Artemether	298.374	

4.2.3 Compounds chemical structures:

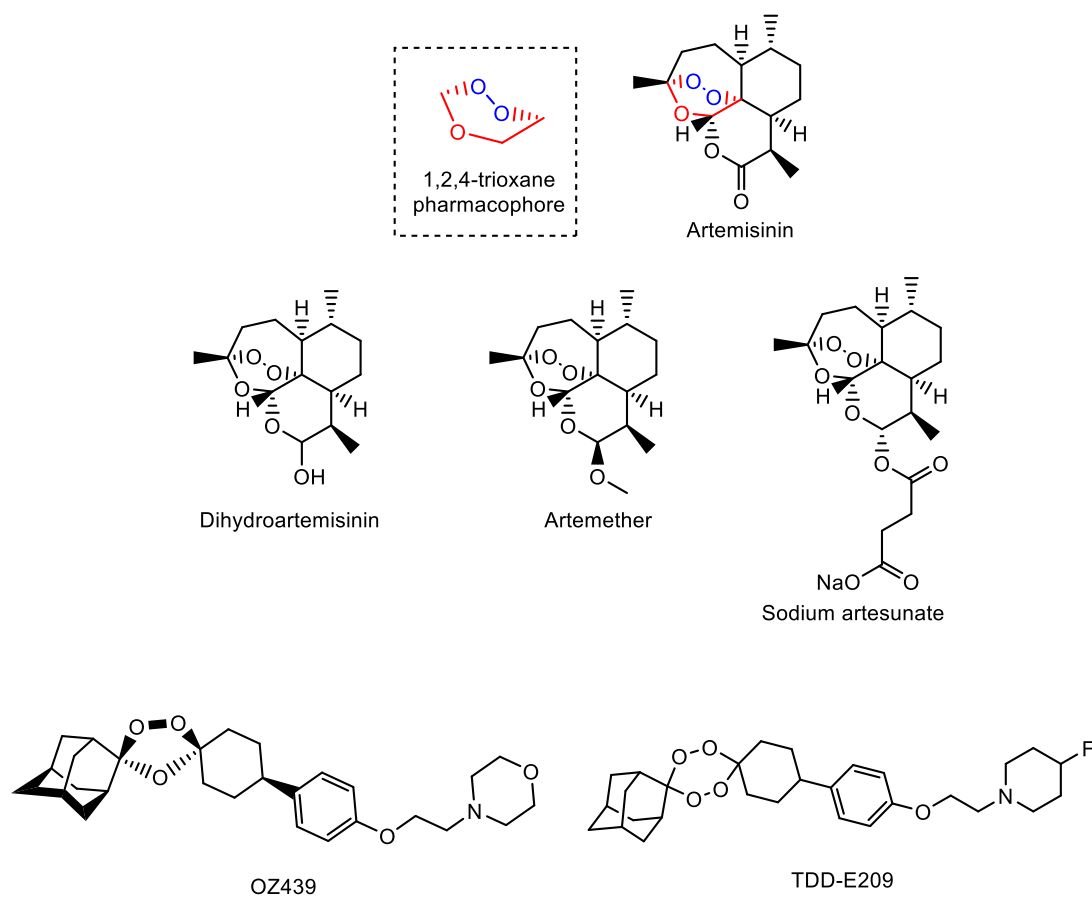


Figure 4.1: Chemical structure of endoperoxides.

Artemisinin, semi-synthetic derivatives (Sodium artesunate, artemether and dihydroartemisinin) and the fully-synthetic trioxane (OZ439) and tetraoxane (TDD-E209).

4.2.4 Methods:

P. falciparum gametocytes culture and gametocytocidal assay were performed as described in methodology chapter 2, section 2.3 and then PK-PD model were performed as described in chapter 2, section 2.4.

4.3 Results:

4.3.1 In-vitro gametocytocidal activity of selected endoperoxide compounds at a single concentration (5 μ M) against specific gametocyte life-cycle stages:

A newly generated luciferase-reporting transgenic parasite line 3D7elo1- pfs16-CBG99 was used to determine the activity of the selected endoperoxides on gametocyte viability at two different life-cycle stages, early gametocytes (stages II-III) and late stages (IV and V). The main findings are the distinct stage-specific kinetics and the dynamics of the compounds' effects (Figure 4.2). It can be clearly seen in Figure 4.2 that all compounds are inhibitory against the early stage gametocytes at 5 μ M, which is considered the high human drug exposure level in the blood. All endoperoxides tested showed >75% inhibition of early-stage viable gametocytes, however, only the semi-synthetic compounds (DHA, artesunate and artemether) exhibited significant potency (>98% inhibition) compared to the other compounds; artemisinin, OZ439 and TDD-E209 showed inhibition in early stage gametocyte viability of 89%, 76% and 88%, respectively (Figure 4.2).

In contrast, the same compounds showed much lower activity against late-stage (V) gametocytes, which are responsible for transmission of malaria to the vector, compared to the early stages (II and III). Only DHA showed significant activity of 72% against the late-stage gametocytes at 5 μ M; artesunate and artemether inhibited the late stages by approximately 40%. Artemisinin and the fully synthetic OZ439 and TDD-E209 behaved the same way showing no significant inhibition of late-stage gametocytes (< 20% inhibition).

The results from this single concentration exposure experiment implies that endoperoxoides have different inhibition profiles against the various gametocyte

stages and suggests their differential potencies. This prompted the examination of these compounds at different concentrations to determine their concentration-responses against gametocytes at early and late stages.

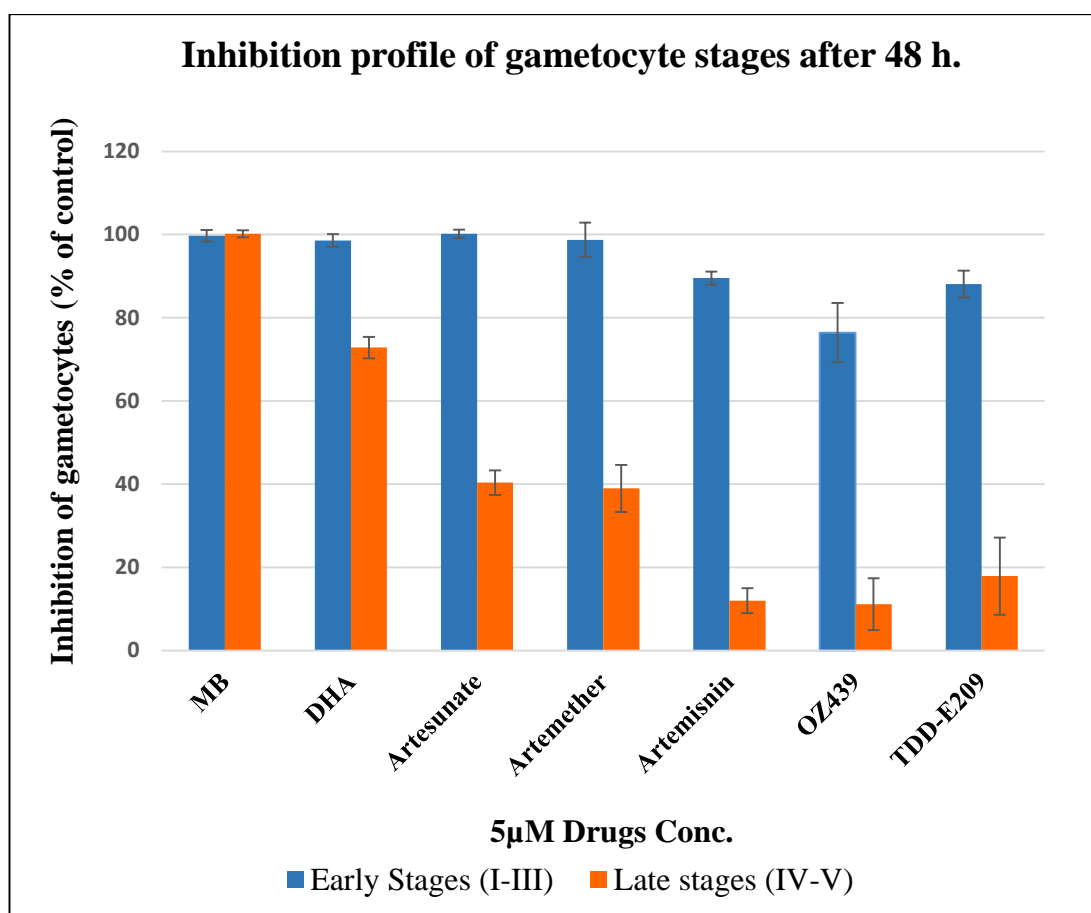


Figure 4.2: Bar chart illustrates the inhibition profile of antimalarial peroxides against early and late gametocyte stages at 5 μ M concentration.

Data show mean \pm SEM from three independent experiments performed in triplicate.

4.3.2 Assessment of the dose-response relationship of selected endoperoxide against early and late stage gametocytes:

A new profiling method was used to assess the IC₅₀ of endoperoxides at distinct gametocyte stages (II-III, IV-V). The activity of four artemisinin based (artemisinin, artesunate, artemether and DHA) and two fully synthetic endoperoxides (OZ439 and TDD-E209) were assessed. The effect of drug on gametocyte viability, measured over 48h was determined for stage II and II gametocytes (collected day 2-4 post invasion) and stage IV and V gametocytes) collected day 10-12 post invasion). The mean IC₅₀ ± SEM values are presented in Table 4.2. In addition, the relative dose-response curves for the selected endoperoxides (artemisinin, OZ439, TDD-E209, DHA, artesunate and artemether) are illustrated in Figure 4.3 and Figure 4.4.

Table 4.2: Gametocytocidal activity of 6 current and candidate endoperoxide drugs.

Compound	IC ₅₀ (nM) ±SEM		%Viability ± SEM	
	Early	Late	Early@ 1 µM	Late@ 10 µM
Artemisinin	13.9 ± 2.8	24.2 ± 1.4	13.3 ± 2.4	81.1 ± 0.3
OZ439	9.4 ± 4.0	63.3 ± 0.7	26.1 ± 2.4	89.9 ± 1.7
TDD-E209	20.3 ± 4.6	168.9 ± 2.3	14.8 ± 2.1	82.9 ± 2.5
DHA	6.19 ± 1.9	54.1 ± 5.5	4.8 ± 3.3	31.1 ± 4.1
Artesunate	6.7 ± 2.04	45.3 ± 1.4	4.5 ± 2.6	56.6 ± 0.8
Artemether	10.6 ± 2.3	51.2 ± 1.7	9.1 ± 5.3	55.9 ± 0.3

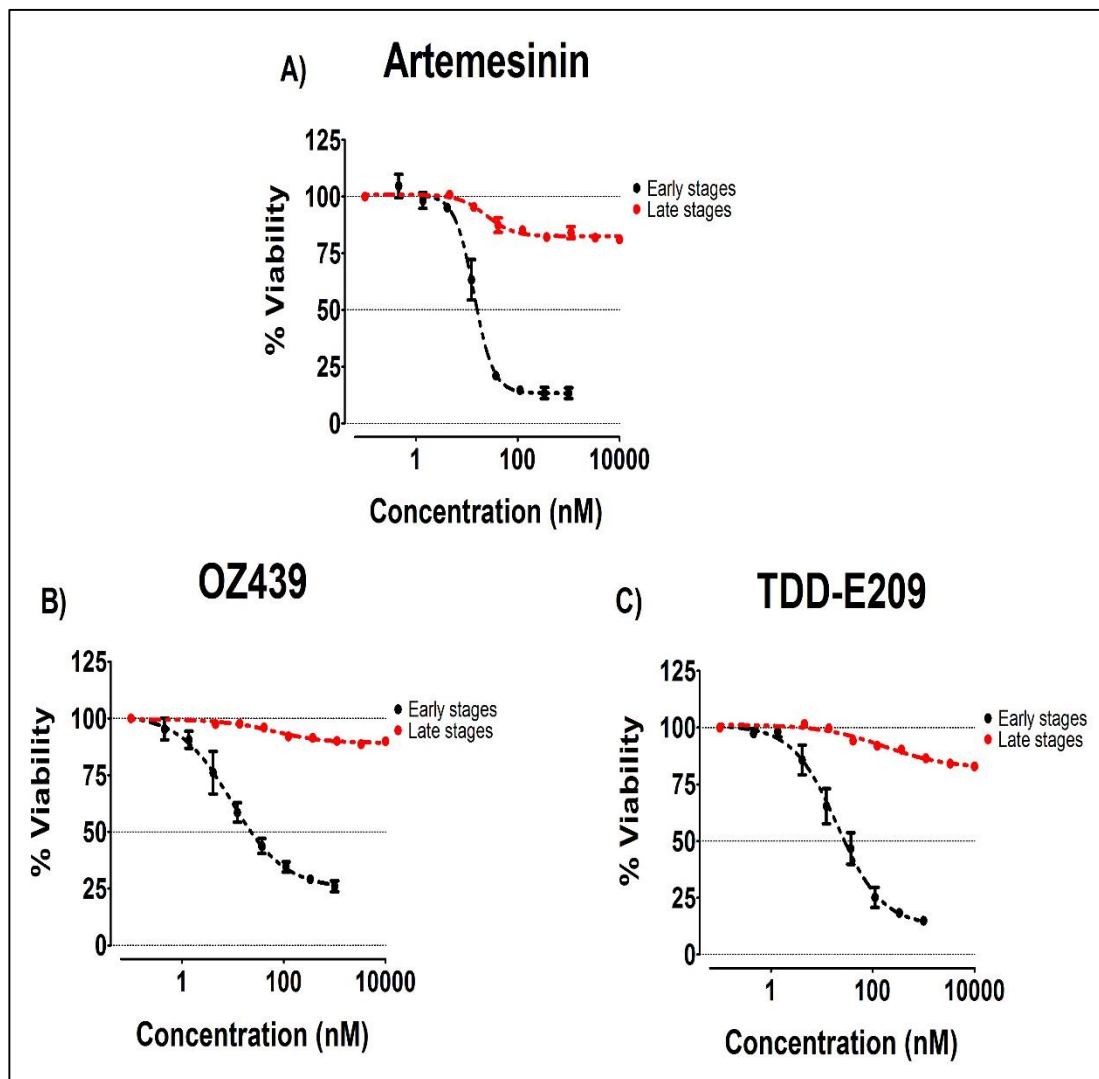


Figure 4.3: Typical curves showing the dose response of (A) artemisinin, and the fully synthetic peroxides (B) OZ439 and (C) TDD-E209 against early (black) and late (red) gametocyte stages.

IC₅₀ values were calculated through nonlinear regression using the “log (inhibitor) vs. response -- Variable slope (four parameter model)” model in Prism 5. The error bars represent the standard error of the mean (SEM) of three independent experiments each in run as triplicates ($n=3$).

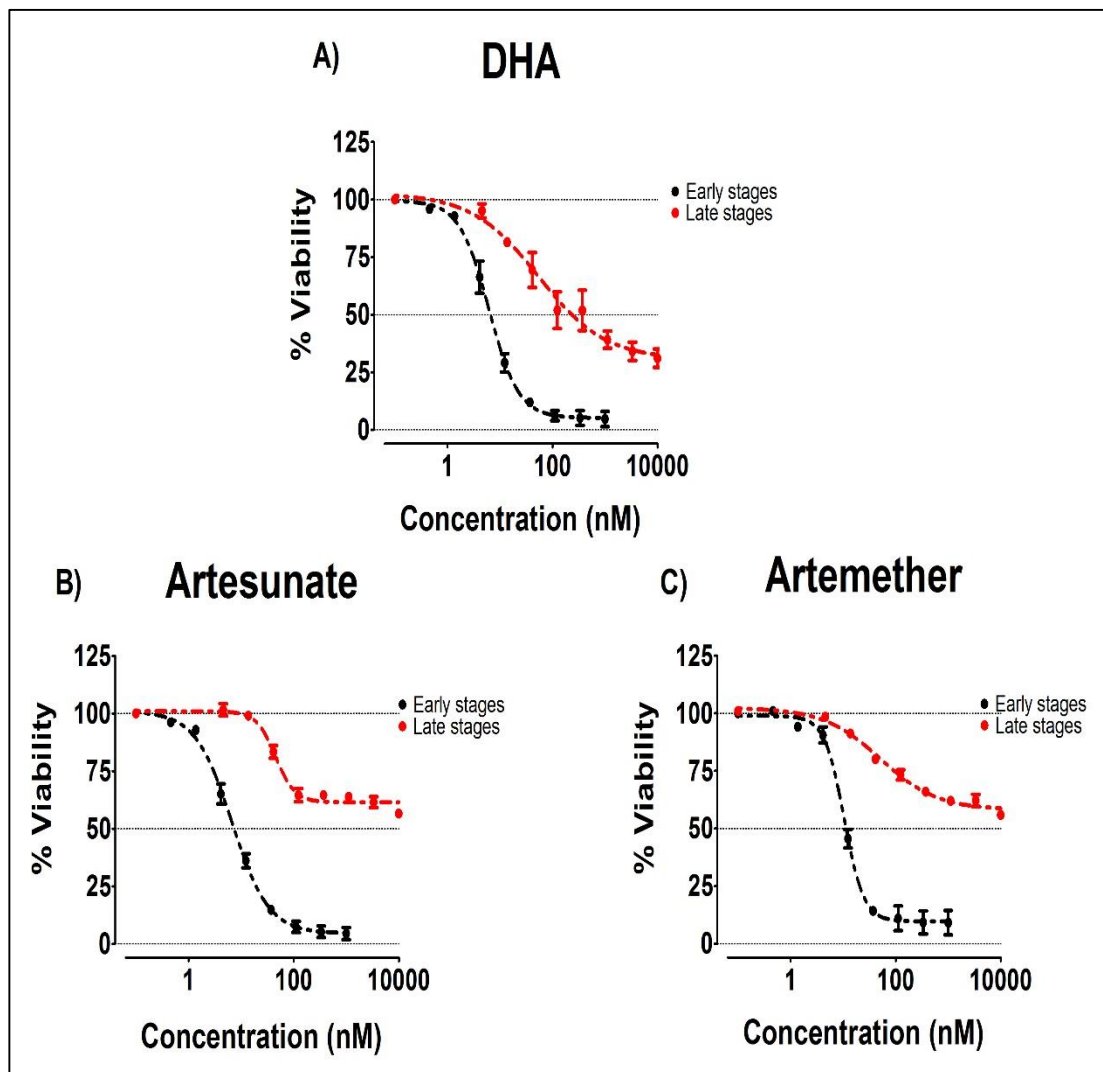


Figure 4.4: Typical curves showing the exposure-effect relationship of the semi-synthetic derivatives of artemisinin (A) DHA, (B) Artesunate and (C) Artemether against early (black) and late (red) gametocyte stages.

*IC*₅₀ values were calculated through nonlinear regression using the “log (inhibitor) vs. response -- Variable slope (four parameters)” model in Prism 5. The error bars represent the standard error of the mean (SEM) of three independent experiments each run in triplicate (*n*= 3).

Table 4.2 shows the sensitivity of the stages of *Plasmodium falciparum* gametocytes to the different endoperoxide compounds including artemisinin, its semi-synthetic derivatives, artesunate, artemether and DHA, as well as the fully synthetic endoperoxide candidates OZ439 and TDD-E209. The table also shows the mean $IC_{50} \pm SEM$ values determined from the dose-response curves from the gametocyte viability assays for both early and late gametocyte stages.

All compounds were potent inhibitors of early gametocyte (stage II-II) development with IC_{50} s in the nanomolar range (6.19 to 20.25 nM). In terms of parasite viability at and exposure of 1 μM the semisynthetic endoperoxides DHA, artesunate and artemether reduced early gametocyte stage viability >90 % with the artemisinin and the synthetic peroxisdes OZ439 and TDD-E209 reducing by 87%, 74% and 85%, respectively.

In comparison there was a substantial decrease in gametocytocidal potency of all the tested endoperoxide compounds against late-stage gametocytes. As shown in Table 4.2 and Figure 4.4, the reduction in potency was least for artemisinin with a reduction of 1.7 fold up to near 9 fold for DHA. In terms of parasite viability, in this instance at a high drug exposure of 10 μM only DHA exhibited a substantial reduction in late-stage gametocyte viability of 31%. The other semi-synthetic compounds, artesunate and artemether, exhibited moderate activity against late-stage gametocytes reducing the parasite's viability to 56.6% and 55.9 % (mean value at the highest test concentration, 10 μM), with IC_{50} s of 45.26 ± 1.35 and 51.21 ± 1.72 nM, respectively. Remarkably, neither artemisinin nor the fully synthetic peroxides, OZ439 and TDD-209, showed any real activity against late-stage gametocyte viability after exposure at 10 μM , with a 20% or less reduction in viability.

4.3.3 Determination of kill rates and characterisation of key pharmacodynamic properties of endoperoxides against late stage gametocytes:

DHA was selected for the pharmacodynamic study from among all the tested endoperoxide drugs as it showed relatively high potency at all gametocyte stages at clinically relevant levels (IC₅₀ late stage: 54 nM). The dynamics of gametocyte kill rate was systematically investigated by analysing the effect of DHA upon gametocyte counts across a range of clinically relevant concentrations (4, 2, 1, 0.5, 0.25, 0.1, 0.05, 0.01 and 0.001 µM) over discrete but therapeutically relevant time intervals (0, 6, 12, 24, 48, 72, 96 h). The kill rate exerted at each concentration was calculated using Prism 5 for Windows through nonlinear regression using the 'plateau followed by one phase decay' built-in equation, as shown in Equation 1 below (Figure 4.5).

$$Gametocyte\ count = (G_0 - Plateau) * e^{(-k \cdot t)} + Plateau \quad \dots \text{Eq. 1}$$

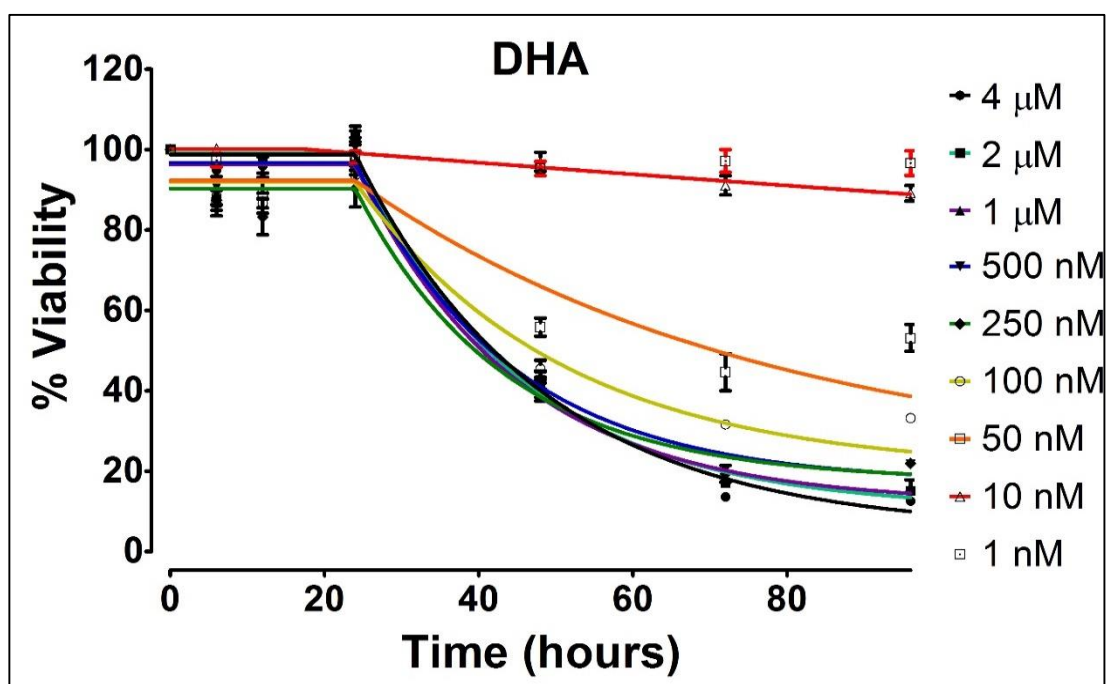


Figure 4.5: Time-dependent killing assay of DHA at different concentrations against late stages gametocytes over discrete time intervals.

Data show mean +/- SEM from three independent experiments performed in triplicate.

Table 4.3: The measured DHA dose dependent kill rates:

DHA conc.	Kill rate ($K \text{ h}^{-1}$)	SEM	N
4 μM	0.041030	0.006182	3
2 μM	0.046640	0.004379	3
1 μM	0.048150	0.009398	3
500 nM	0.050090	0.007175	3
250 nM	0.051820	0.013650	3
100 nM	0.037390	0.009770	3
50 nM	0.018850	0.018340	3
10 nM	0.001517	0.001421	3
0	0.001002	0.0012745	3

Figure 4.5 shows that for the first 24 h of drug exposure in the late gametocyte stages, there was no effect of drug on viability (lag time). Thereafter there was a dose dependent effect on parasite viability. The kill rates (k) achieved by each concentration of DHA (Table 4.3), was used in a pharmacodynamic sigmoidal E_{max} model which calculates the EC_{50} for DHA as well as its maximal kill rate (E_{max}). The kill rates were fitted using Prism 5 by applying the log (agonist) vs. response (three parameter) built-in equation in Prism as shown below in equation 2:

$$\text{Kill rate (hr}^{-1}\text{)} = E_{min} + \frac{(E_{max}-E_{min})}{1+10^{\text{LOG}(EC_{50}-C)}} \quad \dots \text{Eq. 2}$$

The EC_{50} and maximal kill rates (E_{max}) values for DHA were determined in the pharmacodynamic model. DHA EC_{50} was 60.22 ± 2.7 nM and the E_{max} value which represents the maximal kill rate was 0.05182 h^{-1} (Figure 4.6).

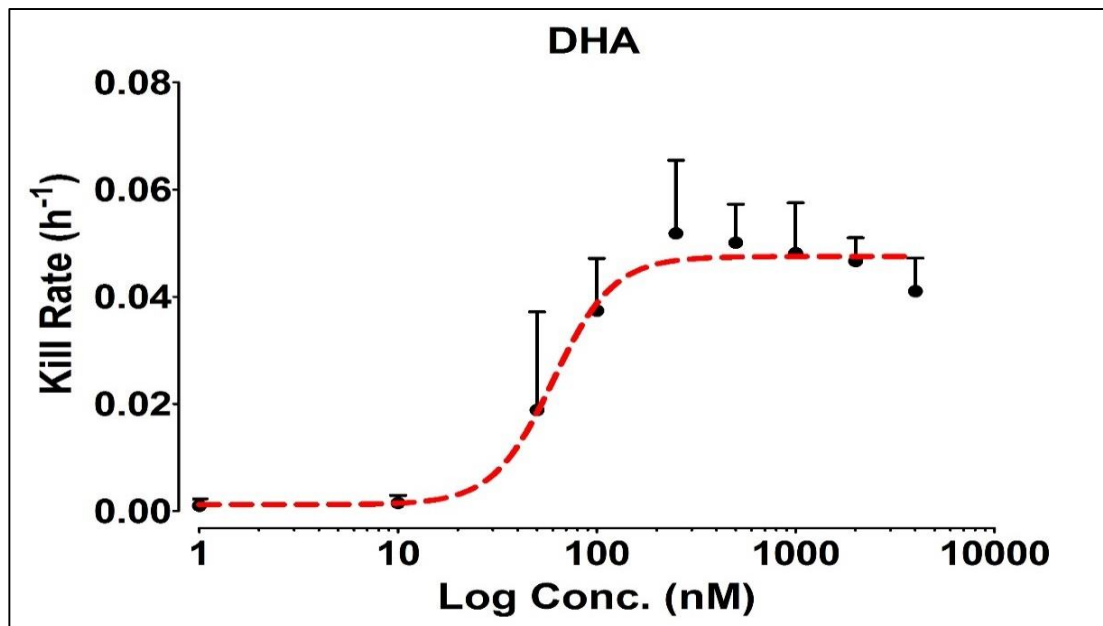


Figure 4.6: Dynamic concentration-kill rate response of DHA.

The figure shows relationship between DHA concentration (X axis) and the kill rate over time exerted at each concentration (Y axis). The dashed red line shows the sigmoidal fit of the data which is used to calculate the EC_{50} and E_{max} values. Data show mean \pm SEM from three independent experiments performed in triplicate.

4.3.4 PK-PD modelling and predictions of clinical drug activity:

4.3.4.1 Pharmacokinetic profile of DHA in human:

The clinical activity of DHA against late gametocyte stages in the blood stream was predicted using Monte-Carlo simulations using the program Pmetrics® (Neely et al., 2012b). The PK parameters of DHA were collected as reported in clinical PK studies in literature as shown in Table 4.4 using a 1 compartment PK model defined by the following two differential equations:

$$\frac{dX_1}{dt} = -k_a \cdot X_1 \quad \dots\dots \text{Eq. 3}$$

$$\frac{dX_2}{dt} = k_a \cdot X_1 - k_e \cdot X_2 \quad \dots\dots \text{Eq.4}$$

Where (X_1) represents drug mass in the gut in grams, K_a the rate of absorption, (X_2) the mass of drug in blood, K_e the rate of elimination and (t) the time in h.

The drug mass of DHA in blood was converted to concentration in molar using the following equation:

$$\text{Drug conc. (M)} = \frac{X_2/V_d}{Mwt} \quad \dots\dots \text{Eq.5}$$

Table 4.4: PK parameters of DHA for adult patients as reported in the literature.

Parameter	Predictable value	References
Dose (mg)	4 mg/kg given once daily for 3 days	(Tarning et al., 2012)
Volume distribution (L/kg)	2.15	
Elimination half-life (h)	1.55	
Elimination rate (k_e) (h^{-1})	0.6	
Clearance (L/h/kg)	1.3	
Absorption rate (k_a) (h^{-1})	1 (fixed)	N/A

The PK exposure simulations in a population of 1000 people with 30% variation of PK parameters was used for determination of DHA PK profile in the human populations. The solid black line in Figure 4.7 shows the median PK exposure whereas dashed red lines show the 5% and 95% percentiles of the population from the same simulation. Median peak concentration (C_{max}) was predicted to be 5.36 μM and time to reach maximum concentration (T_{max}) predicted to equal 1.0 h which agrees with previous literature studies. Antimalarial activity levels of DHA are pointed out in Figure 4.7 between the IC_{50} and IC_{99} levels as dashed blue and green lines, respectively. DHA concentration in the systemic circulation can reach the effective levels with the standard dose (4 mg/kg once daily for 3 days) but those concentrations cannot be maintained above the IC_{99} or IC_{50} levels for the dosing interval due to the drug's short half-life.

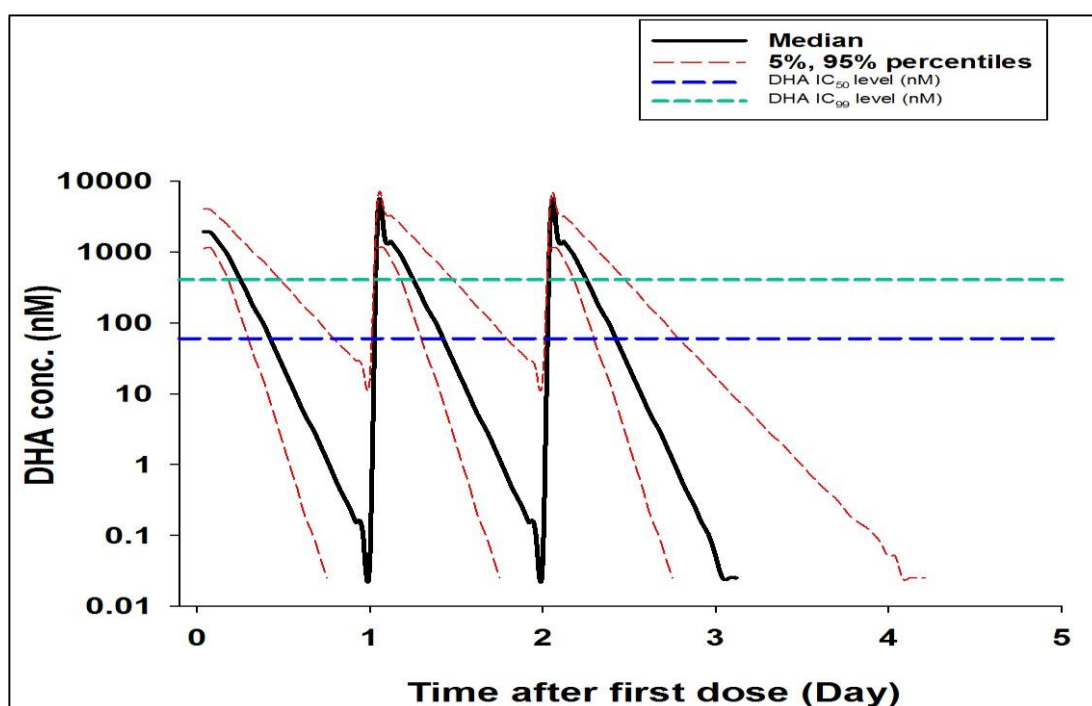


Figure 4.7: Simulation of PK exposure in a population of 1000 people given a 30% variation on all reported PK parameters.

Solid black line shows the median PK exposure whereas dashed red lines show the 5% and 95% values from the same simulation. IC_{50} and IC_{99} levels of DHA are shown in the graph as dashed blue and green lines, respectively.

4.3.4.2 The prediction model of DHA effect on late gametocytes:

The predictive model of DHA gametocyte killing (a measure of gametocyte clearance) is then defined by linking the dynamic drug concentrations (calculated using equations 3-5 for PK profile) to the sigmoidal pharmacodynamic profile (Figure 4.6), characterized in the previous section in equation (2). The final results plotted for the prediction model showing the median profile for gametocyte kill in addition to the 5% and 95% percentiles which represent the range of activity resulting from pharmacokinetic variability in the population are shown in (Figure 4.8). The first 24 h of drug exposure indicated no effect on viable gametocyte counts, this accounts for the lag time that was observed in the in-vitro experiments. After the second and third doses there is a predicted 25% reduction in gametocyte count in each dosing interval, achieving a total 50% reduction in gametocyte viability by the end of the 3 dose standard dosing regimen, with a range of 20-65 % viability reduction (Figure 4.8).

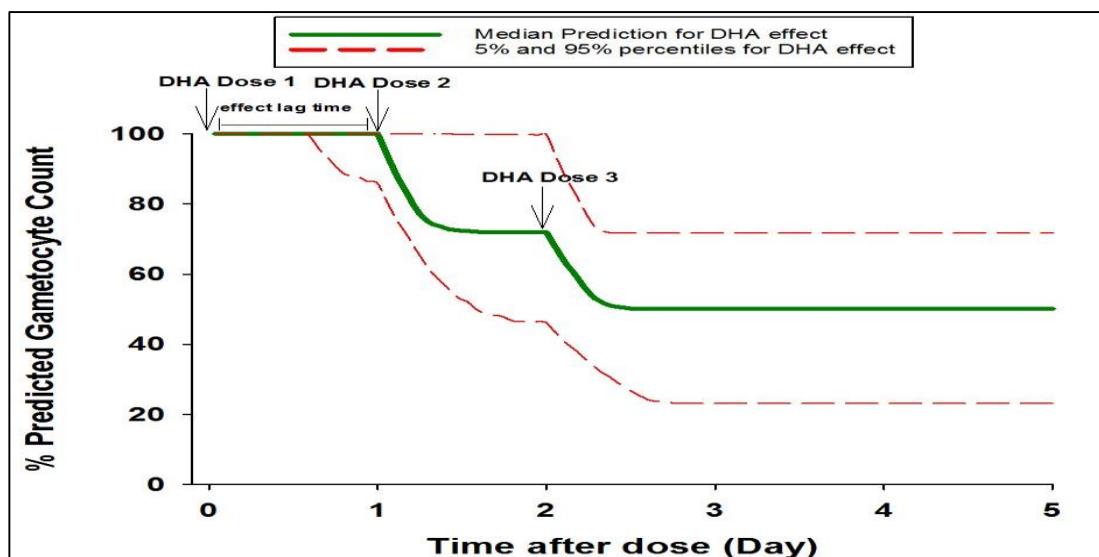


Figure 4.8: PK-PD relationship of DHA and its effect upon gametocytes.

The figure shows a simulation of the DHA effects upon gametocytes with a standard dosing regimen of 4mg/kg for 3 days given the PK and PD properties reported earlier in this chapter. The green line shows the median effect upon viable gametocyte carriage in a population of 1000 people while the dashed red lines show the 5% and 95% percentiles of the same population.

4.4 Discussion:

The global eradication of malaria disease will require new interventions and the development of transmission-blocking drugs to prevent transmission from human hosts to mosquitoes (Alonso et al., 2011). This chapter focused on the *in-vitro* activity of four endoperoxides known to be effective in human malaria infections (artemisinin, artesunate, artemether, and DHA) and two promising fully synthetic endoperoxides (OZ439 and TDD-E209) against *P. falciparum* gametocytes. Here, the relative potency and stage-specific sensitivity of gametocytes is established. The resultant data have been used to develop a comprehensive PK-PD model that predicts the activity of the active metabolite, DHA as a transmission blocking tool under clinically relevant conditions. DHA was selected because it was the most potent of the drugs tested against late stage gametocytes (Table 4.2).

4.4.1 Stage-specific kinetics and dynamics of Endoperoxides:

The most significant activity of all tested endoperoxides was observed against the early stages of gametocytes, whereas late-stage gametocytes displayed relative resistance to all tested endoperoxides except for DHA.

Endoperoxides are thought to exert their pharmacological action through the activation of their peroxide bridge in the presence of heme, which results in the production of cytotoxic metabolites, causing oxidative cell damage (Hartwig et al., 2009, Klonis et al., 2011).

Another theory suggested is that a direct interaction with PfATP6 occurs when endoperoxides interfere with calcium homeostasis inside the parasite (Eckstein-Ludwig et al., 2003, Krishna et al., 2010). Endoperoxides have been known to reduce

gametocyte carriage in infected humans and in early gametocyte stages (Price et al., 1996, Targett et al., 2001, Bolscher et al., 2015, Adjuik et al., 2004). This is consistent with what we have found in this study for the artemisinin based compounds, OZ439 and the newly generated tetraoxane TDD-E209, all of which show significant activity against early stages and show activity patterns that are archetypal of the endoperoxide class.

The early stages (II–III) and asexual stages are thought to share considerable metabolic overlap, including the use of Hb digestion as a source of amino acids (Sinden, 1982). It is the digestion of Hb and the resultant generation of heme and malaria pigment which is thought to be a causative process underpinning the significant activity of the endoperoxides. Once the gametocytes become mature in the late stages (IV–V) and are ready to be infectious, metabolite activity is reduced substantially, Hb digestion is thought to stop (or diminish to minimal levels) and the parasites are deemed to be quiescent reliant on what is called “housekeeping” metabolism, such as the oxidation-reduction activity (Tanaka and Williamson, 2011) and ATP generation (Lelievre et al., 2012).

4.4.2 Dose-Response Endoperoxide Activity Profiles:

The characterization of gametocytocidal activities for these clinical and preclinical endoperoxides demonstrates in all cases a loss of potency as the gametocyte matures that is largely reflected in the viability loss (Figure 4.3 and Figure 4.4). This observation is in agreement with other work in the literature, such as flow cytometry-based and luciferase-based studies that describe the abrupt decline in antimalarial efficacy after stage III gametocytogenesis (day 7), including for the endoperoxides (Lucantoni et al., 2016a, Wang et al., 2014).

The biological activity of endoperoxides depends on the reactivity of the endoperoxide bridge, which is found in all artemisinins and the fully synthetic peroxide compounds (Figure 4.1). Data regarding the activity of the endoperoxides against *P. falciparum* asexual stages (responsible for pathogenesis) have already been reported in the literature. The IC_{50} values are different based on the compounds structure but is generally around 10 nM or less (Mariga et al., 2005, Sharma et al., 2000, Fivelman et al., 2004, Anderson et al., 2005, del Pilar Crespo et al., 2008, Co et al., 2009). In this work, early-stage gametocytes displayed similar drug susceptibilities toward these selected endoperoxides as is seen in asexual stage parasites with IC_{50} in the range of 6–20 nM (Table 4.2). In contrast, marked decreases in sensitivity occurred with all tested endoperoxides in late-stage gametocytes. Only DHA had any real effect on late stage parasites. Interestingly, neither artemisinin nor the full synthetic endoperoxides (OZ439 and TDD-E209) display any significant effect upon that stage. This decrease in drug susceptibility in late stages is clear and reflected in a 5 to 9-fold increase in IC_{50} values in comparison with early stages, and this observation to some extent is in agreement with the findings of recent studies (Lucantoni et al., 2016a, D'Alessandro et al., 2016b) for artemisinin and OZ439 against late stage parasites.

Interestingly, when early-stage gametocytes are still being generated, they will have already produced the main metabolic pathways, particularly Hb digestion by 50% (Sinden, 1982, Klonis et al., 2011, Hanssen et al., 2012). As mentioned earlier the Hb pathway is considered central to the activity of the endoperoxides by providing the iron environment that can activate the peroxide bridge, and hence delivering its pharmacological activity. Conversely, Hb digestion appears to be complete at the late stage gametocyte (stage IV), according to a previous study measuring Hb content by soft X-ray microscopic analysis (Hanssen et al., 2012).

DHA is the primary active metabolite of the semi-synthetic artemisinin and is the most active compound among artemisinins currently used clinically, followed by artesunate and artemether (Klayman, 1985, Haynes et al., 2002, Gautam et al., 2009, Jansen, 2010). It could be argued therefore that the molecular target of DHA in late stages could be different from asexual and early gametocyte stage targets although a more plausible explanation would be around potency and efficiency of iron dependent peroxide lability.

Although gametocyte populations are sexually dimorphic, with males and females, both sexes are required for malaria transmission. In our results, the activity of DHA in late-stage gametocytes was significant, but with no distinguishing findings between male and female sensitivity. Therefore, a better understanding of the drug sensitivity of each sex is important, as all endoperoxides have been reported to be specifically more active against male gametocytes than against female gametocytes (Delves et al., 2013b). Endoperoxides' activity against male gametocytes may target residual heme of male-specific rather than female-specific gametocytes, and resultant toxic species could then affect parasite mitochondrial function (Baker, 2010, Delves et al., 2013b).

4.4.3 PK-PD Modelling of DHA and Gametocytes Clearance Predictions:

In this chapter we have reported the PD effect of DHA on gametocyte clearance using a simulations based on a PK/PD model that can help provide a broad understanding of the effect of DHA on transmission of mature gametocytes in a clinical setting. An *in-vitro* study has been conducted to determine the PD of DHA on mature gametocytes. DHA was selected from all tested endoperoxides because of its significant activity upon mature stages (Table 4.2 and Figure 4.4A).

DHA is the primary active metabolite of all semi-synthetic artemisinins, and is considered to be an antimalarial drug on its own. The dynamics of gametocyte kill

were systematically characterised by analysing the effect of DHA on gametocyte counts and viability at a range of clinically relevant concentrations over discrete therapeutically relevant time intervals. In order to build up the desired model, PD parameters derived from the time- dependent kill assay were used to define gametocyte kill rates over this wide range of concentrations. Interestingly, the dynamic measurements illustrate a 24-h delay (lag time) in gametocyte kill, which would significantly affect the final gametocyte clearance rates measured in patients. PK parameters of DHA were collected from the literature in order to define the concentration-time profile modelled using a one-compartment PK model (Na-Bangchang et al., 2004). Mature, late-stage gametocytes (stage V) are known to be released from the bone marrow and spleen to the bloodstream in order to become infectious to mosquitoes (Smalley and Sinden, 1977, Smalley et al., 1981, Rogers et al., 2000). Here, we are targeting the drug plasma concentration (C_P) in blood circulation, as these transmission competent gametocytes exist in the peripheral bloodstream, by using a one-compartment PK model. The main finding from simulating the PK exposure with a standard dosing regimen of the drug was to show that while 4 mg/kg administered daily can achieve efficacious concentrations, those levels could not be maintained throughout the treatment duration with significant periods of time below the required kill concentrations. This is due to the short elimination half-life of DHA of about 1.2 h (Na-Bangchang et al., 2004, Djimde and Lefevre, 2009). Exposure to sub therapeutic levels of drug could also potentially increase the chances of resistance developing, especially if the drug is used as a monotherapy. To avoid that possibility all artemisinin derivatives should be used in combination with other, long-acting antimalarial agents with totally different modes of action as recommended by the WHO (WHO, 2014).

Linking the PD sigmoidal model of DHA (Figure 4.6) to its clinical pharmacokinetic profile (Figure 4.7) allows the generation of the final PK-PD simulations, which reflects the activity of DHA in reducing gametocyte carriage from patients with standard doses. The lag-time effect still appears in the first 24 h from dose one, indicating that the first day of treatment is not enough to induce any gametocytocidal activity. Thereafter, the 50% reduction in viable gametocyte carriage after a standard three-day treatment predict a moderate effect of DHA on gametocyte clearance and carriage. These results are strongly in agreement with clinical studies which showed that artemether-lumefantrine and other ACTs significantly reduce the post-treatment prevalence of malaria transmission to mosquitoes and can limit or delay the time period of gametocyte carriage after treatment (Sutherland et al., 2005a, Bousema et al., 2006, Group, 2016). Gametocyte-carriage reductions in previous clinical studies is probably because of the fast-acting ability of endoperoxides to eliminate asexual and early-stage gametocytes, especially for long-term detection of late-stage gametocytes. Our data predict that about 50% of late gametocytes would be killed with active metabolite endoperoxide (DHA) and this direct reduction of gametocytes is highly likely to be biased towards inhibition of male gametocytes in exflagellation, as endoperoxides are reported to have more specific activity against male gametocytes than female gametocytes (Delves et al., 2013a).

4.4.4 Conclusion:

In this chapter we aimed to characterise the activity of selected antimalarial endoperoxides against the *P. falciparum* sexual stages (gametocytes) looking toward developing interventions to block malaria transmission. In general, the PD responses of peroxide compounds demonstrate stage-specific efficacy during

gametocytogenesis. All test compounds were potent against early-stage gametocytes, including the fully synthetic peroxides (OZ439 and TDD-E209), which have better PK profiles with longer elimination half-lives than artemisinin and semi-synthetic derivatives (Copple et al., 2012, Moehrle et al., 2013, Wells et al., 2015). All semi-synthetic artemisinins show better activity against late stages than artemisinin and fully synthetic peroxides against that stage. However, only DHA demonstrated potency against gametocyte at all stages and especially late stages that might have important transmission blocking potential.

A time-dependent killing assay has been developed to determine the exposure-effect relationship of DHA. In addition, the PK/PD relationship of DHA in a clinical context has been established in order to estimate clinical gametocyte clearance rates with standard treatment regimes. The predicted model suggests that DHA exposure is suboptimal in terms of gametocyte clearance. The data in this chapter are discussed in the context of the strategies that aim at the discovery and development of new transmission-reducing antimalarial drugs.

Chapter 5

Gametocytocidal activity of primaquine and Its CYP2D6 metabolites

5.1 Introduction:

The 8-aminoquinoline primaquine (PMQ) is the only drug registered by the FDA to treat relapsing malaria, caused by the reactivation of dormant liver forms, or hypnozoites, of *P. vivax* and *P. ovale* (Krotoski et al., 1980, Potter et al., 2015a). Through its usage as a standard treatment of relapsing malaria, PMQ has also become vital tool in blocking the malaria transmission by targeting the stage V gametocytes (Graves et al., 2015b).

PMQ structure consists of an aromatic quinoline as a core main component and two substituents, which are a methoxy group and an amino side-chain at position 6 and 8 respectively (McChesney and Sarangan, 1984). The structure of PMQ allows it to be metabolised in the liver by cytochrome P450 enzymes, and this metabolism appears to be important to its activity against hypnozoite stages (Pybus et al., 2012a, Bennett et al., 2013b, Pybus et al., 2013, Deye and Magill, 2014, Marcsisin et al., 2016, Potter et al., 2015b). In addition, it has been shown that the metabolism of 8-aminoquinolines is dependent on CYP2D for its activated as anti-malarial in the liver stage studies of human and mouse (Pybus et al., 2012b, Pybus et al., 2013, Bennett et al., 2013a, Potter et al., 2015a, Marcsisin et al., 2016). It was shown that PMQ was active only in mice with the capability of metabolizing substrates of CYP2D6. Deleting the mouse enzyme that is closest to human CYP2D6 led to a complete blockage of the liver stage antimalarial activity in vivo (Pybus et al., 2013). Similarly, it was found that phenolic metabolite levels of PMQ were highest in mice with the capability of metabolizing CYP2D6 substrates (Potter et al., 2015b).

PMQ administration in single or low doses is recommended for blocking malaria transmission of *P. falciparum* by reducing the gametocytemia and gametocyte

transmission to mosquitoes in children (Potter et al., 2015a, Goncalves et al., 2016, Dicko et al., 2016). Although the exact mechanism of action of PMQ is not fully understood (Myint et al., 2011a), a recently proposed mode of action for its antimalarial activity in hypnozoites is shown in Figure 5.1. The hypothesised hydroxylation of PMQ by CYP2D6 metabolism results in unstable metabolites (e.g. 5-hydroxyPMQ) with the capability to undergo spontaneous redox cycling, resulting in the production of oxidative stress. The redox recycling and the production of reactive oxygen species, may account for the observed activity and toxicity of PMQ (Bennett et al., 2013b, Pybus et al., 2013, Potter et al., 2015a).

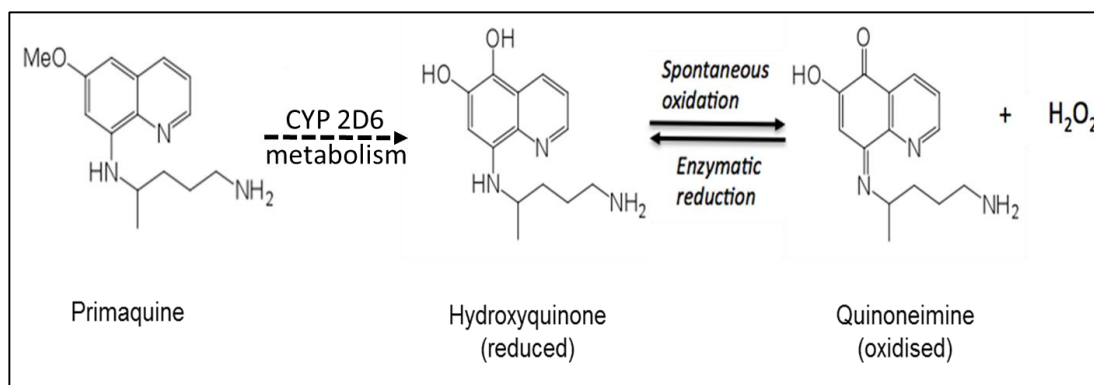


Figure 5.1: The proposed mechanism of action of PMQ bio-activation via CYP 2D6 metabolism and antimalarial efficacy.

The aim in this chapter is to determine the ability of PMQ, and a series of related 8-aminoquinoline compounds to interact with recombinant human cytochrome P450 2D6 (CYP 2D6) enzyme, and to investigate any resulting metabolites generating from either CYP2D6 and liver microsomes (consist of CYP450 enzyme) for gametocytocidal activity. In this chapter, the interaction of PMQ and some proposed metabolites (5 hydroxyPMQ and 5,6 hydroxyPMQ, PQQI and 6 hydroxyPQQI in quinoneimine form and hydroxyl-carboxyPMQ) synthesised in the Chemistry

Department, Liverpool University in collaboration with Prof. Paul O'Neill, with CYP2D6, has been determined via competition assays using the Vivid® CYP450 Screening Kit, and a fluorescent CYP2D6 substrate as a tracer. These experiments were then repeated in the absence of tracer, and any potential CYP and HLM metabolism products were assayed for gametocytocidal, in comparison with parent compounds.

5.2 Material and Methods:

5.2.1 Reagents:

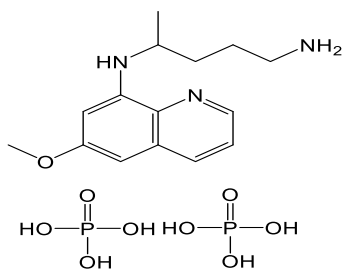
P. falciparum 3D7elo1-pfs16-CBG99 were generously provided by Prof Pietro Alano lab (INBB, Istituto Nazionale di Biostrutture e Biosistemi, 00136 Rome, Italy) (Cevenini et al., 2014). PMQ diphosphate was purchased from sigma and other PMQ metabolite compounds were synthesised in the Chemistry Department by Dr Michael Wong and Dr Shirley Leung (Prof Paul O'Neill group), University of Liverpool, UK (Table 5.1 and Figure 5.2). The Vivid® CYP450 2D6 Screening Kit was obtained from Life Technologies (Paisley, UK). Pooled Human Intestinal Microsomes, NADPH Regenerating System, Solution A and NADPH Regenerating System, Solution B were obtained from Corning ® Gentest™ (Corning B.V. Life Sciences, Netherland). The Oxytherm system and O₂ View software was obtained from Hansatech Instruments Ltd. (King's Lynn, UK). Catalase, from bovine liver was obtained from Sigma (C1345; Dorset, UK).

5.2.2 Test compounds:

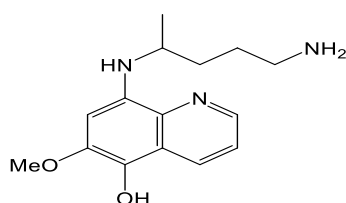
Table 5.1: PMQ and its metabolites used in this chapter.

8-AQ class	Compound name	Molecular Weight	Solvent
Metabolites Compounds	PMQ diphosphate	455.3 g/mol	50 % MeOH
	5-hydroxyPMQ	275.35 g/mol	
	MW-01-034 (PQQI)	273.33 g/mol	
	5,6-hydroxyPMQ	261.33 g/mol	
	MW-01-034 (6OHPQQI)	259.30 g/mol	
	MW-01-036 (5-hydroxy caboxyPMQ)	290.31 g/mol	
	CarboxyPMQ	274.32 g/mol	DMSO

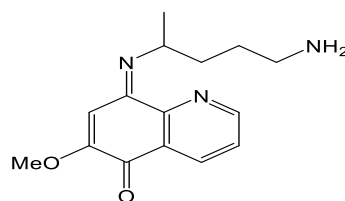
5.2.3 Compounds chemical structures:



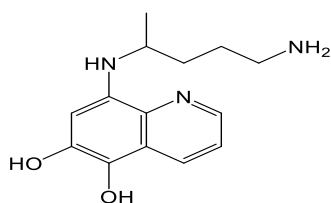
Primaquine diphosphate
Chemical Formula: $C_{15}H_{27}N_3O_9P_3$
Molecular Weight: 455.341



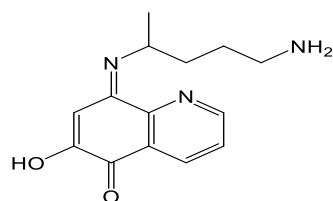
MW-01-019
5-OH PMQ
Chemical Formula: $C_{15}H_{21}N_3O_2$
Molecular Weight: 275.35



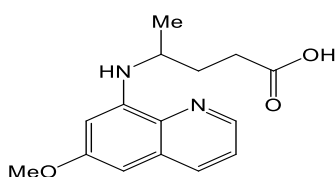
MW-01-034 (PQQI)
Chemical Formula: $C_{15}H_{19}N_3O_2$
Molecular Weight: 273.33



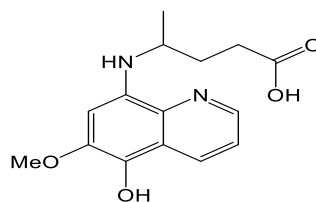
MW-01-014
5,6-OH PMQ
Chemical Formula: $C_{14}H_{19}N_3O_2$
Molecular Weight: 261.33



MW-01-034 (6O-HPQQI)
Chemical Formula: $C_{14}H_{17}N_3O_2$
Molecular Weight: 259.30



MW-01-036 (CarboxyPMQ)
Chemical Formula: $C_{15}H_{18}N_2O_3$
Molecular Weight: 290.31



MW-01-036 (5-OH CarboxyPMQ)
Chemical Formula: $C_{15}H_{18}N_2O_4$
Molecular Weight: 290.31

Figure 5.2: Chemical structure of primaquin and selected PMQ metabolites.

Abbreviations: 5-hydroxyPMQ (5-OH PMQ), PMQ quinoneimine (PQQI), 5,6 hydroxyPMQ (5,6-OH PMQ), 6 hydroxyquinoneimine (6-OH PQQI), carboxyPMQ (carboxyPMQ) and 5 hydroxycarboxyPMQ (5-OH carboxyPMQ).

5.2.4 Experimental Methods:

P. falciparum gametocytes culture gametocytocidal viability assay was performed as described in methodology chapter 2, section 2.3.

5.2.4.1 Enzyme kinetic assay to measure the interaction between test compounds and CYP2D6:

The Vivid® CYP450 Screening Kit, Vivid® CYP2D6 Blue (ThermoFisher, UK) was used to measure the interaction between test compounds and cytochrome P450 enzymes (CYP2D6) according to the manufacturer's protocol. Briefly, Vivid® substrate (CYP2D6) EOMCC was reconstituted using 205 µl anhydrous acetonitrile to make 2 mM stock solution and stored at -20°C. CYP450 BACULOSOMES®, Regeneration System, and NADP⁺ were thawed at room temperature for 10-15 min, then stored on ice until ready to use. In 15 ml falcon tubes, Vivid® CYP450 Reaction Buffer I was prepared by diluting (1:1 v/v): 5 ml buffer I in 5 ml nanopure water to make final concentration of 100 mM (1X buffer). The diluted buffer was used for the preparation of inhibitors, Master Pre-Mix, and Vivid® substrate/ NADP⁺ solutions. To prepare the test compounds, positive inhibition control and solvent control, 2.5 X of positive control (quinidine 10µM), solvent or vehicle control (30µM) and test compounds (serial dilution: 30µM, 10µM, 3.3µM, 1.1µM, 0.11µM) in 1X reaction buffer. Only 40 µl of the 2.5 X solution prepared above was added to clear bottom 96-well plate (black) and the samples were duplicated in each experiment. The Master Pre-Mix was prepared for full 96-well plate as follows: 4800 µl 1X buffer plus 100 µl Regeneration System (100 X) and 100 µl CYP450 BACULOSOMES®. 50 µl of Master Pre-Mix was added to assay plate. The assay plate was then incubated for 10 min at room temperature to allow the interaction between the compounds and P450 in the absence of enzyme turnover. In the meantime, 10X mixture of tracer/substrate

NADP⁺ was prepared by using 50 µl of reconstituted substrate plus 30 µl NADP⁺ added to 920 µl of buffer I. The reaction was started by adding 10 µl per well of the 10X Vivid[®] substrate/ NADP⁺ mixture to assay plate. Within less than 2 min the assay plate was transfer into Varioskan [®] Flash plate reader (Thermo Electron Corporation) and the florescence monitored over time at excitation 415 nm and emission 460 nm. The reading was in 1 min intervals for 120 min as a kinetic assay mode (Table 5.2).

Table 5.2: Summary of CYP2D6 reaction procedure.

Reagents for one 96-well plate		Volume (µl)
Master Pre-Mix preparation		
1	1X Vivid [®] CYP450 Reaction Buffer I	4800
2	Vivid [®] Regeneration System (100 X)	100
3	CYP 2D6 BACULOSOMES [®]	100
Total volume:		5000
10X Vivid[®] substrate/ NADP⁺ mixture		
1	1X Vivid [®] CYP450 Reaction Buffer I	920
2	Vivid [®] substrate EOMCC	50
3	Vivid [®] NADP ⁺	30
Total volume:		1000
1	Test Compounds & Controls	40
2	Master Pre-Mix	50
3	Vivid [®] substrate/ NADP ⁺	10
Total volume:		100

5.2.4.1.1 Endpoint analysis:

The percentage inhibition by the test compounds or positive control at the end point was calculated using the equation below:

$$\% \text{ Inhibition} = \left(1 - \frac{x-b}{a-b}\right) \times 100\%$$

a= the florescence intensity (FI) in the absence of inhibitor (solvent control)

b= the florescence intensity (FI) in the presence the positive control (quinidine).

x= the florescence intensity (FI) in the presence of test compounds.

Then, Michaelis-Menten equation was used to fit the enzyme inhibition and determine the K_m and V_{max} using the following equation:

$$Y = V_{max} * X / (K_m + X)$$

5.2.4.2 PMQ/CYP 2D6-mediated hydrogen peroxide generation:

5.2.4.2.1 Calibration of the Oxytherm System:

The Oxytherm system was calibrated to give the concentration of dissolved O₂ in nmol/ml, by defining the maximum O₂ concentration and the complete absence of dissolved O₂ in distilled water, under normal atmospheric pressure and the appropriate experimental temperature settings. Fully aerated distilled water was produced by adding 5 mL distilled water to a 1 L conical flask, sealing the opening with parafilm, and shaking vigorously for 3 min. 2 mL of fully aerated water was added to the reaction chamber which was then sealed. The calibration mode within the O₂ view software package was enabled and data were recorded until the trace reached a plateau, defining the maximum O₂ concentration. An excess of the reducing agent sodium dithionite was then added and the data were again recorded until the trace reached a plateau, defining the complete absence of dissolved O₂.

5.2.4.2.2 Measurement of PMQ/CYP 2D6-mediated H₂O₂ generation:

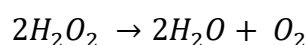
The ability of the PMQ parent drug and the PMQ metabolites to generate hydrogen peroxide after interaction with the VIVID® CYP450 2D6 screening kit was assessed indirectly by measuring catalase-mediated oxygen release. Catalase is an enzyme which catalyses the decomposition of hydrogen peroxide to water and oxygen. Test compound (30 µM) or vehicle control was incubated with the VIVID® CYP450 2D6 screening kit as per the User Guide* in a final volume of 600 µL per reaction at 37 °C for 120 min. At the end of the incubation period, a subsample of the mixture (400 µL) was transferred to the reaction chamber of a previously calibrated Oxytherm System (Hansatech, UK), and recording commenced immediately. Once the kinetic trace for oxygen concentration within the mixture had reached a plateau for at least 3 min, catalase enzyme (from bovine liver, prepared in 50 mM potassium phosphate buffer, pH 7.05; final assay concentration 10 µg/ml) was added, after which data were recorded for a further 6 min. Appropriate controls were performed with each individual reaction, including addition of addition of catalase buffer to assess addition artefact, and a repeat of experiments with non-Vivid® CYP450 2D6-treated test compounds to assess enzyme-independent hydrogen peroxide generation.

*All concentrations are final assay concentrations. The VIVID® CYP450 2D6 screening kit in this instance is comprised of a reaction buffer (100 mM potassium phosphate, pH 8.0), BACULOSOMES® [insect cell-derived microsomes recombinantly expressing human cytochrome P450 2D6 (10 nM) and human cytochrome P450 reductase], regeneration system [glucose-6-phosphate (3.33 mM) and glucose-6-phosphate dehydrogenase (0.3 U/mL) in 100 mM potassium phosphate, pH 8.0] and NADP⁺ (30 µM).

5.2.4.2.3 Data analysis:

Oxygen concentration was recorded as nmol/ml using the O₂ View software package v.2.06 (Hansatech Instruments Ltd., UK). Raw data files were transferred to GraphPad Prism v.5 for graphical presentation. To allow for easier comparison of individual traces, the *x* axis was adjusted by defining the addition of catalase as t=0, and the corresponding *y* axis value defined as 0 nmol/ml.

The following equation describes the decomposition of hydrogen peroxide to water and oxygen, where 2 moles of hydrogen peroxide produce 1 mole of diatomic oxygen.



The concentration of hydrogen peroxide present therefore is twice the peak increase in oxygen concentration, post-catalase addition.

5.2.4.3 Gametocytocidal activity of non-CYP and CYP-treated compounds:

5.2.4.3.1 CYP-treated compounds preparation:

For test compounds and controls, 2.5 X of 30 µM of compounds were prepared (1 µl drug/Control + 133 µl of 1 X reaction buffer in 1.5 ml eppendorf tube, with vortexing). Then, 60 µl of prepared drugs/controls were transfer to 96-well plate. Master Pre-Mix was also prepared to be suitable for experiment as follows: 1200 µl reaction buffer added to 25 µl regeneration system and 25 µl CYP 2D6 BACULOSOMES® and mixed gently by inversion. Then a 75 µl of Master Pre-Mix was transferred to each well in assay plate. The assay plate was incubated for 10 min at 37°C to allow the compounds to interact with CYP2D6 in absence of Vivid® NADP⁺. During the incubation time, the Vivid® NADP⁺ mixture was prepared by adding 7.5 µl of NADP⁺ to 242.5 µl reaction buffer in 1.5ml Eppendorf tube and vortexed in the absence of Vivid® substrate. To start the reaction, 15 µl of NADP⁺ was transferred to each well

in the assay plate. Immediately, the plate was incubated at 37°C for 120 min (Table 5.3).

Table 5.3: Summary of the CYP-reaction constituents used in the late stage gametocyte assay.

Reagents for one 96-well plate		Volume (µl)	Volume/ well in assay plate
Drugs/controls dilution			
1	Drug/ MeOH	1 µl	60 µl
2	1 X Reaction buffer	133 µl	
Master Pre-Mix preparation			
1	1 X Reaction buffer	1200 µl	75 µl
2	Vivid® Regeneration System (100 X)	25 µl	
3	CYP 2D6 BACULOSOMES®	25 µl	
10X Vivid® NADP⁺ mixture			
1	1 X Reaction buffer	242.5 µl	15 µl
2	Vivid® NADP ⁺	7.5 µl	

5.2.4.3.2 Human liver microsoms-treated compounds preparation:

A new protocol for the generation of compound metabolites using HLMs for testing against gametocytes was established. The assay buffer was prepared using potassium phosphate buffers at pH 7.4 as following: 1M of K₂HPO₄ plus 1M of KH₂PO₄ were combined 1:1, and then diluted 10X in distilled H₂O. Typically, 10 ml of assay buffer was prepared. For test compounds and controls, 2 X of 30 µM of compounds were prepared (1.2 µl drug/Control + 200 µl of assay buffer in 1.5 ml eppendorf tube, with vortexing). Then, 50 µl of prepared drugs/controls were transfer to 96-well plate.

Master Pre-Mix was also prepared to be suitable for the experiment as follows: 2075 μl reaction buffer was added to 250 μl regeneration system solution A and 50 μl regeneration system solution B. the mixture was subdivided into two equal parts (1187.5 μl). HLM were mixed gently by inversion. The mixture tubes were labelled as (+) HLM and (-) HLM. To the (+) HLM, 62.5 μl of HLM was added; to (-) HLM 62.5 μl of assay buffer was added. A 50 μl of Master Pre-Mix was transferred to each well in the assay plate and then the reaction was started. Immediately, the plate was incubated at 37°C for 120 min.

5.2.4.3.3 Luciferase assay of CYP-treated and HLM-treated compounds:

After 120 min incubation of parent compounds with VIVID CYP450 kit (subsequently referred to as CYP2D6-treated compounds) or following HLM-treatment as described above, the incubation 96-well plates were centrifuged for 3 min at 1500 rpm. A 125 μl aliquot of reaction product was taken and added to individual wells of a dilution plate containing 62.5 μl per well of complete medium (CM) to give a total volume of 187.5 μl per well (20 μM final compound concentration).

In the luciferase gametocytes plate assay, 50 μl of 20 μM CYP-treated compounds were added in triplicate. After that, 50 μl of 2% haematocrit of 2-3.5 % gametocytemia were added to each well of the assay plate (see section 2.3.1 and 2.3.2). The final volume of each well was 100 μl , which consist of 10 μM of compounds/controls concentration, 1% haematocrit and 2-3.5 % gametocytemia. The plate was then placed in humidified chamber gassed it for at least 2 min and incubated 72 h before reading.

5.2.4.3.4 Gametocyte viability assay reading and data analysis:

Data were generated from three independent biological replicates performed in triplicate are. Luciferase assay after drug-treatment experiments were performed after transferring samples to 96-well white microplates as describe previously (Cevenini et al., 2014). The measurement of samples in the 96- well white microplate to reduce the interfering of luminescence between the samples (Chapter 2, section 2.3.1 and2.3.2). The optimal D-luciferin substrate in citrate buffer 0.1 M, pH 5.2 was 1 mM D-luciferin (final concentration). The substrate was added directly to the samples at a 1:1 ratio, and the plates were read in luminescence reader, Varioskan ® Flash plate reader (Thermo Electron Corporation) at the stable kinetics enzyme at least between 8-18 min after addition D-luciferin substrate (chapter 2, Figure 2.4).

5.3 Results:

5.3.1 CYP2D6 enzyme kinetic inhibition:

To determine the interaction of the 8-aminoquinoline drug PMQ and its proposed metabolites compounds with the human CYP450 2D6 enzyme a Vivid® CYP450 screening fluorescence-based assay was used. Quinidine (10 μM), a known inhibitor of CYP2D6, was used as a positive control, while 50% methanol (solvent) was the negative control. In the absence of any other compound, the recombinant CYP2D6 enzyme metabolises the tracer, producing a kinetic fluorescent signal (Figure 5.3A black trace). The positive control quinidine inhibits the function of CYP 2D6, preventing the metabolism of the tracer and leading to a reduction in the kinetic fluorescent signal (Figure 5.3A green trace). Figure 5.3 A shows clearly that control (methanol). CarboxyPMQ produced very limited inhibition of the tracer metabolism, indicating it does not interact with CYP 2D6 at 10 μM concentration (Figure 5.3, A red trace). PMQ diphosphate (10 μM) inhibited the tracer metabolism by about 50 % (Figure 5.3A purple trace). Figure 5.3 used here as an example to show the inhibition of tracer metabolism of controls and positive (PMQ) and negative (carboxyPMQ) drug response.

Thereafter, the concentration-response data were subjected to a steady-state kinetic analysis using the Michaelis-Menten equation, to determine K_m and V_{max} , as illustrated in Figure 5.3, B. PMQ showed complete inhibition of the tracer metabolism with $V_{max} = 105 \pm 11\%$ and K_m equal to $12.9 \pm 3.1 \mu\text{M}$. This indicates that PMQ can interact with CYP 2D6 and inhibit the tracer.

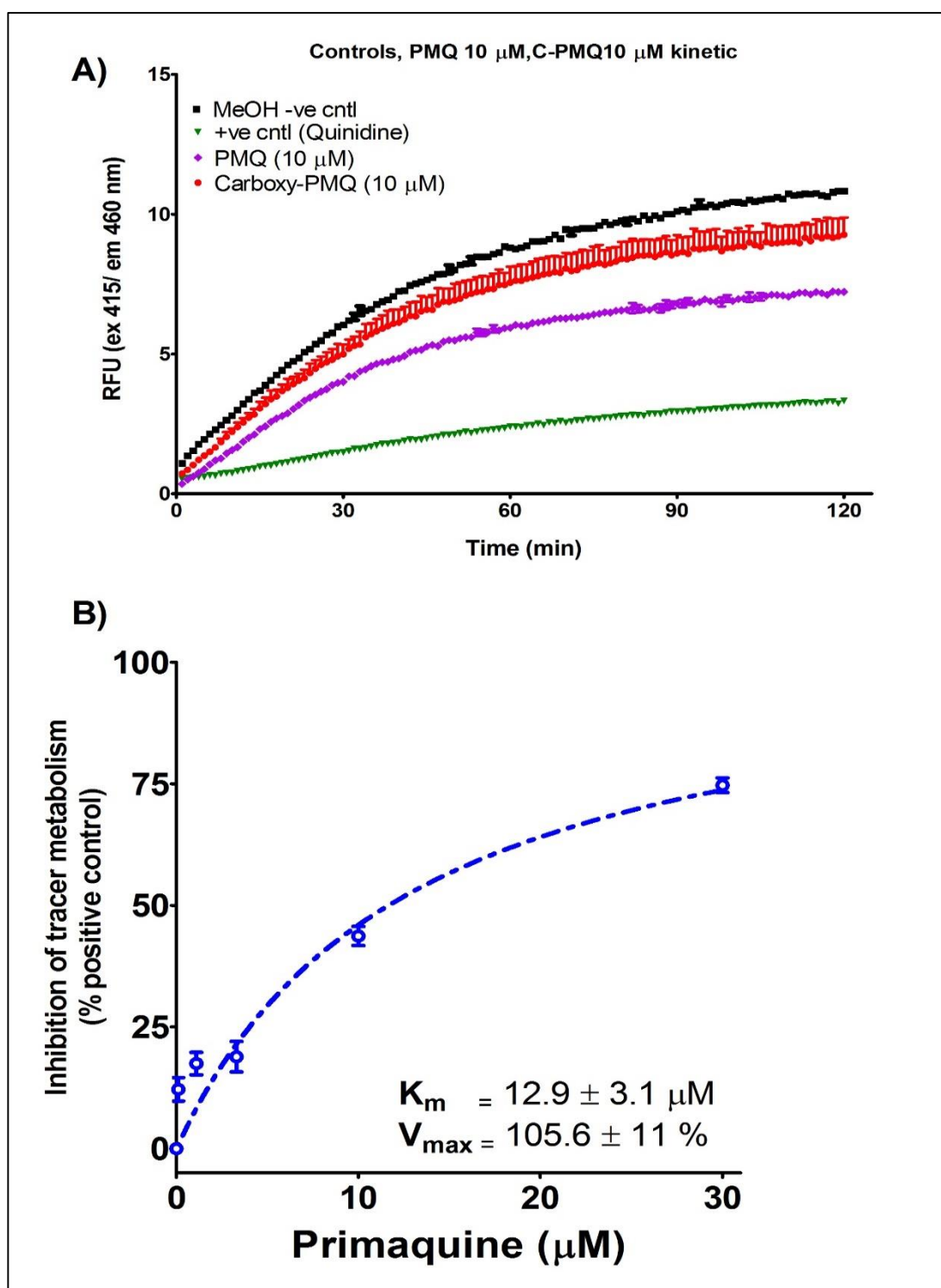


Figure 5.3: CYP2D6 steady-state kinetics and inhibition of tracer metabolism vs. PMQ concentration.

A) 50% methanol (MeOH) and quinidine were used as controls (negative and positive respectively). PMQ and carboxyPMQ shown in this figure as an example of kinetic monitoring over time. B) Concentrations of PMQ were from 0 to 30 μ M. The concentration response data was fitted to the Michaelis-Menten equation.

As shown in Figure 5.4 and Figure 5.5, a concentration-dependent enzyme kinetic assay was performed for the proposed PMQ metabolites compounds to test their interaction with CYP2D6. The data was then subjected to steady-state kinetic analysis to determine K_m and V_{max} . The two hydroxyquinine compounds, 5 hydroxyPMQ and 5, 6 hydroxyPMQ, showed a pronounced inhibition of the tracer metabolism ($K_m = 2.7 \pm 0.4 \mu\text{M}$, $V_{max} = 79 \pm 13\%$ and $K_m = 4.5 \pm 1 \mu\text{M}$, $V_{max} = 90 \pm 5\%$ as seen in Figure 5.4 and Figure 5.5, respectively).

Two other metabolites (quinoneimine) compounds, PQQI and 6-OHPQQI, were tested. These compounds are derived from spontaneous oxidation of the previously hydroxylated compounds, where PQQI is derived from the oxidation of 5 hydroxyPMQ and 6-OHPQQI from 5,6 hydroxyPMQ (Figure 5.4 and Figure 5.5). All compounds displayed an inhibition tracer of the CYP2D6 enzyme with varying potency. With PQQI, the result was $K_m = 2.0 \pm 0.5 \mu\text{M}$, $V_{max} = 58 \pm 2\%$, whereas with 6OH-PQQI, the inhibition of tracer metabolites was higher, with $K_m = 12.0 \pm 8.6 \mu\text{M}$, $V_{max} = 90 \pm 22\%$. Interestingly, the potency of 5, 6-HPMQ and its quinoneimine form (6OH-PQQI) to inhibit the metabolism tracer more than 5-HPMQ and its quinoneimine form (PQQI).

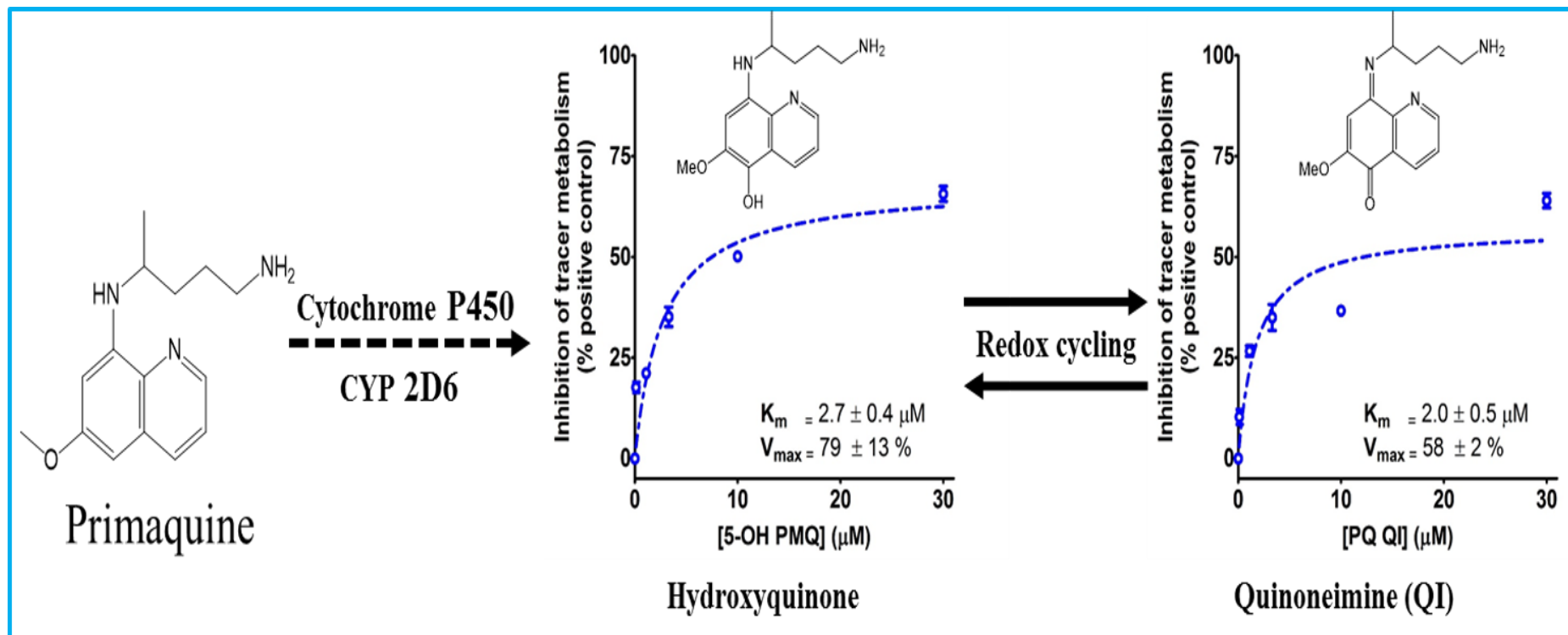


Figure 5.4: CYP2D6 steady-state kinetics of PMQ metabolites 5OH PMQ and PQQI.

Inhibition of tracer metabolism vs. compound concentrations. All compounds concentrations response data were fitted to the Michaelis-Menten equation.

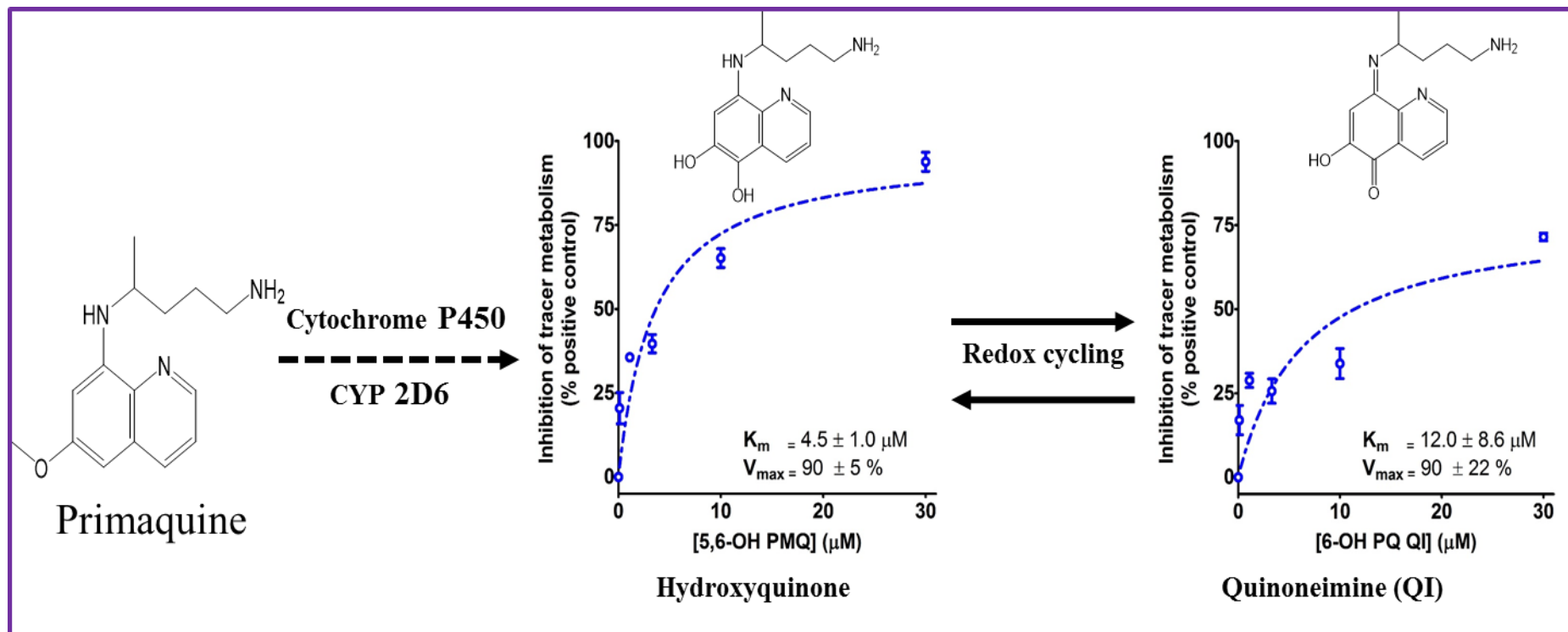


Figure 5.5: CYP2D6 steady-state kinetics of PMQ metabolites 5,6-OH PMQ and 6-OH PQQI.

Inhibition of tracer metabolism vs. compound concentrations. All compounds concentrations response data were fitted to the Michaelis-Menten equation.

In the case of carboxy PMQ (carboxy-PMQ), it was found to be non-toxic (Link et al., 1985) and had no effect on tracer metabolism. In addition, MW-01-036 (hydroxycarboxyPMQ) failed to inhibit the tracer and interact with the CYP2D6 enzyme, as illustrated in Figure 5.6.

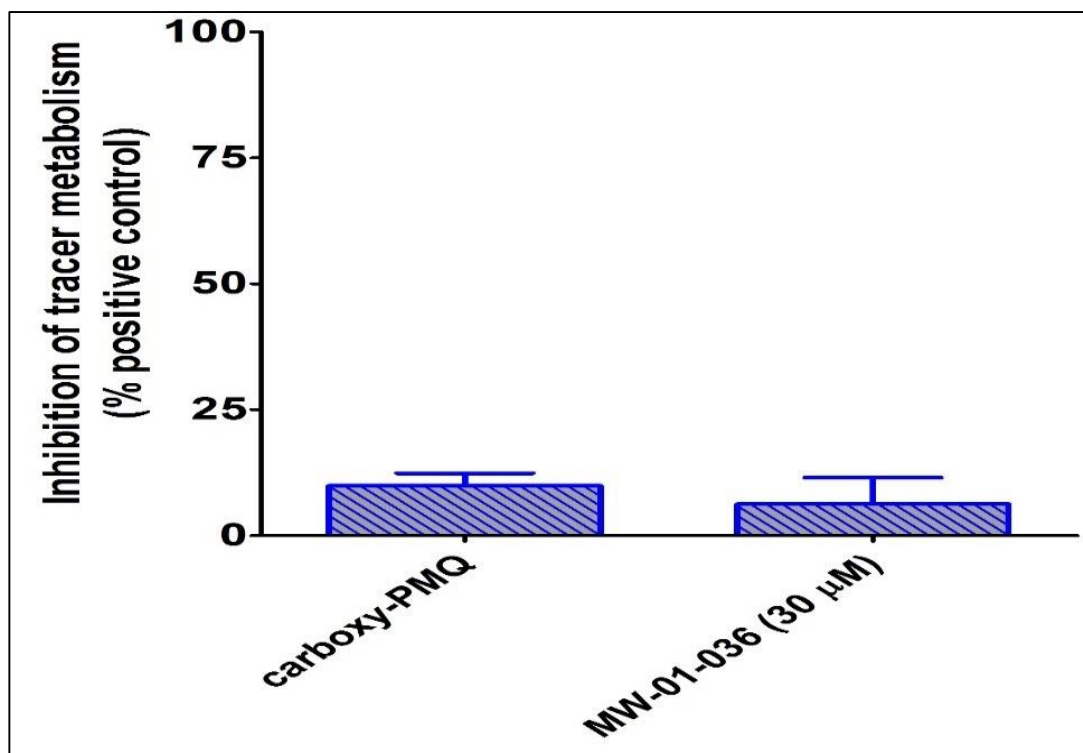


Figure 5.6: CYP2D6 steady-state kinetics of carboxy and 5-hydroxycarboxyPMQ compounds.

Inhibition of tracer metabolism vs. compound concentration at 30 μM (highest drug conc. used), where the CarboxyPMQ and MW-01-036 don't display significant inhibition.

5.3.2 Hydrogen peroxide production through CYP drug metabolites reaction:

Our hypothesis is that the metabolism of PMQ via CYP2D6 results in unstable hydroxylated metabolites with the capability of spontaneous redox cycling and the production of hydrogen peroxide (H_2O_2). To test this, indirect detection of H_2O_2 produced by the compound-CYP2D6 interaction was performed by measuring the oxygen (O_2) released after adding catalase enzyme, using the Oxytherm system. In the case of non-CYP2D6 treated compounds, no O_2 released after catalase addition was detected in all tested compounds, indicating no H_2O_2 production in the absence of CYP2D6 enzyme (Figure 5.7A).

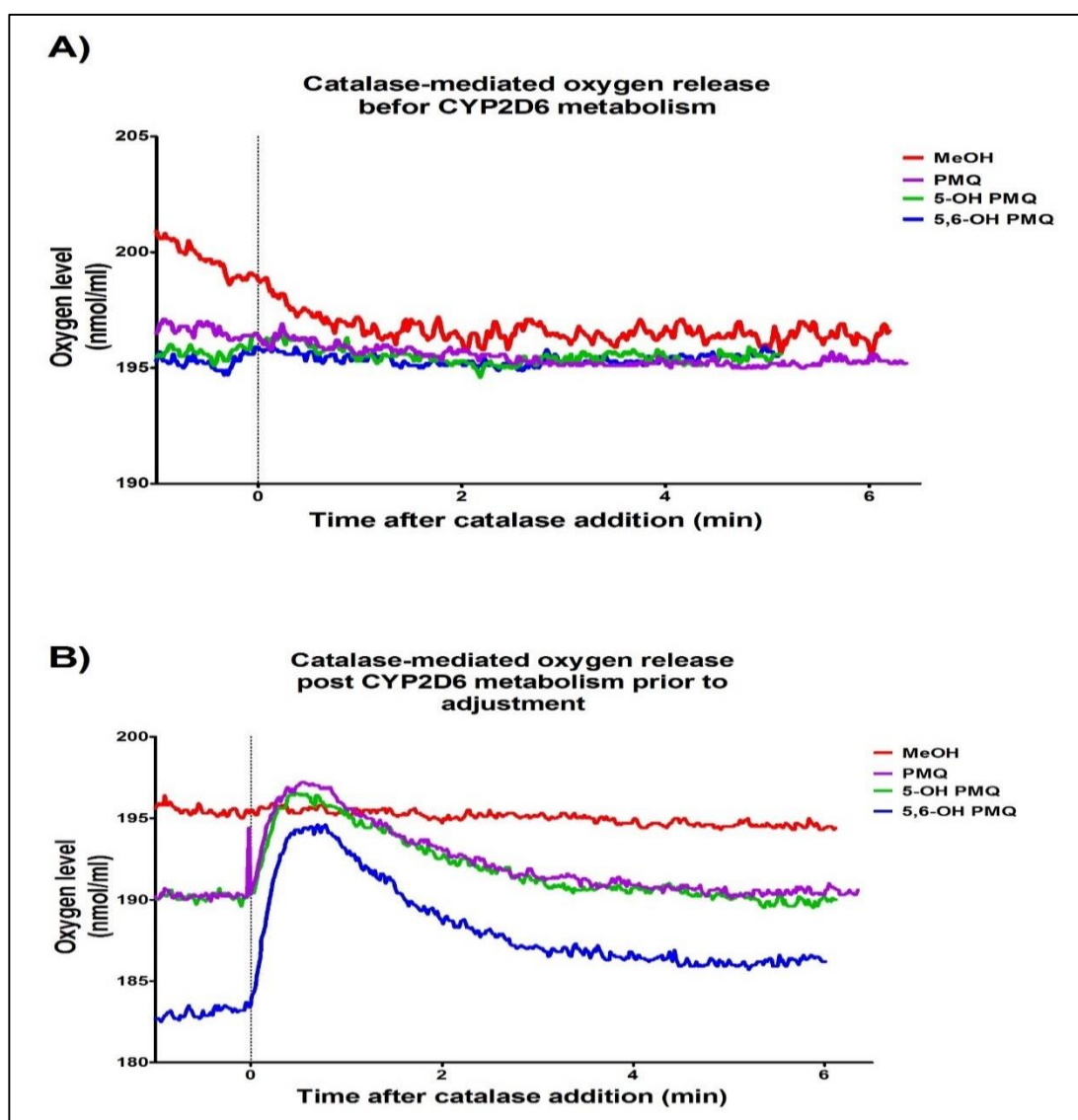


Figure 5.7: Catalase-mediated oxygen release from PMQ and its metabolites compounds before and after CYP2D6 metabolism prior to normalization (adjustment).

Catalase-mediated oxygen release before CYP metabolism (A) and after CYP2D6 metabolism (B) of primquine and its hydroxylated metabolites. The dashed line at 0 min represent the addition of catalase. MeOH in red trace line, PMQ in purple line, 5-OH PMQ in green and 5,6-OHPMQ in blue.

As shown in Figure 5.7B with CYP2D6-treated compounds, there was a release of O_2 after catalase addition. The measurement of the O_2 level for all test compounds and the control was started immediately after two h incubation of the CYP reaction. Interestingly, O_2 level (nmol/ml) for the various compounds, prior to addition of

catalase, was not at the same level Figure 5.7B. The starting O₂ level of negative control 50% methanol (MeOH) was 195 nmol/ml, whereas, PMQ and 5-OH PMQ were at 190 nmol/ml and 5,6-OH PMQ at 183 nmol/ml. To compare the maximum level of O₂ released after catalase addition for all test compounds, the y-axis was normalized to zero and oxygen released after adding catalase plotted as shown in Figure 5.8. 50% methanol was used as solvent negative control in CYP2D6-treated condition and adding catalase to it showed no O₂ released. However, addition of catalase to the CYP 2D6-treated compounds PMQ and 5 hydroxyPMQ produced a clear O₂ release of 6.1nmol/ml and 6.3 nmol/ml, respectively (Figure 5.8, purple and green trace). Furthermore, addition of catalase to the CYP 2D6-treated compound of 5,6 hydroxyPMQ produced a clear O₂ release of 11 nmol/ml with 2 fold more than parent compounds (Figure 5.8, blue trace).

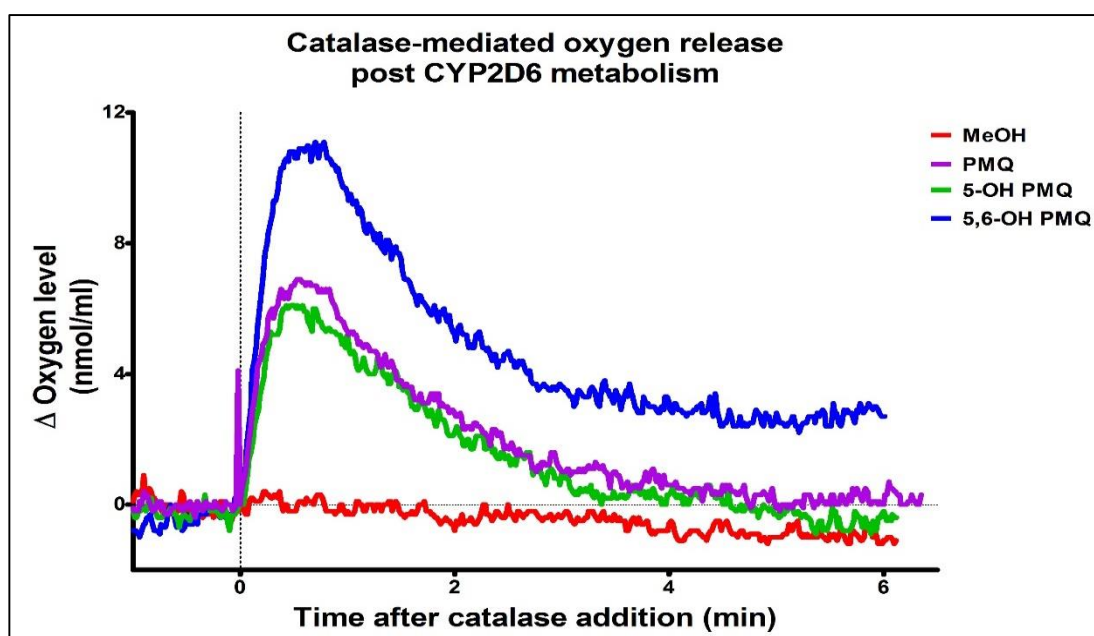


Figure 5.8: Normalized catalase-mediated oxygen release post CYP2D6 metabolism of PMQ and its metabolites.

The dashed line at 0 min represent the addition of catalase. MeOH in red trace line, PMQ in purple line, 5-OH PMQ in green and 5,6-OH PMQ in blue.

5.3.3 Gametocytocidal activity of PMQ compounds vs CYP 2D6-treated compounds:

PMQ was tested against late-stage *P. falciparum* gametocytes *in vitro*, using the compound before and after metabolism by the CYP2D6 reaction system. It was clearly observed that after 72 h incubation, PMQ at 10 μ M displayed only moderate inhibitory activity against gametocytes with mean of 70.13% viability. However, following prior incubation with CYP2D6, PMQ displayed significantly more potent inhibition of gametocytes, with a measured viability of 38.7% after 72h incubation (Figure 5.9).

The results obtained from the luciferase-based gametocytocidal assay of proposed PMQ metabolites showed pronounced enhancement of gametocytocidal activity of CYP-treated compounds, compared to the non-CYP condition. To be specific, 5-OH PMQ and its quinoneimine form, PQQI, exhibited a similar pattern of activity with the CYP-treated condition with mean values of 62.25% and 57.3% viability, respectively. The non-CYP-treated PQQI showed little effect (86.2% viability) and no activity at all with the non-CYP-treated 5-OH PMQ.

It can also be clearly seen that 5, 6-OH PMQ and its quinoneimine, 6-OH PQQI, showed the same pattern of response activity against the gametocytes. For the CYP-treated compounds at 10 μ M, the mean viability values of 5, 6-OH PMQ and 6OH-PQQI were 29.25% and 46.9% respectively. The same non-CYP treated compounds also showed gametocytes viability with mean values of 66.2% and 77.16% viability against gametocytes. Remarkably, carboxyPMQ did not show any activity against gametocytes in both conditions for the CYP-treated and non-CYP compounds with mean of 95.25% and 94.38 % gametocytes viable, respectively.

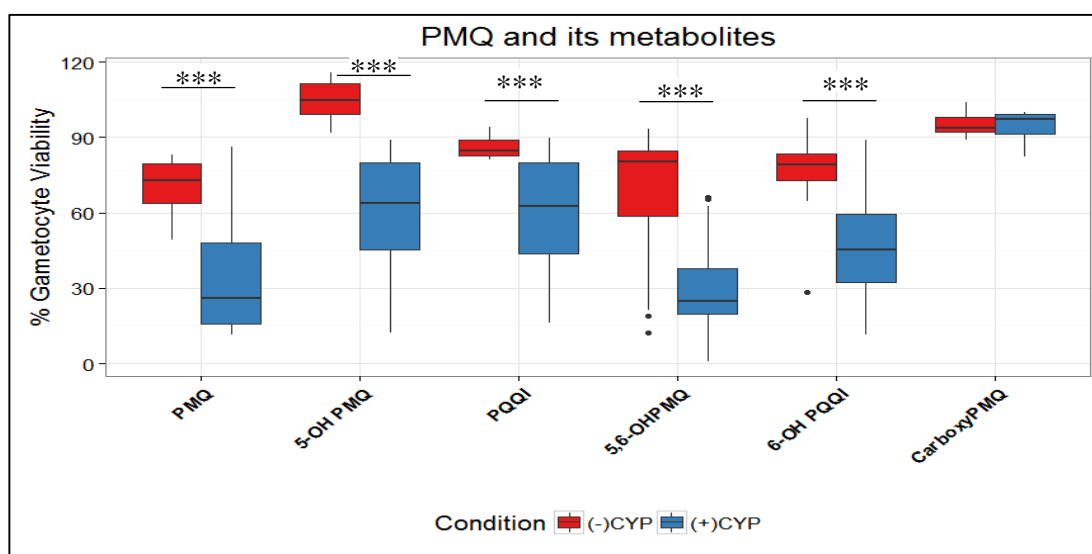


Figure 5.9: %Viability of *P. falciparum* gametocytes after exposed to PMQ metabolites compounds in the context of CYP 2D6 metabolism.

PMQ and its metabolites were tested at a concentration of 10 μ M in two conditions: non-CYP compound and drug after metabolites activation CYP. The data represent comparison between conditions in each compounds. The midline of each box-plot is the median, with the edges of the box representing 1st and 3rd quantiles. Whiskers delineate the 5th and 95th percentiles. *** indicates $P < 0.001$, (Welch's *t*-test, two tail, $n=7$ separate experiments, each in triplicates).

From the statistical view, the results obtained from the gametocytocidal assay for PMQ and the proposed metabolites activity against gametocytes, showed there was a statistically significant increase in the gametocytocidal activity of all the CYP-treated compounds, compared to the non-CYP condition, except for carboxy PMQ, which failed to kill the gametocytes in either condition (.

Table 5.4).

Table 5.4: The summery of statistical significances between (-) CYP and (+) CYP activity against late gametocytes (Welch's *t*-test, two tail).

Compound @ 10 μ M	% Survival No CYP (Mean)	%Survival CYP (Mean)	P-value	95% CI	Significance
PMQ	70.1	38.7	0.00049	16.1, 46.8	***
5-OHPMQ	104.8	62.3	5.74E-07	31.3, 53.9	***
PQQI	86.3	57.3	0.00049	15.5, 42.4	***
5,6-OHPMQ	66.2	29.3	0.00035	19.6, 54.3	***
6OH-PQQI	77.2	46.9	0.00046	15.6, 44.8	***
C-PMQ	95.3	94.4	0.82101	-6.4, 8.1	ns

*** Statistically significant at $p < 0.001$.

p values were corrected for false positives using the benjamini and hochsberg method.

5.3.4 Gametocytocidal activity of PMQ compounds vs HLM-treated compounds:

To further investigate the physiological relevance of PMQ metabolism in relation to inhibitory gametocytocidal activity, the experiments were repeated with compounds incubated with HLM. Unlike the baculosomes, the HLM contains a full complement of CYP450 enzymes. The 8-aminoquinoline PMQ and its proposed metabolites were tested against late-stage *P. falciparum* gametocytes *in vitro*, both before and after incubation with the human HLM reaction system. As with the previous experiments using the VIVID system, the gametocytocidal activity of each compound was tested under two conditions: (-) HLM that contains the compound and reaction mixture without liver microsomes and (+) HLM that have the compound and reaction mixture with liver microsomes.

After 72 h incubation, PMQ in the condition of (-) HLM at 10 μ M did show moderate activity against gametocytes (53.32% gametocytes viability) as seen in Figure 5.10. Incubation of PMQ with the HLM, prior to exposure to the parasites, produced a significant increase in the compound gametocytocidal activity with survival mean of 18.6%, viability (Table 5.5. and Figure 5.10).

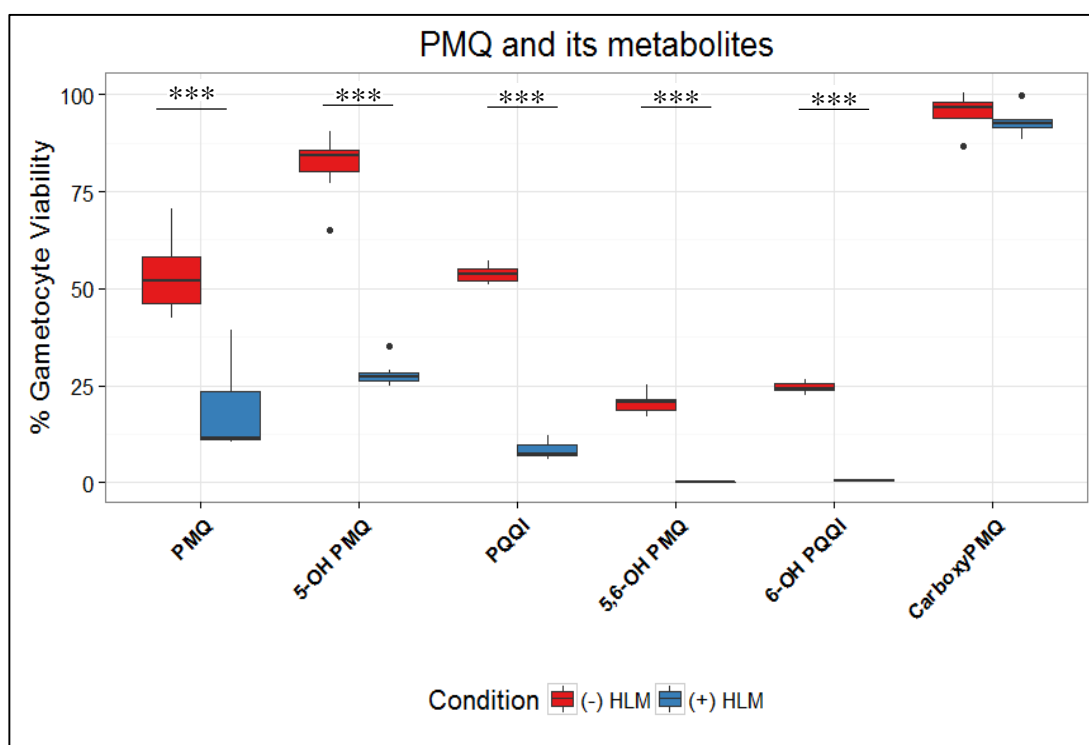


Figure 5.10: %Viability of *P. falciparum* gametocytes after exposed to PMQ metabolites compounds in the context of HLM metabolism.

PMQ and its metabolites were tested at a concentration of 10 μ M in two conditions: (-) HLM compound and drug after metabolites activation (+) HLM. The data represent comparison between conditions in each compounds. The midline of each box-plot is the median, with the edges of the box representing the 1st and 3rd quartiles. Whiskers delineate the 5th and 95th percentiles. *** indicates $P < 0.001$, (Welch's t-test, two tail, $n=3$ separate experiments, each in triplicates).

5-OH PMQ and its quinoneimine form (PQQI) exhibited statistical significant activity in (+) HLM-treated condition by a mean of 27.9 % viability and 8.3 %, respectively. In the (-) HLM-treated condition of the former two metabolites drugs, PQQI showed moderate activity against gametocytes by reducing the viability to 53.7% and only 81.6 % in 5-OH PMQ (Figure 5.10).

5,6-OH PMQ and its quinoneimine, 6-OH PQQI, exhibited a similar pattern of gametocytocidal activity. Both (+) HLM-treated compounds at 10 μ M, were able to totally kill gametocytes with 0.05% and 0.5% gametocytes viability, respectively. The

(-) HLM-treated compounds also reduce the gametocytes viability to the mean value of 20.4% and 24.6% respectively.

As in the recombinant CYP 2D6-treated experiments, carboxy PMQ did not show any activity against gametocytes in both conditions. All the statistical differences between the two conditions in all compounds are listed in the Table 5.5.

Table 5.5: Summary of statistical significances between (-) HLM and (+) HLM activity against late gametocytes (Welch's *t*-test, two tail).

Compound @ 10µM	% Survival (-) HLM (Mean)	%Survival (+) HLM (Mean)	P-value	95% CI	Significance
PMQ	53.3	18.6	1.78E-04	21.3, 48.1	***
5-OHPMQ	81.6	27.9	8.85E-07	45.7, 61.6	***
PQOI	53.8	8.4	1.12E-12	42.7, 48.1	***
5,6-OHPMQ	20.4	0.1	2.32E-06	17.9, 22.8	***
6OH-PQOI	24.6	0.5	2.81E-08	22.7, 25.5	***

*** Statistically significant at $p < 0.001$.

p values were corrected for false positives using the benjamini and hochsberg method.

5.4 Discussion:

PMQ is a vital component in the fight against malarial disease, as it is the only registered drug that is used to treat relapsing malaria infection caused by *P. vivax* and *P. ovale*. Also, in view of its gametocytocidal efficacy against *P. falciparum* late stages, it is strategically important in malaria eradication/ elimination programs by blocking parasite transmission (Maude et al., 2012, Eziefula et al., 2012, Graves et al., 2015a). Although the actual mode of action of PMQ is not well understood, its efficacy is thought to be linked to biotransformation of the compound to its active metabolites (Vasquez-Vivar and Augusto, 1992, Tekwani and Walker, 2006, Vale et al., 2009, Pybus et al., 2012a, Bennett et al., 2013b). The suspected candidates for the PMQ activity are phenolic metabolites (hydroxylated), as they have been linked to hemolytic toxicity in G6PD-deficient individuals (Link et al., 1985, Bolchoz et al., 2002). In this chapter, PMQ and different selected metabolites were investigated in the context of their ability to interact with CYP 2D6, their capability to generate hydrogen peroxide via the recombinant CYP2D6 system and the effect of CYP2D6 and HLM metabolism on their gametocytocidal activities.

5.4.1 PMQ and its phenolic metabolites can inhibit the metabolism of a CYP2D6 enzyme substrate and generate H₂O₂:

The steady-state enzyme kinetic data of PMQ and its proposed phenolic metabolites showed a clear interaction between the drugs and the human CYP 2D6 enzyme. CYP2D6 BACULOSOME (microsomes prepared from insect cells expressing a human P450 CYP 2D6 isozyme) offer a discrete benefit over HLM as they express only a specific CYP450, thereby avoiding metabolism by any other CYP450 enzyme. The main reason for investigating the role of CYP 2D6 in isolation is because of its

suspected role in PMQ metabolism to redox active metabolites which lead to the antimalarial activity of this compound (Vale et al., 2009, Pybus et al., 2012a, Bennett et al., 2013a). CYP 2C19 and CYP 3A4 show the capacity to metabolise PMQ and PMQ metabolism mostly derives through mono-amine oxidase (MAOs) pathways, however the generation of the proposed redox active metabolites is thought to proceed via CYP 2D6 (Baker et al., 1990, Pybus et al., 2012b). The most abundant PMQ metabolite in human plasma is carboxy-PMQ, which is generated through the MAO-A pathway, and therefore carboxy-PMQ is the main elimination metabolite of PMQ (Mihaly et al., 1984a, Pybus et al., 2012b). In the present study, we have tested the ability of PMQ and the proposed metabolites to interact with the CYP 2D6 isoenzyme and then tested the generated products for their gametocytocidal activity.

The steady state data showed PMQ inhibited the tracer metabolism by CYP2D6 with the highest V_{max}/K_m compare to its metabolites (Figure 5.3). To some extent, this result supports the catalytic efficiency of CYP2D6 towards PMQ reported in a previous study which demonstrated the highest V_{max}/K_m ratio for 2D6 compared to other CYP450 isoenzymes (Pybus et al., 2012a). In fact, CYP2D6 concentrations tend to be comparatively lower than other relevant CYP450s isoenzyme in liver, however it is still thought to be notes the important.

An orthoquinone of hydroxyl metabolites was identified as the major metabolites produced by CYP2D6 and is considered a marker of 5-OH PMQ generation (Fasinu et al., 2014). In this study, the hydroxylated metabolite (5-OH PMQ) and its quinoneimine PQQI inhibited CYP2D6 dependent metabolism.

The second hydroxylated metabolite 5,6-OH PMQ and its oxidised quinoneimine form 6-OH PQQI also displayed inhibition the CYP2D6 isoenzyme, but in this case the inhibition was greater (by about 90 %) compared with 5-OH PMQ and PQQI.

The oxidation reduction cycling of hydroxylated metabolites is thought to generate H₂O₂ molecules and it is suggested that this H₂O₂ represents one of main oxidative agents responsible for the hemolytic effects of the 8-aminoquinolines (Vasquez-Vivar and Augusto, 1992, Vasquez-Vivar and Augusto, 1994). The indirect (sensitive) and direct measurement methods used for determining H₂O₂ production through CYP2D6 in this chapter were used to confirm the capability of redox cycling of hydroxylated metabolites to generate oxidative agent H₂O₂. The 5,6-OH PMQ generated much higher amounts of H₂O₂ than other tested compounds.

It is known that the generation of dihydroxy PMQ is stereospecific and as such each enantiomer behaves differently (Fasinu et al., 2014, Tekwani et al., 2015). Interestingly, in the case of CYP2D6 metabolite generation, the dihydroxyPMQ that is then converted to a dihydroxy product could only be identified with the - enantiomer (Fasinu et al., 2014).

Through the MOA-A pathway, carboxyPMQ, the major human plasma metabolite is generated (Mihaly et al., 1984a, Pybus et al., 2012a). Again enantiomeric differences on PQ metabolism have been reported previously (Schmidt et al., 1977, Fasinu et al., 2014) and it was noted that carboxyPMQ is a non-active metabolite (Peters W, 1984, Link et al., 1985, Fasinu et al., 2014). Our results indicate that carboxyPMQ and its oxidative form hydroxy carboxyPMQ (MW-01-036) fails to interact with CYP2D6 and cannot generate H₂O₂. Carboxy PMQ is known to be further metabolized to ring-hydroxylated forms (Fasinu et al., 2014) which, similar to hydroxycarboxyPMQ (MW-01-036) compound, acted similarly as carboxy PMQ and all of them failed to interact with CYP2D6.

5.4.2 Gametocytocidal activity of PMQ is CYP2D6-dependant metabolism:

A single dose of PMQ has a clinical impact on gametocyte carriage reduction and a clear impact on microscopically identifiable gametocytes when given in combination with ACTs (Bousema et al., 2010, Smithuis et al., 2010b, Okebe et al., 2016). It has been indicated previously that the efficacy of PMQ is reliant upon biotransformation of the parent compound to generate the active redox cycling metabolites via a CYP 2D6-dependent pathway (Tekwani and Walker, 2006, Vale et al., 2009, Myint et al., 2011b, Pybus et al., 2012a, Bennett et al., 2013b). Phenolic metabolites, that are mostly produced via the CYP2D6-dependant pathway (Pybus et al., 2012a), are highly likely to demonstrate such activity due to their reactive nature of oxidation reduction cycling thereby generating oxidative stress within the parasite.

In this chapter, we have used recombinant CYP2D6 and HLM to generate PMQ metabolites *in-vitro* and harvested the product to investigate their gametocytocidal activity against late stages of *P. falciparum* gametocytes. Here, incubation of parent drug and metabolites with either HLMs or the CYP2D6 metabolising system significantly reduced gametocyte viability with exception of carboxyPMQ, which was failed to kill the gametocytes in all conditions. The effects for PMQ and CYP2D6 were significant (.

Table 5.4). All other hydroxylated metabolites and their quinoneimine equivalents also exhibited significantly greater activity after CYP2D6-treatment. A similar pattern was observed using HLM to drive metabolism. Although there appears to be some inhibitory effect of the buffer components of the non-HLM-treated compounds on gametocytes viability, the efficacy of (+) HLM-treated compounds were always statistically significant. One possible cause of the inhibitory effect in the control (-) HLM treatments, is the presence of NADP⁺ reductase activity in the regeneration

system, which may in itself generate reactive oxygen species. These results are in strong agreement with the effect of PMQ in reducing the gametocyte carriage *in-vivo* as well as their link to biotransformation of the compound to its active phenolic metabolites via interaction with CYP 450s particularly CYP2D6 isoenzyme.

5.4.3 Conclusion:

PMQ is an important antimalarial drug for the treatment of relapsing infections (*P. vivax* and *P. ovale*) and its potential clinical impact in reducing gametocyte carriage. The effectiveness of PMQ has been linked to Cytochrome P450 (CYP)-mediated metabolism, and recent studies suggest that in particular, the CYP isoform 2D6 is important to generate redox-active metabolites. Although the mechanism of action of this class of antimalarial drugs (8-AQ) is still not well understood, the interaction of PMQ and its metabolites with CYP2D6 were conducted to measure the inhibition potential of each compound against CYP2D6 as a surrogate for being a potential substrate. The results indicate there to be interaction between the CYP2D6 enzyme with PMQ and its phenolic metabolites and this linked with their capacity for redox cycling of hydroxylated metabolites to generate the oxidative agent H₂O₂. Carboxy PMQ and MW-01-036 failed to inhibit the tracer metabolism at all, which indicated the failure of the interaction with the CYP2D6 enzyme.

To investigate the efficacy of 8-AQ metabolites against late-stage gametocytes, an *in vitro* metabolite activation method was developed under two conditions that mediated metabolism, i.e. CYP2D6-mediated and HLM-mediated metabolism. In general, PMQ and its hydroxylated and quinone imine compounds showed significant activity against gametocytes in both conditions. Carboxy PMQ and MW-01-036 had no activity against gametocytes in all conditions. These data were discussed in the context

of understanding the mechanism of action of PMQ and its metabolites which are important for developing and designing new transmission-reducing antimalarial drugs.

Chapter 6

**Tafenoquine and novel 8-aminoquinoline analogues:
Gametocytocidal activity, haemolytic toxicity in the role of
CYP2D6-dependent drug metabolism**

6.1 Introduction:

The 8-aminoquinoline class are an old and important antimalarial drug class that have proven activity against the exoerythrocytic liver stages of malaria, supporting their potential use in the treatment of relapsing malaria (Krotoski et al., 1980, Potter et al., 2015a). PMQ as the only drug actually registered to treat malarial relapsing, has been proposed as a vital tool in malaria transmission, blocking malaria and control strategies (Graves et al., 2015b, Rieckmann et al., 1968, Pukrittayakamee et al., 2004, Shekalaghe et al., 2007). However, long term safety concerns over PMQ, especially in G6PD deficient patients, has prompted the development of new 8-aminoquinoline analogues with a better safety and tolerability profile (Vennerstrom and Eaton, 1988, Bray et al., 2005a, Pradines et al., 2006).

Tafenoquine (TF), although invented several decades ago by the US military, is seen as a new 8-aminoquinoline analogue related to PMQ that has progressed to Phase III clinical trials as a potential new anti-relapse and chemoprophylactic agent (Dow et al., 2014, Llanos-Cuentas et al., 2014). This analogue has a significantly longer elimination half-life more than that in PMQ (Brueckner and Fleckenstein, 1991, Brueckner et al., 1998, Li et al., 2014). Therefore, single dose of TF has used for radical cure in humans (Llanos-Cuentas et al., 2014). Another advantage, and unlike primaquine, TF is active against asexual stages of *P. falciparum* at pharmacologically achievable blood concentrations (Vennerstrom et al., 1999, Pradines et al., 2006).

In spite of the pharmacokinetic benefits of TF compare to PMQ, both drugs seem to have similar pharmacogenomic dependency on CYP2D6-mediated metabolism for their hypnozoite (dormant liver) stage activity (Pybus et al., 2013, Pybus et al., 2012a,

Marcisin et al., 2014) and both drugs can cause haemolytic toxicity in G6PD deficient patients (Miller et al., 2013a).

Although the precise mechanism of action of TF is not yet known, it is thought to share features with PMQ. The accepted view is that 8-aminoquinoline metabolism generates as yet unidentified unstable metabolites with the capacity to undergo spontaneous redox cycling with *P. falciparum* enzymes such as ferredoxin-NADP⁺ reductase and diflavin reductase enzymes, resulting in the production of oxidative stress (hydrogen peroxide and hydroxyl radicals). The spontaneous redox recycling and the production oxidative stress via metabolism can be used to describe the antimalarial activity and the toxicity of the 8-aminoquinolines (Researches council UK (2016).

Like blood schizonticides such as the 4-aminoquinoline CQ, TF can inhibit asexual parasite growth via inhibitions of heme polymerisation as another mode of action in *P. falciparum* asexual stages. This could explain the ability of TF to be active against blood stages while PMQ is not (Vennerstrom et al., 1999).

The main aim of this chapter is to determine the ability of TF and a series of novel, but related structurally to 8-aminoquinoline, to kill malaria gametocytes and by use of recombinant enzymes and liver microsomes to establish the importance of human cytochrome P450 2D6 (CYP 2D6) enzyme in antimalarial activity and red cell hemolytic toxicity. In addition to TF, the structural analogues SL-6-41, SL-6-46 and SL-6-56 were also investigated for their gametocytocidal activity and the role of CYP2D6 metabolism in their antimalarial activity.

6.2 Material and Methodes:

6.2.1 Reagents:

P. falciparum 3D7elo1-pfs16-CBG99 were generously provided by Prof Pietro Alano lab (INBB, Istituto Nazionale di Biostrutture e Biosistemi, 00136 Rome, Italy) (Cevenini et al., 2014). TF succinate was purchased from Sigma and other novel 8-aminoquinoline compounds were synthesised in the Chemistry Department by Dr Michael Wong and Dr Shirley Leung (Prof Paul O'Neill group), University of Liverpool, UK (Table 6.1 and Figure 6.1). The Vivid® CYP450 2D6 Screening Kit was obtained from Life Technologies (Paisley, UK). Pooled Human Intestinal Microsomes were generated in Liverpool and a NADPH Regenerating System was obtained from Corning ® Gentest™ (Corning B.V. Life Sciences, Netherland). The Oxytherm system and O₂ View software was obtained from Hansatech Instruments Ltd. (King's Lynn, UK). Catalase, from bovine liver, was obtained from Sigma (C1345; Dorset, UK).

6.2.2 Test Compounds:

Table 6.1: TF and the newly synthetic 8-aminoquinoline compounds studied.

Class	Compound name	Molecular Weight	Solvent
8-aminoquinoline	TF	581.58 g/mol	50 % MeOH
	SL-6-41	275.35 g/mol	
	SL-6-56	273.33 g/mol	
	SL-6-46	261.33 g/mol	

6.2.3 Compounds chemical Structures:

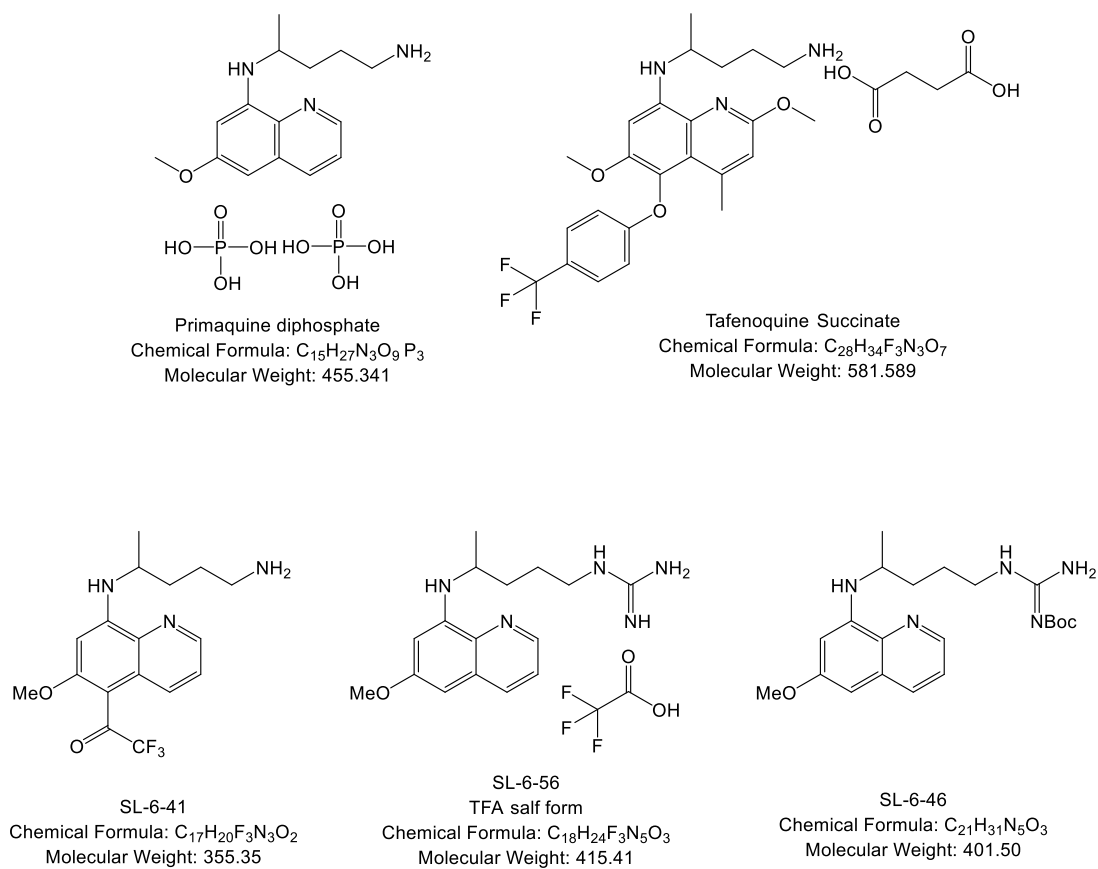


Figure 6.1: Chemical structure of PMQ, TF and newly synthetic 8-aminoquinoline (SL-6-41, SL-6-46 and SL-6-56).

6.2.4 Methods:

P. falciparum gametocytes culture gametocytocidal viability assay was performed as described in methodology chapter 2, section 2.3. Enzyme kinetic assay, H₂O₂ generation of CYP-treated compounds and gametocytocidal assay of CYP-treated and HLM-treated compounds were conducted as described in detail previously in chapter 5 section 5.2.4.

6.2.4.1 *In-vitro* hemolysis assay:

Test and control compounds at 20µM concentration were prepared in isotonic phosphate buffer under two different conditions: with and without recombinant CYP2D6 enzyme system, and diluted 2:3 in isotonic phosphate buffer (150mM NaCl in 10 mM phosphate buffer). Incubations were carried out at 37°C for 24 h in a shaking water bath, with a suspension of human erythrocytes (RBC, 1% hematocrit) obtained from either normal or glucose-6-phosphate dehydrogenase (G6PD)-deficient donors. The reaction was stopped by cooling at 4°C and the mixtures were centrifuged at 1000 g for 10 min, and the absorbance of the supernatants was measured at 540 nm in a plate reader, Varioskan ® Flash plate reader (Thermo Electron Corporation). The hemolytic rate was calculated using 10 mM phosphate buffer as the positive control for hemolysis of human RBC, which was considered 100% hemolysis (Fraser and Vesell, 1968, Wang et al., 2010).

6.3 Results:

6.3.1 TF autofluorescence spectrum:

The overlapping of the TF auto-fluorescence could interfere with fluorescence-based assay (Vivid® CYP450 screening assay). Using a Varioskan ® Flash plate reader (Thermo Electron Corporation) multimode reader, fluorescence intensity was monitored to detect the precise excitation and emission wavelength characteristic of TF (Figure 6.2).

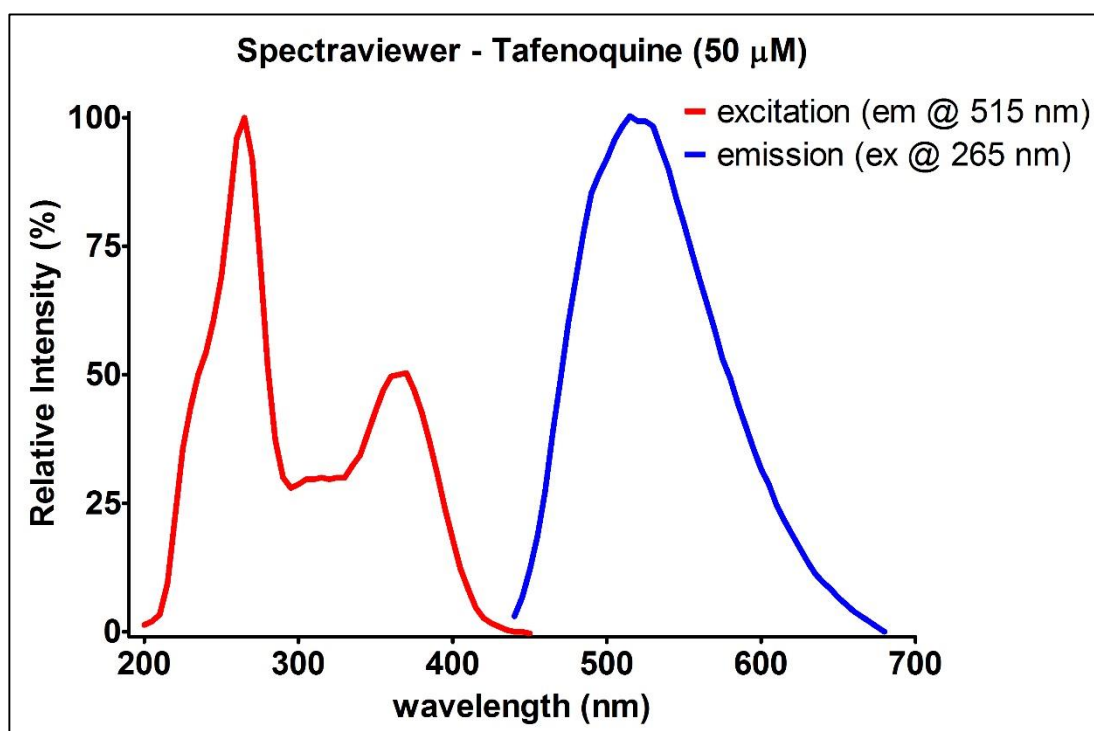


Figure 6.2: Spectrum auto fluorescence excitation and emission detected at high concentration of TF.

6.3.2 CYP2D6 enzyme kinetic inhibition:

To determine the interaction of the 8-aminoquinoline drug TF and other 8-aminoquinoline analogues with the human CYP450 2D6 enzyme, a Vivid® CYP450 screening fluorescence-based assay was used. Quinidine (10 μ M), a known inhibitor of CYP2D6, was used as a positive control, while 50% methanol (solvent) was the negative control. In the absence of any other compound, the recombinant CYP2D6 enzyme metabolises the tracer, producing a fluorescent signal that changes with time (Figure 6.3, A & B silver trace). The positive control quinidine inhibits the function of CYP 2D6, preventing the metabolism of the tracer and leading to a reduction in the kinetic fluorescent signal (Figure 6.3, A & B black trace). TF produced auto-fluorescence that overlapped with the tracer metabolism (Figure 6.3). To correct for the auto-fluorescence of TF, the experiment was conducted with and without the tracer substrate in the incubation as shown in Figure 6.3, A. The inhibition kinetics caused by TF was established after subtracting the auto-fluorescence contribution from the total signal as shown in Figure 6.3, B. Inhibition of CYP2D6 dependent substrate metabolism was minimal up to 30 μ M TF (mean inhibition of $67.7\% \pm 3.7$ (Figure 6.3, B red trace).

Thereafter, the concentration-response data was subjected to a steady-state kinetic analysis using the Michaelis-Menten equation, to determine K_m and V_{max} , as illustrated in Figure 6.3, C. TF showed “non-specific” inhibition of tracer metabolism and the typical Menten curve could not be generated from the data collected.

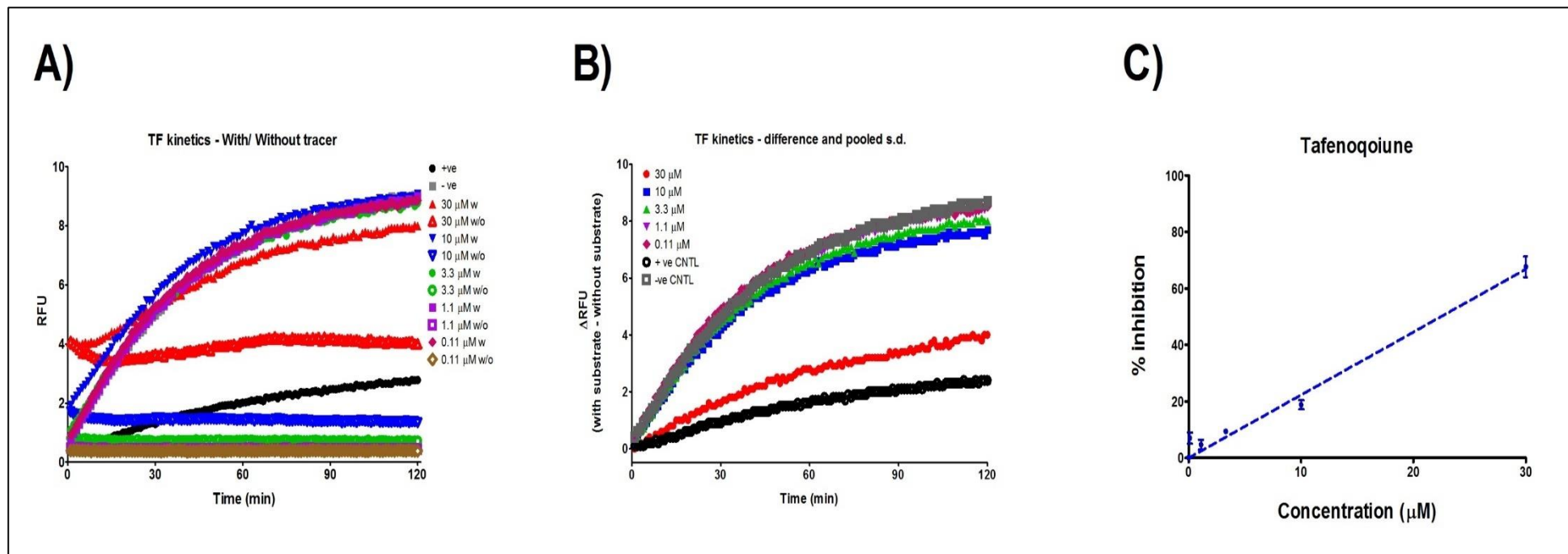


Figure 6.3: The ability of TF to compete for CYP2D6 via competition with the metabolism of a fluorescent probe substrate.

A. shows the florescent signal generated by TF alone and in the presence of the CYP2D6 substrate (with tracer). B. shows the kinetics of TF induced inhibition of CYP2D-dependent tracer metabolism after subtracting the autoflourescence signal from Tafenoauine. C. Michaelis-Menten fit of the data.

Figure 6.4, highlights the Michaelis Menton curves generated using the novel 8-aminoquinolines in the CYP2D6 metabolism experiment. SL-6-46 and SL-6-56, showed a pronounced inhibition of the tracer metabolism ($K_m = 6.7 \pm 1.2 \mu\text{M}$, $V_{max} = 96 \pm 2\%$ and $K_m = 0.5 \pm 0.1 \mu\text{M}$, $V_{max} = 92 \pm 1\%$, respectively as seen in). The inhibitory potency of the structural analogue SL-6-56 was superior to SL-6-46 achieving V_{max} at $10 \mu\text{M}$ (Figure 6.4B & C).

In the case of SL-6-41, the compound had no clear effect on tracer metabolism for $10 \mu\text{M}$ or less. However, SL-6-41 display non-specific inhibition at higher concentration ($30 \mu\text{M}$) by mean $66.7\% \pm 2.67$ (Figure 6.4, A) similar to the profile seen with TF.

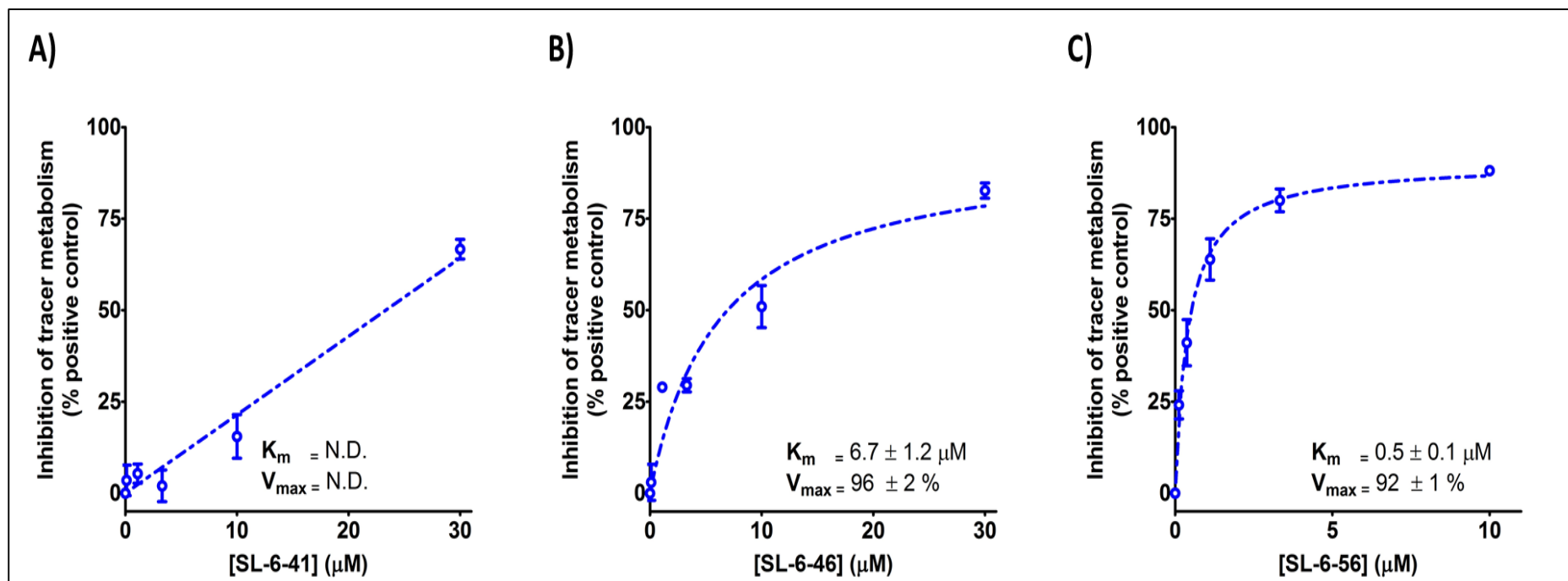


Figure 6.4: The ability of three 8-minoquinoline PMQ analogues to compete for CYP2D6 via competition with the metabolism of a fluorescent probe substrate.

Inhibitor concentrations ranged from 0 to 30 μM . SL-6-41 display non-specific inhibition at higher concentration (30 μM). All compounds concentrations response data were fitted to the Michaelis-Menten equation.

6.3.3 CYP metabolism dependent hydrogen peroxide production:

One proposed hypothesis is that the metabolism of TF, like PMQ, via CYP2D6 generates metabolites with the capacity for spontaneous redox cycling leading to the production of hydrogen peroxide (H_2O_2) which contributes the drugs mechanism of action. To test this, the Oxytherm system was used to indirectly measure H_2O_2 production. The assay measured oxygen released in the presence of catalase. In the absence of the CYP2D6 drug metabolising system, there was no oxygen released when incubated with the control methanol of TF or PMQ (Figure 6.5, A).

However, in the presence of the CYP2D6 enzyme, there was a measurable release of oxygen (Figure 6.5, B) in support of the working hypothesis.

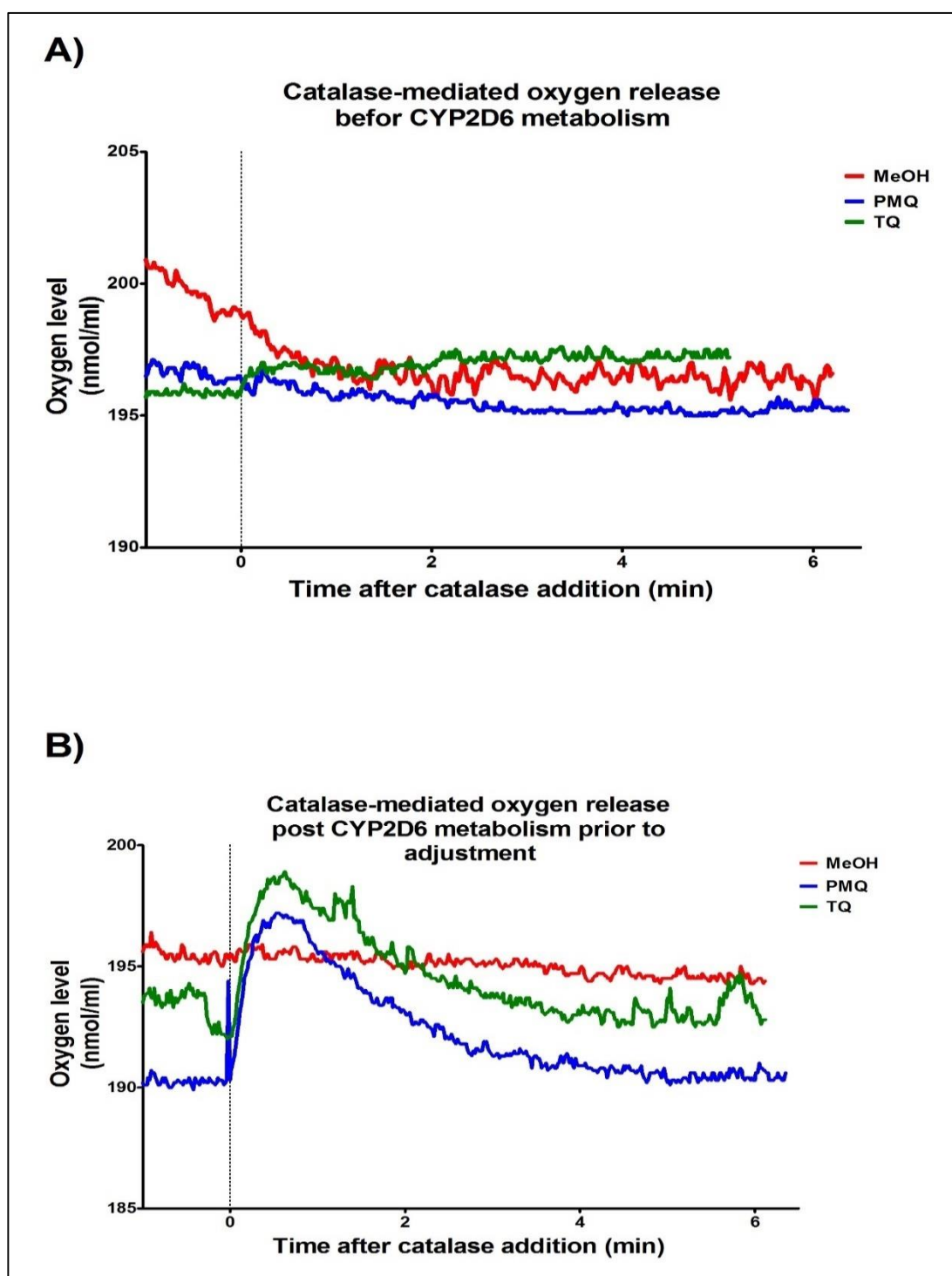


Figure 6.5: Catalase-mediated oxygen release before and after CYP2D6 metabolism of TF compared to PMQ prior to normalization (adjustment).

Catalase-mediated oxygen release before CYP metabolism (A) and after CYP2D6 metabolism (B) of primiquine and its hydroxylated metabolites. The dashed line at 0 min represents the addition of catalase. MeOH as control in red trace line, PMQ in blue line and TF in green.

To directly compare the peak O₂ release after catalase addition for TF and primaquine, the y-axis was normalized to zero and the oxygen released after adding catalase plotted as shown in Figure 6.6. Methanol 50% was used as solvent negative control and in the CYP2D6 assays after the addition of catalase there was no O₂ released. However, addition of catalase to the CYP 2D6-treated compounds TF and PMQ produced a clear O₂ release of 6.08nmol/ml and 6.1 nmol/ml, respectively (Figure 6.6, green and blue trace).

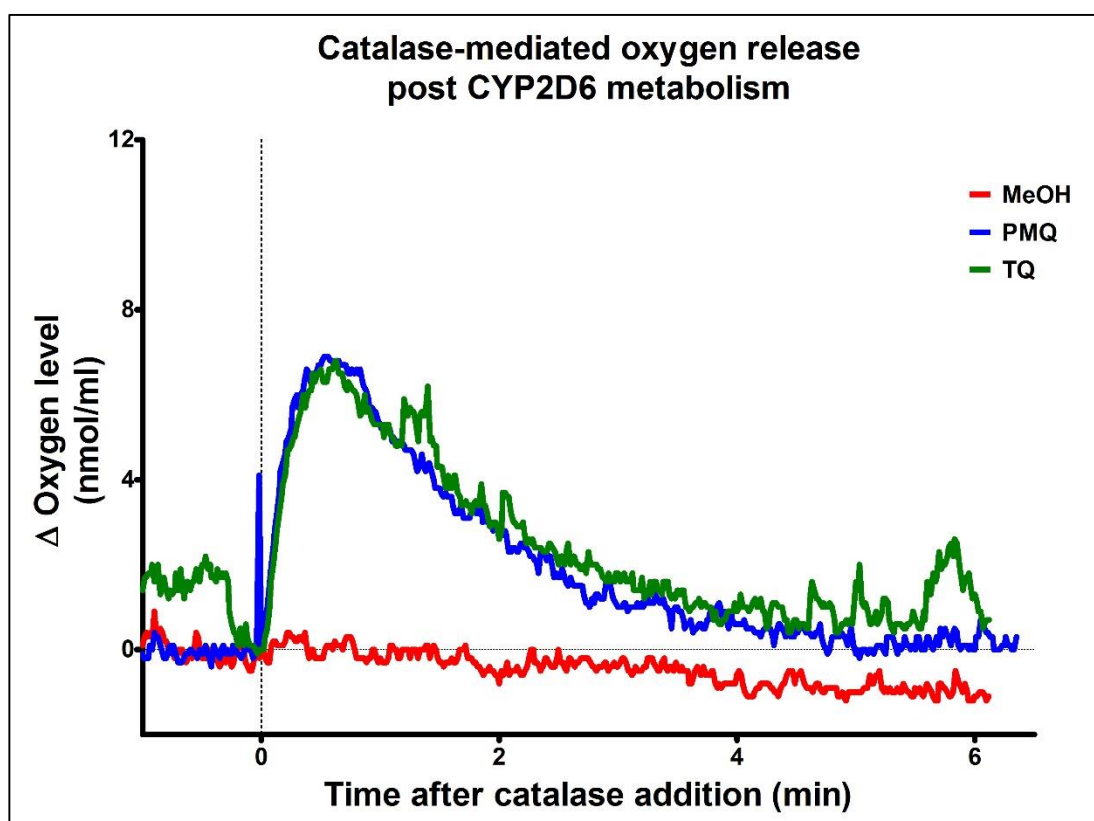


Figure 6.6: Normalized catalase-mediated oxygen release post CYP2D6 metabolism of TF compare to PMQ.

The dashed line at 0 min represents the addition of catalase. MeOH as control in red trace line, PMQ in blue line and TF in green.

6.3.4 Hemolysis in red blood cell G6PD deficient:

The hemolysis of human red blood cells (normal and G6PD-deficient) was investigated after incubation with 10 μ M drug in the absence or presence of a CYP2D6 drug metabolising system. Compared to the negative control (50% methanol), PMQ (at 10 μ M) did not cause any hemolysis either in normal or G6PD blood donors either in the presence or absence of the metabolising system (Figure 6.7). In contrast TF in the absence of the metabolising system induced pronounced lysis in both normal and G6PD deficient red cells that was significantly reduced in the presence of the CYP2D6 metabolising system (Figure 6.7).

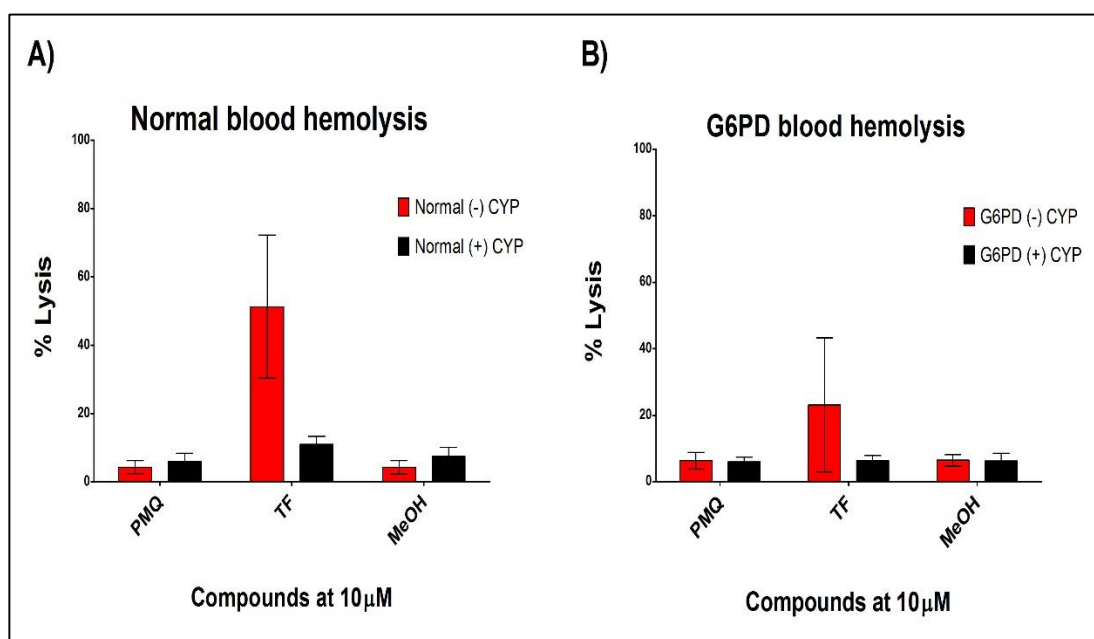


Figure 6.7: In-vitro hemolysis induced by PMQ and TF.

The figure illustrates the haemolytic effect of PMQ and TF in either normal or G6PD deficient red cell under two reaction conditions i.e. in the presence (black) or absence (red) of the CYP2D6 drug metabolising system (A) normal blood donors and (B) G6PD blood donors.

6.3.5 The role of CYP2D6 specific metabolism and gametocytocidal activity:

TF was tested against late-stage *P. falciparum* gametocytes *in vitro*. In the absence of any CYP2D6 metabolising system it can be seen that after 72 h incubation tafenoquine (10 μ M) reduced gametocyte viability substantially (mean viability reduction of 22.06%). Under equivalent conditions PMQ was much less potent with viability reduced to a mean of 70.12%. In the presence of the CYP2D6 drug metabolising system the effect of TF on gametocyte viability was significantly reduced ($P < 0.001$), while that of PMQ was enhanced (Figure 6.8). Using the 8-aminoquinoline analogues in similar experiments demonstrated that SL.6.41 was gametocytocidal in its own right, reducing viability by 37.26% in the absence of the CYP2D6 system and 22.45% in the presence of CYP2D6 (Figure 6.9). SL-6-46 had a weak effect on gametocyte viability in either the presence (81.99%) or absence (83.08) of the metabolising system. Finally, SL-6-56, exhibited no effect on gametocyte viability in the absence of the enzyme system but reduced viability to 73.36% in the presence of the metabolising system (Figure 6.8, Table 6.2).

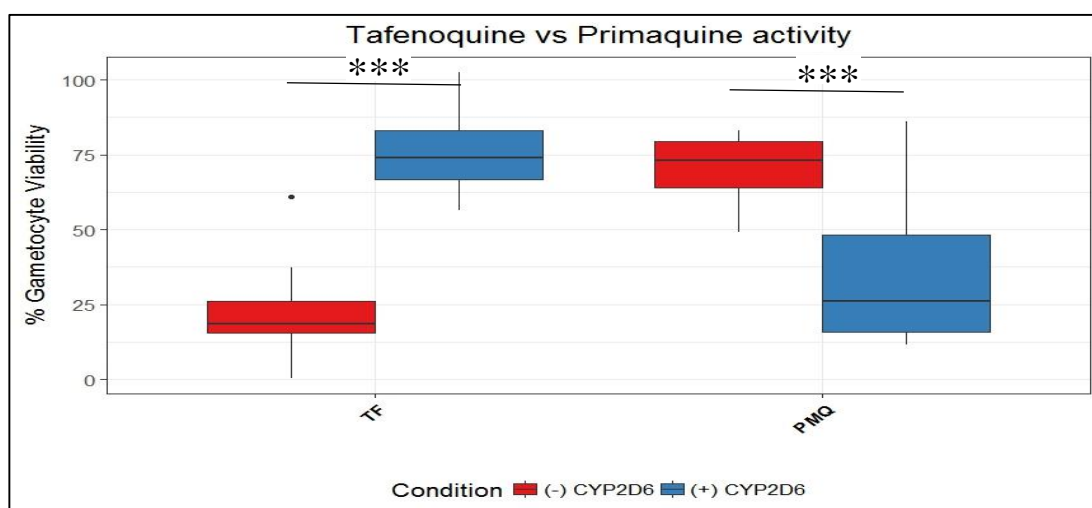


Figure 6.8: The effect of CYP2D6-dependent metabolism on the viability gametocytes after exposure to TF vs PMQ.

TF and PMQ were tested under two conditions: without exposure to a CYP2D6 drug metabolising system and after incubation with a CYP2D6 drug metabolising system. The data represent comparison between conditions in each compound. The midline of each box-plot is the median, with the edges of the box representing the 1st and 3rd quartiles. Whiskers delineate the 5th and 95th percentiles. *** indicates $P < 0.001$, (Welch's t-test, two tail, $n=7$ separate experiments, each in triplicates).

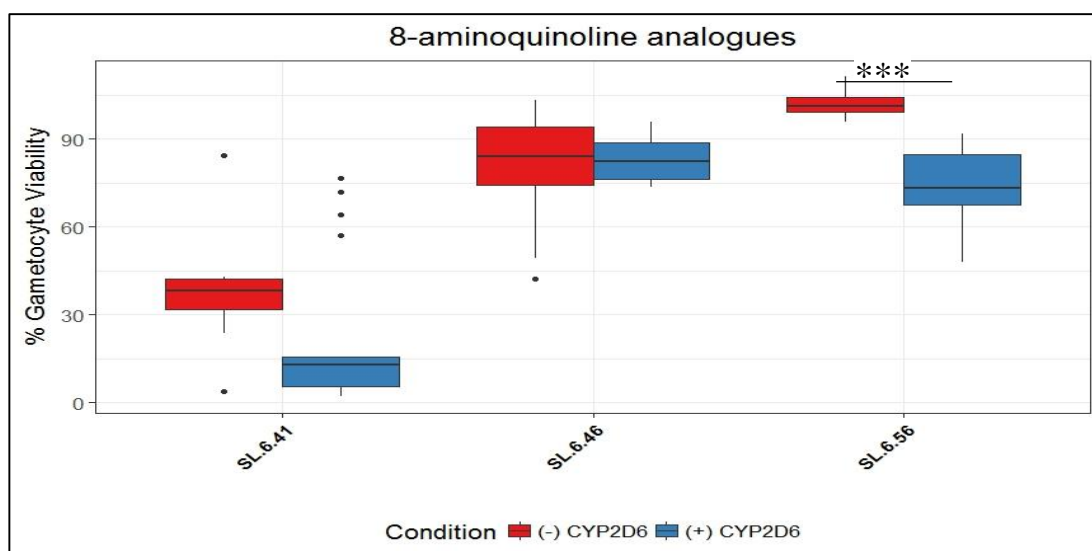


Figure 6.9: The effect of CYP2D6-dependent metabolism on the viability of gametocytes after exposure to novel synthetic 8-aminoquinoline analogues.

8-aminoquinoline analogues were tested under two conditions: without exposure to a CYP2D6 drug metabolising system and after incubation with a CYP2D6 drug metabolising system. The data represent comparison between conditions in each compound. The midline of each box-plot is the median, with the edges of the box representing the 1st and 3rd quartiles. Whiskers delineate the 5th and 95th percentiles. *** indicates $P < 0.001$, (Welch's t-test, two tail, $n=7$ separate experiments, each in triplicates).

Table 6.2: The summary of statistical analysis between (-) CYP and (+) CYP incubations with TF, primaquone and the 8-aminoquinoline analogues in terms of effects on viability of late gametocytes (Welch's *t*-test, two tail).

Compound	% Survival No CYP (Mean)	%Survival CYP (Mean)	P-value	95% CI	Significance
TF	22.1	74.8	2.45E-12	-61.6, -43.7	***
PMQ	70.1	38.7	0.00049	16.1, 46.8	***
SL-6-41	37.3	22.5	0.0710	-0.46, 30.1	ns
SL-6-46	81.9	83.1	0.821	-11, 8.8	ns
SL-6-56	102.3	73.4	2.01E-07	21.8, 35.9	***

*** Statistically significant at $p < 0.001$.

p values were corrected for false positives using the benjamini and hochsberg method.

6.3.6 The role of microsomal metabolism on gametocytocidal activity:

To further investigate the physiological relevance of metabolism of 8-aminoquinolines and their gametocytocidal activity, the experiments outlined in section 1.3.5 were repeated with compounds incubated with HLM in place of a specific recombinant CYP2D6 system. Unlike the Baculosome CYP2D6 system, the HLM contain a full complement of CYP450 enzymes. TF and other 8-aminoquinoline structural analogues were tested against late-stage *P. falciparum* gametocytes *in vitro*, both before and after incubation with the HLM reaction system. As with the previous experiments using the VIVID system, the gametocytocidal activity of each compound was tested under two conditions: (-) HLM that contains the compound and reaction mixture without liver microsomes and (+) HLM that have the compound and reaction mixture with liver microsomes.

After a 72 h incubation, TF (at 10 μ M) under both conditions did not show significant activity against gametocytes with mean parasite viabilities of 85.2% and 87.5%. In contrast, PMQ still exhibited statistically significant activity ($P < 0.001$) in (+) HLM-

treated condition reducing mean parasite viability to 18.6% compared to HLM- 53.3% viability in the absence of HLM (Figure 6.10).

The structural analogue, SL-6-41, again exhibited statistically significant ($P < 0.001$) activity in the absence of HLM (mean of 23.1 % viability) (Figure 6.11). In the (+) HLM-treated condition of the former analogue, showed moderate activity against gametocytes by reducing the viability to 51.9%. In the case of SL-6-46, this structural analogue exhibited a similar pattern of gametocytocidal activity before and after HLM incubation with mean viability of 63.05% in (-) HLM and 57.6% in (+) HLM. Similarly, the structural analogue, SL-6-56, displayed slight gametocytocidal activity in (-) HLM compare to (+) HLM condition with mean of 61.5% and 74.4% viable gametocytes, respectively (Figure 6.11).

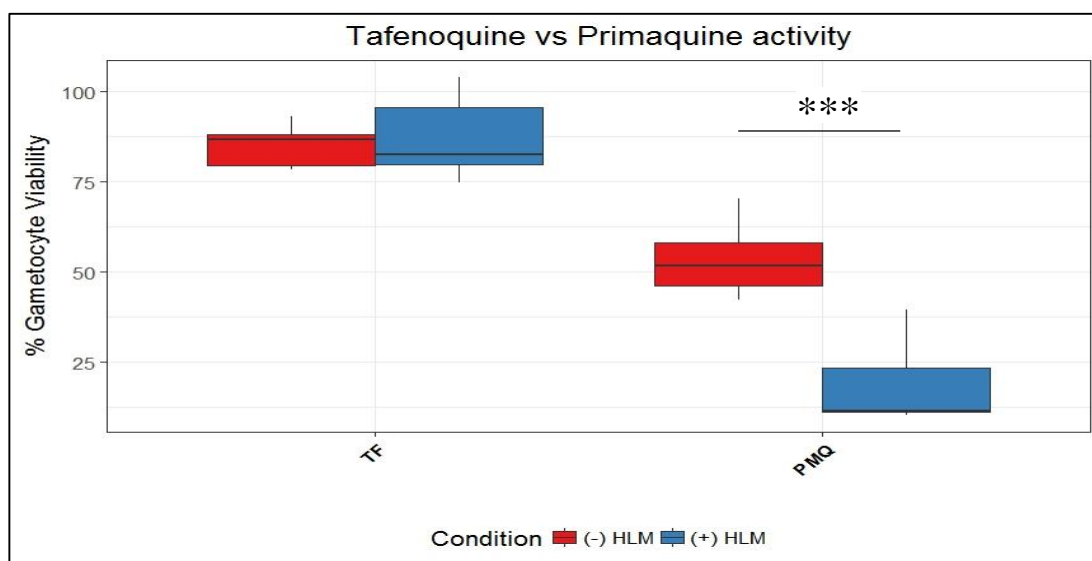


Figure 6.10: The effect of HLM-dependent metabolism on the viability gametocytes after exposure to TF vs PMQ.

TF and PMQ were tested under two conditions: without exposure to a HLM drug metabolising system and after incubation with a HLM drug metabolising system. TF were tested in two conditions: (-) HLM compound and drug after metabolites activation (+) HLM. The data represent comparison between conditions in each compound. The midline of each box-plot is the median, with the edges of the box representing the 1st and 3rd quartiles. Whiskers delineate the 5th and 95th percentiles. *** indicates $P < 0.001$, (Welch's t-test two tail, $n=3$ separate experiments, each in triplicates).

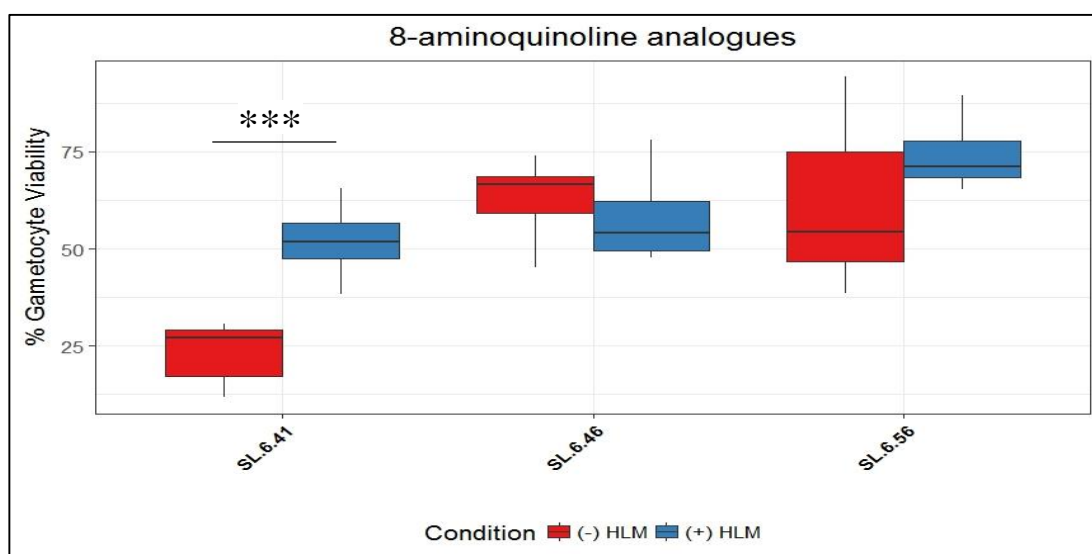


Figure 6.11: The effect of HLM-dependent metabolism on the viability gametocytes after exposure to new synthetic 8-aminoquinoline analogues.

8-aminoquinoline analogues were tested under two conditions: without exposure to a HLM drug metabolising system and after incubation with a HLM drug metabolising system. The data represent comparison between conditions in each compound. The midline of each box-plot is the median, with the edges of the box representing the 1st and 3rd quartiles. Whiskers delineate the 5th and 95th percentiles. *** indicates $P < 0.001$, (Welch's *t*-test two tail, $n=3$ separate experiments, each in triplicates).

All the statistical differences between the two conditions in all compounds are listed in the Table 6.3.

Table 6.3: The summary of statistical significances between (-) HLM and (+) HLM of TF, primaquone and 8-aminoquinoline analogues against late gametocytes (Welch's *t*-test, two tail).

Compound	% Survival No HLM (Mean)	%Survival HLM (Mean)	P-value	95% CI	Significance
TF	85.2	87.5	0.67402	-14.1, 9.5	ns
PMQ	53.3	18.6	0.000178	21.3, 48.1	***
SL-6-41	23.2	51.9	8.27E-05	-38.9, -187	***
SL-6-46	63.1	57.7	0.416085	-7.2, 17.9	ns
SL-6-56	61.5	74.5	0.265975	-34.7, 8.8	ns

*** Statistically significant at $p < 0.001$.

p values were corrected for false positives using the benjamini and hochsberg method.

6.4 Discussion:

TF is the only new 8-aminoquinoline that is close to clinical use in the field having come a long way in clinical trials to demonstrate its potential to be a radical cure for human malaria. It is proposed as a single dose treatment that is an alternative analogue to PMQ. The longer half-life that allows single dose use is a big advantage over PMQ. However, the requirement for hepatic metabolism in the mechanism of action and toxicity of TF is poorly understood and in comparison with PMQ it is a differentiation in these areas of pharmacology that will be important clinically (Marcsisin et al., 2014, Vuong et al., 2015).

The definite connection between TF liver stage anti-malarial activity and the need for hepatic metabolism to active metabolites (via CYP 2D6 or other enzymes) has so far been established in humans but is currently being actively investigated (St Jean et al., 2016). Thus, the aim of this chapter is to investigate if TF and a small number of other novel 8-aminoquinolines can interact with CYP2D6, measured through inhibition of a CYP2D6 probe substrate in a CYP2D6 recombinant enzyme assay described in section 5.2.4.1. Thereafter, the link between the metabolism of these potential gametocytocidal drugs (investigated using HLMs and a specific CYP2D6 system) and their effects on late stage gametocyte viability were studied.

6.4.1 The interaction between selected 8-aminoquinolines and CYP2D6:

Clinical data indicate that PMQ is metabolised by CYP2D6 in man (Tekwani and Walker, 2006, Vale et al., 2009, Pybus et al., 2012a, Deye and Magill, 2014, Marcsisin et al., 2016). Furthermore, CYP2D6 poor metabolisers fail to achieve radical cure with PMQ (Bennett et al., 2013a) suggesting an intimate relationship between antimalarial effects in the liver and CYP2D6 specific routes of metabolism (De Gregori et al., 2010,

Marcisin et al., 2016). The question that remains is whether this is the case for all 8-aminoquinolines and is it important for their effects against gametocytes. The in vitro enzyme inhibition data suggest that all the 8-aminoquinolines can interact with CYP2D6 but with different mechanisms. As mentioned in chapter 5, using BACULOSOME (microsomes expressing a human CYP2D6 isozyme) offers a benefit of using only the metabolism from a specific CYP450 in isolation. The data generated indicate that TF causes a non-specific inhibition of tracer metabolism at 30 μ M. Failure of inhibition of tracer metabolism by TF at lower concentrations demonstrates that TF might not be dependent on CYP2D6 metabolism to the same level as PMQ and the failure to show pure competition suggests it may not be a 2D6 substrate although this cannot be definitively concluded. This data are in agreement with a recent study in which it was concluded that TF activity against *P.vivax* might not be linked to CYP2D6 metabolism (St Jean et al., 2016). In addition, the reduced metabolism in CYP2D6 from the same study was not linked to relapse among intermediate metabolizers following the administration of TF for *P. vivax* (St Jean et al., 2016). In contrast to this clinical data other studies have supported a link between TF liver stage anti-malarial activity and CYP 2D6 metabolism based on altered TF pharmacokinetics dependent on CYP 2D metabolizer status in mice (Vuong et al., 2015). Similarly, TF achieved the efficacy in CYP 2D metabolizer status in mice and also in humanized CYP2D6 mice with high dose (2-fold of ED₁₀₀) (Marcisin et al., 2014).

We have extended the investigation of CYP2D6 to look at other related 8-aminoquinolines currently under investigation in Liverpool as alternatives for development. Two analogues, SL-6-46 and SL-6-56, clearly inhibited the metabolism of the tracer, and thus showed their capability to interact with CYP2D6 and possibly be substrates. However, the structural analogue SL-6-41 displayed non-specific

inhibition of tracer metabolism at 30 μ M, similar to that observed with TF. This results could guide structure activity studies. The common trifluoromethyl (CF₃) at 5 position could be driving the orientation of the interaction with CYP2D6.

It is hypothesised that redox cycling of hydroxylated metabolites generated from 8-aminoquinoline metabolism is capable of generating H₂O₂ which has a role in drug action. Furthermore, it is suggested that this H₂O₂ represents one of main oxidative agents responsible for unacceptable toxic hemolytic effects associated with PMQ and related molecules (Vasquez-Vivar and Augusto, 1992, Vasquez-Vivar and Augusto, 1994). In this study, we have been able to confirm that the CYP2D6 dependent generation of metabolites from both PMQ and TF that lead to the formation of the oxidative agent H₂O₂. It is worth noting the reported stereoselective differences seen with PMQ (Schmidt et al., 1977, Fasinu et al., 2014), which should be studied in the future using TF enantiomers.

6.4.2 Gametocytocidal activity of TF is not associated with CYP2D6-dependent metabolism:

In comparison to PMQ, TF has a significantly longer elimination half-life (Brueckner and Fleckenstein, 1991, Brueckner et al., 1998, Li et al., 2014), thus, a single dose is used for radical cure in humans (Llanos-Cuentas et al., 2014). Another advantage is the ability of TF to be active against asexual stages of *P. falciparum* (Vennerstrom et al., 1999, Pradines et al., 2006). TF has also shown clear activity as a gametocytocidal compound and we looked closely at the dose response of this drug in early and late stages gametocytes in chapter 3.

The conclusive link between TF liver stage anti-malarial activity and CYP 2D6 metabolic activation has yet to be firmly established in humans, although it is currently

a hot topic of investigation (St Jean et al., 2016), however the emerging data from human trials and preclinical models are somewhat contradictory (Vuong et al., 2015, Marcsisin et al., 2014).

In this chapter, we have used recombinant CYP2D6 and HLM (HLM) to generate natural metabolites of TF and other structural analogues in-vitro. These metabolic products have then been used to investigate their gametocytocidal activity compared to relevant controls. In the context of CYP2D6 metabolism, and unlike PMQ, the activity of normal drug exposure without the CYP2D6 metabolising system indicated that TF was statistically significant active against late stages gametocyte compared to the situation following incubation with the CYP2D6 system as shown in Figure 6.8. However, this was not the case when using HLM to generate TF metabolites (+) HLM and with (-) HLM. A possible explanation of this is that CYP2D6 converts TF to metabolites with inferior gametocytocidal properties compared to parent drug and as such it is protective. In the case of HLM it must be assumed that the 2D6 pathway is relatively trivial for TF with other routes predominating and producing inactive metabolites. However, this does not explain why in type CYP2D6 environment without the enzyme we observed significant reductions in gametocyte viability that was not present in the HLM cultures without the HLPs. This will require further investigation of the effects of the underlying culture conditions.

SL-6-41 is the only structural analogue that has clear activity against late stage gametocytes. However, unlike TF, the activity of this analogue was increased following incubation with CYP2D6. Under HLM incubation conditions the analogue still demonstrated significant activity in (-) HLM, however after addition of the microsomes, the gametocyte viability was reduced to 51.9 %.

SL-6-56 showed some effect on gametocyte viability that was enhanced in the presence of CYP2D6. Compared to PMQ, SL-6-56 and SL-6-46 failed to demonstrate gametocytocidal activity even though they showed a clear interaction with CYP2D6. Hemolytic toxicity, measured as hemolysis was studied after addition of drug (10 μ M) in the presence and absence of a CYP2D6 metabolising system. Remarkably, TF demonstrated the ability to lyse erythrocytes of normal and G6PD-deficient blood even without metabolic activation. Whereas, PMQ did not displayed lysis effect compare to negative control in any set of conditions. This is strongly in agreement with a very recent study which revealed that TF can trigger eryptosis or suicidal erythrocyte death in normal blood samples. TF-treated blood sample collected in this study were tracked by the presence of the two main markers for the eryptosis; cell membrane scrambling with phosphatidylserine translocation and cell shrinkage (Al Mamun Bhuyan et al., 2016). This eryptosis effect due to TF dose may be increased in some clinical conditions such as malaria and G6PD deficiency (Lang and Lang, 2015). However, our observation from TF and PMQ at specific concertations, after CYP2D6-mediated metabolism, failed to show hemolytic toxicity.

6.4.3 Conclusion:

8-aminoquinoline antimalarial drugs play an important role in the treatment of relapsing infections and in reducing the gametocyte carriages of *P. falciparum*. The activity of 8-aminoquinoline is thought to be linked to Cytochrome P450 metabolism, in particular, CYP isoform 2D6 that is thought to be important in the generation of redox-active metabolites. The main finding from this chapter is the converging evidence that TF gametocytocidal activity is not totally reliant on CYP2D6 metabolism. TF, after CYP2D6 metabolism, can generate H₂O₂. In addition, TF, under

the conditions used here can lysis erythrocytes of normal and G6PD blood even in the absence of CYP2D6.

SL-6-41 shows clear activity against gametocytes with notable improvements in gametocyte activity after CYP2D6 metabolism. Both TF and SL-6-41 demonstrate a similar pattern of interaction with CYP2D6 (non-specific inhibition of tracer metabolism at 30 μ M). The structural analogues SL-6-46 and SL-6-56, confirmed as inhibitors of CYP2D6 dependent metabolism failed to have any they have impact on gametocytocidal activity.

Overall, some but not all 8-aminoquinolines are potential CYP2D6 metabolism. Metabolism can have a profound impact on both gametocytocidal activity and haemolytic toxicity which is drug specific. The big question is whether it is possible to find an 8 amnioquinoline that can kill gametocytes in a non CPYP2D6 dependent manner without causing red cell toxicity.

Chapter 7

Conclusion, Limitations and Future Perspectives

7.1 Summary and conclusion:

The aim of this chapter is to summarise the main findings from the thesis and set out how the information can be integrated to gain a better understanding of the important features of gametocyte-active antimalarials of *P. falciparum* with a focus on understanding of the mechanisms underpinning the activity of some of the agents tested.

Malaria still main reasons a disease that causes serious mortality and morbidity in the world, especially in tropical areas. With the ambition of accomplishing a sustainable strategy for malaria eradication/elimination, malarial transmission has to be a priority within an integrated approach that will include vector control, mass treatment etc. Therefore, targeting gametocytes remains pharmacologically attractive and strategically essential in the current efforts to eliminate malaria.

Starting with the screening of selected reference antimalarial drugs as well as new potential compounds against two distinct stages of the gametocyte life cycle (early stage II and III and late stages IV and V), the main findings are that all compounds known to be active have stage specific activity profiles, even within a specific class there are subtle differences. This suggests differences in the underlying biochemical pathways that are exploitable in early and late stage gametocytogenesis. This is in line with accepted thinking that the early stages are metabolically very active whereas the late stages are essentially almost dormant and just carrying out essential functions to remain viable prior to ingestion by the next feeding mosquito. These pharmacodynamic differences could be helpful in directing future studies aimed at providing a deeper understanding of the mechanisms underpinning the activity of specific compounds. Briefly, MB, the spiroindolones and MPS1 inhibitors showed

significant gametocytocidal activity against all four stages. TF was the only 8-aminoquinoline that demonstrated gametocytocidal activity during all four studied gametocyte stages. However, even then, TF was much more potent against late stage gametocytes in comparison to early gametocytes. This is the only molecule with this type of profile, for all other compounds active against early and late stages, it was the early stages that appeared to be most susceptible. Lumefantrine displayed moderate gametocytocidal activity against early stages, inhibiting gametocyte viability by 52% compared to about 25 % inhibition of the mature late stages. This indicates that lumefantrine as a partner drug of choice with artemisinin (Coartem), the most commonly used ACT (Wells et al., 2009), would display moderate gametocytocidal activity through the actions of both components. In contrast, the 4-Aminoquinolines, ATQ and the antifolate (pyrimethamine) failed to display any substantial activity against gametocytes, apart from CQ which caused 50 % inhibition against early gametocyte stages, which is in line with data in the literature (Chapter 3).

The PD responses of the endoperoxide compounds also demonstrated stage-specific differences during gametocytogenesis. All test compounds were potent against early-stage gametocytes. This included the fully synthetic peroxides (OZ439 and TDD-E209), which have better PK profiles and significantly longer elimination half-lives (>1day) than artemisinin and the semi-synthetic derivatives (Copple et al., 2012, Moehrle et al., 2013, Wells et al., 2015). However, all the semi-synthetic artemisinins showed better activity against late stages than artemisinin and fully synthetic peroxides against that stage. DHA, the common antimalarial metabolite of the semi-synthetics and an antimalarial in its own right, demonstrated potency against gametocytes at all four stages. This was particularly noticeable against late stages and would support the argument that DHA (either from the pro-drugs artesunate or

artemether or directly) might have an important role in disease eradication based on its transmission blocking potential (Chapter 4).

The newly developed of time-dependent killing assay was used to investigate the exposure-effect relationship and gametocyte kill rates of MB and DHA against *P. falciparum* late stage gametocytes. Subsequently, the PK/PD relationship of the drugs as used in a clinical context was used to estimate the rate of clinical gametocyte clearance that could be achieved after clinically relevant treatment doses. The predicted gametocyte clearance potential with MB was total clearance of gametocytes from the blood stream in less than 3 days, whereas with DHA current dosage regimens are predicted to be suboptimal in terms of gametocyte clearance, although they would have a minor impact on carriage (Chapter 3 & 4).

A significant portion of the thesis is dedicated to the study of the 8-minoquinolines notably PMQ and its key predicted metabolites, TF and three novel 8-aminoquinoline analogues. The stage-specific gamatocytocidal activities of all compounds was assessed and differences in potency and stage specificity were observed between the analogues. There is strong evidence that the 8-aminquinolines exert their antimalarial effects via one or more metabolites and this has been linked to Cytochrome P450 metabolism, in particular, the CYP isoform 2D6, an enzyme that is polymorphic in humans with populations identified as either poor or extensive metabolisers of substrates that utilise this enzyme. Recent clinical evidence suggests that metabolism through CYP2D6 is important to the eventual generation of redox-active metabolites that kill the parasite.

Although the detailed mechanism of action of this class of antimalarial drugs (8-AQ) is still not well understood even after nearly 70 years of investigation, a role for CYP2D6-dependent metabolism era to be important. As a consequence, the interaction

of PMQ and its key metabolites with purified human CYP2D6 was investigated. The idea was to see if any of these molecules could inhibit the metabolism of a test substrate that was metabolized by this enzyme. Any type of inhibition was interpreted as meaning that the compound interacted with the enzyme but it could not be interpreted definitively as being a substrate for the enzyme. The results indicated that with the exception of the inactive carboxy metabolite, PMQ and all its metabolites could inhibit CYP2D6 activity to some degree. Furthermore, the suggested interaction of PMQ and its hydroxylated metabolites with CYP2D6 was shown to liberate redox cycling of hydroxylated metabolites to generate the oxidative agent H₂O₂. To investigate the activity of 8-AQ metabolites generated by CYP2D6 or HLMs against late-stage gametocytes, an *in vitro* assay allowing metabolite generation and activation was developed. Using this procedure to generate active metabolites from a starting substrate (PMQ or its known metabolites), the overall pattern was that enzyme activation increased gametocyte activity compared to that observed with the substrate prior to metabolic activation. The implication of this observation was discussed in the context of understanding how PMQ and its metabolites work and highlights some important features that should be considered when designing the next generation of transmission-reducing antimalarial drugs (Chapter 5).

An intriguing finding was several lines of evidence that suggest that TF gametocytocidal activity is not totally reliant on CYP2D6 metabolism. In comparison to PMQ, TF has demonstrated minimal hepatic metabolic turnover in many recent studies (Pybus et al., 2013, Pybus et al., 2012b, St Jean et al., 2016). This is in keeping with its long half-life. This would support the argument that in contrast to PMQ, metabolism of TF may not be as important a factor in the drug's activity either against gametocytes, as seen in the *in-vitro* data presented in this thesis or *in vivo* based on

data with *P. vivax* presented in the recent study of St Jean and his colleagues (St Jean et al., 2016). However, as note of caution, TF after CYP2D6 incubation with TF was shown to generate H₂O₂, indicating there may be some overlap with PMQ in the area of redox cycling of metabolites under certain conditions.

Further evidence that TF activity might be independent of CYP2D6 activation was the demonstration that TF was capable of causing lysis of erythrocytes from normal and G6PD deficient patient in the absence of the metabolising system. This is strongly in agreement with very recent study which revealed that TF can trigger eryptosis or suicidal erythrocyte death in normal blood samples (Al Mamun Bhuyan et al., 2016). Of the new 8-aminoquinoline analogues investigated, SL-6-41 showed a clear activity against gametocytes with a notable enhancement in the presence if the CYP2D6 metabolising system. Both TF and SL-6-41 analogue demonstrated a similar pattern of inhibition against CYP2D6 (non-specific inhibition of tracer metabolism at 30µM). The structural analogues, SL-6-46 and SL-6-56, validate their ability to inhibit the tracer metabolism and interact with CYP2D6, however failed to demonstrate clear activity against gametocytes (Chapter 6). These data are discussed in the context of understanding the mechanism of action of 8-aminoquinolines may provide important insights for developing and designing new transmission-reducing antimalarial drugs (Chapter 6).

7.2 Study limitations and future perspectives:

Although this work generated data that contributed to the understanding of the pharmacodynamic response of *P. falciparum* gametocytes to a range of compound classes and gives new insights into the mechanisms of action and predicted PK-PD modelling, there are limitations in the study which need to be considered in the design and development of the future experiments. These include:

- I. The thesis provides data that confirms the stage specific effect of different drugs against early and late stage gametocytes, but it doesn't really shed much light on the underlying biochemical processes that are being targeted. It should be possible to build on these observations by employing some new technologies that are currently being validated in the lab. These include metabolomics and proteomic studies of different gametocyte stages in the presence and absence of drugs and metabolites. A good study example of this approach using a chemical proteomic approach with asexual stages parasites from our group research has recently been described for the endoperoxides (Ismail et al., 2016).
- II. All the experiments carried out in this thesis were performed using one selected method using a luciferase-based transgenic parasite line to test for gametocytes viability. This limits the studies that can be carried out and excludes studies with patients isolates or other strains with interesting drug susceptibility phenotypes.
- III. The PK-PD model offers an interesting way to assess the potential impact of drugs and drug combinations on transmission blocking. It will be important to look at all currently available drugs and drug combinations in use and rank

their potential for transmission blocking prior to formal evaluation in patients and vector populations.

- IV. It is noted that all the thesis PD findings were obtained from gametocytes in vitro using two different defined age ranges stages during the gametocytogenesis (early stage II and II and late stage IV and V). Whilst there is nothing wrong with this, it would be a more complete analysis with broader relevance if these findings could be replicated through to direct measurement of transmission-blocking potential. Most important will be the demonstration of the ability of these compounds to prevent transmission to the mosquito using the standard membrane feeding assay or other currently validated methods that measure the functional viability of the *P. falciparum* mature gametocytes (functional viability of the *P. falciparum* mature male and female stage V gametocytes) such as the *P. falciparum* dual gamete formation assay developed by Ruecker and his colleagues (Ruecker et al., 2014).
- V. The need for understanding the mechanism of action and activation of 8-aminoquinolines appears to be important due to different PD responses of PMQ and TF against gametocytes. TF did not need any metabolic activation to show activity against malaria blood stages, where PMQ did need this activation via CYP450 enzymes, particularly CYP2D6. TF is known to demonstrate minimal hepatic metabolic turnover compare to PMQ as mentioned in recent studies (Pybus et al., 2013, Pybus et al., 2012b, St Jean et al., 2016). Therefore, a comprehensive study to identify the metabolites of this drug using recent advanced technologies in liquid chromatography-mass spectrometry instrumentation after activation via recombinant CYP2D6 isoenzyme and HLM need to be conducted. Currently, some initial data from

our group indicate the involvement of two parasite enzymes in the mode of action of PMQ. This enzyme known as *P. falciparum* ferredoxin-NADP⁺ reductase (PfFNR) and a novel diflavin reductase (PfCPR). This should be the starting point for more detailed mechanism of action studies.

There has been nearly 70 years of intense study looking at the biochemistry, cell biology and pharmacology of drugs active against asexual malaria parasites. Studies focussing on gametocytes have been rare. If we wish to generate new drugs that specifically target these stages it will be important to develop new ways of investigating this challenging life cycle stage of the parasite and invest time and resources in understanding their biochemistry.

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