Early detection of reduced artemisinin susceptibility in plasmodium falciparum using novel phenotypic indicators

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2019

Xiong, A. (2019). Early detection of reduced artemisinin susceptibility in plasmodium falciparum using novel phenotypic indicators. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/106390>

<https://doi.org/10.32657/10220/49591>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**EARLY DETECTION OF REDUCED ARTEMISININ
SUSCEPTIBILITY IN *PLASMODIUM FALCIPARUM* USING
NOVEL PHENOTYPIC INDICATORS**

XIONG AOLI

SCHOOL OF BIOLOGICAL SCIENCES

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NOVEL PHENOTYPIC INDICATORS**

XIONG AOLI

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Peter R. Preiser

Authorship Attribution Statement

This thesis contains material from 1 paper published in the following peer-reviewed journal where I was the co-author.

Part of Chapter 3 is published as Thamarath, Smitha Surendran, Aoli Xiong, Po-Han Lin, Peter Rainer Preiser, and Jongyoon Han. "Enhancing the sensitivity of micro magnetic resonance relaxometry detection of low parasitemia *Plasmodium falciparum* in human blood." Scientific reports 9, no. 1 (2019): 2555.

The contributions of the co-authors are as follows:

- The original study was designed by S.T.S., J.H. and P.R.P.
- The saponin lysis of parasites, sample preparation and MRR experiments were performed by S.T.S.
- X.A. and P.H.L. were involved in preparing *P. falciparum* parasite samples.
- P.H.L. performed MRR measurements on lysed schizont stage iRBC samples.
- P.R.P. and J.H. supervised the whole work.
- S.T.S., X.A., P.R.P. and J.H. wrote the paper.
- The data was analysed by all authors and checked the manuscript.

04-Jul-2019
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Acknowledgement

I would like to first thank my supervisors, Prof. Peter Preiser and Prof. Jongyoon Han, who have been giving me countless inspirations and supports throughout my PhD journey. I entered as a fresh graduate from University, knowing nothing about bioengineering and malaria research. Thanks to my supervisors, I have gained much experience and knowledge on research, career and life after these years as a PhD student. They have been supportive to all my new and naïve ideas. Having them as my supervisors is one of the greatest fortunes of my life.

I would like to thank Nanyang Technological University (NTU) and Singapore-MIT alliance for research and technology (SMART) for the SMART fellowship that funded my PhD.

Thanks to Prof. Konstantin Pervushin, Prof. Rajesh Chandramohanadas and Dr. Wengkung Peng for their great support as my thesis advisory committee members. Special thanks to Dr. Wengkung Peng and Mr. Chen Lan who closely guided me on the MRR research when I first joined the group. I would also like to thank Dr. Yin Lu for all the constructive discussions and advices on experiments, thesis writing and career choices. I would like to thank Dr. Charles Woodrow for providing all the clinical samples, relevant information and supportive discussions and thank Prof. Olivo Miotto for the information on the clinical samples that helped the genetic analysis at the CometChip chapter. Also thank Prof. Guillaume Thibault for the insightful discussions on the eIF2 α work.

Thank Prof. David Fidock for providing the Dd2 resistant strains and Prof. Zybneck Bozdech for providing the non-K13 mediated resistant strains used in the thesis. Special thanks to Ms. Xu Min, my old friend from Nanjing University, for all the constructive advices on genetic analysis. Thanks to Prof. Lu Lanyuan and Dr. Zhu Lei for fruitful discussion on Biostatistics and Bioinformatics involved in this thesis.

Tremendous thanks to Prof. Bevin Engelward who hosted me during my MIT visit, helped me adapt the CometChip to malaria research and provided me experiment space and all the fruitful discussions. Great thanks to all the lab members from Prof. Engelward's group, Christy, Lizzie, Josh, Ian, Jenny and Kathleen, who gave me a great time in the lab and helped me in the experiments and life around MIT. I would also like thank Prof. Jacquin Niles for providing me space for malaria culture in MIT and giving me all those inspiring discussions. Great thanks to Sebastian, Charisse, Mai, Sumanta, Aleja, Lisl and all other members from Nile's lab for all the help, discussion and happy times we had together.

I would also like to thank all the former and current lab members from Prof. Peter Preiser's group, Annie, Anthony, Regina, Omar, Rebecca, Xue Yan, Sally, Amy, Tate, Kripa, Weijian, Yaw, Han Ping, Donald, Ameya, Marvin, Radek, Chee Sheng, Yu Bin, Jenny, Charmaine, Kong Fang, Jing Wen, for all the discussion, troubleshooting, experiment work and happy moments. Many thanks to lab members in Prof. Jongyoon Han's group at MIT, Debbie, Lidan, Sha, Ouyang, Kyungyong, Taehong, Aniruddh, Hyunryul, Sung Hee, and others for all the help,

discussion and great times during my stay in MIT as well as in the group. Enormous thanks to previous and current colleagues in SMART BioSym, Bala, Averil, Zhengyang, Vicky for always helping me out in the lab, Yin Lu, Evan, Yan Teck, Bee Luan, Teng Yang, Kerwin, Smitha, Tian Fook, Ching Ann, Menglin, Chen Lan, Brian (Weng Kung Peng), Bena, Yasaman, Zhang Xing and others for all the collaborative work, insightful discussions and great times together.

Many thanks to my dear friends, Li, Luis, Weng Zhe, Anna and Anna, Nini, Gao Yang, Ma Liya, Kong Deqing, Xue Zeya, Zhi Yaqing and those have been mentioned above for sharing all the ups and downs, happy and sad moments we have in life. With you, I know I never walk alone. I hope we can maintain the friendship many years after.

I would also like to thank my beloved family for their love and support, my father, sister, brother-in-law, brother, sister-in-law and three adorable nephews. Without you, I would not have persisted through this. Lastly, I would like to thank my late mother, Zhang Juan, who passed away on the second year of my PhD, but her love has been holding me through all the difficulties of life. Thank you, Ma.

Contents

Acknowledgement	I
List of figures.....	IX
List of tables	XI
Abbreviations	XII
Abstract.....	XV
Chapter 1 Introduction	1
1.1 Malaria and malaria drug resistance.....	2
1.1.1 Malaria as a global health threaten	2
1.1.2 The life cycle of <i>P. falciparum</i>	3
1.1.3 Anti-malarial drugs and anti-malaria drug resistance	5
1.2 Artemisinin and artemisinin resistance.....	11
1.2.1 Artemisinin and its anti-malarial mechanism	11
1.2.2 Artemisinin resistance.....	16
1.3 Current detection methods for artemisinin resistance.....	21
1.4 Research rationale and objectives	23
Chapter 2 Method and materials	25
2.1 General parasite culture and synchronization	26
2.1.1 <i>In vitro</i> culture of <i>P. falciparum</i>	26
2.1.2 Magnetic purification.....	28
2.1.3 Drug preparation.....	28

2.2 Methods used in detection based on micro-magnetic resonance relaxometry (MRR)	29
2.2.1 MRR sample preparation and detection	29
2.2.2 MRR measurement	29
2.2.3 MRR phenotypic assay	30
2.3 Methods used in detection based on phosphorylation of eukaryotic initiation factor 2 α	31
2.3.1 Drug treatment regime	31
2.3.2 Ring-stage survival assay (RSA)	31
2.3.3 Sample preparation for eIF2 α detection	32
2.3.4 Immunoblotting and analysis	32
2.4 Methods used in detection based on CometChip	33
2.4.1 Fabrication of CometChip	33
2.4.2 Alkaline CometChip	34
2.4.3 Data analysis	35
2.4.4 Enrichment of live ring stage parasites using streptolysin O (SLO)	35
2.4.5 On-chip treatment using hydrogen peroxide	36
2.4.6 Off-chip treatment using artesunate	37
2.4.7 SNPs analysis	37
Chapter 3 Detection of artemisinin resistance based on Magnetic resonance relaxometry (MRR)	38
3.1 Introduction	39

3.2 Results.....	42
3.2.1 Improving the stability of MRR detection: introduction of A-ratio	42
3.2.2 Detection of <i>P. falciparum</i> 3D7 infection in continuous culture.....	43
3.2.3 MRR phenotypic assay for early detection of reduced artemisinin susceptibility	46
3.2.4 Further improvement of MRR detection: a lysis approach	51
3.2.5 Detection of reduced artemisinin susceptibility based on the lysis method: a preliminary study	55
3.3 Discussion.....	58
Chapter 4 Detection of reduced artemisinin susceptibility based on the phosphorylation of <i>P. falciparum</i> eukaryotic initiation factor 2 α (eIF2 α)	63
4.1 Introduction	64
4.2 Result	68
4.2.1 Phosphorylation of eIF2 α in wild-type malaria parasites by dihydroartemisinin (DHA)	68
4.2.2 Difference in the phosphorylation of eIF2 α between K13 mediated resistant and wild type malaria parasites	69
4.2.3 Detection of DHA resistance in clinical isolates based on the phosphorylation level of eIF2 α	74
4.2.4 Phosphorylation of eIF2 α in non-K13-mediated ART-resistance	75
4.3 Discussion.....	77
Chapter 5 Detection of reduced artemisinin susceptibility based on the level of DNA damage	82

5.1 Introduction	83
5.2 Results.....	86
5.2.1 Cometchip detection of DNA damage in early and late stage <i>P. falciparum</i>	86
5.2.2 DNA damage induced by artemisinin in <i>P. falciparum</i>	89
5.2.3 Correlation between DNA damage and reduced artemisinin susceptibility	90
5.2.4 Correlation between DNA damage and artemisinin resistance at genetic level.....	94
5.3 Discussion.....	106
5.4 Appendix 1 List of known DNA repair genes	112
Chapter 6 General conclusions and future directions	114
6.1 $\Delta\Delta A$ -ratio detection based on MRR	116
6.1.1 Key findings:	116
6.1.2 Future works:	118
6.2 Phosphorylation of eukaryotic initiation factor 2 α subunit (eIF2 α).....	118
6.2.1 Key findings:	119
6.2.2 Future works:	120
6.3 Level of DNA damage based on CometChip.....	121
6.3.1 Key findings:	121
6.3.2 Future works:	123
6.4 Significances of the work	124
Chapter 7 References.....	126

List of figures

Figure 1-1 Life cycle of <i>P. falciparum</i>	4
Figure 1-2 Plant <i>Artemisia annua</i> and the chemical structure of artemisinin.	12
Figure 3-1 T_1 (A), T_2 relaxation time (B) and normalized ΔA -ratio index (C) as a function of parasitemia level.	43
Figure 3-2 Characterization of <i>P. falciparum</i> infection in continuous culture.	44
Figure 3-3 The change of ΔA -ratio during or after drug treatment.	46
Figure 3-4 A. Comparison of ΔA -ratio of sensitive (black) and resistant (grey) strains at 6 hours and 24 hours.	47
Figure 3-5 Blind test for ΔA -ratio of clinical isolates at time point of 6 hours (A) and 24 hours (B).	48
Figure 3-6 Illustration of MRR system and lysis methods.....	52
Figure 3-7 R_2 measurements of unlysed iRBCs (A) and saponin lysed RBCs (B).....	53
Figure 3-8 Detection of reduced artemisinin susceptibility using lysis method.	57
Figure 4-1 Integrated stress responses and life cycle regulation by eIF2 α phosphorylation.....	67
Figure 4-2 eIF2 α phosphorylated after DHA treatment.....	69
Figure 4-3 Lower level of phosphorylated eIF2 α in the resistant Dd2 I543T parasites and Cambodian clinical isolates KH004-003.	71
Figure 4-4 The impact of parasite age (A) and treatment time (B) and different K13 mutation (C) on detecting artemisinin resistance using the level of phosphorylated eIF2 α	72
Figure 4-5 RSA survival rates of Dd2 WT, C580Y, I543T, KH004-041 and KH004-003.....	73
Figure 4-6 Characterization of eIF2 α profile (A) and RSA survival rate (B) in other clinical isolates.	74
Figure 4-7 Lower level of phosphorylated eIF2 α in non-K13-mediated artemisinin resistant parasites.....	76
Figure 4-8 Role of UPR and proteasome degradation pathway in artemisinin resistance.	78
Figure 4-9 Illustration of the possible layout of a dipstick-form device.....	80

Figure 5-1 CometChip for high-throughput assessment of DNA damage.....	84
Figure 5-2 Optimization of CometChip.	88
Figure 5-3 Artesunate induced DNA damages in <i>P. falciparum</i> 3D7.	89
Figure 5-4 Artesunate induced DNA damages in sensitive and resistant parasites.....	91
Figure 5-5 Artesunate induced DNA damages in KH strains.	92
Figure 5-6 Correlation between DNA damages level at 1000nM artesunate and parasite clearance half-life in multiple clinical isolates.....	93
Figure 5-7 Phylogenetic tree based on SNPs profile of samples from Pailin, Cambodia 2011. .	96
Figure 5-8 Area profile of K13 mutation prevalence (pie chart of WT (blue) and K13 (magenta)) and distribution of the 'ResisDNArep genotype' in parasites carrying wild-type K13 (WT, upper panel) and parasites carrying K13 mutations (K13, lower panel) in Southeast Asia and Africa..	99
Figure 5-9 Temporal change of the frequency of the 'ResisDNArep genotype' in K13 parasites from 2011 (black bar) to 2012 (grey bar) in Pailin, Cambodia.	100
Figure 5-10 P values from Chi-square analysis of gene association in different population and sub-populations.	102
Figure 5-11 Distribution of the 'ResisDNArep genotype' in K13 C580Y parasites and parasites with other K13 mutations in e-GMS (A) and w-GMS (B).	105
Figure 6-1 Comparison of <i>in vitro</i> culture time for different indicators.	124

List of tables

Table 1-1 Summary of main anti-malarial drugs [30].	10
Table 5-1 List of mutations in the 'ResisDNArep genotype'..	97
Table 5-2 Distribution of mutations in 'ResisDNArep genotype' in the KH and Dd2 strains.	98

Abbreviations

μl	microliter
A	Alanine
ACT	Artemisinin-based combination therapies
ANOVA	Analysis of variance
ARMD	accelerated resistance to multiple drugs
arps10	apicoplast ribosomal protein S10
AUC	area under curve
C	Cysteine
crt	chloroquine resistance transporter
DFO	deferoxamine
DHA	dihydroartemisinin
DMSO	Dimethyl sulfoxide
E	Glutamic acid
eIF2α	eukaryotic initiation factor 2 α subunit
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ETC	mitochondrial electron transport chain
FACS	Fluorescence-activated cell sorting
fd	ferredoxin
GMS	Greater Mekong Subregion
h	hour(s)
H	Histidine
Hb	haemoglobin
HIV	human immunodeficiency virus
HPI	hours post invasion
I	Isoleucine

IC50	half maximal inhibitory concentration
iRBCs	infected red blood cells
K13 mutations	mutations in the PF3D7_1343700 kelch propeller domain
kDa	Kilodalton
LD	Linkage disequilibrium
LMPA	low-melting point agarose
mdr2	multidrug resistance protein 2
met-Hb	methaemoglobin
MRR	magnetic resonance relaxometry
N	Asparagine
NADH	nicotinamide adenine dinucleotide
nM	nanomolar
NMR	nuclear magnetic resonance
PfATP6	SERCA-type Ca ²⁺ -ATPase orthologue
<i>pfcr1</i>	<i>P. falciparum</i> chloroquine resistance transporter
<i>PfDHFR</i>	<i>P. falciparum</i> dihydrofolate reductase
<i>PfDHPS</i>	<i>P. falciparum</i> dihydropteroate synthetase
PfEMP1	<i>P. falciparum</i> erythrocyte membrane protein 1
PfExp1	<i>P. falciparum</i> Exported Protein 1
<i>pfmdr1</i>	<i>P. falciparum</i> multidrug resistance transporter 1
<i>pfmhe1</i>	<i>P. falciparum</i> sodium/proton exchanger 1
PfPI3K	phosphatidylinositol 3-kinase
PI3P	phosphatidylinositol 3-phosphate
PVM	parasitophorous vacuolar membrane
Q	Glutamine
R	Arginine
RBCs	red blood cells
ROC curves	receiver operating characteristic curves
ROS	reactive oxygen species
RSA	ring-stage survival assay

RSA _{0-3h}	ring-stage survival assay with synchronized ring stage parasites (0-3hour post invasion)
S	Serine
SEA	Southeast Asia
SERCA	sarco/endoplasmic reticulum Ca ²⁺ -ATPase
SLO	streptolysin O
SNPs	single nucleotide polymorphisms
T	Threonine
T ₁ /R ₁	longitudinal relaxation time/rate
T ₂ /R ₂	transverse relaxation time/rate
TVM	tubovesicular membrane
UPR	unfolded protein response
uRBCs	uninfected red blood cells
WHO	world health organization
Y	Tyrosine
μM	micromolar

Abstract

Artemisinin-based combination therapies (ACT), the first-line anti-malarial treatments recommended by World Health Organization (WHO), have achieved great success globally since their introduction. However, emerging artemisinin resistance in Southeast Asia is posing a real threat as it would have a significant impact on malaria control, treatment and the global elimination efforts. Early and reliable detection of reduced susceptibility to artemisinin is critical for the effective treatment of the disease, as well as surveillance and containment of artemisinin resistant parasites. Currently the most widely used methods for the detection of artemisinin resistance include parasites clearance rate measurements, the ring-stage survival assay (RSA) and DNA based approaches to detect mutations in the K13-propeller gene. All these tests are limited in their usefulness for a rapid and accurate diagnosis due to their intrinsic disadvantages: clearance rate is measured post-treatment; RSA is time-consuming and labor-intensive; test for existing K13 mutations is not only time consuming but may also miss new resistance-conferring mutations. Therefore, the need for an early and reliable phenotypic indicators of artemisinin resistance is compelling. In this thesis, three potential phenotypic indicators were investigated, including the magnetic properties in the magnetic resonance relaxometry (MRR), level of phosphorylated eukaryotic initiation factor 2 α subunit (eIF2 α) and the level of artemisinin induced DNA damage.

The initial focus of this thesis was to track the magnetic changes in the MRR signal caused by the growth of resistant parasites. An index $\Delta\Delta A$ -ratio was defined and shown to be capable of distinguishing well characterized resistant and sensitive lab strains and one clinical isolate. However, it rendered suboptimal detection accuracy when applied to a wider range of clinical isolates. A new sample preparation approach was later applied and shown to greatly increase the sensitivity of MRR detection. However, this did not improve the detection of artemisinin resistance by MRR.

Therefore, alternative approaches focusing on rapid cellular changes triggered by artemisinin were investigated. Phosphorylation of eIF2 α and DNA damage were previously shown to be rapidly induced after artemisinin treatment. Their potential as phenotypic indicators of artemisinin resistance were thus investigated. A reproducible reduction in artemisinin-induced phosphorylated eIF2 α was observed in multiple resistant parasites compared to their sensitive counterparts, including non-K13-mediated resistant strains, providing a valid basis for further development of rapid diagnostic assays. Alternatively, a lower level of DNA damage was also observed in multiple schizont stage resistant strains compared to the sensitive strains suggesting that DNA damage could serve as another phenotypic marker of resistance. However, this result was complicated by an outlier clinical isolate KH44 that was found to be sensitive to artemisinin but experienced reduced DNA damage. This observation indicated a potential link between the acquisition of artemisinin resistance and DNA damage repair. This link was confirmed by surveying genetic variations in genes involved in DNA repair between resistant and sensitive parasites, providing new

insights into broader genetic changes the parasite needs to undergo to acquire artemisinin resistance.

In conclusion, this work investigated new phenotypic indicators for early detection of artemisinin resistance. The changes in eIF2 α phosphorylation and DNA damage, appearing shortly after *in vitro* drug treatment, were successfully detected in malaria parasites, and correlated with artemisinin resistance, demonstrating their potentials in future clinical applications.

Chapter 1 Introduction

1.1 Malaria and malaria drug resistance

1.1.1 Malaria as a global health threaten

Malaria has threatened human life for hundreds of thousands of years. The record of the disease can trace back to Ancient Greece and China. Despite great progress in fighting malaria has been made in the past few decades, the disease is continuing to pose a severe public health challenge to the world. Nearly half of the world population (~3.3 billion people) is at the risk of infection, with 214 million infection cases and 438000 deaths reported in 2015 [8]. In the sub-Saharan Africa area, it is the fourth largest cause of death for children [8].

Human malaria is commonly caused by six species of parasites that belong to genus *Plasmodium* - *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, *P. knowlesi* and *P. cynomolgi* - and is transmitted by female mosquitoes of the genus *Anopheles* [8]. Among these species, *P. falciparum* is the most lethal [9].

Clinical symptoms of uncomplicated malaria, common to different malaria species, include headache, fever, chills, fatigue, sweating, nausea, etc., which are often wrongly diagnosed as diseases like influenza by physicians who see malaria infrequently [10]. The symptoms and severity of the infection differ from patient to patient, depending on factors like parasite load, immune status, age and cumulative exposure to malaria of the patient [11]. However, without correct diagnosis and prompt treatment, uncomplicated malaria, especially *P. falciparum* malaria, can progress rapidly to severe malaria or even death [10].

In the case of *P. falciparum* malaria, the infected red blood cells (RBCs) may cytoadhere to the wall of small blood vessels, block the circulatory system, cause considerable obstruction to tissue perfusion, leading to complications like cerebral malaria and severe organ failure [10, 12]. If infected by the parasites during pregnancy, the infected RBCs can cytoadhere to the syncytiotrophoblasts of the placenta which may lead to maternal and fetal death. Infants born with mothers infected with human immunodeficiency virus (HIV) and malaria parasites often have low birth weight and high mortality rate [10].

1.1.2 The life cycle of *P. falciparum*

The life cycle of *P. falciparum* consists of asexual and sexual stages in the human and mosquito hosts. The life cycle of the asexual stage in the human host begins when female mosquitos bite human and inject sporozoites (the form of parasite in the salivary gland of mosquitos) into human blood circulatory system or lymph system. The sporozoites travel to the liver, where they invade hepatocytes and mature into schizonts, which produce thousands of merozoites in 5 to 6 days. The merozoites burst out of hepatocytes, enter the bloodstream and invade RBCs, indicating the start of the blood stage. Within approximately 48 hours post invasion (HPI) of a blood stage cycle, a merozoite inside RBC develops from the ring stage to the trophozoite stage, to the mature schizont stage. A matured schizont stage parasite will finally burst and release many new merozoites to invade more RBCs. Alternatively, merozoites inside RBCs can also

develop into sexual forms known as gametocytes, which are picked up by female mosquitos during a blood meal, initiating the sexual life cycle in the mosquito host. Male and female gametes merge to form zygotes in the mosquitos' midgut, which then develop into motile ookinetes, penetrate the midgut wall and become oocysts. Oocysts eventually mature and release sporozoites, which migrate to the salivary glands of mosquito and are capable of infecting other human hosts during the next blood meal (Figure 1-1).

Figure 1-1 Life cycle of *P. falciparum*. When an infected female mosquito bites human, the sporozoites in its salivary gland enter circulatory or lymph system of the human host and travel to liver, where they invade and further develop in the hepatocytes. In 5 to 6 days, the infected hepatocyte can produce thousands of merozoite, which then enter the blood stream and initiate the blood stage. Within approximately 48 hours, the parasite develops from ring stage, to trophozoite stage and eventually matures to schizont stage. Matured schizonts rupture and release many new merozoite to the blood stream to invade RBCs. Parasite in RBCs can also develop into gametocytes, which will be picked up by a mosquito during a blood meal and initiate life cycle in the mosquito host. *Figure reproduced from Centers for Disease Control and Prevention with minor modifications. Reprinted with permission.*

1.1.3 Anti-malarial drugs and anti-malaria drug resistance

Despite the potentially lethal outcome of malaria infection, with correct and prompt diagnosis, malaria is a curable disease. Even prior to the discovery of malaria parasites, people had found ways to treat the disease. The bark of cinchona tree, which is the origin of quinine, and the herb plant Qinghao, from which artemisinin is extracted, had been used to treat fever and chill [13, 14]. When people switched from herbal remedies to pure chemical compounds and later to synthetic drugs, the treatment of malaria also underwent dramatic development. Unfortunately, resistance to anti-malarial drugs often emerged rapidly and spread to most of the malaria-endemic areas (Table 1-1). In an effort to reduce the emergence rate of drug-resistant malaria parasites, most of the anti-malarial drugs are now used as part of a combination therapy where each drug has a different mode of action in the parasites.

Quinine

Quinine is one of the oldest anti-malarial drugs and has been used for centuries by native Peru people in the form of the bark of cinchona tree to treat fever. The chemical compound was identified and extracted in 1820 and was used as the main anti-malarial drug until World War II when Japanese occupied the cinchona tree areas in Southeast Asia and cut off the supply of the drug [13]. It was later replaced by chloroquine, a synthetic compound that has a lower cost,

higher effectiveness, and fewer side effects. Until now, quinine is still used to treat severe or resistant malaria in many parts of the world, and the combination of quinine with antibiotics is recommended by WHO as the second-line anti-malarial treatment when the first line treatments, ACTs, are not available [15]. The mode of action of quinine against the malaria parasite is only partially understood. Similar to chloroquine, it was shown to specifically accumulate inside the digestive vacuole of parasites and interfere with the synthesis of hemozoin, which is an important detoxification step for parasite [16].

Resistance to quinine was first documented in 1910 and described as diminished drug effectiveness instead of total failure of the drug. Resistance has been related to multiple genes including *pfcr1* (*P. falciparum* chloroquine resistance transporter), *pfmdr1* (*P. falciparum* multidrug resistance transporter 1), and *pfhhe1* (*P. falciparum* sodium/proton exchanger 1), which may lead to the reduction of the drug inside the food vacuole of malaria parasites by reducing the abundance of the drug transporter or extrusion of drug via a mutated transporter [17-21]. In addition to the resistance, the ineffectiveness of quinine treatment might also be resulted from variations of the drug pharmacokinetics in different people, poor drug quality, poor drug compliance due to prolonged treatment course and severe side effects [13].

Chloroquine

Chloroquine, a synthetic derivative of quinine, was discovered in 1934 [13]. Instead of immediate application for anti-malarial treatment, chloroquine

became only widely used to treat malaria after 1946 when the United States re-evaluated it and designated it as anti-malarial drug [22]. It soon became the gold standard for malaria treatment because of its effectiveness, affordability, and safety, even during pregnancy [23]. The mode of action of chloroquine has been extensively studied for decades. It was found to be accumulated inside the food vacuole of parasites and bind to hemozoin. Hemozoin is an intermediate during the detoxification of heme to hemozoin. The binding of chloroquine to hemozoin interferes with the formation of hemozoin, leading to the accumulation of toxic heme that permeabilizes the food vacuole membrane and leads to the eventual death of the parasites [23].

However, after about 10 years of massive use, during the late 1950s to 1960s, chloroquine resistance developed. The resistance was found to be related to multiple genes including *pfcr1* and *pfmdr1*. Nowadays, chloroquine is not recommended in most of the world to treat *P. falciparum* malaria due to the development of complete resistance. It is only used, often in combination with primaquine, to treat *P. vivax* worldwide and uncomplicated *P. falciparum* in some areas of South America [15].

Other anti-malarial drugs

Lumefantrine, mefloquine, amodiaquine, piperaquine and sulfadoxine-pyrimethamine (under trade name Fansidar®) are currently used in artemisinin combination therapies (ACTs), the first-line antimalarial treatments recommended by World Health Organization (WHO) [24]. Lumefantrine and mefloquine, like quinine, are aryl-amino alcohols. Amodiaquine and

piperaquine also have similar structures to chloroquine. They are believed to also kill the parasites by preventing the formation of hemozoin in the food vacuole [25]. Mefloquine was also shown to inhibit the endocytosis of the parasites [26].

Lumefantrine has not been used in monotherapy, which may help slow down the development of resistance to this drug. Resistance to mefloquine was related to the amplification of *pfmdr1*, which results in the overexpression of this transporter at the food vacuole membrane [27]. Amodiaquine resistance was also related to mutations in *pfcr1* and *pfmdr1* while piperaquine resistance was linked to the mutations in *pfcr1* and amplification of *plasmepsin 2-3* [28, 29]. Although cross-resistance to chloroquine exists, amodiaquine and piperaquine were found to be effective against chloroquine-resistant parasites [24].

Sulfadoxine and pyrimethamine are antifolate drugs that inhibit the synthesis of folic acid in the parasites. Antifolate drugs also include proguanil. Sulfadoxine targets dihydropteroate synthetase (PfDHPS) while pyrimethamine and proguanil targets dihydrofolate reductase (PfDHFR) [30]. Point mutations in the target enzymes of sulfadoxine and pyrimethamine emerged soon after the introduction of the drugs and led to resistance [31].

Atovaquone is a hydroxynaphthoquinone that has broad-spectrum antiparasitic effect and can kill apicomplexan parasites including *Plasmodium*, *Toxoplasma*, *Theileria* and *Babesia* [32]. It was shown to target the cytochrome *bc₁* complex in the inner membrane of mitochondria and thus inhibit the respiratory chain and depolarize mitochondria membrane potential [32]. Resistance to

atovaquone can be easily conferred by single nucleotide polymorphisms (SNPs) in the cytochrome *b* gene [33]. Due to its high cost and easily arising resistance, atovaquone is mainly used in combination with proguanil (under trade name Malarone®) as prophylaxis for malaria [24].

Artemisinin

Artemisinin was identified and extracted around 1970s when resistance had developed to most of the anti-malarial drugs available then [34, 35]. Artemisinin, also known as qinghaosu, is from a sweet wormwood plant named *Artemisia annua*, an herb used in traditional Chinese medicine. ACT is the current standard treatment for uncomplicated malaria recommended by WHO [8]. The appearance of resistance to artemisinin was first documented around 2006 at the Cambodia-Thailand border [36], the same place where chloroquine resistance was first reported, implying an important role of the unique social and economic factors, such as highly mobilized labor population, use of single dose and often cheap and counterfeit drug, and poor access to medical service, in this area [36-38]. The anti-malarial mechanisms of artemisinin and possible underlying causes of artemisinin resistance have attracted much attention in the field of malaria research.

Table 1-1 Summary of main anti-malarial drugs [30]. *Lumefantrine has never been used as monotherapy. The first introduction of Lumefantrine was in the artemisinin combination therapy whereby Lumefantrine was used as the partner drug for Artemether, sold under the trade name Coartem.

Category	Name	Mechanism(s)	Resistance-related gene(s)	Resistance-related mechanism(s)	Year of first introduction	Year of resistance reported
Quinolines	Quinine	Interfere with hemozoin biosynthesis pathway	<i>pfcr1, pfmdr1, pfhhe1</i>	Changes in related drug transporters	1630s	1910
	Chloroquine		<i>pfcr1, pfmdr1</i>		1945	1957
	Mefloquine		<i>pfmdr1</i>		1970s	1982
	Amodiaquine		<i>pfcr1, pfmdr1</i>		1948	1971
	Piperaquine		<i>pfcr1, plasmepps1n 2-3</i>		1970s	1990
	Lumefantrine		<i>pfmdr1</i>		1992*	N.A.
Antifolates	Sulfadoxine	Inhibit the synthesis of folic acid	<i>pfhps</i>	Mutations in the targeted enzymes that involve in the synthesis of folic acid	1967	1967
	Pyrimethamine		<i>pfhfr</i>			
	Proguanil				1948	1949
Hydroxynapht hoquinone	Atovaquone	Inhibit the mitochondria respiratory chain and depolarize mitochondria membrane potential	<i>cytochrome b</i>	Mutations in the targeted cytochrome b	1996	1996
Endoperoxide	Artemisinin	Attack targets including heme, PfATP6, membrane, mitochondria, protein and DNA	<i>kelch 13</i>	Enhanced cellular stress responses	1970s	2008

1.2 Artemisinin and artemisinin resistance

1.2.1 Artemisinin and its anti-malarial mechanism

Artemisinin has an extremely short half-life in the human body, typically from half an hour to a few hours [39], but kills all blood stage parasites rapidly. The chemical structure of artemisinin contains a sesquiterpene lactone backbone with an endoperoxide bridge, which is believed to be critical for the anti-malarial effect of the drug (Figure 1-2). The backbone structure of the drug can vary dramatically without losing anti-malarial efficacy, such as the synthesized drugs, OZ209 and OZ277 [40]. However, deoxy-artemisinin, lacking the endo-peroxide bridge, has no anti-malarial effect [40].

Artemisinin is rapidly concentrated inside the parasites by 8 to 300 folds, compared with the drug concentration in the host RBC cytoplasm and the surrounding culture medium [41, 42], and distributed in all organelles of the parasites [43]. Activation of artemisinin to generate toxic radical is closely related to hemoglobin digestion [44]. Host hemoglobin is transported to the food vacuole of the parasite and enzymatically digested to release free heme, which can activate artemisinin [45, 46]. The abundance of free heme inside the parasites may contribute to the selectivity of artemisinin towards infected RBCs over normal RBCs [14]. Although the iron (II) molecule in heme alone, as well as

Figure 1-2 Plant *Artemisia annua* and the chemical structure of artemisinin. Photo of plant *Artemisia annua* was original work by Jorge Ferreira shared via Wikimedia Commons. Red arrow refers to the endoperoxide bridge of artemisinin.

hemin and hemoglobin, were also shown capable of activating artemisinin [41], heme derived from hemoglobin digestion was found to be mainly responsible for the activation of artemisinin [46, 47]. The activation of artemisinin can be prevented by deferoxamine (DFO), an iron chelator selective to non-heme free irons, further indicating that non-heme iron is involved in the activation of artemisinin, such as the iron originating from electron transport chain in the inner membrane of mitochondria [40, 43]. Activated artemisinin releases free radicals, such as reactive oxygen species (ROS) and superoxide, which lead to many detrimental events inside the parasites [40, 48, 49], including the alkylation of heme [41, 43, 50], the inhibition of PfATP6 [51], the depolarization of plasma[49, 52] and mitochondria membrane [40, 49, 53, 54], massive covalent bond to protein [46, 55] and DNA damages[48, 56].

Potential targets of artemisinin and its derivatives

Heme

Artemisinin was found to alkylate heme and form an artemisinin-hemin adduct, which is non-polymerizable and redox-active. The accumulation of this adduct will lead to the death of the parasite. The artemisinin-hemin adduct was first identified by mass spectrometry [41] and was later found in the spleen and urine of mice infected with *P. vinckei* and treated with artemisinin [50]. However, the presence of the adduct can be a result of the reaction between artemisinin and the deposited hematin in mice liver and spleen *ex vivo*. In addition, artemisinin was found to be distributed over the whole parasite instead of being solely concentrated in the food vacuole [43] and can kill the early ring stage parasite where little free heme or hemozoin exists, suggesting that the hemozoin synthesis pathway if targeted by artemisinin, is not an exclusive target.

Inhibition of PfATP6

Artemisinin was also found to inhibit PfATP6, the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) orthologue in *P. falciparum*, with the same potency of another well-known SERCA inhibitor, thapsigargin [51]. SERCA is responsible for reducing cytosolic free calcium concentration by actively pumping calcium into membrane-bound stores, which is important for cellular survival. The inhibition is highly specific because no other transporters were found to be affected [51]. Deoxyartemisinin, which lacks the endoperoxide bridge and antimalarial activity, also lacks the ability to inhibit PfATP6, suggesting PfATP6 as an important target of artemisinin in the parasite. However, it may not be the only and most important one [14, 54, 57]. While the drug inhibiting PfATP6 was expected to affect the morphology of endoplasmic reticulum (ER) and thapsigargin has a

significant effect on the morphology of the ER at 10 times its half maximal inhibitory concentration (IC_{50}), there is no change in ER morphology caused by artemisinin. Moreover, studies also showed contradictory evidence which suggested no antagonism between thapsigargin and artemisinin, indicating the existence of other targets for artemisinin [51, 54].

Parasite membranes

It was shown that artemisinin can impair the parasite membrane via ROS [49], not by damaging the lipid structure of the parasite membrane, but by increasing *P. falciparum* Exported Protein 1 (PfExp1) budding along the periphery of the parasite [52] as well as depolarizing the membrane potential [49]. Since PfExp1 is a 25kDa protein localized to the parasitophorous vacuolar membrane (PVM)-tubovesicular membrane (TVM) domain, it suggested that artemisinin can affect certain membrane transport properties of the PVM and TVM. Moreover, these changes are not the results of non-specific targeting since the Maurer's cleft was not affected.

Mitochondria

Artemisinin was found to disturb mitochondria function by depolarizing their membrane potential in both *P. berghei* and *P. falciparum* parasites [40, 49] without affecting the morphology of the mitochondria [54]. The depolarization can be reversed by washing off the drug and incubating parasites with culture medium [40], suggesting that the depolarization by artemisinin is an early event and not a secondary effect to cell death. These evidences showed that mitochondrion is a direct target of artemisinin, if not the sole one. Similar to the

previously proposed model/target, this process was also found to be mediated by ROS and the endoperoxide bridge played a key role in the depolarizing action. Despite the depolarization of mitochondria membrane potential, no or minimal inhibitory effect was found on the major mitochondrial respiratory chain components [49]. Meanwhile, the inhibition of the mitochondrial electron transport chain (ETC), specifically the NADH dehydrogenase, was found to antagonize the action of artemisinin. However, the ETC is not a direct target but more like an activator or mediator for the generation of ROS because no inhibitory effect of ETC can be observed under low concentration of artemisinin [40]. The mitochondrial membrane potential was found to be maintained in a small subset of artemisinin treated, morphologically dormant but viable parasites [58], suggesting the mitochondria potential as a critical marker of parasite viability in artemisinin resistance. Atovaquone, a mitochondrial inhibitor, can delay or stop the recovery of dormant parasites [58].

Protein

Artemisinin was shown to alkylate and then covalently bond to around 124 proteins involved in various biological processes that are essential for the survival of parasites, including the digestion of hemoglobin, the synthesis and metabolism of amino acid and ribonucleotide, the assembly of ribonucleoprotein, the regulation of pH, translation and phosphate metabolic process [46, 55]. Importantly, the previously identified artemisinin target, PfATP6, was also found to be covalently bound by artemisinin. In a study that explores the anti-cancer potential of artemisinin, the covalent binding of

proteins was even found in artemisinin treated cancer cells [46], suggesting the covalent protein binding as an essential step in artemisinin killing process. The process on how cells deal with damaged protein might be key for final survival and thus the resistance to the drug.

DNA

Accumulation of DNA damage was observed when treating parasites with increasing concentration of artesunate (a derivative of artemisinin) [48]. It was also found to be mediated by ROS because mannitol, a ROS scavenger, can effectively prevent DNA damage caused by artemisinin treatment. Parasites were able to repair the artemisinin induced DNA damages and achieve as high as 90% recovery from the treatment [48]. The ability of the parasite to repair DNA damage after exposure to artemisinin suggests that the repair processes may play an important role in determining the susceptibility of parasite towards artemisinin.

1.2.2 Artemisinin resistance

ACTs have been the first-line treatment for uncomplicated malaria for over a decade. It is also the last-line treatment against malaria due to the lack of an equally effective drug. The emergence of artemisinin resistance in Southeast Asia has thus greatly challenged the control and elimination of malaria globally.

The first report of suspected artemisinin resistance was from drug efficacy surveillance studies in Cambodia in 2006, in which abnormally high recrudescence of *P. falciparum* parasite after ACT treatment was observed [36, 37]. Later studies found that in some patients, a subpopulation of parasites had reduced susceptibility to artesunate (a derivative of artemisinin) and delayed parasite clearance time at adequate plasma drug concentration [59]. The relationship between artemisinin resistance and delayed parasite clearance was concluded in a study in 2009, which found that with the same dosage regime applied, the parasite clearance time in Palin, Cambodia (84 hours) was much longer than that in Wang Pha, Thailand (48 hours), and correlated with the observed ACT efficacy [60].

The reduced susceptibility to artemisinin was only observed in ring-stage resistant parasites, especially in the early rings (0-3 HPI), after exposure to short drug pulse [61]. The ring-stage-specific resistance may result from an intrinsic lower sensitivity of parasites at the ring stage against artemisinin treatment. For the same sensitive strain, compared with its trophozoite and schizont stage, ring-stage exhibited more than 100-fold lower sensitivity to short artemisinin pulse, although a sub-population of rings (2-4 HPI) was found to be hypersensitive [62, 63].

The short half-life of artemisinin *in vivo* (0.5 hour to a few hours) is also key for resistant parasites to escape from the treatment [61, 64]. Artemisinin-resistant parasites were shown to be able to survive short artemisinin treatment but could be killed by prolonged drug treatment *in vitro* [60]. The mechanism of

ring-stage resistant parasites surviving under short drug pulse was associated with the developmental arrest at this stage. A small percentage of early ring-stage parasites (~0.1% parasitemia, 0-3 HPI) could enter a 'dormant' status under the short drug pressure and resume the cell cycle after the removal of the drug, while the parasites at other stages were killed.

The selective survival of ring stage parasites led to changes in life cycle progression of many resistant parasites. Even in the absence of drug exposure, some resistant strains exhibited a prolonged ring stage and remarkably shortened trophozoite stage, compared to sensitive clones. A resistant clone was even found to have highly accelerated development of all blood stages, with trophozoite stage significantly shortened [3, 65]. By extending the less sensitive ring stage and reducing the more susceptible trophozoite stage, resistant parasites gain higher opportunities to survive until the drug concentration declines.

Genotypic changes of artemisinin resistant parasites

Artemisinin resistance is highly heritable; 56-58% of the variation in the parasite clearance rate can be attributed to parasite genetics [66]. However, artemisinin resistance cannot be explained by genetic polymorphisms in *pfcr*, *pfmdr1* or *pfatp6*, which are the possible cause of chloroquine resistance or the proposed target of artemisinin [60, 67-70]. Later mutations in the PF3D7_1343700 kelch propeller domain (K13 mutations) were associated with artemisinin resistance in Southeast Asia in terms of ring stage survival rate *in vitro* and parasite clearance rate *in vivo* [71]. Until now, sixteen mutant alleles were identified.

Among them, E252Q and A578S were validated as not associated and Y493H, R539T, I543T, N458Y, and C580Y were validated as associated, with C580Y being most prevalent [71, 72]. Removal of the K13 mutations from resistant parasites decreased ring stage survival rate from 13-49% to 0.3-2.4%; insertion of K13 mutations to wild-type sensitive parasites significantly increased the survival rate [73]. These evidences strongly suggested that K13 mutations conferred the artemisinin resistance in *P. falciparum* malaria in Southeast Asia. Although numerous polymorphisms in the K13 locus were also observed in the Sub-Saharan Africa region, they were not associated with artemisinin resistance [74]. This suggested that the development of Artemisinin resistance might require K13 mutations in a particular genetic background where nonsynonymous polymorphisms in fd (ferredoxin), arps10 (apicoplast ribosomal protein S10), mdr2 (multidrug resistance protein 2) and crt (chloroquine resistance transporter) exist [75]. These genetic background markers might be used to assess the risk of a sensitive strain developing into a resistant strain.

Mechanisms of artemisinin resistance

In early ring stage parasites, K13 mediated artemisinin resistance was associated with down-regulation of genes involved in DNA replication and upregulation of genes involved in protein folding and repair pathways, such as unfolded protein response (UPR) [76]. Resistant parasites exhibit decelerated ring stage development and enhanced cell stress response, compared with sensitive strains [3, 65, 76]. Decelerated development of resistant ring-stage parasites indicates a lower level of hemoglobin digestion and thus lower level

of heme-mediated activation of artemisinin. Upregulated UPR could increase the capability of parasites to repair or degrade the protein damaged by artemisinin. Phosphorylation of eukaryotic initiation factor 2 α (eIF2 α), an important signaling event in UPR response, was also suggested to contribute to artemisinin resistance. eIF2 α phosphorylation was found to be responsible for the latency of malaria parasites after artemisinin treatment [77]. Pre-existing phosphorylated eIF2 α was found in early ring-stage resistant parasite (0-3 HPI) but not in the drug-sensitive parasite, implicating the important protective role of phosphorylated eIF2 α against artemisinin treatment in resistant parasite [77].

Although the detailed mechanism remains unclear [78], K13 protein was found to concentrate at phosphatidylinositol 3-phosphate (PI3P)-tubules/vesicles of the ER of parasites and increase phosphatidylinositol 3-kinase (PfPI3K) in ring stage *P. falciparum* to produce higher PI3P, which promotes the survival of parasites from drug [79, 80]. Synthetically elevated PI3P can render resistance even without K13 mutations [79]. K13 was also found to be co-purified with *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), the major virulence adhesin of parasites, in the exported vesicles. The export of PfEMP1 dropped dramatically in wild-type parasites while sustained in K13 mutants after drug treatment, which may contribute to the survival of the parasites and indicate a role for the host RBCs in artemisinin resistance [79].

In conclusion, unlike other antimalarial drugs, artemisinin kills parasites at all blood stage, including early ring stage, and targets many important cellular processes inside the parasites, which make it one of the most successful

antimalarial drugs to date. Alternatives as powerful as artemisinin are currently unavailable. The emergence of artemisinin resistance in Southeast Asia deeply threatens the global effort of malaria containment and elimination. If artemisinin fails, the partner drugs in ACT will face severe selection pressures that lead to the quick development of resistance to those partner drugs and the failure of most anti-malarial treatments. It is thus important to monitor and contain artemisinin resistance as much as possible.

1.3 Current detection methods for artemisinin resistance

Early diagnosis of artemisinin-resistant malaria is critical for appropriate treatment of malaria as well as the successful surveillance and containment of the resistance. Although no case of complete failure of artemisinin was observed so far, identification of resistant case early enough could help doctors to choose an appropriate treatment plan for the patients. The change of treatment upon a resistant case may help slow down the development of resistance.

Parasite clearance rate and K13 mutations are used as clinical indicators of artemisinin resistance in Southeast Asia. WHO defines confirmed endemic artemisinin resistance as $\geq 5\%$ of patients carrying K13 resistance-confirmed mutations, all of whom having either persistent parasitemia by microscopy on day 3 or a 50% parasite clearance time ≥ 5 hours after ACT or artesunate monotherapy [72]. However, the clinical prediction and confirmation of artemisinin resistance for individual cases based on K13 mutation and parasite clearance rate are complicated due to practical reasons. Firstly, the newly

identified K13 mutations need to be individually validated for their correlation with artemisinin resistance [72]. Secondly, the parasite clearance rate is not only dependent on artemisinin, but also significantly affected by initial parasitemia, the timing of sampling, sample size, and individual differences in immunity and drug metabolism, etc. [81-83]. These factors sometimes obscure the effect of artemisinin, so that neither increasing the drug dose nor splitting the dose could improve the parasite clearance rate [83, 84].

In vitro or *ex vivo* detection of artemisinin-resistant parasites has been conducted using ring-stage survival assay (RSA_{0-3h}) via lab culture, in which 0-3 HPI ring stage parasites (*In vitro*) or circulating blood containing ring stage parasites from patients (*ex vivo*) are treated with dihydroartemisinin (DHA, a derivative of artemisinin) for 6 hours and the survival rate is measured by Giemsa-based microscopy at 72 hours. RSA has been improved for fast and accurate detection and easy implementation. Giemsa-based microscopy was replaced by dual-color Fluorescence-activated cell sorting (FACS) detection method to improve detection throughput and accuracy [85]. The time required for synchronizing 0-3 HPI ring stage parasites with Percoll-sorbitol method could be shortened by using simple filtration or sorbitol synchronization method [86].

Compared with the clinical measurement of parasite clearance rate, which requires frequent parasite density count from the same patient, RSA could be performed with one-time isolation of parasites from individual patients, and its outcome correlated better to the K13 mutations than the clinical parasite clearance rate [61, 64]. However, RSA intrinsically depends on the growth and

replication of parasites *in vitro*, requiring culture adaptation and extended culture time. The Giemsa-based microscopy used in classic RSA may lead to underestimation of the survival rate of the resistant strains in cases where some dormant ring stage parasites were mistakenly considered dead due to pyknotic cell morphology [65]. Therefore, the identification of new phenotypic indicators for more rapid and accurate detection of reduced artemisinin susceptibility has great clinical significance.

1.4 Research rationale and objectives

Artemisinin, since its discovery in the 1970s, has achieved huge success in treating malaria. ACT is the first line antimalarial treatment recommended by WHO now. It is also the last line of fighting against malaria since no replacement drug as powerful as artemisinin is available.

Unfortunately, resistance to artemisinin started to emerge. First suspicious cases were reported in 2006 in the Greater Mekong area of Southeast Asia [36] and the development of resistance was confirmed in 2008 [60]. The resistance was associated with delayed parasite clearance in patients and K13 mutations [71]. It is considered as partial resistance since reduced susceptibility to artemisinin was only observed at ring stage resistant parasites, which can still be killed by prolonged artemisinin *in vitro*. However, without effective surveillance and containment of current artemisinin resistance, full resistance to artemisinin will soon develop. If artemisinin fails, parasites will be exposed to partner drugs alone, leading to tremendous selection pressure on the partner

drugs, and thus the rapid development of resistance to those drugs and eventually failure of most antimalarial treatments.

Current indicators for monitoring artemisinin resistance include K13 mutations, which is believed to be the major genetic marker of artemisinin resistance; parasite clearance half-life (rate) and RSA survival rate, which reflect the susceptibility of parasites to artemisinin. Although widely used, detection of genetic marker like K13 mutations is resource demanding. Moreover, the polymorphism of K13 mutations and the possible K13-independent artemisinin resistance could lead to potential false-negative detections. However, methods for phenotypic surveillance of reduced artemisinin susceptibility, although more informative, suffers from other disadvantages. Parasite clearance half-life, as a post-treatment measure, cannot be used as a diagnostic standard for patients. RSA is time-consuming and requires culture-adaptation of parasites.

Therefore, this project aimed to identify phenotypic indicators that enable early, easy and reliable detection of reduced artemisinin susceptibility. Three phenotypic changes, which may take place quickly after artemisinin treatment, were investigated. They are:

- 1) Change of magnetic properties in magnetic resonance relaxometry (MRR)
- 2) Change of the phosphorylation level of eIF2 α
- 3) Change of DNA damage level

For each phenotypic change, the relationship to artemisinin resistance was characterized. The reliability of the changes as indicators for early detection of artemisinin resistance was assessed.

Chapter 2 Method and materials

2.1 General parasite culture and synchronization

2.1.1 In vitro culture of *P. falciparum*

P. falciparum strain 3D7 (The Malaria Research and Reference Reagent Resource Center (MR4), USA) was cultured using fresh erythrocytes at 2.5% hematocrit in RPMI medium 1640 (Gibco, Thermo Fisher Scientific, USA) supplemented with 2.3 g/L sodium bicarbonate (Sigma-Aldrich, USA), 2.5 g/L AlbuMAX® (Gibco, Thermo Fisher Scientific, USA), 0.05 g/L of hypoxanthine (Sigma-Aldrich, USA) and 10mg/L gentamicin (Gibco, Thermo Fisher Scientific, USA) (Noted as 'complete RPMI'). Complete RPMI without AlbuMAX® supplemented was noted as 'incomplete RPMI'. The parasites were incubated at 37 °C with 5% CO₂, 3% O₂, and 92% N₂. Synchronized ring-stage parasites were obtained using 5% D-sorbitol (Sigma-Aldrich, USA) and schizont stage parasites were purified using Percoll centrifugation as described [87, 88]. Percoll solution contains 63% Percoll® (pH 8.5-9.5 (20 °C), Sigma-Aldrich, USA), 27% 10X PBS and 10% incomplete RPMI.

Parasitemia was quantified either by standard Giemsa based microscopy or flow cytometry. In Giemsa based microscopy, blood smears were fixed and permeabilized using 100% methanol and stained with 1X Giemsa stain (Sigma-Aldrich, USA). Parasitemia was then manually estimated at 1000X magnification using light microscope Olympus IX63 (Olympus, Japan). In the flow cytometry analysis of parasitemia, parasites were washed with incomplete RPMI twice and

stained with 1X SYBR™ Green I (Invitrogen™, Thermo Fisher Scientific, USA) in complete RPMI for 10min in dark. Parasites were then washed with incomplete RPMI for 3 times, resuspended and diluted in complete RPMI and subjected to flow cytometry analysis on an Accuri C6 flow cytometer (Becton Dickinson, USA).

Dd2 wild-type, I543T and C580Y strains used in this project were obtained from Prof. David Fidock (Columbia University, USA), which were generated in the study named “K13-propeller mutations confer artemisinin resistance in plasmodium falciparum clinical isolates” [73].

6A-R and 11C-R were obtained from the study of Prof. Zbynek Bozdech’s group (Nanyang Technological University, Singapore) named “Oxidative stress and protein damage responses mediate artemisinin resistance in malaria parasites” [89]. 6A-R and 11C-R may maintain the resistant phenotype for less than two weeks if not under pulse-based drug challenge at each life cycle. Therefore, all the experiments were done within one week to minimize the possibility of phenotype loss.

KH004 serial clinical isolates were obtained from Dr. Charles J. Woodrow (Mahidol Oxford Tropical Medicine Research Unit, Thailand and the Centre for Tropical Medicine, UK), which were collected from Cambodia for the clinical trial entitled “A multicenter, randomized trial to detect *in vivo* resistance of Plasmodium falciparum to artesunate in patients with uncomplicated malaria (TRAC Study)” [90]. Cryo-preserved parasites were thawed using sorbitol gradient and cultured as described in previous section. Genotype information of the strains were obtained from Dr. Charles J. Woodrow and Prof. Olivo Miotto

(Mahidol–Oxford Tropical Medicine Research Unit, Thailand and Nuffield Department of Medicine, the Medical Research Council Centre for Genomics and Global Health, University of Oxford and the Wellcome Trust Sanger Institute, Hinxton, UK).

2.1.2 Magnetic purification

Ring stage parasites were purified with MACS system (25 LS columns, Miltenyi Biotec, Germany). LS columns were held with Quadro MACS magnetic support and preloaded with complete RPMI. The RBC suspension at 5% hematocrit was passed through the column. Normal RBCs and ring stage parasites passed through the column while the late stage (trophozoite and schizont) parasites and free hemozoin were retained in the column.

2.1.3 Drug preparation

Dihydroartemisinin (DHA) (Sigma-Aldrich, USA) was dissolved to 200mg/ml in Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA). Artesunate (Sigma-Aldrich, USA) was dissolved to 50mM in 7.5% sodium bicarbonate solution (Sigma-Aldrich, USA). Aliquots of both drugs were stored at -20°C and used one-time only without re-freezing.

2.2 Methods used in detection based on micro-magnetic resonance relaxometry (MRR)

2.2.1 MRR sample preparation and detection

In prior to MRR detection, samples were washed with 1X Phosphate buffered saline (PBS) (Lonza, USA) for three times and resuspended in PBS at 10% hematocrit. Samples were well mixed at room temperature through low speed vortex and transferred into a heparinized micro-capillary tube (Fisherbrand™, Thermo Fisher Scientific, USA). The capillary tube was sealed with an inert tube sealant (CritoSeal®, Krakerler Scientific Inc., USA) at the end where sample was loaded and centrifuged using a Sorvall Legend Micro 21 microcentrifuge (Thermo Fisher Scientific, USA) at 3000g for 1min. The tube was then loaded to MRR coil for detection at the concentrated RBC band as described to enhance the detection sensitivity [91].

2.2.2 MRR measurement

The ¹H MRR measurements of RBCs were performed at resonance frequency of 21.56M Hz inside the MRR device as described [91]. The T₂ relaxation time was measured by standard Carr-Purcell-Meiboom-Gill (CPMG) train pulse (200 microsecond (μs) of inter-echo time) consisting of 3000 echoes. 64 scans were

performed to obtain average signal, which was processed by Prospa 3.12 program (Magritek, New Zealand) using exponential fit to get the T_2 relaxation time. The recycle delay was set to 2 seconds, so that all the spins had sufficient time to return to thermal equilibrium state. T_1 relaxation time was measured using the standard inversion recovery method with additional CPMG detection, which is often used for T_1 measurement in an inhomogeneous magnetic environment. For bulk RBC detection, CPMG pulse with echo-time of 200 μ s consisting of 1000 echoes were performed 18 times with different delay times between the extremes. The minimum and maximum gap between 180- and 90-pulse (refer to 'Minimum delay and maximum delay') are 100 milliseconds (ms) and 3000 ms, respectively, for all the RBC detection except otherwise mentioned. The inter-measurement time was set to 1 second.

2.2.3 MRR phenotypic assay

Late schizont stage parasites were inoculated into fresh RBCs, which were washed using incomplete RPMI twice in prior to use. After 12 hours, the culture was synchronized using 5% D-sorbitol and purified by MACS system to remove the remaining schizont and free hemozoin. The synchronized ring-stage parasites were then treated with vehicle control (0.0015% sodium bicarbonate) or 1000nM artesunate, respectively, for 6hrs. The parasites were then washed with incomplete RPMI and put back to culture or proceed to MRR detection.

2.3 Methods used in detection based on phosphorylation of eukaryotic initiation factor 2 α

2.3.1 Drug treatment regime

Before treatment, parasites cultures were purified with MACS, and the parasitemia was determined by flow cytometry as described above. The culture was adjusted to 1% parasitemia and 2.5% hematocrit using fresh RBCs before drug treatment. 1000X DHA solution was prepared by diluting the DHA stock solution with DMSO. The solution was further diluted with complete RPMI medium to reach the final concentration. Vehicle control group contains 0.1% DMSO.

2.3.2 Ring-stage survival assay (RSA)

The ring-stage survival assay was carried out as described [61]. Briefly, ring stage parasites were exposed to 700nM DHA or vehicle control (0.1% DMSO) for 6hrs. The parasites were then washed and cultured for another 66hrs. Survival rates were assessed using flow cytometry as described with modifications [85]. Both control and drug treated parasites were stained with 1X SYBR™ Green I (Invitrogen™, Thermo Fisher Scientific, USA) and 1.2 μ M MitoTracker™ Deep Red FM (Invitrogen™, Thermo Fisher Scientific, USA) in dark for 15min, and washed

with incomplete RPMI. Samples were then resuspended and diluted in complete RPMI and subjected to analysis on an Accuri C6 flow cytometry (Becton Dickinson, USA).

2.3.3 Sample preparation for eIF2 α detection

Antiprotease mixture (APM) was made by 1X cOmplete™ Protease Inhibitor Cocktail (Roche, Switzerland), 20mM N-ethylmaleimide (NEM) and 2mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, USA) in Hanks' Balanced Salt Solution (HBSS) (Life technologies, Thermo Fisher Scientific, USA). PMSF and NEM were fully dissolved in 100% ethanol before added to APM. 0.15% saponin solution was freshly prepared using saponin powder (Sigma-Aldrich, USA) and 1X HBSS supplemented with APM and kept on ice. Mock or drug treated parasites were collected using centrifuge and lysed with 0.15% saponin at volume ratio of 1 (parasites pellet) to 2 (saponin solution) for 10 minutes on ice. Pellets were then washed with 1X HBSS for three times, re-suspended in APM and store at -20° until further analysis.

2.3.4 Immunoblotting and analysis

Samples were first mixed with Laemmli sample buffer (Bio-Rad, USA) supplemented with 5% 2-mercaptoethanol (Bio-Rad, USA) and heat at 95°C for 10 minutes. Proteins were separated by 10% SDS/PAGE gel and transferred to

PVDF (Bio-Rad, USA) or low fluorescent PVDF membrane (Bio-Rad, USA). The membranes were blocked for 1 hour at room temperature with 5% skim milk in tris-buffered saline with 0.05% Tween® 20 (TBST) in chemiluminescence western blot or TBS-based LI-COR blocking buffer (LI-COR Biosciences, USA) in fluorescent western blot. Rabbit anti-phosphorylated eIF2 α , mouse anti-total eIF2 α and mouse anti- β -actin (Cell Signaling Technology, USA) antibodies were diluted 1:1000 in 1% BSA in TBST or Licor blocking buffer with 0.2% Tween 20 (Sigma-Aldrich, USA). HRP conjugated anti-mouse IgG and anti-rabbit IgG (Cell Signaling Technology, USA) were diluted 1:5000 in 1% BSA in TBST in the case of chemiluminescent western blot. Membranes were then treated with ECL substrate (Bio-Rad, USA) and exposed with Lucent Blue X-Ray film (Advansta, USA). Images were quantitatively analyzed using ImageJ [92]. IRDye 800CW Donkey anti-rabbit IgG and IRDye 680RD Goat anti-mouse IgG (LI-COR Biosciences, USA) were diluted 1: 10,000 in fluorescent western blot. Membranes were scanned with Odyssey CLx Imager (LI-COR Biosciences, USA). Images were analyzed using Imagestudio (LI-COR Biosciences, USA).

2.4 Methods used in detection based on CometChip

2.4.1 Fabrication of CometChip

CometChip assay was performed as described [5, 93]. A homemade PDMS stamp with an array of micropillars was obtained from Professor Bevin Engelward (Department of Biomedical Engineering, Massachusetts Institute of

Technology, USA). Briefly, 3% w/v agarose (Bio-Rad, USA) was dissolved in phosphate-buffered saline (PBS) (Lonza, USA) and applied to the hydrophilic side of GelBond® film (Lonza, USA). The PDMS stamp was pressed on top of the molten agarose gel to generate arrays of microwells with around 40-50 micrometer (μm) in both diameter and depth, 240 μm space. The agarose gel was allowed to solidify for 15 minutes before the stamp was removed. A bottomless 96-well plate was applied on top of the agarose gel chip and secured by clips to form 96 macrowells, with an array of ~300 microwells in each.

Cell suspension was added to macrowells of the chip and incubated at 37°C for 15 minutes in the incubator. Cells were captured in microwells by gravity and excess cells were washed off with PBS (Lonza, USA). After wash, the chip was overlaid with 1% w/v low-melting point agarose (Thermo Fisher Scientific, USA) prepared in PBS (melted and stabilized to 37°C before use). The chip was then kept at 4°C for 4 minutes for completely gelation of the overlaid agarose before use

2.4.2 Alkaline CometChip

Alkaline lysis for the CometChip was performed at 4°C overnight. Alkaline lysis buffer (pH ~ 10) consisted of 2.5 M Sodium chloride, 100 mM disodium EDTA, 10 mM Trizma® base, and 1% v/v Triton X-100 (Sigma-Aldrich, USA) in deionized H₂O. After alkaline lysis, the chip was submerged in cold alkaline electrophoresis buffer (pH ~ 13.5), containing 0.3M sodium hydroxide and 1mM disodium EDTA,

to unwind the nuclei at 4°C for 40min. Electrophoresis was then performed in the same buffer at 4°C for 30 minutes at 1 V/cm and ~300 mA. The chip was washed twice at room temperature with neutralization buffer containing 0.4M Trizma® HCl (Sigma-Aldrich, USA). The CometChip was stained with 1X of SYBR™ Gold (Invitrogen™, Thermo Fisher Scientific, USA) for 30 minutes at room temperature in dark. Fluorescent images of the comets were captured at 40X magnification using epifluorescence microscopes, Nikon Eclipse 80i (Nikon, Japan) or Olympus IX83 (Olympus, Japan), with a 480-nm excitation filter.

2.4.3 Data analysis

Images were analyzed using Guicometalyzer, a customized software developed in MATLAB (The MathWorks Inc., USA) as previously described [5]. Median of comets in each well was calculated from results generated in Guicometalyzer and converted into excel file using Comettoexcel, a program developed in Python.

2.4.4 Enrichment of live ring stage parasites using streptolysin O (SLO)

25,000 units of Streptolysin O (Sigma-Aldrich, USA) was dissolved in 2.25 ml PBS (Lonza, USA) and stored at -80°C as stock solution. 1 M dithiothreitol (DTT) (Sigma-Aldrich, USA) was prepared in deionized water and stored at -20°C.

Hemolytic unit (HU) was determined as described [94]. SLO was activated by mixing 1 M DTT with SLO stock solution at 1:10 ratio and incubating at room temperature for 15 minutes. 20 µl of compact RBCs were lysed with different concentrations of activated SLO for 6 minutes, and centrifuged. Absorbance at 412 nm wave length of the supernatant was measured by infinite M200 pro plate reader (Tecan, Switzerland). The 1 HU is defined as the amount of SLO required to cause 50% lysis of RBCs and was determined for each batch of SLO. Live ring stage parasites were enriched as described previously [95]. Briefly, synchronized ring stage culture with more than 10% parasitemia was lysed with 4 HUs of activated SLO and washed with 1X PBS until the supernatant turned colorless. The enriched parasites were resuspended with complete RPMI for further experiments.

2.4.5 On-chip treatment using hydrogen peroxide

Parasites enriched using SLO lysis or Percoll gradient centrifugation were resuspended in complete RPMI at 0.5% v/v. 100ul cell suspension was added to each macro-well. After LMPA was laid onto the chip, a new bottomless 96-well plate was applied onto the chip. Hydrogen peroxide solution, stored at 4°C and protected from light, was diluted with cold PBS and kept on ice. Cells were treated with 100ul of different concentrations of hydrogen peroxide for 20min at 4°C. The chip was submerged to cold alkaline lysis buffer immediately after treatment.

2.4.6 Off-chip treatment using artesunate

Enriched parasites were resuspended in complete RPMI at 0.5% v/v. 10X artesunate or mock solutions were prepared by diluting the stock with complete RPMI. Cell suspension were incubated with different concentration of 1X drugs in 96-well plate for 1 hour at 37 °C. Treated cells were transferred to CometChip and proceeded as describe previously.

2.4.7 SNPs analysis

VCF files containing SNP information of various parasite strains were obtained from open source Pf3K project release 5.1 (Wellcome Trust Sanger Institute, UK), processed using GATK tools (Broad Institute, USA) and vcftools [96], annotated using SnpEff [6]. Phylogenetic tree based on SNPs was generated using SNPhylo [97].

**Chapter 3 Detection of reduced
artemisinin susceptibility based on
Magnetic resonance relaxometry (MRR)**

3.1 Introduction

A benchtop system, magnetic resonance relaxometry (MRR), was previously developed for label-free detection of malaria infection in both blood culture as well as animal model [1]. It is based on nuclear magnetic resonance (NMR) and exploits hemozoin, a naturally present and highly paramagnetic metabolic byproduct in the parasites, as the basis of detection. As malaria parasites grow, they consume hemoglobin and release heme, which is toxic to the parasite. The toxic heme is then converted to the highly paramagnetic hemozoin and stored in the food vacuole of the parasite, known as 'parasite pigment'. The presence of hemozoin results in high magnetic susceptibility of the malaria parasites infected blood, and induces a significant change in the proton transverse relaxation rate (R_2) of the blood relative to the R_2 of healthy blood, which can be picked up by the MRR [1]. Compared with conventional bulky NMR systems, this bench-top system is portable and inexpensive, yet offers highly sensitive detection of malaria with parasitemia as low as 0.0001% (less than 10 parasites/microliter (μl)) in small blood volume (few μl).

Currently, such highly sensitive detection was made possible by comparing the R_2 of infected red blood cells (iRBCs) with that of healthy RBCs from the same source of blood samples. However, the absolute R_2 value of healthy RBCs varies significantly due to factors like metabolic differences between donors, aging of RBCs caused by storage and potential *in vitro* culture, which leads to the possibility of false positive/negative diagnosis due to the presence of different paramagnetic hemoglobin (Hb) states (e.g. presence of methemoglobin (met-

Hb)). To overcome this challenge and thereby improve the quality of MRR based diagnosis of malaria in clinical settings, it is necessary to overcome the variations in the baseline R_2 value that is not directly attributable to parasite infections.

In addition to the transverse relaxation time/rate (T_2/R_2), MRR can also measure the longitudinal relaxation time/rate (T_1/R_1) (the relaxation rate is the reciprocal of the relaxation time). Increased paramagnetism of samples results in decrease of both T_2 and T_1 , often at different rates. While studies use solely T_2 (R_2) or T_1 (R_1) to characterize the properties of samples [98-100], the relationship between them can be informative [101-103]. The change of the ratio of T_2 and T_1 related to malaria infection and its potential in improving detection sensitivity and reliability were thus investigated in this chapter.

Meanwhile, since the background in MRR assessment of malaria infection is mainly caused by RBCs, the removal of RBC contents from the sample may greatly increase the signal to noise ratio. Here we incorporated a saponin lysis step into the sample preparation procedure and investigated whether it could improve the sensitivity and specificity of MRR detection.

With improved detection sensitivity and accuracy, MRR may be capable of examining artemisinin susceptibility in malaria parasites. Better survival of the resistant parasites after artemisinin exposure should correlate with higher amount of hemozoin in blood while the amount of hemozoin in sensitive parasites will remain the same due to the death of parasites. A highly sensitive MMR system capable of detecting the difference in the level of hemozoin between sensitive and resistant parasite strains after artemisinin exposure,

would hold great clinical significance in rapid screening of artemisinin resistance in field.

3.2 Results

3.2.1 Improving the stability of MRR detection: introduction of A-ratio

To overcome the disadvantages of using absolute value of T_2 relaxation time, a new index, A-ratio, defined as T_1 relaxation time divided by T_2 relaxation time, was introduced and used to detect malaria infection.

To avoid the effect of blood aging due to *ex vivo* storage, freshly drawn RBCs were inoculated with late stage parasites one day before the assay. Synchronized ring-stage *P. falciparum* 3D7 parasites were obtained using 5% D-sorbitol treatment to remove late stage parasites and magnetic purification to remove any residual hemozoin. To eliminate effects from different blood sources, RBCs from different donors, namely, sample A, B and C, were used. Both T_1 and T_2 were normalized by subtracting the value of uninfected RBCs (uRBCs) from the value of corresponding iRBCs. The normalized T_1 relaxation time ($T_{1\text{ (iRBCs)}} - T_{1\text{ (uRBCs)}}$) showed fluctuations as parasitemia increased (Figure 3-1A). The normalized T_2 relaxation time ($T_{2\text{ (iRBCs)}} - T_{2\text{ (uRBCs)}}$) exhibited correlation with the parasitemia but at low parasitemia the T_2 relaxation time overlapped with the uRBCs control (grey area) (Figure 3-1B). The normalized Δ A-ratio ($A\text{-ratio}_{\text{ (iRBCs)}} - A\text{-ratio}_{\text{ (uRBCs)}}$) showed strong correlation with the parasitemia in all three samples, respectively, and high sensitivity at low

parasitemia ($R^2 > 0.99$, Figure 3-1C). These data suggested A-ratio as a more reliable indicator of parasite load in the blood compared to solely T_1 or T_2 relaxation time.

Figure 3-1 T_1 (A), T_2 relaxation time (B) and normalized ΔA -ratio index (C) as a function of parasitemia level. The experiments were carried out on three independently cultured *P. falciparum* with blood from three different donors, namely, Sample A (square), B (round) and C (triangular). The grey area refers to the value range of uninfected RBCs (uRBCs). More than two measurements were taken at each data point. All data are shown as means $\pm$ standard error of the mean (SEM).

3.2.2 Detection of *P. falciparum* 3D7 infection in continuous culture

The growth of *P. falciparum* 3D7 in continuous culture was tracked by measuring the change of T_1 , T_2 , and A-ratio. First day fresh RBCs from healthy

Figure 3-2 Characterization of *P. falciparum* infection in continuous culture. Profiling of T_1 relaxation time (A), T_2 relaxation time (B) and A-ratio index (C) against days after infection. The percentage numbers listed above each day (C) indicate the total parasitemia and the corresponding percentage of ring (R) and trophozoite (T). More than three measurements were taken at each data point. All data were shown in means $\pm$ SEM.

donors were inoculated with late stage schizont (denote as “day 0”) and cultured overnight. Synchronized ring stage parasites were obtained on Day 1 using 5% sorbitol and magnetic purification. From day 1 to day 3, T_1 and T_2 decreased as the results of increased parasitemia (Figure 3-2A, B). On Day 5, the absolute value of T_1 and T_2 exhibited slight increase or no change as the parasitemia continued to increase. Moreover, the absolute T_2 value of uRBCs showed decrease on day 5. In contrast, A-ratio steadily increased in continuous *in vitro* culture with increasing parasitemia (Figure 3-2C). The composition of

ring and trophozoite stage parasites fluctuated slightly with no significant impact on the change of A-ratio index (Figure 3-2C). Taken together, compared with T_1 or T_2 relaxation time, A-ratio index showed higher accuracy in change of parasitemia in continuous *in vitro* culture, indicating higher potential in detection of reduced artemisinin susceptibility which required sensitive detection of parasitemia change.

3.2.3 MRR phenotypic assay for early detection of reduced artemisinin susceptibility

The potential of using MRR to quickly detect reduced artemisinin susceptibility in *P. falciparum* was further investigated. $\Delta\Delta A$ -ratio index was introduced and defined as the difference between A-ratios of artesunate treated and untreated iRBCs ($A\text{-ratio}_{(iRBC + 1000nM \text{ artesunate})} - A\text{-ratio}_{(iRBC \text{ control})}$). *P. falciparum* Dd2 wild-type (denoted as 'WT') and resistant Dd2 K13 I543T mutant (denoted as 'I543T'), 3D7 wild-type (denoted as '3D7'), and artemisinin-resistant clinical isolate KH004-003 (denoted as "KH03"), were tested with MRR [73].

Figure 3-3 The change of $\Delta\Delta A$ -ratio during or after drug treatment. A. Schematic illustration of drug treatment and time point for test. Moderately synchronized ring stage parasites (0-12 HPI) at 1% parasitemia were treated with 1000 nM artesunate or vehicle control (0.00015% sodium bicarbonate) for 6 hours. After which, the drug was washed off and parasites were returned to culture. **B.** In vitro $\Delta\Delta A$ -ratio change of WT and I543T within 6 hours of artesunate treatment and at 24 hours. **C.** Change of $\Delta\Delta A$ -ratio in resistant KH004-003 and sensitive 3D7 after 6hrs exposure to artesunate. Red square area indicates the 6 hours of drug treatment. $N \geq 2$. Dotted line refers to $\Delta\Delta A$ -ratio equals to zero. * $p < 0.05$, ** $p < 0.01$, two-way analysis of variance (ANOVA), p value adjusted after Šidák correction. All data were shown in means $\pm$ SEM.

To mimic the real clinical situation whereby the age distribution of circulating parasites ranging from 0 to 18 HPI [63], the late stage parasites were inoculated into fresh blood and synchronized 12 hours later. The moderately synchronized ring stage parasites (0-12 HPI) were then purified using the same method described (denoted as “0-hour”) and treated with artesunate or vehicle control for 6 hours (Figure 3-3A). The drug was then washed off and the parasites were returned to culture.

By the end of the 6-hour treatment, the $\Delta\Delta A$ -ratio of WT and I543T started to show difference (Figure 3-3B). Difference in the $\Delta\Delta A$ -ratio between 3D7 and KH03 was also observed at 6-hour and persisted till 48-hour (Figure 3-3C). These results indicated the possibility of using $\Delta\Delta A$ -ratio to diagnose artemisinin resistance within 24 or even 6 hours.

Figure 3-4 A. Comparison of $\Delta\Delta A$ -ratio of sensitive (black) and resistant (grey) strains at 6 hours and 24 hours. Dotted line refers to $\Delta\Delta A$ -ratio equal to zero. Each dot represents an independent MRR assay. *** $p < 0.001$, two-way analysis of variance (ANOVA), p value adjusted after Šidák correction. Normality was tested before ANOVA. B. Receiver operating characteristic (ROC) curves base on MRR tests at 6- or 24-hour. The area under curve (AUC) calculated from the ROC was 0.975 ± 0.0354 at 6-hour and 0.950 ± 0.0586 at 24-hour.

Comparison of $\Delta\Delta A$ -ratios was then made between sensitive and resistant strains at 6 and 24 hours for all the independent MRR assays (Figure 3-4A). Resistant and sensitive parasites showed significant difference at both 6- and 24-hour time point, whereby the $\Delta\Delta A$ -ratios of resistant parasites are higher than those of sensitive parasites (Figure 3-4A). The receiver operating characteristic (ROC) curves, which used to illustrate the diagnostic capability of a binary classifier system as its discrimination threshold is varied, were drawn

Figure 3-5 Blind test for $\Delta\Delta A$ -ratio of clinical isolates at time point of 6 hours (A) and 24 hours (B). Moderately synchronized ring stage clinical isolates (0-12 HPI) at 1% parasitemia were treated with 1000 nM artesunate or vehicle control (0.00015% sodium bicarbonate) for 6 hours. After which, the drug was washed off and parasites were returned to culture. MRR measurements were taken at 24-h. Dotted line refers to $\Delta\Delta A$ -ratio equals to zero. 'S' refers to artemisinin sensitive and 'R' refers to artemisinin resistant. All data were shown in means $\pm$ SEM. C. Comparison between resistant and sensitive parasites at 6- or 24-hour. n.s. = non-significant, two-way analysis of variance (ANOVA), p value adjusted after Šidák correction. Normality was tested before ANOVA. D ROC curve of the blind test at 6- or 24-hour. calculated from the ROC was 0.625 ± 0.235 at 6-hour and to 0.542 ± 0.220 at 24-hour.

for detections at 6- and 24-hour time point, respectively (Figure 3-4B). The area under curve (AUC) calculated from the ROC was 0.975 at 6-hour (standard error = 0.0354) and 0.950 at 24-hour (standard error = 0.0586), indicating great diagnostic potential of $\Delta\Delta A$ -ratio in differentiating artemisinin resistant parasites from sensitive ones.

The use of MRR phenotypic assay for early detection of reduced artemisinin susceptibility was further explored by blindly testing six Cambodian clinical isolates. Unlike previous characterization, the $\Delta\Delta A$ -ratio of each strain at both 6- and 24-hour exhibited large fluctuation (Figure 3-5A, B) and no clear separation between resistant and sensitive strains (Figure 3-5C). ROC curves were also drawn based on individual MRR assays at 6- and 24-hour time point, respectively (Figure 3-5D). The AUC of the ROC significantly dropped to 0.625 at 6-hour (standard error = 0.235) and to 0.542 at 24-hour (standard error = 0.220), suggested a limited capability of $\Delta\Delta A$ -ratio in distinguishing resistant and sensitive clinical isolates in the blind test.

There are important differences between the initial studies focusing on well characterized lab adapted strains like *P. falciparum* Dd2 (Figure 3-4) and the subsequent blinded study involving the more poorly characterized clinical isolates (Figure 3-5) that could explain the differences observed. These include potential parasite heterogeneity, differences in growth rates as well as other so far unknown factors relating to hemoglobin digestion that would lead to complications on the change of $\Delta\Delta A$ -ratio. In addition, with the majority of sample consisting of uRBCs especially after drug treatment, the number of

viable parasites is extremely low, this would significantly impact on the overall parasite derived signal detectable making measurements unreliable and even being the reliable limit of detection of $\Delta\Delta A$ -ratio. Therefore, further effort was made to improve the sensitivity of MRR by removing the bulk uRBCs and to see if successful detection of reduced artemisinin susceptibility could be achieved with improved sensitivity.

3.2.4 Further improvement of MRR detection: a lysis approach

Acknowledgement: This section contains excerpt from our paper entitled 'Enhancing the sensitivity of micro magnetic resonance relaxometry detection of low parasitemia Plasmodium falciparum in human blood', published in Scientific Report, 2019.

The lysis approach was primarily configured by Prof. Jongyoon Han and Dr. Smitha Thamarath Surendran. The malaria samples were prepared by Dr. Smitha Thamarath Surendran and me; the lysis and MRR measurements were performed by Dr. Smitha Thamarath Surendran and Mr. Po-Han Lin. The manuscript was prepared and edited among all authors.

To further improve the sensitivity of MRR measurement, we found it necessary to remove background from RBCs that are not directly attributable to parasite infections. In previous MRR detection, intact iRBCs or uRBCs pellet was separated from the plasma or supernatant of blood sample to the 4mm detection range of the detection coil of the MRR by using a micro centrifuge (Figure 3-6A, B). The new approach applied a saponin lysis step to the blood samples to remove the host RBC components and uRBCs. In addition, different methods in how the samples were then prepared for MRR measurements (spinning and suspension) were investigated and compared to the previous MRR measurements.

Schizont stage *P. falciparum* 3D7 parasites were purified using a Percoll gradient centrifugation to remove uRBCs and obtain a relatively pure schizont stage fraction (approximately 90% schizont stage). The schizont fraction was then diluted with uRBCs to generate a final parasitemia of 10%. This 10% parasitemia stock solution was subsequently further diluted with uRBCs to create final parasitemia concentrations between 0.0001% – 10%.

Figure 3-6 Illustration of MRR system and lysis methods. A. Micro MRR system [1] consists of a portable permanent magnet which provides strong magnetic field. A home build radiofrequency (RF) detection probe is connected to RF spectrometer. This probe transmits and receives RF signal from the spectrometer for MRR analysis of the sample inside the RF detection micro coil. The sample, iRBCs/uRBCs pellet was separated from plasma of blood sample to 4mm detection range of detection coil by using a micro centrifuge. Red colour in 4 mm range is the nRBCs/iRBCs pellet separated from PBS (blue colour) by micro centrifuge. B. Sample preparation of unlysed nRBCs/iRBCs to micro capillary tube for MRR measurements. C. Saponin lysis of iRBCs [2]. Sample preparation of lysed uRBCs/iRBCs (D) spin- down method: spin down all the pellet (green colour) into a micro capillary tube and (E) suspension method: dilute the pellet in 20ul of PBS and fill the 4 mm detection range (brown colour) of micro capillary tube.

100 μ l iRBCs were lysed using 0.15% of saponin and parasite pellets were collected after repeated washing [2]. Saponin permeabilizes the RBC membranes while leaving the malaria parasite membrane intact (Figure 3-6C). By repeated washing after saponin lysis, pure parasite pellet was collected. This purified parasite pellet was then processed using two different procedures. For the spinning method (Figure 3-6D), the parasite pellet is spun down in the micro capillary tube while for the suspension method (Figure 3-6E), the parasite pellet

Figure 3-7 R_2 measurements of unlysed iRBCs (A) and saponin lysed RBCs (B). A. R_2 measurements of unlysed iRBCs. iRBCs were spiked to 100 μ l of uRBCs to make parasitemia of 10 to 0.0001% (red) and corresponding uRBCs (black) in which blood samples from different healthy blood donors were used. B. The R_2 measurements of saponin lysed RBCs. Same set of RBC samples (uRBCs and iRBCs with parasitemia ranging from 10% to 0.0001%) were lysed by 0.15% saponin, prepared by suspension method (green) and by spin-down method (red). C is the enlarged version of B at low parasitemia region. Gray area refers to uRBCs control.

is diluted in 20 μ l PBS and the micro capillary tube is filled with the parasite suspension to the 4mm detection range.

To compare the sensitivity of detection of the three different approaches, 100 μ l of *P. falciparum* iRBCs with a parasitemia of 10%, 1%, 0.1%, 0.01%, 0.001% and 0.0001% along with an uRBCs control were analysed to obtain the R_2 values using the MRR. In addition to account for variations in donor uRBCs that may lead to differences in the measurements obtained parasites were diluted using blood from 6 different donors and the data is shown reflects all the 6 different measurements. Unlysed uRBCs and iRBCs show significant variations in the R_2 values from donor to donor making it difficult to accurately distinguish iRBCs from uRBCs until parasitemia levels reach between 0.1-1% (Figure 3-7A). The R_2 values fluctuation of uRBCs (control/baseline values) most likely can be attributed to different metabolic conditions and different uncontrolled Hb states present in the different donors. Saponin lysis and subsequent washing of the samples to obtain purified parasites is a way to reduce or remove donor derived fluctuations. As can be seen both the spinning as well as the suspension method (Figure 3-7B) show little variation between the R_2 signal of uRBCs as well as iRBCs from different donors. The uRBCs R_2 value is stable across multiple measurements and donors and increase in R_2 obtained in as little as 0.0001% parasitemia can be clearly distinguished from the uRBCs baseline. Importantly, there is a clear linear correlation in both methods between the detected R_2 value and parasitemia which would indicate that it is possible to directly extrapolate parasitemia in a diagnostic sample from a single R_2 measurement.

3.2.5 Detection of reduced artemisinin susceptibility based on the lysis method: a preliminary study

Acknowledgement: This section was collaboratively done with Dr. Smitha Thamarath Surendra. I performed drug assays; the lysis and MRR measurements were done by Dr. Smitha Thamarath Surendra.

Since the R_2 value after saponin lysis of the samples showed great correlation with parasitemia change, we decided to investigate its potential in detecting the growth of artemisinin resistant malaria parasites after drug treatment. Moderately synchronized ring stage *P. falciparum* Dd2 WT and I543T parasites were treated with 700nM DHA for 6 hours. The drug was then washed off and the iRBCs were returned to culture. At 24-hour time point, 100 μ l iRBCs were collected, lysed and measured using spinning method as described above. The R_2 of the drug treated group is expected to be lower than the R_2 of the control group due to the death of parasites in the treated group and the increased amount of hemozoin as the parasites grow in the control group. As expected, the R_2 of drug treated WT is relatively lower than that of control group, suggested the death of parasites (Figure 3-8). Meanwhile, drug treated resistant I543T has a relatively similar R_2 compared with its control group. However, no statistical significance can be established. ΔR_2 , defined as the difference between R_2 of drug treated and untreated infected RBCs (R_2 (iRBC + 700nM DHA) - R_2

(iRBC control)), was calculated and drawn for each MRR comparison test. Among the three tests, only two showed expected lower ΔR_2 in WT and no statistical significance can be established.

Since in real clinical settings, diagnosis often relies on one-time test, especially in resource limited areas, the requirement of test sensitivity and specificity is often high. If considering each MRR test as an independent 'diagnosis', the current poor sensitivity and specificity of ΔR_2 rendered it as a suboptimal indicator for differentiating resistant and sensitive parasites. Since the principle of the detection relies on death and growth of parasites after drug treatment, significantly different R_2 between control and drug treated group (100% death) should be firstly established to provide sufficient detection range for any growth of the resistant parasites. Therefore, further effort could be made on removing the bulky background signal contributed by the dead parasites, likely by microfluidic sorting of treated cells. If such difference can be established, potential detection for drug resistance based on parasite growth could be investigated.

Figure 3-8 Detection of reduced artemisinin susceptibility using lysis method. A. Change of R_2 after DHA treatment in WT and I543T. Moderately synchronized ring stage WT and I543T (0-12 HPI) were treated with vehicle control (0.1% DMSO) or 700nM DHA for 6 hours. After which, the drug was washed off and parasites were returned to culture. MRR measurements were taken at 24-h. N=3. Two-way ANOVA. Multiple comparisons were made between control and DHA in WT and I543T, respectively. n.s. = Non-significant. p value adjusted after Šidák correction. Data were shown in means $\pm$ SEM. B. Comparison of ΔR_2 of WT and I543T. Connected lines indicate three paired independent experiments between WT and I543T. Dotted line was drawn at ΔR_2 equal to zero. Wilcoxon matched-pairs signed rank test was performed to test the significance.

3.3 Discussion

Rapid, sensitive and reliable early detection of malaria parasite is critical for timely treatment and global elimination of malaria. MMR has been demonstrated to provide sensitive and early detection of malaria infections from microliter volume of blood sample. In this chapter, as a new indicator of parasite load, A-ratio index was compared with previously used T_1 or T_2 relaxation time and showed better detection capability at low parasitemia (Figure 3-1A, B). As a measurement of proton (^1H), the absolute values of T_1 and T_2 relaxation time depend significantly on the water content in the samples and thus can be affected by factors like osmotic pressure of cells instead of exclusively by changes in the hemozoin content. The values of uRBCs can vary from sample to sample and lead to low sensitivity and a difficulty in detecting low parasitemia and poor correlation with parasitemia change. On the other hand, the A-ratio, reflecting the relative change between T_1 and T_2 , is less affected by the physiological conditions of the RBCs and thus improved the detection sensitivity of MRR (Figure 3-1C).

In the case of continuous *in vitro* culture, while A-ratio index steadily increased as parasitemia increased with days, the absolute value of T_1 and T_2 relaxation time decreased as expected from day 1 to day 3 but lost the trend on day 5. The baseline value established on uRBCs also skewed on day 5 (Figure 3-2B, C), indicating a significant impact of *in vitro* culture conditions on the RBCs. Unlike the *in vivo* condition where the body has various mechanisms to maintain the health of RBCs, the *in vitro* culture condition, especially for malaria parasites

where hypoxia is applied, MRR measurement of RBCs could be significantly affected by factors such as changes in osmotic pressure and increase in met-Hb. This change in the RBCs themselves significantly impacts on the accuracy of the absolute value of T_1 and T_2 relaxation time in reporting parasitemia change while the A-ratio index is less affected (Figure 3-1C, Figure 3-2C).

The improved ability of the A-ratio to detect small changes in parasite levels and growth may provide an avenue for rapid assessment of the impact of drugs on parasite growth. This is particularly relevant for the detection of artemisinin resistant parasites where current assays are complex and highly time consuming. We explored the possibility of utilizing changes in the A-ratio to assess the effectiveness of artemisinin on sensitive and resistant parasites.

$\Delta\Delta$ A-ratio was thus derived and tested. While $\Delta\Delta$ A-ratios successfully distinguished resistant and sensitive parasites using the well characterized lab strains and one clinical isolate (Figure 3-3B, C), these results could not be reliably repeated in blinded tests of other Cambodian clinical isolates, indicating that the current approach needs to be further refined to be suitable for future clinical application. There may be a number of factors that contribute to this: Firstly, although A-ratio showed a better correlation with parasitemia changes, the biological implication of it is not fully understood. The change of A-ratio may not be solely due to the increased amount of hemozoin but also other cellular changes. It was unclear what and how these contributed to the change in the A-ratio. Subsequently, $\Delta\Delta$ A-ratio, which reflects the relative change of artesunate treated samples to untreated control, may not be solely attributable to the

growth or death of parasites in the treated group. For example, while a negative $\Delta\Delta A$ -ratio should always be obtained since drug treatment would be expected to inhibit the growth of parasites and the synthesis of hemozoin, $\Delta\Delta A$ -ratios were shown to be positive in resistant parasites, indicating the existence of unknown artesunate induced change in magnetic properties of the resistant parasites. Thirdly, the outcome of $\Delta\Delta A$ -ratio may be further complicated by the fact that artemisinin resistant parasites appear to have changes in cell cycle progression and may enter dormant status upon drug treatment [62, 65] which could further impact hemozoin synthesis and thus the observed A-ratio. The results from blind test might therefore be a reflection of the lack of understanding of the biological complexity that is measured by the $\Delta\Delta A$ -ratio as well as the potential trivial increase of the amount of hemozoin that can be hidden by the RBCs background in the 4 μl of intact blood. For this reason, it was necessary to explore options that further reduce the impact of any contribution to the A-ratio by the RBC rather than the parasite makes to the overall signal, by removing the RBC background to increase the sensitivity of MRR in detecting parasitemia change.

Saponin lysis was applied to blood samples to permeabilize the membranes of iRBCs and uRBCs while leaving the parasite membrane intact [2]. Combining this with a rigorous washing step allowed the removal of most of the host cell contents thereby removing potential confounding factors like cell-free Hb, RBC ghosts, white blood cells, etc., leaving only the parasite pellet behind. Based on this concept two slightly different approaches were used to prepare the parasite pellet for MRR analysis, the spinning method and the suspension method. Both

approaches greatly improve the ability of MRR to detect the parasite and to remove confounding factors. Lower R_2 values of the suspension method compared to the spinning method are observed at each parasitemia samples (Figure 3-7C). This can be explained that the spinning method (Figure 3-6D) results in a highly concentrated parasite pellet in the microcapillary tube near the detection coil (bottom of the capillary tube) compared to a more diluted but more evenly distributed parasite suspension in the suspension method (Figure 3-6E).

The improved sample preparation method (spinning method) was then evaluated for the rapid detection of reduced artemisinin susceptibility. In an initial preliminary study, no clear difference between resistant and sensitive parasites could be reliably detected (Figure 3-8B). The assay was not being further pursued due to considerations in two perspectives. First, in terms of current results, difference between control and drug treated WT (considered as '100% death') is not significant and large enough to establish a detection range to sensitively detect any growth in the resistant parasites (Figure 3-8A). With increased sample size, the difference may become statistically significant but will not lead to larger detection range. The high R_2 in drug treated group could relate to the fact that hemozoin from killed parasites was not removed in the *in vitro* system and would continue to contribute to the total signal. Further effort could be made by removing the dead parasite from the system, using techniques like microfluidic sorting, and having only alive parasites for MRR readout. Secondly and more importantly, current protocol design for MRR phenotypic assay still suffers from the same problem as other growth-based

assays: time consumption. Although relapse after drug treatment is the most straightforward marker for drug resistance, assays based this principle are often time-consuming. 24-hour detection may provide benefit over 72-hour RSA but would not be ideal for diagnosis. Therefore, the following chapters will explore detection methods based on drug induced instant biochemical changes in the parasites, which may provide more direct diagnostic basis for reduced artemisinin susceptibility.

**Chapter 4 Detection of reduced
artemisinin susceptibility based on the
phosphorylation of *P. falciparum*
eukaryotic initiation factor 2 α (eIF2 α)**

4.1 Introduction

In artemisinin resistance, it was suggested that same amount of artemisinin was activated in resistant and sensitive parasites, but the time gap between drug activation and onset of parasite killing was longer in resistant parasites [3]. Moreover, genes involved in oxidative stress responses were shown to be upregulated at the transcriptional level in resistant field isolates as compared to sensitive ones [76]. It is therefore believed that artemisinin resistance is mainly mediated by enhanced cellular stress responses.

Stress responses in *P. falciparum*, especially the unfolded protein response (UPR), were shown to be involved in artemisinin resistance [76]. UPR is a collective term for pathways that are activated when unfolded or misfolded proteins overly accumulate in the endoplasmic reticulum (ER). Activated UPR down-regulates general protein synthesis and up-regulates folding, trafficking and degradation machineries in the ER to re-establish homeostasis of the cell. If homeostasis cannot be re-established, cell death will be triggered. In mammalian cells, UPR was shown to be mediated by Protein kinase RNA (PKR)-like ER kinase (PERK), activating transcription factor-6 (ATF6) and inositol-requiring protein-1 (IRE1)[104]. In *P. falciparum*, only the homologue of PERK, PfPK4, and its substrate, eukaryotic initiation factor 2 α subunit (eIF2 α), have been identified by *in silico* analysis [105].

In fact, eIF2 α is the key signal transducer of several cellular stress responses such as UPR and amino acid starvation and play an important role in the life cycle progression of parasites. In addition to PfPK4, two other eIF2 α kinases

have also been identified in *P. falciparum*: Pfk1K1 (also termed as UIS1), which responds to amino acid starvation [106], and Pfk1K2, which controls the latency of sporozoites in the mosquito salivary glands [107]. Upon sensing of stress, eIF2 α is phosphorylated by its kinases [108, 109]. The phosphorylation of eIF2 α increases the binding affinity of eIF2 to its GTP exchange factor, eIF2B, and slows down the GTP-GDP exchange, which suppress the function of eIF2 and thus inhibits global protein synthesis. Alternatively, retarded eIF2 enables the selective expression of cyto-protective genes and helps re-establish homeostasis (Figure 4-1).

The hibernation response of artemisinin resistant parasites towards artemisinin treatment resembles the response to amino acid starvation, whereby the parasites exhibit eIF2 α phosphorylation mediated developmental arrest [62, 106, 110]. It was thus proposed that eIF2 α phosphorylation may play an important role in the parasite's response to artemisinin and maybe in artemisinin resistance [111]. Later, the PfPK4-eIF2 α pathway was shown to be quickly triggered by DHA treatment in *P. falciparum*, leading to the developmental arrest of the parasite [77, 112]. In addition, phosphorylated eIF2 α induced by salubrinal in sensitive strain was shown to be protective against artemisinin treatment [77], suggesting the potential role of eIF2 α phosphorylation in artemisinin resistance. As a critical stress response event to artemisinin treatment, eIF2 α phosphorylation is quickly triggered by the drug treatment and may be related to the final survival outcome of parasites, indicating the great potential of phosphorylated eIF2 α in serving as an indicator for early detection of artemisinin resistance.

When this work was being pursued, another study by Zhang et al. showed that the phosphorylation of eIF2 α was induced by DHA treatment [77]. Consistent with what has been observed, in this chapter, DHA triggered phosphorylation of eIF2 α in the ring stage parasites was validated. The study was then deepened in the direction to further investigate the relationship between the level of artemisinin induced phosphorylated eIF2 α and reduced artemisinin susceptibility and to examine if the level of artemisinin induced phosphorylated eIF2 α could serve as a reliable phenotypic indicator for early detection of reduced artemisinin susceptibility.

Figure 4-1 Integrated stress responses and life cycle regulation by eIF2α phosphorylation. eIF2α, is the α subunit of eukaryotic initiation factor 2 (eIF2, with α, β, γ subunits). eIF2 mediates the binding of methionyl tRNA to the 40s ribosomes and recognizes the mRNA to initiate translation, during which the GTP on eIF2 is hydrolyzed to GDP. GDP on eIF2 must subsequently be exchanged to GTP by the GTP-exchange factor, eIF2B, at the beginning of each initiation cycle. Phosphorylation of eIF2α under stress conditions increases the binding affinity between eIF2 and eIF2B, slows down GDP-GTP exchange and thus decelerates the overall protein expression [6]. Phosphorylated eIF2α in turn also leads to activates the translation of factors like activating transcription factor 4 (ATF4) to up-regulate stress response related genes and control the negative feedback of phosphorylation [4].

4.2 Result

4.2.1 Phosphorylation of eIF2 α in wild-type malaria parasites by dihydroartemisinin (DHA)

To determine whether eIF2 α pathway is activated by artemisinin exposure, moderately synchronized ring stage (0-12 HPI) *P. falciparum* Dd2 wild-type parasites were treated with 700nM DHA or vehicle control (0.1% DMSO). Phosphorylated eIF2 α was observed after DHA treatments for 1 to 3 hours while no significant difference was observed in the total eIF2 α level (Figure 4-2A), implying that DHA triggered the phosphorylation of pre-existed eIF2 α in *P. falciparum*. In addition, the phosphorylation of eIF2 α was found to be dose-dependent (Figure 4-2B). The level of phosphorylated eIF2 α gradually increased in response to DHA treatment from 0 to 700nM. In contrast, none of these concentrations of DHA had significant impact on the level of total eIF2 α (Figure 4-2B). These results confirmed the induction of eIF2 α phosphorylation by DHA treatment in ring stage *P. falciparum* parasites, consistent with the findings where asynchronized or trophozoite parasites were used [77, 112].

Figure 4-2 eIF2α phosphorylated after DHA treatment. A. Dd2 WT parasites (0-12 HPI) either treated with vehicle control (0.1% DMSO) or 700nM DHA for 1, 2, 3 hours, respectively. B. Dd2 WT parasites (0-12 HPI) treated different concentration DHA for 2 hours. 0nM refers to control group treated with vehicle control (0.1% DMSO).

4.2.2 Difference in the phosphorylation of eIF2α between K13 mediated resistant and wild type malaria parasites

Artemisinin sensitive *P. falciparum* Dd2 wild-type (denoted as 'WT') and resistant K13 I543T mutant (denoted as 'I543T') were compared for the phosphorylation of eIF2α after DHA treatment. In RSA_{0-3h}, only tightly synchronized early ring stage resistant parasites (0-3 HPI) showed significantly distinguishable survival rate from the sensitive ones [61]. However, the age of circulating parasites in patients ranges from 0h to 18h at the time of clinical presentation [63]. To mimic the real clinic situation, moderately synchronized ring-stage WT and I543T parasites (0-12 HPI) were used in this study. The level of total and phosphorylated eIF2α were analyzed against β-actin loading control. Quantitative analysis of western blots showed that resistant I543T did not express significantly different level of total eIF2α before or after treatment,

compared to the WT strain (Figure 4-3A, C, D). In contrast, a significant lower level of phosphorylated eIF2 α were observed in the I543T artemisinin-resistant parasites after 2-hour drug treatment (Figure 4-3A, B).

Clinical isolates from Pailin, Cambodia were used to test the potential use of phosphorylated eIF2 α as an indicator for the field artemisinin resistance. KH004-041 (denoted as 'KH41') is a wild-type clinical isolate and KH004-003 (denoted as 'KH03'), carrying K13 C580Y mutation, is a resistant clinical isolate. Both strains were treated with 700nM DHA for 2, 3 hours, respectively. Quantification results showed significantly lower level of phosphorylated eIF2 α in the resistant KH03 after 2 hours drug treatment compared to the sensitive KH41 (Figure 4-3E).

Figure 4-3 Lower level of phosphorylated eIF2α in the resistant Dd2 I543T parasites and Cambodian clinical isolates KH004-003. A-C. Sensitive Dd2 WT and resistant Dd2 K13 I543T mutant (0-12 HPI) were treated with vehicle control (0.1% DMSO) or 700nM DHA for 1, 2, 3 hours, respectively. Samples were subjected to western blot and quantitative analysis. A. Representative western blot. B. Change of phosphorylated eIF2α after DHA treatment. C. Basal level of total eIF2α. D. Change of total eIF2α after treatment. E. Change of phosphorylated eIF2α after DHA treatment in clinical isolates KH004-041 and KH004-003. Both were treated with vehicle control (0.1% DMSO) or 700nM DHA for 2, 3 hours, respectively. N=3. * $p < 0.05$, ** $p < 0.01$, two-way ANOVA. Multiple comparisons were made between WT and I543T at each time point, respectively. p value adjusted after Šidák correction. Data were shown in means $\pm$ SEM.

To test whether parasites age affects the difference in phosphorylation of eIF2 α between wild type and resistant strains, tightly (0-3 HPI) and moderately (0-12 HPI) synchronized ring stage WT and I543T were compared. Results showed that the difference in eIF2 α phosphorylation level between WT and I543T was significant in moderately synchronized parasites, but not in the tightly synchronized ones (Figure 4-4A), which is likely due to the low level of phosphorylated eIF2 α at early ring stage in both wild type and resistant parasite.

Figure 4-4 The impact of parasite age (A) and treatment time (B) and different K13 mutation (C) on detecting artemisinin resistance using the level of phosphorylated eIF2 α . A. Tightly (0-3 HPI) and moderately (0-12 HPI) synchronized ring stage WT and I543T were treated with vehicle control (0.1% DMSO) or 700nM DHA for 2hours. B. Parasites (0-12 HPI) were treated with vehicle control (0.1% DMSO) or 700nM DHA for 15min or 2hours. N $\geq$ 3. * p <0.05, n.s. = non-significant, * p <0.05, ** p <0.01, two-way ANOVA. Multiple comparisons were made between WT and I543T at each condition. p value adjusted after Šidák correction. Data were shown in means $\pm$ SEM. C. Lower level of phosphorylated eIF2 α in Dd2 C580Y. Moderately (0-12 HPI) synchronized ring stage WT and C580Y were treated with vehicle control (0.1% DMSO) or 700nM DHA for 2hours. N=3. All data were shown in means $\pm$ SEM. ** p <0.01, Student's t -test, 2-tailed, paired (comparison between sensitive WT and resistant C580Y).

To investigate if the difference can be detected at an earlier timepoint, 15-minute treatment established by Zhang et al. [77] was compared with 2-hour treatment using moderately synchronized ring stage WT and I543T. Results showed more significant and robust difference between WT and I543T with 2-hour treatment (Figure 4-4B), further confirming 2-hour as the optimal detection timepoint. The most prevalent K13 mutant strain in Southeast Asia, C580Y, was also shown to have lower level of phosphorylated eIF2 α after 2-hour DHA treatment (Figure 4-4C), demonstrated that the difference in eIF2 α phosphorylation in comparison to wild-type is common to different K13 mutants. Meanwhile, the gold standard RSA was carried out on moderately

Figure 4-5 RSA survival rates of Dd2 WT, C580Y, I543T, KH004-041 and KH004-003. N $\geq$ 3. *p<0.05, n.s. = non-significant, one-way ANOVA, p values were adjusted after Holm-Šidák correction. Multiple comparisons were only made between WT-I543T, WT-C580Y, KH004-041 – KH004-003, respectively. ‘S’ refers to sensitive while ‘R’ refers to resistant. All data were shown in means $\pm$ SEM.

synchronized ring stage parasites (0-12 HPI) of different strains, and results only showed significant difference between Dd2 WT and I543T (Figure 4-5).

Together these data suggested that the level of phosphorylated eIF2 α in moderately synchronized ring parasites after 2-hour 700nM DHA treatment *in vitro* exhibited significant and robust difference between resistant and sensitive parasites, suggesting it as a potentially promising phenotypic indicator for early detection of K13-mediated artemisinin resistance.

4.2.3 Detection of DHA resistance in clinical isolates based on the phosphorylation level of eIF2 α

Another six clinical isolates were tested to verify the accuracy of the eIF2 α phosphorylation level in detecting reduced artemisinin susceptibility. Similarly,

Figure 4-6 Characterization of eIF2 α profile (A) and RSA survival rate (B) in other clinical isolates. A. Correlation of the change of eIF2 α phosphorylation level after DHA treatment with clearance half-life. Clinical isolates were treated with vehicle control (0.1% DMSO) or 700nM DHA for 2 hours. Samples were subject to western blot and quantitative analysis. Dotted line refers to the average eIF2 α phosphorylation level of KH004-014. B. Correlation between ring stage survival rate and clearance half-life. Dotted line refers to the average survival rate of KH004-044. Resistant (grey square) and sensitive (black circle) parasites were separated at clearance half-life equals to 5 hours (pink background). N=3. All data were shown in means $\pm$ SEM.

moderately synchronized ring stage parasites were treated with 700nM DHA for 2 hours. Among these parasite, two sensitive strains, KH004-042 and KH004-044 were shown to have higher level of phosphorylated eIF2 α after DHA treatment than the four resistant strains, consistent with their reported field clearance half-life [64, 76] (Figure 4-6A). RSA results of these parasites also showed positive correlation with the clearance half-life (Figure 4-6B). However, while there was clear overlap in the survival rate among the resistant and sensitive strains (Figure 4-6B), the overlap in the phosphorylation level of eIF2 α was relatively lower (Figure 4-6A). It suggested that the phosphorylation level of eIF2 α , as a phenotypic indicator of reduced artemisinin susceptibility, had similar, if not higher, sensitivity than RSA on moderately synchronized clinical isolates. Compared with RSA, which takes 72 hours and requires culture adaptation [61], the phosphorylation level of eIF2 α measured after 2-hour *in vitro* incubation with drug provides much earlier signal for detection and less possibility of false negative results due to culture failure.

4.2.4 Phosphorylation of eIF2 α in non-K13-mediated ART-resistance

Though K13 mutation is commonly found to be associated with artemisinin resistance in Southeast Asia, sensitive strains carrying K13 mutations and slow-clearing strains without K13 mutation have been reported in Africa [74, 113], which has raised the concern that artemisinin resistance in Africa, if any, may not be K13-mediated. Therefore, it is important to test if the phosphorylation

level of eIF2 α is capable of detecting non-K13-mediated artemisinin resistant strains.

P. falciparum 6A-R and 11C-R, two lab generated non-K13-mediated artemisinin resistant strains derived from *P. falciparum* 3D7, were tested [89]. Similarly, artemisinin did not trigger any detectable change of the level of total eIF2 α (Figure 4-7A). The phosphorylation of eIF2 α was observed in all three strains but the level of phosphorylated eIF2 α was much lower in the resistant 6A-R and 11C-R strain (Figure 4-7A, B). In contrast, the average survival rate of 6A-R and 11C-R, though higher than the sensitive 3D7 strain, was not statistically different

Figure 4-7 Lower level of phosphorylated eIF2 α in non-K13-mediated artemisinin resistant parasites. 6A-R and 11C-R are lab-generated resistant strains derived from two isogenic clones of *P. falciparum* 3D7. Parasites were treated with vehicle control (0.1% DMSO) or 700nM DHA for 2 hours and subjected to western blot and quantitative analysis. A. Representative western blot. B. the change of eIF2 α phosphorylation level after DHA treatment. C. RSA survival rates. N=3. * p <0.05, n.s. = non-significant, one-way ANOVA, p values were adjusted after Holm-Šidák correction. Multiple comparisons were only made between 3D7-6A-R and 3D7-11C-R, respectively. All data were shown in means $\pm$ SEM.

(Figure 4-7C), further demonstrated the level of phosphorylated eIF2 α as a reliable phenotypic indicator even in non-K13-mediated artemisinin resistant strains.

4.3 Discussion

eIF2 α was shown to be phosphorylated in *P. falciparum* in response to DHA exposure at ring stage (Figure 4-2A, B), trophozoite stage [112] and asynchronized parasites [77]. Quantitative characterization of eIF2 α phosphorylation level in ring stage parasites after DHA treatment showed lower level of phosphorylated eIF2 α in two lab-generated resistant K13 mutants (I543T and C580Y) (Figure 4-3B, Figure 4-5B), five resistant clinical isolates which carry the K13 C580Y mutation (Figure 4-5C, Figure 4-6A), and two non-K13-mediated resistant strains (Figure 4-7A, B), compared with their respective sensitive references. In contrast, no significant difference in the level of total eIF2 α (Figure 4-3C) nor the change in the level of total eIF2 α after treatment (Figure 4-3D) was observed between the resistant and sensitive strains. These evidences suggested that the resistant parasites respond to DHA differently from wild type with a reduced level of eIF2 α phosphorylation but not the changes in the overall expression of eIF2 α .

The PfPK4-eIF2 α pathway is known to respond to ER stress by increasing the capacity of degrading unfolded or misfolded protein and reducing the protein translation [104, 114, 115]. In sensitive parasites, pre-exist phosphorylated eIF2 α induced by salubrinal was shown to be protective against artemisinin treatment [77]. Therefore, the PfPK4-eIF2 α pathway would be expected to be

Figure 4-8 Role of UPR and proteasome degradation pathway in artemisinin resistance. Artemisinin disturbs the protein synthesis and lead to accumulation of unfolded or misfolded proteins in the ER lumen. Those proteins trigger the UPR and thus the eIF2 α phosphorylation. At the same time, misfolded proteins are retro-transport back to cytosol and ubiquitinated for proteasome degradation. In artemisinin resistant parasites, both protein ubiquitination [3] and eIF2 α phosphorylation (this study) were shown to be attenuated compared with sensitive parasites and were closely linked to the survival outcome of parasites.

upregulated in resistant parasites to survive from artemisinin treatment.

However, relative lower level of phosphorylated eIF2 α was observed in all resistant strains tested. Furthermore, the level of phosphorylated eIF2 α between resistant strains and their sensitive counterparts after 15 minutes and 1-hour treatment did not show significant difference (Figure 4-3B, Figure 4-4B), indicating no early up-regulation of phosphorylated eIF2 α in resistant parasites.

Nevertheless, the reduced level of phosphorylation of eIF2 α in resistant parasites compared to sensitive ones appear to provide a survival benefit after DHA treatment (Figure 4-5, Figure 4-6A). The downstream effect of eIF2 α phosphorylation in UPR can be complicated. The pathway not only promotes

survival but also triggers cell death. The switch between survival and death was shown to be determined by the crosstalk among various other cellular processes like autophagy, ER-associated degradation (ERAD), mitochondria function, AKT pathway etc. [108, 114, 116-118]. It is likely that the overly upregulated eIF2 α phosphorylation in sensitive parasites, together with massive signal feedback from the dysregulated ERAD [112], mitochondria [40] and other cellular processes like PfPI3K [80], eventually triggers cell death. On the other hand, resistant parasites do not respond dramatically to artemisinin treatment (Figure 4-8). The K13 mutation leads to alterations in protein ubiquitination and degradation pathways, which likely allow the parasites to deal with damaged proteins without triggering much ubiquitination and degradation [3, 112]. Less phosphorylated eIF2 α helps resistant parasites to avoid switching to the cell death pathway but rather enable them to enter hibernation and only resume cellular activities after the drug has been cleared. Therefore, the higher level of phosphorylated eIF2 α in artemisinin treated sensitive parasites is a “death signal”, representing the massive malfunction of cellular processes induced by the drug.

Regardless of the mechanism by which resistant parasite may escape from the drug pressure, the phosphorylation level of eIF2 α after 2 hours treatment of 700nM DHA was shown to be a reliable phenotypic indicator to distinguish sensitive and resistant parasites. Compared with genetic detection on K13 mutations, this phenotypic indicator is advantageous in distinguishing the non-K13-mediated artemisinin resistant ones (Figure 4-7) and thus more powerful in the surveillance of artemisinin resistance. Compared with the gold standard,

RSA survival rate, this method requires only 2 hours of *in vitro* drug treatment to achieve similar detection sensitivity (Figure 4-5, Figure 4-6), enabling potentially faster detection and diagnosis of artemisinin resistance in the field.

Although the utility and the advantages of the novel phenotypic indicator for reduced artemisinin susceptibility have been explored in this study, further effort needs to be taken to apply it for clinical use. Firstly, a pre-purification step which removes schizont and gametocyte from the test samples is essential (Figure 4-9). Since phosphorylated eIF2 α are critical for life cycle progression [119, 120], large amount of phosphorylated eIF2 α exists in schizont and gametocyte, which would weaken the detection accuracy of the indicator with blood containing mixed stage parasites. Secondly, western blot needs to be replaced by quantitative detection methods such as enzyme-linked immunosorbent assay (ELISA), which might give faster and more accurate detection and more practical for clinical use. Based on ELISA, further development of a diagnostic kit could be realized with help from microfluidic devices by incorporating the sample purification, drug incubation and signal detection into one dipstick-like form device (Figure 4-9). Furthermore, to further

Figure 4-9 Illustration of the possible layout of a dipstick-form device. Blood draw from infected patients must undergo purification step to remove the gametocyte. Purified blood will be incubated with drug for 2-hour *in vitro* in the reservoir. After incubation, the red blood cells will be lysed and removed. Parasites lysate then can be analyzed for eIF2 α phosphorylation level using ELISA based colorimetric assay.

validate the robustness of this indicator and establish database for diagnostic use, test on different treatment conditions mimicking clinical situation like low parasitemia and poor culture setups, and more clinical isolates including resistant isolates with different K13 mutations or even without K13 mutations, from different origins etc. Lastly, multi-center clinical trial with massive screening of patient samples can be conducted.

**Chapter 5 Detection of reduced
artemisinin susceptibility based on the
level of DNA damage**

5.1 Introduction

Southeast Asia, where artemisinin resistance was first reported, has been the hotbed of drug resistance development. Earlier research attributed this phenomenon to the hypermutability or accelerated resistance to multiple drugs (ARMD) phenotype, whereby the parasite exhibited 1000-fold higher rates of resistance to anti-malarial drugs compared with wild-type strains. The phenotype was primarily linked to defects in DNA repair, most likely DNA damage sensing. Although the debate on the relationship between the ARMD phenotype and drug resistance development in Southeast Asia has continued, mutations in DNA repair genes have been linked to the resistance development in Southeast Asia [121, 122].

Artesunate (derivative of artemisinin) was reported to cause DNA damage in trophozoite stage malaria parasites after only 1-hour treatment [48, 123, 124]. DNA repair pathways were shown to be activated and able to repair damage caused by sub-lethal dose of artesunate [56]. The relationship between artemisinin resistance and DNA damage and repair has yet to be resolved, however, to survive artemisinin treatment, artemisinin resistant parasites must have either successfully repaired the artemisinin induced DNA damages or suffered sub-lethal amount of DNA damage from the treatment. Therefore, they may exhibit substantial lower level of DNA damages compared with sensitive parasites. Since successful replication of parasite requires repair of DNA damage,

Figure 5-1 CometChip for high-throughput assessment of DNA damage.

A. CometChip fabrication. 1) A PDMS stamp with an array of micropillars is pressed into molten agarose. Once the agarose cools down and solidifies, the stamp is lifted to reveal an array of microwells (~40-50 μm in both diameter and depth, spaced 240 μm from each other). 2) Cells in suspension are loaded directly into microwells via gravity. 3) Excess cells are washed off by shear force, revealing an array of micropatterned cells. 4) Low-melting point (LMP) agarose kept molten at ~37°C is placed on top of the micropatterned cells and allowed to solidify by a brief incubation at 4°C (~two minutes). B. Macrowells are formed by clamping a bottomless 96-well plate on top of a microwell array. The bottom surface of each macrowell contains up to 300 microwells. Macrowells can be used both to load multiple cell types at the same time and to perform parallel treatments. C. Example fluorescent images of comets on alkaline CometChip. Images were taken at 40X magnification. Each frame can capture ~60 to more than 100 comet images. Left: untreated TK6 cells yield comets with little to no tail. Right: comets from TK6 cells treated with a high dose of a DNA damaging agent (50 μM H_2O_2) have visibly large tails. (Image adapted from previous publications [4, 5]).

the difference in DNA damage levels between resistant and sensitive parasites must be apparent before the start of the next life cycle, providing an early detectable signal thereby providing a potential faster indicator of reduced artemisinin susceptibility as compared to 72-hour RSA.

Quantitative assessment of DNA damage can be performed with CometChip, a high throughput platform based on the well-established comet assay [5, 125]. Briefly, untreated or treated cells are captured into customized micro-wells (50 μm) on the agarose gel of a CometChip and covered with a layer of low-melting point agarose (LMPA) to seal the cells in the CometChip (Figure 5-1A). Cells are

subsequently lysed to release DNA. After electrophoresis, damaged DNA strands are pulled away from the bulky nuclear DNA ('comet head') and revealed as a 'comet tail' by fluorescent staining (Figure 5-1C). The intensity of the 'comet tail' relative to the 'comet head' are then automatically quantified by customized software [5] to reflect the level of DNA damage. Compared with the traditional comet assay which is low throughput, involves laborious imaging and analysis methods, the fixed pattern of cell seeding on the CometChip effectively reduces overlapping of comets, which not only enhances the reproducibility of the assay, but also facilitates the automated imaging-based analysis of the comets (Figure 5-1C). In addition, CometChip can be carried out in a 96-well form with different cells and reagents being applied in different macro-wells, enabling high-throughput analysis of different conditions on one chip (Figure 5-1B).

The aim of this chapter is to adapt the CometChip for quantification of DNA damages in early and late stage malaria *P. falciparum* parasite after drug treatment, to examine the reliability of drug induced DNA damage level in reporting reduced artemisinin susceptibility, and to further investigate the relationship between the level of DNA damage and artemisinin resistance.

5.2 Results

5.2.1 Cometchip detection of DNA damage in early and late stage *P. falciparum*

The CometChip platform developed for mammalian cells [5] was modified for malaria parasites. To optimize the assay, hydrogen peroxide was used to induce DNA damage in *P. falciparum* 3D7. The percentage of DNA in the 'comet' tail (% tail DNA) was used to represent the level of DNA damages.

Due to the relatively small genome size (23 Mb, human: 3,000 Mb), it is necessary to concentrate the parasites in the infected blood samples for detection. To ensure the successful detection of hydrogen peroxide induced DNA damage, the time gap between treatment and detection must be minimized, because the DNA damages caused by hydrogen peroxide is transient [126]. Therefore, live ring stage parasites were firstly concentrated using the previously described streptolysin O (SLO) method [94, 95] (Figure 5-2A, C). The highly enriched ring stage parasites were then loaded onto the CometChip, sealed with LMP agarose, treated with hydrogen peroxide on chip and followed by immediate alkaline lysis (Figure 5-2A). An observable 'comet' started to show up after treatment with 100 μ M hydrogen peroxide and increased as the drug concentration increased (Figure 5-2B). The % tail DNA was quantified, and the

results showed dose-dependent increase of % tail DNA in response to hydrogen peroxide in ring stage parasites (Figure 5-2D).

Schizont stage parasites were concentrated using Percoll gradient centrifugation and treated with hydrogen peroxide on the chip. Although small tails were detected even in the untreated group, a clear increase of the % tail DNA was observed as the concentration of hydrogen peroxide increased (Figure 5-2B). Quantification showed statistical difference between untreated parasites and parasites treated with hydrogen peroxide above 100 μ M and the detected DNA damage level reached maximum at 500 μ M treatment (Figure 5-2D). With optimized platform, its potential in detecting artemisinin induced DNA damage was subsequently investigated.

Figure 5-2 Optimization of CometChip. A. Schematic comparison of Off chip treatment using saponin and on chip treatment using Streptolysin O (SLO). B. Representative pictures of 'comet' of ring and schizont stage *P. falciparum* 3D7 after on-chip hydrogen peroxide treatment. C. Ring stage parasites enriched after SLO lysis. D. Quantification of 'comet' after treatment. Y-axis represents the percentage of DNA at 'Comet' tail. N=3. * $p<0.05$, ** $p<0.01$, one-way ANOVA. Dunnett's multiple comparison tests were performed between vehicle control (0 μM) and respective concentrations. All data were shown in means $\pm$ SEM.

5.2.2 DNA damage induced by artemisinin in *P. falciparum*

Artesunate was reported to cause DNA damage in trophozoite stage parasites [48]. It is unknown whether DNA damage is also induced in ring and schizont stage parasites and whether it can be detected using CometChip. Ring and schizont stage *P. falciparum* 3D7 parasites, enriched by SLO method or Percoll

A

B

Figure 5-3 A. Artesunate induced DNA damages in *P. falciparum* 3D7.

Ring and schizont stage *P. falciparum* 3D7 parasites were treated with different concentration of artesunate. Data was normalized within each treatment group. N=3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA. Dunnett's multiple comparison tests were performed between vehicle control (0 μ M) and respective concentrations. All data were shown in means $\pm$ SEM. B. CometChip images and illustration of the detachment of tail (grey hollow shape) from 'Comet' head (grey solid circle) under higher concentration of drug. If too much DNA damages were induced, the tail will be pulled further away from the head by electrophoresis and cannot be picked up by the software during analysis, which may lead to the false decrease of %Tail DNA.

gradient centrifugation as described above, were treated with different concentrations of artesunate for 1 hour *in vitro* and subsequently transferred onto CometChip. The time gap between artesunate treatment and on-chip alkaline lysis was limited to 5 minutes. Clear DNA damage showed up in parasites treated with 100 nM and higher concentrations of artesunate for 1 hour in both ring and schizont stage parasites (Figure 5-3A), which is consistent to the fast-acting feature of the artesunate [48]. The damage, as manifested by %Tail DNA, saturated at higher concentration. A decrease in %Tail DNA were even observed at 1000nM in both ring and schizont stage (Figure 5-3A), which is likely caused by the excessive DNA damages induced by high dose of artesunate. As illustrated in Figure 5-3B, at high concentration of artesunate, the tail of the comet has separated too far from the head and thus cannot be fully recognized by the software during the analysis. Despite the level of damage plateauing at high concentrations, the artesunate induced DNA damage was clearly detected and quantified on CometChip in a high throughput manner.

5.2.3 Correlation between DNA damage and reduced artemisinin susceptibility

With CometChip being optimized and validated to detect the artesunate induced DNA damages at both ring and schizont stage, resistant and sensitive parasites were tested to see whether any difference in the drug induced DNA damage level can be seen. Lab strains *P. falciparum* Dd2 wild-type (denoted as 'WT') and K13 I543T mutant (derived from Dd2 wild-type, denoted as 'I543T'),

as well as two clinical isolates KH004-041 (sensitive, denoted as 'KH41') and KH004-003 (resistant, denoted as 'KH03') were investigated for the relationship between the level of DNA damage and K13-mediated artemisinin resistance.

No artesunate induced DNA damage can be observed in ring stage WT and I543T (Figure 5-4A). The sensitive clinical isolate KH41 at ring stage showed increase

A

B

Figure 5-4 Artesunate induced DNA damages in sensitive and resistant parasites. Ring (A) and schizont stage (B). Dd2 WT, I543T, sensitive KH004-041 and resistant KH004-003 were treated with different concentration of artesunate for 1 hour. $N \geq 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-way ANOVA. Comparisons were made between WT and I543T (left panels), between KH004-041 and KH004-003 (right panels), at each concentration and p values were adjusted after Šidák correction. All data were shown in means $\pm$ SEM.

of DNA damage at higher concentrations ($\geq 250\text{nM}$) of artesunate (Fig. 3A). On the other hand, ring stage resistant isolate, KH03, appeared to show a slight increase in DNA damage at higher concentration, however, no statistical significance could be established (Figure 5-4A). Between ring stage KH41 and KH03, significant difference can only be seen at concentration of 250nM. Unlike ring stage, both schizont stage sensitive parasites, WT and KH41, exhibited a dose-dependent increase of the DNA damage while their resistant counterparts, I543T and KH03, showed a significantly lower amount of DNA damage (Figure 5-4B). Compared with ring stage, schizont stage parasites with 1-hour artesunate treatment exhibited distinct difference between resistant and sensitive strains on the malariaCometChip and thus may be the stage where the

Figure 5-5 Artesunate induced DNA damages in KH strains. Schizont stage KH strains treated with different concentration artesunate for 1 hour. 'R' represents artemisinin resistant strain and 'S' represents sensitive strains. N=3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-way analysis of variance (ANOVA). Comparisons were made between vehicle control and each concentration and p values were adjusted after Šidák correction. All data were shown in means $\pm$ SEM.

relationship between DNA damage level and reduced artemisinin susceptibility can be reliably revealed.

Therefore, another 6 culture-adapted clinical isolates from Pailin, Cambodia were tested at schizont stage using malariaCometChip. Sensitive strain KH004-042 showed significantly higher DNA damage at 1000 nM drug concentration. Resistant strains, KH004-032 and KH004-048, and sensitive strain KH004-044 (KH44) showed a slight or no change on the level of DNA damage after artesunate treatment at all doses. Resistant KH004-014 (KH14) showed a reduce in DNA damage at higher concentration of artesunate treatment (Figure 5-5). The normalized levels of DNA damage at 1000 nM artesunate treatment in different KH strains were found to be inversely correlated with their

Figure 5-6 Correlation between DNA damages level at 1000nM artesunate and parasite clearance half-life in multiple clinical isolates. Dotted line is the linear fit-curve. Value of tail DNA used was normalized within the population. Four zones were divided by 5 hours clearance half-life and 21.7% tail DNA and anti-clockwise labeled as I, II, III, IV from upper left corner. 21.7% tail DNA is the value on the fit-curve when clearance half-life equal to five.

corresponding reported clinical clearance half-lives[76] (Figure 5-6). Based on clinical criteria, the resistant parasites are separated from sensitive ones at clearance half-life > 5 hours. With 21.7% normalized tail DNA as separation criteria on y-axis, four zones were created and anti-clockwise labeled as I, II, III and IV. With zone II represent false-negative and zone IV represent false-positive, 5 out of 6 strains tested showed the results consistent with their clearance half-life, except for KH44.

In conclusion, the level of %Tail DNA after 1-hour 1000nM artesunate treatment exhibited good correlation with the clearance half-life (Figure 5-6), with one exception, indicating its potential in detecting artemisinin resistance in the field. On the other hand, the uniqueness of KH44 subsequently became the critical puzzle in unraveling the relationship between artemisinin resistance and the level of DNA damage revealed by CometChip.

5.2.4 Correlation between DNA damage and artemisinin resistance at genetic level

To understand why KH44 experienced lower level of DNA damage as compared to other sensitive strains, genetic analysis of the clinical isolates was carried out with SNPs data from the Pf3K/TRAC database [75, 90]. A phylogenetic tree was constructed based on the full SNPs data of samples from Pailin, Cambodia, 2011, where the KH samples came from (Figure 5-7). Among the KH strains, KH41, KH42 and KH44 have wild-type *kelch13* and are considered as artemisinin

sensitive. KH41 and KH42, which showed DNA damage response to artesunate (Figure 5-4B, Figure 5-5), were closely related to each other and distantly related to the resistant KH strains. However, the other sensitive strain KH44, which showed no DNA damage at high concentration of artesunate treatment, fell into the cluster of resistant strains and was most closely related to the resistant strain KH14, which showed a similar though more significant decrease of DNA damage upon artesunate treatment (Figure 5-5).

The mutations in known DNA repair genes (Appendix 1) in these clinical isolates were subsequently compared. Considering KH44 as 'resistant' due to the low level of DNA damage it experienced after artemisinin treatment (Figure 5-5), seven nonsynonymous mutations, including three SNPs and four deletions, in 7 genes, PF3D7_0710400, PF3D7_1006000, PF3D7_1106000, PF3D7_1368800, PF3D7_1429900, PF3D7_1455300, were found different between the five 'resistant' KH strains and sensitive KH41, KH42 (Table 5-1). Among these genes, PF3D7_0710400, PF3D7_1106000 and PF3D7_1368800 were found to be highly essential [7]. For ease of explanation, these mutations were defined as 'ResisDNArep genotype' and their counterparts in wild-type K13 carrying parasites (WT) as 'SensiDNArep genotype'. Notably, Dd2 WT, the ARMD strain which showed lower level of DNA damages compared with KH41 at both ring and schizont stage, possess 4 of the 8 mutations in the 'ResisDNArep genotype' (Table 5-2), suggesting a potential link between the 'ResisDNArep genotype' and ARMD phenotype.

Figure 5-7 Phylogenetic tree based on SNPs profile of samples from Pailin, Cambodia 2011. CometChip tested sensitive strains were labeled in black square and resistant strains were labeled in grey square.

Table 5-1 List of mutations in the ‘ResisDNArep genotype’. Mutation type, variant type, impact and amino acid change were predicted using SnpEff [6] with latest *P. falciparum* 3D7 genome data from PlasmoDB. Information of gene product, predict function and gene essentiality [7] were extracted from PlasmoDB.

Gene ID	Position	Reference	Variant	Type	Variant type	Impact	Amino acid change	Product	Predict function	Gene essentiality
PF3D7_0710400	474979	TA	*	DEL	missense_variant	Moderate	-	DNA repair protein	Nucleotide excision repair, DNA damage recognition	High
	475135	A	G	SNP	missense_variant	Moderate	Ile171Met	RAD14, putative		
PF3D7_1006000	257175	TAATCTTCCCT	T	DEL	frameshift_variant	High	Lys164fs	IMP1-like protein,	Unknown function	Low
PF3D7_1106000	257027	T	C	SNP	missense_variant	Moderate	Asn39Asp	RuvB-like helicase 2	ATP-dependent 5'-3' DNA helicase activity	High
PF3D7_1368800	2731444	TCGGTGGTCCAT TTTTTCAACTTC	*	DEL	frameshift_variant missense_variant	High	Glu1567fs	DNA repair endonuclease XPF, putative	DNA repair, nuclease activity	High
PF3D7_1429900	1178944	TGATAAG	T, TGAT AATG	DEL	frameshift_variant missense_variant	High	Asn725fs	ADP-dependent DNA helicase RecQ	ATP-dependent 3'-5' DNA helicase activity	Low
PF3D7_1455300	2260945	T	G	SNP	missense_variant	Moderate	Met494Arg	conserved Plasmodium protein	mismatch repair, single-stranded DNA 5'-3' exodeoxyribonuclease activity, flap endonuclease activity	Low

Table 5-2 Distribution of mutations in ‘ResisDNArep genotype’ in the KH and Dd2 strains. Artemisinin sensitive (red) and resistant (black) strains were compared. Mutant, heterozygote and wild-type were labeled in orange, yellow and white, respectively.

Gene ID	Position	KH004-041	KH004-042	Dd2 WT	KH004-044	KH004-003	KH004-014	KH004-040	KH004-048	KH004-032
PF3D7_0710400	474979 475135									
PF3D7_1006000	257175									
PF3D7_1106000	257027									
PF3D7_1368800	2731444									
PF3D7_1429900	1178944									
PF3D7_1455300	2260945			TGATAATG	*	*	T	T	T	*
Wild type										
Heterozygote										
Mutant

Figure 5-8 Area profile of K13 mutation prevalence (pie chart of WT (blue) and K13 (magenta)) and distribution of the 'ResisDNAREp genotype' in parasites carrying wild-type K13 (WT, upper panel) and parasites carrying K13 mutations (K13, lower panel) in Southeast Asia and Africa. X-axis and legend of the bar chart were label at the bottom of the figure. Areas belong to e-GMS were labeled in orange square.

To investigate if the 'ResisDNArep genotype' is correlated to K13 mutations and artemisinin resistance, the distribution of the 'ResisDNArep genotype' in clinical isolates from different areas with different K13 mutation prevalence were analyzed (Figure 5-8). In Pailin, where around 90% of the parasites carry K13 mutations, mostly C580Y, a high proportion of 'ResisDNArep genotype' was observed in the K13 parasites (Figure 5-8). As the prevalence of K13 mutations decreased in the study sites, the percentage of K13 parasites having 'ResisDNArep genotype' decreased. For example, in Pursat, Cambodia, which has a lower prevalence of K13 mutations (77.32%) compared to Pailin, more than 60% of the K13 parasites have the mutations in the 'ResisDNArep genotype' except for PF3D7_1368800 (41.67%) (Figure 5-8). In Phuoc Long, where half of the population carry K13 mutations, only 4 out of 7 mutations in the 'ResisDNArep genotype' was found to be over 50% in the K13 population (Figure

Figure 5-9 Temporal change of the frequency of the 'ResisDNArep genotype' in K13 parasites from 2011 (black bar) to 2012 (grey bar) in Pailin, Cambodia.

5-8). From a temporal perspective, most of the resistant parasites from Pailin, 2011, including the four KH resistant strains investigated in CometChip, shared similar 'ResisDNArep genotype' (average around 80%), especially for PF3D7_1006000 (95.65%) and PF3D7_1455300 (100%) (Figure 5-9). In 2012, as the frequency of K13 mutations remained high and the K13 C580Y mutation dominated in the area, the K13 parasites from Pailin further progressed with increased percentage of resistant parasites possessing towards the 'ResisDNArep genotype' (average around 90%) (Figure 5-9), indicating an interplay between K13 mutations, especially C580Y, and the 'ResisDNArep genotype' in the K13-mediated artemisinin resistance.

The distribution of the 'ResisDNArep genotype' in WT parasites was also analyzed to further investigate the relationship between the 'resisDNArep genotype' and the development of K13-mediated artemisinin resistance. The distribution of the 'ResisDNArep genotype' in WT parasites showed similarity between areas in Southeast Asia but is different in Africa. In places like Pailin and Pursat, where artemisinin resistance is prevalent, more than half of the WT parasites were found to possess the majority of the 'ResisDNArep genotype' (Figure 5-8). In Ramu, Bangladesh, while geographically close to Pailin but with no K13 mutation were documented in 2012, 4 out of the 7 mutations in the 'resisDNArep genotype' were found in more than half of the WT parasites. In contrast, in Africa, where only one K13 mutation was found and with clear

geographic separation from Southeast Asia, only 1 out of the 7 mutations in the 'ResisDNArep genotype' were found in more than half of the WT population.

To understand how well these mutations are associated with the resistance-rendering K13 mutations, To investigate if the 'ResisDNArep genotype' is linked to K13 mutations and artemisinin resistance, linkage disequilibrium (LD) was assessed between K13 and each resisDNArep mutation using Chi-squared analysis, respectively, and the P-values were plotted (Figure 5-10). Heterozygotes (due to mixed infection) were excluded from the analysis. With Bonferroni correction on the P-value, five out of seven mutations were found to be significantly associated with K13 mutations in Southeast Asian (SEA) population, except for SNPs at PF3D7_0710400-474979 and PF3D7_1455300. Previous studies suggested a strong regional difference between parasite

Figure 5-10 P values from Chi-square analysis of gene association in different population and sub-populations. SEA refers to Southeast Asia population, which was further divided into eastern Great Mekong Sub-regions (e-GMS) and western Great Mekong Sub-regions (w-GMS). The line was drawn at P value equal to 0.00625 (the threshold after Bonferroni correction).

populations from the eastern and western Great Mekong Sub-regions (GMS) in Southeast Asia [75, 127]. LD was thus assessed between 'ResisDNArep genotype' and K13 mutations after further dividing the SEA population in to eastern and western GMS (e-GMS and w-GMS) groups (Figure 5-8). Consistent with previous observation, differences in gene associations were observed between the two regions. In e-GMS, where the KH strains analyzed in CometChip come from, six out of seven mutations were found to be highly associated with K13 mutations, except for SNPs at PF3D7_1455300 (Figure 5-10). On the contrary, only three out of seven mutations, including the SNPs at PF3D7_1106000 and the deletions in PF3D7_1006000 and PF3D7_1429900, were found to be associated with K13 mutations in w-GMS (Figure 5-10).

Studies showed that the K13 C580Y mutation gradually dominated other K13 mutations in the resistant population, which is likely linked to the higher fitness or transmission of the C580Y strains [128]. Temporal analysis in Pailin also showed increased proportion of 'ResisDNArep genotype' in the K13 population when C580Y became the only K13 mutation in the Pailin population from 2011 to 2012 (Figure 5-9). To investigate if the 'ResisDNArep genotype' is associated with C580Y, the C580Y carrying parasites in the two sub-populations were examined. The results showed a higher percentage of parasites carrying the 'ResisDNArep genotype' in the C580Y parasites, especially in e-GMS (Figure 5-11). Analysis of LD between C580Y and other K13 mutations showed a close correlation between K13 C580Y mutation and the SNPs at PF3D7_0710400-475135 and PF3D7_1106000 and the deletions at PF3D7_0710400-474979 and

PF3D7_1368800 in e-GMS. In w-GMS, no statistically significant association with C580Y were found (Figure 5-11).

In conclusion, consistent with previous observations, results here indicate a highly conserved, maybe pre-existing, genetic background is associated with K13-mediated artemisinin resistance in Southeast Asia, especially e-GMS. In addition to previous identified mutations [75], mutations in DNA repair genes, such as those in the 'ResisDNArep genotype', may be part of this predisposing genetic background.

Figure 5-11 Distribution of the 'ResisDNArep genotype' in K13 C580Y parasites and parasites with other K13 mutations in e-GMS (A) and w-GMS (B). &, $P < 0.00625$ (value after Bonferroni correction), Chi-squared analysis (between C580Y and other K13 mutations within K13 population). The dotted line was drawn at 50%.

5.3 Discussion

The emerging artemisinin resistance in Southeast Asia has led to the concern about the consequent massive selection pressure on the partner drugs and thus the complete failure of ACTs [64]. If ACTs fail, no alternative as good as ACTs is available for treating malaria. To aid in the surveillance, containment and prevention of artemisinin resistance worldwide, we explored the role of DNA damage repair in artemisinin resistance development in Southeast Asia to identify potential phenotypic indicator as well as genetic factors for faster artemisinin resistance detection and resistance risk assessment.

Previous work has indicated that the genetic background of the parasites found in Southeast Asia may make the parasite more likely to develop resistance to a wide range of drugs [56, 75, 129]. One hypothesis suggested that the ARMD phenotype, the ability of parasite to rapidly repair DNA damage at the cost of loss of fidelity and therefore a higher mutation rate was the basis of the high proportion of parasite drug resistance that developed in SEA[56, 122, 129]. In the initial GWAS study of artemisinin resistance examining the associations with delayed parasite clearance[130], SNPs lie in or near genes involved in DNA repair pathways were identified. Therefore, we investigated the ability of both lab adapted parasites as well as clinical isolates to deal with artesunate induced DNA damage using the high throughput optimized malariaCometChip for quantitative analysis of DNA damage [5].

It was found that while at schizont stage artemisinin can induce substantial high levels of DNA damage in 3D7 and some sensitive Cambodian field isolates with

as little as a 1-hour *in vitro* treatment, it caused insignificant level of DNA damage in the K13-mediated resistant parasites (Figure 5-3Figure 5-4B,Figure 5-5). The level of DNA damage at 1000nM artesunate treatment of most of the Cambodian clinical isolates is negatively correlated to their reported clinical clearance half-lives (Figure 5-6Figure 5-6).

It was believed that the resistance happened at ring stage parasites, especially early ring stage, where significantly improved survival of resistant parasites was observed [60, 61, 78, 111]. Yet our results demonstrated a significant improvement in the ability of artemisinin resistant parasites to deal with DNA damage even at schizont stage with short artesunate exposure, suggested the reduced susceptibility to artemisinin maybe developing at the late stage of life cycle and the standard drug susceptibility assay has limited capability to detect. The phenomenon, as a result of factors contributing to current ring-stage-specific resistance, may also indicate the ongoing development of full resistance to artemisinin. From the perspective of monitoring, controlling and even preventing the development of resistance, phenotypical assessment of susceptibility to artemisinin induced DNA damages by malariaCometChip could have enormous implications in global drug resistance containment and malaria elimination.

The improved ability to deal with DNA damage is also observed in an artemisinin sensitive clinical isolate KH44 as well as the lab adapted Dd2 parasite that has previously been linked to the ARMD phenotype. The existence of KH44 proved the important role of DNA repair in drug response and resistance development.

KH44 possess SNPs in *fd*, *mdr2*, *crt* and *arps10*, which are the markers of the genetic background for the contemporary artemisinin resistance development [75] and could be the culprit for the resistance to artesunate induced DNA damage. However, this unique strain and the resistant KH03 suffered no DNA damage from short MMS treatment, indicating their intrinsic greater ability to deal with DNA damages instead of specifically dealing with artesunate induced ones. In addition, although drug induced DNA damages was observed at schizont stage Dd2 WT strain, it consistently exhibited lower level of DNA damage compared to KH41 at both ring and schizont stage (Figure 1D, Figure 2B).

The experimental data showing differences in DNA repair suggested that there are differences in the genetic background of these parasites that may provide them with an advantage for the development of artemisinin resistance in Southeast Asia. Here we identified seven mutations, including nonsynonymous SNPs and deletions, in known DNA damage repair genes that may contribute to providing the right genetic background for the development of artemisinin resistance in Southeast Asia.

Three out of seven mutations from the 'resisDNArep genotype' (Supplementary table 1) were found in Dd2WT, indicating a potential link between the 'ResisDNArep genotype' and the accelerated development of drug resistance. As one of the ARMD strains, Dd2 strain was shown to have defects in sensing DNA damage and triggering DNA repair [56] and therefore has a higher tolerance to damages, which led to higher mutation rates and accelerated

development of resistance to multiple drugs [121, 129]. Although there have been debates on the existence of ARMD phenotype in Southeast Asia [131, 132], both previous studies [122, 127] and evidence here suggested potential links between mutations in DNA repair genes, accelerated rates of resistance development and the field artemisinin resistance development.

Except for the SNP in PF3D7_1106000, other mutations have not been reported with association to artemisinin resistance. Top-ranked LD with K13 mutations was found in deletions in PF3D7_1006000 (IMP1-like protein) and PF3D7_1429900 (ADP-dependent DNA helicase RecQ) in both e- and w-GMS. Protein coded by PF3D7_1006000 was found to have mRNA binding activity at trophozoite and schizont stage without information on protein structure. The deletion in PF3D7_1429900 is predicted to be in the intrinsically disordered region (IDR) of the protein. IDRs often serve as flexible platforms for protein-protein interaction[133], indicating a possible impact of the deletion on the protein function and the pathway its involved. In fact, these genes appear to have low essentiality and high mutant fitness score. Having them mutated could help parasites rapidly adapt to the environment, which may partially explain the loss of resistance for field isolates under long term in vitro culture. The SNP in PF3D7_1455300 has a high frequency in SEA population, especially e-GMS, and thus appears to be not in LD with K13 mutations. However, it cannot be found in African population. This SNP could be fixed in the population due to selection and become a signature for the SEA population.

The SNP in PF3D7_0710400-475135 and deletions found in PF3D7_0710400-474979 and PF3D7_1368800 were only significantly associated with K13 in e-GMS. The regional differences observed between e-GMS and w-GMS may be partially related to the hard selective sweep that took place in the area, whereby a resistant C580Y lineage, originated from Pailin, spread to North-eastern Thailand and Southern Laos, outcompeted other parasites and dominated the area [75, 127, 128, 134]. It was suggested this C580Y lineage may possess higher fitness or transmission than other resistant parasites, which thus led to the dominance. In the analysis of Pailin K13 population, where the K13 C580Y mutation became the only K13 mutation type in 2012, the percentage of K13 parasites possessing the 'ResisDNArep genotype' further increased to around 90%, indicating a close relationship between the 'ResisDNArep genotype' and the dominating C580Y lineage (Supplementary figure 3). The C580Y parasites from w-GMS were shown to have a different origin, explaining why these parasites have a different association pattern between the 'ResisDNArep genotype' and K13 mutations.

The functional alterations (in DNA repair) caused by these mutations and how they contributed to overall fitness or the transmission capability of the K13 parasite is worth further exploration. Although the seven mutations found in the 'ResisDNArep genotype' were in known DNA repair genes, the changes caused by these mutations may go beyond the DNA repair pathways they are involved in. The mutations may affect the upstream and downstream genes involved in different cellular functions as listed in Supplementary table 2. In addition, in an expression quantitative trait loci (eQTL) analysis based on

Southeast Asian samples[134], the SNPs at gene PF3D7_0710400 position 475135 and gene PF3D7_1455300 position 2260945 were linked to gene PF3D7_0710500 and gene PF3D7_1029900, respectively. Both targeted genes encode conserved Plasmodium proteins with unknown function. The SNP at gene PF3D7_1106000 position 257027 were found to be associated with gene PF3D7_0923200, which encodes a nitric oxide synthase.

In conclusion, consistent with previous observations[75, 127, 134], the existence of parasites like KH44 indicated a founder population for drug resistance in the GMS with pre-deposited genetic factors including mutations in DNA repair genes. Those mutations may compromise accuracy with increased repair speed, lead to higher mutation rate in the genome and thus benefit the development of resistance, in this case, to artemisinin. From the perspective of monitoring, controlling and even preventing the development of resistance, phenotypical and genetical identification of such resistant-prone parasites like KH44 could have enormous implications in global malaria elimination. Together with previous identified genetic factors [75, 127, 134], the DNA damage phenotype measured by malariaCometChip and the 'ResisDNArep genotype' identified in this study may aid in the identification and surveillance of areas that are particularly at risk to develop artemisinin resistance.

5.4 Appendix 1 List of known DNA repair genes

Gene ID	Gene group	Gene function
PF3D7_0107800	GO	GO-0006281-BP-DNA repair
PF3D7_0107800	GO	GO-0006302-BP-double-strand break repair
PF3D7_0203300	GO	GO-0006289-BP-nucleotide-excision repair
PF3D7_0206000	GO	GO-0006281-BP-DNA repair
PF3D7_0217400	GO	GO-0006281-BP-DNA repair
PF3D7_0305600	GO	GO-0006281-BP-DNA repair
PF3D7_0408500	GO	GO-0006281-BP-DNA repair
PF3D7_0416400	GO	GO-0006281-BP-DNA repair
PF3D7_0505500	GO	GO-0006281-BP-DNA repair
PF3D7_0505500	GO	GO-0006298-BP-mismatch repair
PF3D7_0509500	GO	GO-0006281-BP-DNA repair
PF3D7_0513600	GO	GO-0006281-BP-DNA repair
PF3D7_0514100	GO	GO-0006281-BP-DNA repair
PF3D7_0605800	GO	GO-0006302-BP-double-strand break repair
PF3D7_0614800	GO	GO-0006284-BP-base-excision repair
PF3D7_0614800	GO	GO-0006281-BP-DNA repair
PF3D7_0619100	GO	GO-0006281-BP-DNA repair
PF3D7_0619100	GO	GO-0009380-CC-excinuclease repair complex
PF3D7_0628600	GO	GO-0006281-BP-DNA repair
PF3D7_0706700	GO	GO-0006298-BP-mismatch repair
PF3D7_0710100	GO	GO-0006281-BP-DNA repair
PF3D7_0710400	GO	GO-0006289-BP-nucleotide-excision repair
PF3D7_0725000	GO	GO-0006298-BP-mismatch repair
PF3D7_0726300	GO	GO-0006298-BP-mismatch repair
PF3D7_0803400	GO	GO-0000724-BP-double-strand break repair via homologous recombination
PF3D7_0910500	GO	GO-0006281-BP-DNA repair
PF3D7_0917100	GO	GO-0006284-BP-base-excision repair
PF3D7_0917100	GO	GO-0006289-BP-nucleotide-excision repair
PF3D7_0934100	GO	GO-0006289-BP-nucleotide-excision repair
PF3D7_1003700	GO	GO-0006281-BP-DNA repair
PF3D7_1006000	GO	GO-0006284-BP-base-excision repair
PF3D7_1008200	GO	GO-0006281-BP-DNA repair
PF3D7_1011700	GO	GO-0006289-BP-nucleotide-excision repair
PF3D7_1012000	GO	GO-0006281-BP-DNA repair
PF3D7_1037600	GO	GO-0006281-BP-DNA repair
PF3D7_1037600	GO	GO-0006289-BP-nucleotide-excision repair
PF3D7_1106000	GO	GO-0006281-BP-DNA repair
PF3D7_1107400	GO	GO-0006281-BP-DNA repair
PF3D7_1112600	GO	GO-0006281-BP-DNA repair

PF3D7_1117800	GO	GO-0006298-BP-mismatch repair
PF3D7_1129500	GO	GO-0006284-BP-base-excision repair
PF3D7_1140300	GO	GO-0006281-BP-DNA repair
PF3D7_1215700	GO	GO-0006298-BP-mismatch repair
PF3D7_1244200	GO	GO-0006281-BP-DNA repair
PF3D7_1250800	GO	GO-0006289-BP-nucleotide-excision repair
PF3D7_1303800	GO	GO-0006281-BP-DNA repair
PF3D7_1304100	GO	GO-0006281-BP-DNA repair
PF3D7_1314900	GO	GO-0006281-BP-DNA repair
PF3D7_1316900	GO	GO-0006281-BP-DNA repair
PF3D7_1332100	GO	GO-0006281-BP-DNA repair
PF3D7_1332600	GO	GO-0006281-BP-DNA repair
PF3D7_1338400	GO	GO-0006281-BP-DNA repair
PF3D7_1353500	GO	GO-0006281-BP-DNA repair
PF3D7_1368300	GO	GO-0006281-BP-DNA repair
PF3D7_1368800	GO	GO-0006281-BP-DNA repair
PF3D7_1405400	GO	GO-0006298-BP-mismatch repair
PF3D7_1415000	GO	GO-0006284-BP-base-excision repair
PF3D7_1427500	GO	GO-0006298-BP-mismatch repair
PF3D7_1429900	GO	GO-0006281-BP-DNA repair
PF3D7_1430600	GO	GO-0006281-BP-DNA repair
PF3D7_1432600	GO	GO-0006289-BP-nucleotide-excision repair
PF3D7_1441900	GO	GO-0006289-BP-nucleotide-excision repair
PF3D7_1455300	GO	GO-0006298-BP-mismatch repair
PF3D7_1467100	GO	GO-0006284-BP-base-excision repair
PF3D7_1471600	GO	GO-0000724-BP-double-strand break repair via homologous recombination

Chapter 6 General conclusions and future directions

Artemisinin-based combination therapies (ACT) have achieved remarkable success globally for more than a decade and are now the first-line anti-malarial treatment recommended by WHO [72]. However, emerging artemisinin resistance has been reported in Southeast Asia [64]. If ACTs fail, no alternative as good as ACTs is currently available. Effective surveillance in combination with containment of artemisinin resistance are essential so that the recent gains in reducing malaria burden would not be lost.

Currently, three indicators are used for monitoring artemisinin resistance in the field, which are K13 mutations, the parasite clearance half-life and ring stage survival rate in the ring-stage survival assay (RSA). For K13 mutations, in addition to demands for DNA sequencing setup, newly identified K13 mutation often need to be investigated before categorizing it to resistance-conferring mutation [72]. The potential existence of K13-independent artemisinin resistance in Africa area also diminishes the use of K13 mutations in that area [113]. On the other hand, monitoring reduced artemisinin susceptibility is more indicative but current indicators have limited diagnostic capability. Parasite clearance half-life [72], measured after treatment, cannot be used for diagnostic purpose and the results are often obscured by factors like the spleen clearance rates and immunity status of patients [83]. RSA, which involved 6 hours of drug treatment and another 66 hours of *in vitro* culture, may suffer from culture failure and result in an invalid test [61]. It is also time-consuming, resource-intensive and impractical for large-scale surveillance. Therefore, better phenotypic indicators for early and reliable detection of reduced artemisinin susceptibility are needed. In this project, three phenotypic indicators were proposed and investigated: 1.

the $\Delta\Delta A$ -ratio based on MRR; 2. phosphorylation of eukaryotic initiation factor 2 α subunit (eIF2 α); 3. DNA damages level based on CometChip.

6.1 $\Delta\Delta A$ -ratio detection based on MRR

The novel bench top MRR system enables rapid label-free detection of malaria parasites [1]. However, the absolute value of T_2 relaxation time of the blood sample in the MRR measurement is often influenced by factors other than infection, such as the blood aging due to *ex vivo* culture and storage. Therefore, to improve the stability of MRR measurement, a new parameter, the A-ratio, was defined and investigated. With the aid of the A-ratio, the potential of MRR in detection of reduced artemisinin susceptibility was investigated. Later, to further the application of MRR, the RBCs background, consist of signals from uninfected and host RBCs, were removed by a new sample preparation method. The potential of MRR in combination with the new sample preparation method for reduced artemisinin susceptibility detection was also evaluated.

6.1.1 Key findings:

- 1) The A-ratio, defined as T_1 relaxation time divided by T_2 relaxation time, was found to be well correlated with parasitemia change in both calibration and continuous culture (Figure 3-1).
- 2) $\Delta\Delta A$ -ratio, defined as A-ratio of artemisinin treated iRBCs minus the A-ratio of control iRBCs ($A\text{-ratio}_{(iRBC + 1000nM \text{ artesunate})} - A\text{-ratio}_{(iRBC \text{ control})}$), was shown to be positive in resistant parasites and negative in sensitive

parasites during calibrations (Figure 3-4). However, $\Delta\Delta A$ -ratio failed in the blind test of multiple clinical isolates (Figure 3-5) due to the intrinsic unstable magnetic properties of intact RBCs.

- 3) A new sample preparation method was established to remove the background signals from RBCs, whereby the uninfected RBCs and host RBCs were lysed by saponin and the parasites remained intact (Figure 3-6C). By doing so, large amount parasites can be purified and concentrated into the small volume in the detection coil of MRR, which provided clearer and stronger signal for detection of malaria parasites.
- 4) Two approaches for MRR measurements after lysis (spinning and suspension) were investigated and compared to the previous MRR measurements (Figure 3-6D, E). Both methods were proven to have better sensitivity and higher reliability (Figure 3-7A, B). Compared to suspension method, the spinning method exhibited lower limit of detection (Figure 3-7C).
- 5) The potential of the spinning method in helping detect reduced artemisinin susceptibility was subsequently explored (Figure 3-8). However, no reliable difference can be established between resistant and sensitive parasites. The reason remains unclear but is likely linked to the remaining hemozoin from the dead parasites keeps contributing to the overall signal. The altered life cycle progression and drug induced dormancy in the resistant parasites may also contributed to the inconclusive results.

6.1.2 Future works:

The aim of this part is to explore the potential application of the novel MRR system on the detection of reduced artemisinin susceptibility in malaria *P. falciparum*. However, neither new parameter nor new sample preparation method help establish a consistent difference between resistant and sensitive parasites on the MRR system. Further improvement of the sensitivity of MRR in detecting the growth of live resistant parasites can be done by removing the huge number of dead parasites in the samples. This approach is under exploration with aid of microfluidic devices, in collaboration with Dr. Zeming Kwek and Dr. Smitha Thamarath Surendran from Singapore MIT alliance for research and technology (SMART) center.

6.2 Phosphorylation of eukaryotic initiation factor 2 α subunit (eIF2 α)

Previous transcriptomic evidence showed that the unfolded protein response (UPR) is linked to artemisinin resistance in Southeast Asia [76]. The phosphorylation of eukaryotic initiation factor 2 α subunit (eIF2 α), as a critical signaling event of UPR, was closely investigated.

6.2.1 Key findings:

- 1) The phosphorylation of eIF2 α was induced in DHA treated ring stage parasites (0-12 HPI) and persisted as the treatment continued (Figure 4-2).
- 2) The eIF2 α phosphorylation level of ring stage parasites after 2 hours drug treatment was found to be lower in two lab-generated resistant strains with different K13 mutations (I543T and C580Y) (Figure 4-3B, Figure 4-5B), five resistant clinical isolates which carries K13 C580Y mutation (Figure 4-3E, Figure 4-6A) and two lab-generated non-K13 mediated artemisinin resistant strains (Figure 4-7) compared with their corresponding sensitive references.
- 3) The level of total eIF2 α showed no difference between resistant and sensitive parasites as the life cycle progress nor after drug treatment in most strains tested (Figure 4-3C, D).
- 4) The level of phosphorylated eIF2 α after drug treatment was found to negatively correlated with the corresponding RSA survival rate (Figure 4-6A) and performed as good as RSA in distinguish moderately synchronized ring stage resistant and sensitive parasites (Figure 4-6B).

6.2.2 Future works:

To bring this newly identified phenotypic indicator closer to clinical application in early detection of K13 mediated as well as non-K13 mediated artemisinin resistance, short-term tasks are:

- 1) Instead of semi-quantitative western blot, establish the difference of phosphorylated eIF2 α between resistant and sensitive strains using quantitative methods like ELISA.
- 2) Test more non-K13 mediated artemisinin resistant strains to establish the relationship between the change of phosphorylated eIF2 α level and different resistant mechanisms.
- 3) Test recently adapted clinical isolates to further validate the correlation between the change of phosphorylated eIF2 α level and field artemisinin resistance.
- 4) Test under mimicked clinical settings like low parasitemia (0.1%), room temperature culture, culture with or without candle jar etc.

If a quantitative and rapid method for detecting phosphorylated eIF2 α is established, further clinical studies could be carried out as below:

- 1) Establish range of phosphorylated eIF2 α for wild-type parasites and artemisinin resistant parasites by testing known samples. Sample volume should be big enough to build up reliable confidential intervals.
- 2) Blindly test samples and compared with results from gold standard RSA and K13 mutation screen. Diagnostic randomized clinical trials could be

carried out at this stage. Patient samples should be tested with all three indicators. Meanwhile, since clearance half-life is considered as a primary clinical indicator, it should be measured post-treatment and served as one of the comparators in the trial. The treatment decision, however, should be carefully considered based on the severity of artemisinin resistance when the trial is undergoing. If adherence to ACTs in resistance cases has led to treatment failure and mortality, alternative anti-malarial treatments should be considered.

6.3 Level of DNA damage based on CometChip

Previously it has been shown that DNA damages was induced in malaria parasites by artesunate. To survive from drug treatment, resistant parasites must have suffered lower amount of DNA damages and/or repaired the damages more efficiently than the sensitive ones. With the aid of high throughput DNA damage detection platform, CometChip, artesunate induced DNA damage level in sensitive and resistant parasites were investigated.

6.3.1 Key findings:

- 1) High throughput CometChip was optimized to use in malaria parasites by combining it with SLO lysis method (for ring stage) or Percoll separation (for schizont stage) (Figure 5-2).
- 2) DNA damages induced by artesunate were detected by CometChip in both ring and schizont stage parasites (Figure 5-3).

- 3) No reliable difference in DNA damage level was observed between artesunate treated ring stage resistant and sensitive parasites (Figure 5-4A).
- 4) DNA damage level of artesunate treated schizont stage parasites measured by CometChip was found to be lower in multiple K13-mediated resistant and susceptible resistant-prone parasites (Figure 5-4B, Figure 5-5).
- 5) The DNA damage level tested in clinical isolates is well correlated with their reported clearance half-life, except for one outlier, the sensitive clinical isolate KH44 (Figure 5-6).
- 6) Considering KH44 as 'resistant' due to the low level of DNA damage it experienced upon treatment, 8 mutations in 7 DNA repair genes were identified to be different between 'resistant' and sensitive clinical isolates and defined as 'ResisDNArep genotype' (Table 5-1).
- 7) The 'ResisDNArep genotype' was found to be closely associated with K13 mutations in Southeast Asia, especially e-GWS (Figure 5-10), and may be an important part of the predisposing genetic background in Southeast Asia which contributed to the development of K13-mediated artemisinin resistance.

6.3.2 Future works:

This part established a high throughput platform for detection of DNA damages in malaria *P. falciparum* and identified phenotypic alterations and gene mutations associated to K13-mediated artemisinin resistance with the aid of the CometChip. Results suggested the existence of parasites, sensitive under current criteria, is resistant to drug-induced DNA damages. Early identification of those parasites may greatly facilitate the containment of drug resistance development.

To further understand the relationship between DNA damage level and artemisinin resistance and its development, potential future directions are:

- 1) Investigate the specific phenotypic alterations caused by the gene mutations in the 'ResisDNArep genotype', especially those closely associated K13 mutation and gene deletions identified in this study.
- 2) Explore the contribution of the 'ResisDNArep genotype' to the overall fitness or transmission capability of the parasites.
- 3) Extend the CometChip screening to recently adapted clinical isolates from different study sites. This phenotypic screening is important for understanding the prevalence of parasites resistance to drug-induced DNA damage in Southeast Asia and the link between phenotype and genotype.

6.4 Significances of the work

Figure 6-1 Comparison of *in vitro* culture time for different indicators. Time '0' refers to the time when ring stage parasites were taken from the peripheral blood of patients. Time of detection is when the indicator is available for detection method instead of time when the readout of detection result.

In this work, three perspectives were explored to identify early phenotypic indicators for reduced artemisinin susceptibility. Although a consistent and reliable difference between resistant and sensitive parasites cannot be established on MRR system yet, differences in the drug induced eIF2α phosphorylation level and DNA damage level were reliably detected. Both the eIF2α phosphorylation level and the DNA damage level were shown to be well correlated with the reported clinical clearance half-life and the gold standard RSA survival rate in multiple resistant and sensitive parasites (Figure 4-6, Figure 5-6). Compared with the gold standard RSA, these two indicators reduced much time for *in vitro* culture (Figure 6-1): the measurement of eIF2α phosphorylation level was taken after only 2 hours *in vitro* drug treatment of ring stage parasites and the CometChip took place only after 1-hour drug treatment of schizont stage parasites. With much reduced *in vitro* culture time, these two indicators were less susceptible to invalid test due to culture failure like in RSA and enabled potential earlier detection of artemisinin resistance. Moreover, CometChip may

detect not only artemisinin resistant but also resistant-prone parasites. The eIF2 α indicator can potentially detect non-k13 mediated artemisinin resistance. The extended detection spectrum of these indicators suggested their great potential in future clinical applications.

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