Investigation of azithromycin analogues and proteasome-like inhibitors as quick-killing antimalarials

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October 2019

A thesis submitted by

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Bachelor of Science (*Honours)*

to

The University of Adelaide,

For total fulfilment of the requirements for the degree

Doctor of Philosophy in Biological Sciences

in the Department of Molecular and Cellular Biology,

School of Biological Sciences.

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Abstract.

Malaria is caused by mosquito-borne parasites of the genus *Plasmodium* which were responsible for ~435,000 of deaths annually, with >90% caused by the deadliest species, *P. falciparum.* Over the last two decades, global implementation of vector control and artemisinin combination therapies have resulted in significant reductions in the global burden of malaria. Of current concern is the spread of multi-drug resistant parasites that have severely limited the efficacy of antimalarials, including front-line artemisinins, highlighting the urgent need to identify new antimalarials for use as treatments. The aim of this thesis was to investigate novel antimalarial development avenues and identify new chemotypes that could be used in the near future as treatments.

The macrolide antibiotic azithromycin is known to target the malaria parasites remnant plastid organelle (the apicoplast's) bacterial-like ribosome and causes slow-killing 'delayed death', where the parasite dies in the second replication cycle (4 days). Azithromycin has also been shown to inhibit invading merozoites and kill blood stages within the first replication cycle (2 days) via an unidentified mechanism, proposed to be independent of delayed death. Thus, we hypothesised that azithromycin could be redeveloped into an antimalarial with two different mechanisms of action against parasites: delayed death and quick-killing.

Over 100 azithromycin analogues that featured a high proportion of different structural profiles were obtained, leading to improved quick-killing activities over azithromycin. Quick-killing was also confirmed to be completely unrelated to delayed death, as blood stage parasites lacking the apicoplast were equally susceptible to quick-killing of azithromycin and analogues. Two different avenues were also confirmed for azithromycin's antimalarial re-development: delayed death and quick-killing or quick-killing only, which could be modulated depending on the location of added functional groups. Azithromycin and analogues were found to be active across blood stage development, with only short treatments required to kill parasites. The metabolomics signatures of parasites treated with azithromycin and analogues suggested that quick-killing acts multi-factorially, with the parasite's food vacuole and mitochondria being likely targets.

Finally, *in vitro* activities of two subtypes of tri-peptide proteasome-like inhibitors, vinyl sulfone and aldehydes, were addressed against *P. falciparum* and the zoonotic malaria parasite

P. knowlesi. All compounds exhibited low-nanomolar activities against both *Plasmodium* spp. and showed excellent selectivity for parasites over human cells, suggesting these inhibitors provide viable chemical scaffolds for optimisation. There was no evidence of increased protein ubiquitination upon treating parasites with these compounds, suggesting they do not target the proteasome. We also investigated whether hypoxia inducible pro-drug proteasome-like inhibitors could be used to reduce host toxicity of antimalarials. However, these pro-drugs could be not activated in *in vitro* culture conditions and there was limited evidence suggesting this strategy would be applicable in malaria.

These studies build on previous findings on the drug-killing efficacy, mechanism of action and possible application of redeveloping azithromycin analogues as new and improved antimalarials. I also identified new proteasome inhibitor-like scaffolds as starting points for further development. This body of work provides thorough biological characterisation of a panel of compounds that could lead to new avenues for antimalarial development.

Declaration.

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Amy Lee Burns

Date: 28th October 2019

Preface.

A statement of authorship declaring the contribution from each author has been included for the relevant Chapters written as manuscripts (Chapter 4 and Chapter 5) as well as the review that was published during my candidature (Appendix I). This preface declares the contributions received from others for other Chapters:

i) Figures used in Chapter 1, 3, 5 and Appendix 1 were generated with assistance from Mr. Juan Miguel Balbin (University of Adelaide, Research Centre for Infectious Diseases).

As Chapter 6 is written as a thesis chapter, a statement of contribution each author has also been included for this Chapter. Briefly, I would like to acknowledge contributions received from others for this chapter.

i) The proteasome-like inhibitors and proteasome pro-drugs used in Chapter 6 were synthesised in-house by Mr. Aniket Kulkarni and Prof. Andrew Abell (School of Physical Sciences, University of Adelaide, Australia). Mr. Aniket Kulkarni and Prof. Andrew Abell also advised on the structural activity relationship of these compounds.

ii) Tissue culture and cytotoxicity analysis of Huh-7D cells described in Chapter 6 were performed with assistance from Sonja Frölich (Research Centre for Infectious Diseases, University of Adelaide, Australia).

iii) Western blot and analysis described in Chapter 6 were performed with assistance from Xinli Liang (Research Centre for Infectious Diseases, University of Adelaide, Australia).

Acknowledgements.

First and foremost, I would like to give my most sincere thanks to Dr. Danny Wilson for your continuous guidance and support throughout my PhD. My journey over these years has been a roller-coaster, but there were far more ups than downs. I am especially grateful for your compassion, support and reassurance at times when I was feeling overwhelmed and during the difficult times, it made all the difference.

I would also like to extend my thanks to my secondary supervisors, Prof. James Paton, Prof. Geoff McFadden and Prof. James Beeson for all your guidance and support throughout my PhD. A very special thank you to Dr. Brad Sleebs and Dr. Dean Goodman for all of your advice and support you have provided over my candidature.

Special thank you to Ben Liffner. For the last few years, you have been a constant source of encouragement, passion and knowledge. I am extremely grateful to have worked alongside you in such a fantastic and fun environment during my PhD.

To Issy, Miguel, Sonja and Ornella. Thank you all so, so much for all your support and friendship. You guys have been like a second family and I am so grateful for all of the help and advice that you have provided over the last few years.

To all past and present members of the Paton Lab, especially Cathy, Kim, Shannon, Lauren and Erin. Thank you all so much for your support, friendship and company. It has been a pleasure to work with you and share a lab and it has been a wonderful experience working with all of you.

Dear Mum, Dad and Taylor, there is no possible way I could have finished my PhD without the three of you. Mum and Dad, you have both been so supportive of my ambitions and I am very fortunate to have such a loving family. Thank you for your patience and support during the difficult times I have endured over my PhD, I greatly appreciate you all being there. I love you dearly.

Publications.

Published manuscripts arising during this candidature:

Burns AL, Dans MG, Balbin JM, deKoning-Ward T, Gilson PR, Beeson JG, Boyle MJ, Wilson DW. Targeting malaria parasite invasion of red blood cells as an antimalarial strategy. FEMS Microbiol Rev. 2019.

Conference attendance and presentations.

Oral presentations:

Annual Australian Society of Parasitology (ASP) Conference. Adelaide, Australia. 2019. Burns, A.L., Liffner B.S., Harvey, R., Sleebs, B., Goodman, C.D., McFadden, G.I., Beeson G.B., Wilson, D.W. Retargeting the antibiotic azithromycin as a dual-modality antimalarial.

International Congress of Parasitology (ICOPA). Daegu, South Korea. 2018. Burns, A.L., Liffner B.S., Harvey, R., Sleebs, B., Goodman, C.D., McFadden, G.I., Beeson G.B., Wilson, D.W. Retargeting the antibiotic azithromycin as a dual-modality antimalarial.

South Austrian Australian Society of Parasitology: Parasites in Motion. 2018. Burns, A.L., Host cell invasion as an antimalarial target.

Malarial in Melbourne (MiM). Melbourne, Australia. 2017. Burns, A.L., Liffner B.S., Harvey, R., Sleebs, B., Goodman, C.D., McFadden, G.I., Beeson G.B., Wilson, D.W. Retargeting the antibiotic azithromycin as a dual-modality antimalarial.

University of Adelaide Post-Graduate Symposium: 3 Minute Thesis heats. 2017. Burns, A.L., Retargeting the antibiotic azithromycin as a dual-modality antimalarial.

Attendance:

Molecular Approaches to Malaria Conference (MAM). 2016.

Australian Society of Parasitology: Concepts in Parasitology. 2017.

Poster Presentations:

University of Adelaide Post-Graduate Symposium – Poster presentation. 2017. Burns, A.L., Retargeting the antibiotic azithromycin as a dual-modality antimalarial.

Prizes and awards.

15-minute oral presentation student award.

Annual Australian Society of Parasitology Conference. 2019. Adelaide, Australia.

Esther Bright Travel Award.

Soroptimist International Travel Grant ($1000) awarded to attend and present at the 14th International Congress of Parasitology (ICOPA). 2018.

Australian Postdoctoral Scholarship

Department of Industry, Innovation Science, Research and Tertiary Education, Australian Government. 2016.

Abbreviations.

ACT	Artemisinin combinational therapies
AMA	Apical membrane antigen
AsnEDA	Asparagine ethylenediamines
Al	Aldehyde
ATP	Adenosine triphosphate
AZ	Azithromycin
AZCQ	Azithromycin/chloroquine
azidoF	azido-phenylalanine
BBB	Blood brain barrier
Ca^{2+}	Calcium
Cbz	Carboxybenzyl
CC	Cytotoxicity Concertation
CHAPS	3-(cyclohexylamino)-1-propanesulfonic acid
$CHCl_3$	Chloroform
Cl^{+}	Mono-protonated chloroquine
Cl^{++}	Di-protonated chloroquine
cm^2	Centimetre squared
CO_2	Carbon dioxide
CSA	Chondroitin Sulfate A
CQ	Chloroquine
cytB	Cytochrome b, complex III
cytoD	Cytochalasin D
D	Aspartic acid (asp)
DHA	Dihydroartemisinin
DHFR	Dihydrofolate reductase
DHODH	Dihydroorotate dehydrogenase
DHPS	Dihydropteroate synthetase
DMAPP	Dimethylallyl diphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DTT	1, 4-dithiothreitol
E	Glutamic acid (glu)
EPO	Erythropoietin
erm	erythromycin ribosomal methylation
EtBr	Ethidium bromide
ETC	Electron Transport Chain
F	Phenylalanine (phe)
FASII	Type II fatty acid
FBS	Fetal Bovine Serum
FDA	US Food & Drug Administration
Fe^{3+}	$Iron^{3+}$
Fe-S	Sulphur-ion
FIC	Fractional Inhibitory Concentration
FIC_{50}	Fractional IC_{50}
FPs	Falcipains
FSC	Forward Scatter
g	Gram
GFP	Green Fluorescent Protein
GMS	Greater Mekong sub-region
GPI	Glycosylphosphatidylinositol
GSK	GlaxoSmithKline
H+	Hydrogen
HAPs	Hypoxia-activated pro-drugs
HB	Haemoglobin
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HESI	Electro-spray ionization source
hERG	human Ether-a-go-go-Related Gene
HIV	Human Immunodeficiency Virus (AIDS)
hrs/hr	Hour(s)
HPLC	High-Performance Liquid Chromatography
IC_{50}	50% Inhibitory Concentration

Abbreviations

IC_{90}	90% Inhibitory Concentration
IRS	Indoor Residual Sprays
IPP	Isoprenoid Pyrophosphate
IPT	Intermittent Preventive Treatment
IPTp	Intermittent Preventative Treatment for malaria in pregnancy
ITNs	Insecticide-treated bed nets
K^+	Potassium
KEGG	Kyoto Encyclopedia of Genes and Genomes
K13	*P. falciparum* kelch-13 propeller domain
L	Leucine (leu)
LC-MS	Liquid Chromatography-Mass Spectrometry
LLINs	Long lasting Insecticidal Nets
MACs	Magnet activated cell sorting
malERA	Malaria Eradication Agenda Drugs Consultative Group
MAPK	Mitogen-Activated Protein Kinase
MDGs	Millennium Development Goals
mef	Macrolide efflux pump
MEP/DOXP	MEP (2-C-methyl-d-erythritol 4-phosphate) / DOXP (1-deoxy-d-xylulose 5-phosphate), non-mevalonate pathway
MIC	Minimum Inhibitory Concentration
mins	Minutes
mL	Millilitres
MDA	Mass drug administration
MMV	Medicines for Malaria Venture
MQO	Malate quinone oxidoreductase
MSP	Merozoite surface protein
MVA	Mevalonate pathway
N_2	Nitrogen
Na^+	Sodium
$NaHCO_3$	Sodium bicarbonate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
O_2	Oxygen

OAA	Oxaloacetate
P	Peptide
PAM	Pregnancy associated malaria
PBQC	Pooled biological quality control
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-tween
PEP	Phosphoenolpyruvate
PEXEL	*Plasmodium* EXport ELement
PI	Proteasome inhibitor
PIPES	1,4-piperazinediethanesulfonic acid
PI_{PRO}	Proteasome inhibitor prodrug
Plm	Plasmepsins
PlmIII/HAP	Plasmpsin III / Histoaspartic protease
%	Percentage
pk	*Plasmodium knowlesi*
pf	*Plasmodium falciparum*
PfATP4	*Plasmodium falciparum* plasma membrane P-type cation translocating ATPase
PfCARL	*Plasmodium falciparum* cyclic amine resistance
PfCRT	*Plasmodium falciparum* Chloroquine Resistance Transporter
PfEMP1	*Plasmodium falciparum* Erythrocyte Membrane Protein 1
pfmdr1	*Plasmodium falciparum* multidrug resistant transporter 1
pfmrp	*Plasmodium falciparum* multidrug resistance-associated protein
pfnhe1	*Plasmodium falciparum* sodium/proton exchanger 1
PfPI4K	*Plasmodium falciparum* phosphatidylinositol-4-OH kinase
PfSPZ	*Plasmodium falciparum* sporozoite vaccine
PTEX	Parasite-derived translocon of exported proteins
Q	Glutamine (gln)
QC	Quality control
QT prolongation	Abnormal heart rhythms
R	Arginine (arg)
R(number)	Modification site
RBC	Red Blood Cell

Abbreviations

iRBCs	infected RBCs
uRBCs	uninfected RBCs
RBV	Recombinant Blood stage Vaccines
rcf	relative centrifugal force
RH	Reticulocyte-Binding Protein Homologue
RNA	Ribonucleic acid
rRNA	ribosomal RNA
tRNA	transfer RNA
rp1	ribosomal protein gene
RSA	Ring-stage Survival Assay
RSD	Relative Standard Deviation
S. Figure	Supplementary figure
S. Table	Supplementary table
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SERCaP	Single Encounter Radical Cure and Prophylaxis
SNPs	Single Nucleotide Polymorphisms
SP	Sulfadoxine–Pyrimethamine
SUB1	Serine protease 1
TCA	Tricarboxylic acid cycle
TCP	Target candidate profiles
TPP	Target product profiles
UPS	Ubiquitin-proteasome system
vs	Vinyl sulfone
v/v	volume/volume
W	Tryptophan (trp)
WH	Warhead group
WHO	World Health Organization
w/v	weight/volume
Y	Tyrosine (tyr)
α	Alpha
β	Beta

x	times or amino acid

Chapter 1. Introduction.

1.1 Background.

1.1.1 Global burden of malaria.

Malaria is a significant human disease that is caused by mosquito-borne apicomplexan parasites belonging to the genus *Plasmodium*. Currently, 87 tropical and sub-tropical countries and territories, are considered malaria endemic and almost half of the world's human population are potentially at risk from this disease (**Figure 1.1**). In 2017, the World Health Organization (WHO) estimated that ~219 million new cases of malaria infection occurred world-wide, resulting in ~435,000 deaths. Around 90% of malaria related deaths occurred in the sub-Saharan Africa region with children under 5 years of age accounting for ~61% of deaths (1). However, it is believed by many that the true burden of malaria morbidity and mortality may be substantially higher than the best available WHO estimates (2).

1.1.2 *Plasmodium* spp. parasites.

Six *Plasmodium* genus protozoan parasites are considered to be significant human pathogens. Four of these species; *Plasmodium falciparum, P. vivax, P. malariae, P. ovale* (comprised of two different subspecies; *P. ovale curtisi* and *P. ovale wallikeri* (3)), transmit between humans through the bite of an infected female *Anopheles* mosquito vector. In contrast, the other two malaria parasites, *P. knowlesi* and *P. simium*, are zoonotic and are transmitted to humans from a monkey host via the mosquito (4, 5). *P. falciparum* and *P. vivax* are the most prevalent *Plasmodium* species and cause the greatest burden of disease, with *P. falciparum* the causative agent of the majority of global malaria mortality (cause of >90% of deaths) (1, 2). As most people in endemic countries have limited access to health and treatment services, malaria can impose a heavy burden on individuals, households and entire regions (6) (reviewed in (7)). Moreover, it is estimated that this disease alone reduces the economic growth of highly endemic African counties by up to 1.3% annually (6) (reviewed in (8)).

1.2 Lifecycle of *Plasmodium* spp. parasites.

Plasmodium spp. parasites progress though a complex lifecycle that involves both a mosquito vector and human host (**Figure 1.2**).

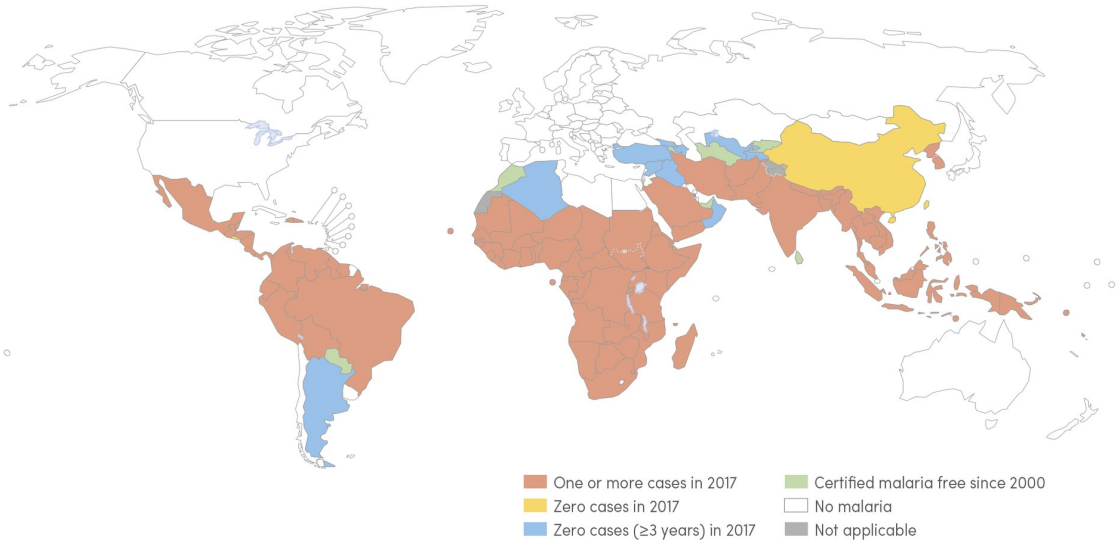

Figure 1.1 Status of malaria endemic countries and regions classified by the World Health Organisation.

Countries in red are classed as malaria endemic in 2017 (1 or more cases), while counties in yellow, China and El Salvador, were malaria endemic in 2016, but reported zero indigenous cases in 2017. Counties in blue have not reported indigenous cases of malaria for more than 3 years and counties in green have been declared malaria free since 2000. Figure from the World Health Organisation's 2018 World Malaria report (1).

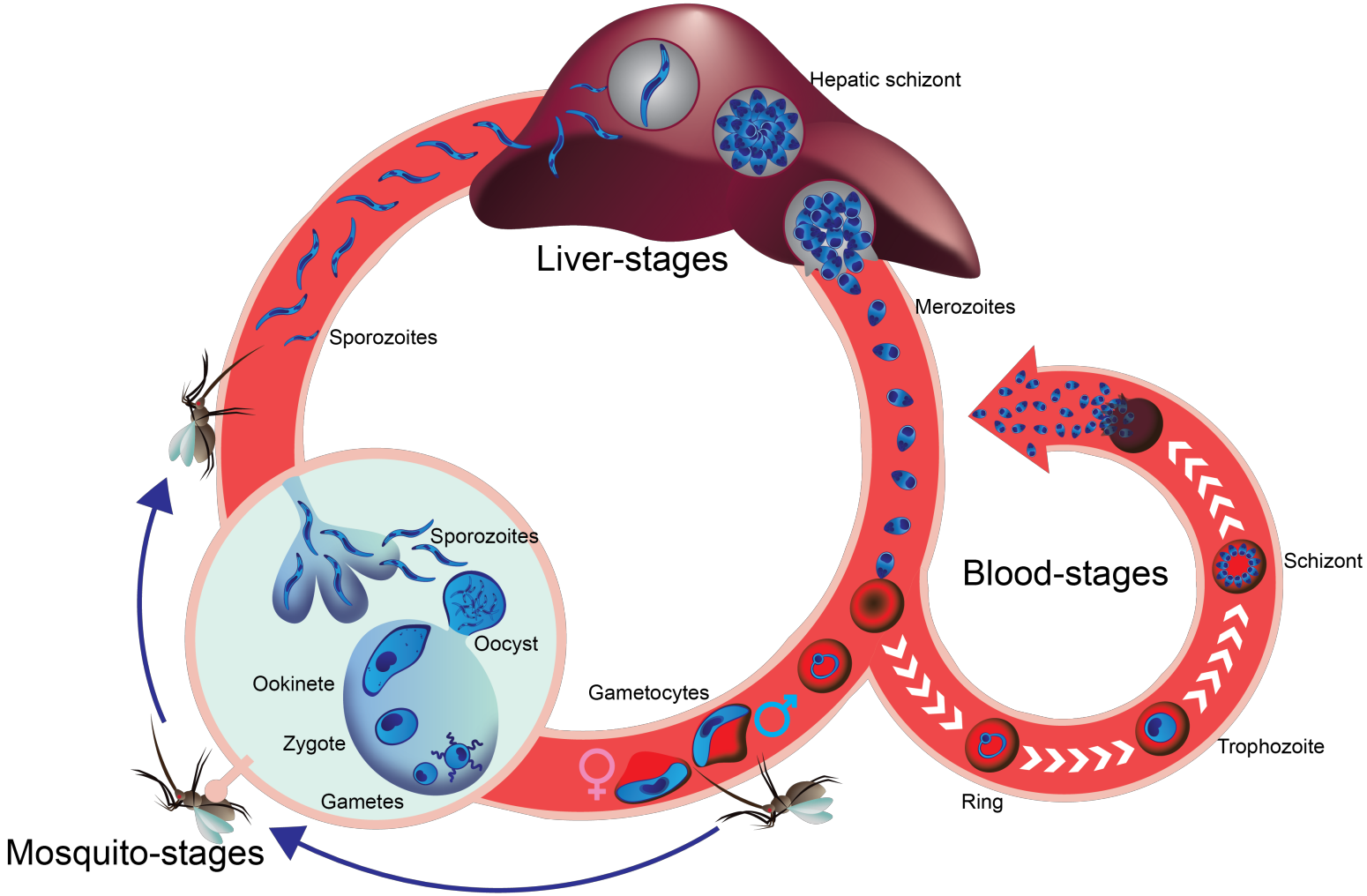
Hepatic schizont
Liver-stages
Merozoites
Sporozoites
Sporozoites
Oocyst
Ookinete
Zygote
Gametes
Gametocytes
Blood-stages
Schizont
Trophozoite
Ring
Mosquito-stages

Figure 1.2 Lifecycle of *Plasmodium.*

Plasmodium parasites are transmitted between hosts by infected female *Anopheles* mosquito vectors. Upon feeding of blood meal, motile sporozoites are released from the mosquito's salivary glands into the dermal capillary beds of a human (reviewed in (9)). The sporozoite migrates to the liver (1-3 hrs) and invades liver hepatocytes before asexually multiplying to form a hepatic schizont (9-16 days). *P. vivax* and *P. ovale* can also form dormant hypnozoites in the liver that can re-activate months or years later (10, 11). Mature hepatic schizonts release tens of thousands of daughter merozoites into the bloodstream which then initiate the asexual blood stages through invasion into the RBC (12, 13). During the blood stage lifecycle, the parasite progresses though ring and trophozoite stages before maturing into a schizont containing merozoites (14, 15). The schizont ruptures, releasing 16-32 daughter merozoites into the bloodstream to initiate the next cycle of replication after 24-72 hrs, depending on the *Plasmodium* species (4, 10, 11, 16, 17). During the blood stages, a subset of ring stage parasites (<1%) commit to sexual development into gametocytes and are ingested by the mosquito for transmission. Sexual reproduction takes place within the mosquito's mid-gut, where the male and female gametocytes fuse. The resulting zygote differentiates into an ookinete and traverses the mid-gut wall to form an oocyst on the outer mid-gut wall. The oocyst then asexually divides and releases thousands of sporozoites that travel to the mosquito's salivary glands where they await transmission to a new host (9, 18).

In humans, following the bite of an infected *Anopheles* mosquito, motile sporozoites are injected into the subcutaneous tissue and breach the endothelial barrier to enter a capillary before circulating to the liver where the parasites invade liver cells (hepatocytes) (reviewed in (9)). These liver-stages (pre-erythrocytic stages) then asexually multiply forming a hepatic schizont containing >10,000 daughter merozoites. Upon maturity, the hepatic schizont ruptures and releases merozoites into the bloodstream.

The asexual blood stage lifecycle (intraerythrocytic stages or blood stages) begins with the merozoite invading a host red blood cell (RBC) (**Figure 1.3**). Merozoite invasion of RBCs is a complex process involving a succession of co-ordinated interactions that occur in remarkably distinct phases (reviewed in (12) and (13)). i) Upon egress, the free merozoite travels through the bloodstream and establishes primary contact with an uninfected RBC. ii) The merozoite then re-orientates such that the apical tip contacts the RBC membrane. iii) Invasion ligands within specialised secretory organelles, the micronemes and rhoptries, situated at the merozoites's apical tip, are secreted and the tight (or moving) junction is established. iv) The RBC membrane is pulled around the invading merozoite via the action of an actin-myosin motor complex and a series of protease cleavage events cleave off merozoite surface proteins near the tight junction as invasion proceeds. v) The invasion pore fuses behind the merozoite's posterior end and a parasitophorous vacuole forms, in which the parasite will reside for the remainder of its blood stage lifecycle (~48 hours (hrs) for *P. falciparum,* reviewed in (12) and (19)) (**Figure 1.3**).

Following the completion of invasion, the merozoite flattens into a biconcave ring-shape, appearing thicker closer to the elongated nucleus (**Figure 1.2**). The parasite then begins to dynamically modify the host cell to obtain nutrients from the surrounding environment and avoid the host's immune response. In order to transport proteins though the parasitophorous vacuole to the RBC's cytoplasm and/or membrane, the parasite establishes a membranous-like structures within the RBC cytoplasm (Maurer's clefts) that act as platforms for exporting parasite proteins involved in virulence, host cell modification and trafficking (reviewed in (20)).

For *P. falciparum,* ~400 parasite proteins are trafficked into the RBC cytosol over the first 20 hrs of growth to establish, amongst other things, new permeability pathways for the accumulation of essential nutrients, export of metabolic waste out of the cell and modification of the ionic composition of the RBCs cytoplasm (reviewed in (21) and (22)).

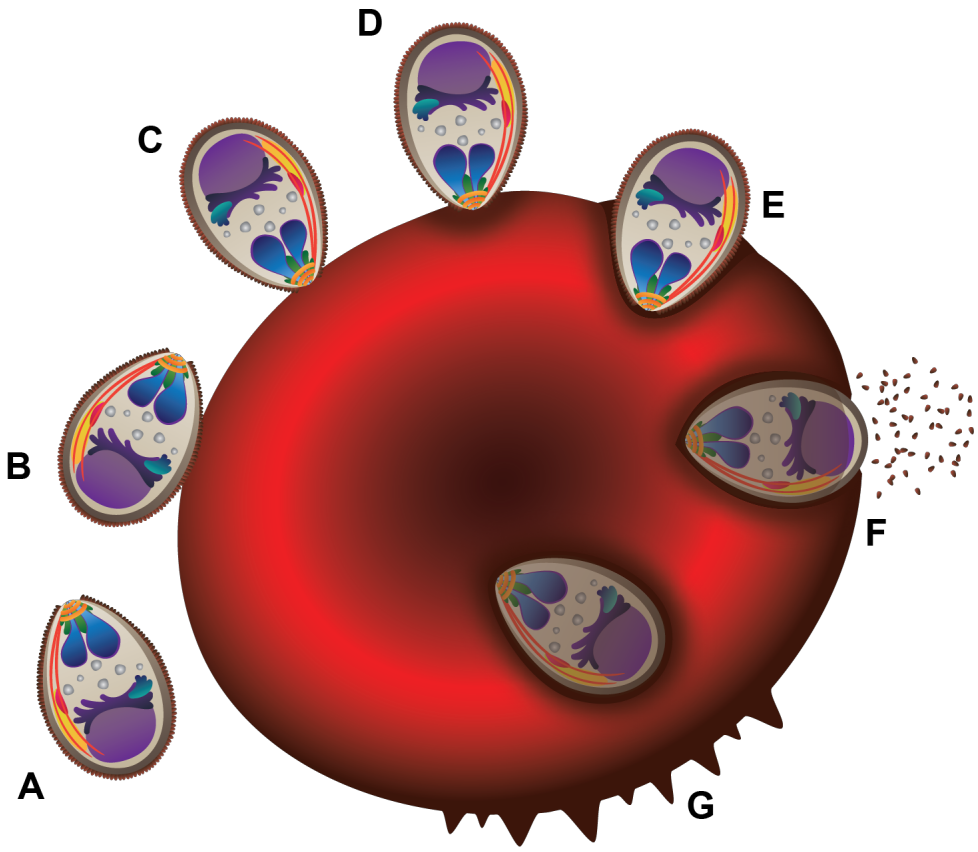

Figure 1.3 Merozoite invasion of the RBC.

A) Free merozoites in the bloodstream post schizont egress. **B**) Initial attachment between the merozoite and host receptors that requires low affinity interactions. **C**) The merozoite re-orientates such that the apical tip attaches to the RBC surface and invasion ligands are secreted from specialised invasion organelles (micronemes and rhoptries). **D**) Interactions between merozoite and RBC ligands establishes an irreversible tight junction. **E**) The merozoite enters the RBC by the action of the actin-myosin motor complex. **F**) Merozoite surface proteins coating the merozoites surface are shed and the invasion pore fuses to form a parasitophorous vacuole. **G**) A portion of the RBC membrane temporally deforms (echinocytosis), believed to be the consequence of rhoptry or calcium flux from the parasite (23, 24). Adapted from (19) (Appendix 1).

In addition to acquiring external nutrients, the parasite also obtains free amino acids by digesting large amounts of haemoglobin from the RBCs cytoplasm within an acidic food vacuole. Exactly how the food vacuole forms remains unclear (25-27). Although initial haemoglobin uptake and catabolism is proposed to occur in early-ring stages (25, 27), the full formation of this organelle is only observed in mid-ring stage parasites (~12-18 hrs post invasion) (27, 28). As haemoglobin digestion liberates chemically reactive free-haem by-products (α-haematin) (29), the parasite neutralises this toxicity by sequestering two (or more) free haem molecules together (chemically identical to β-haematin) and transforms it into an inert hemozoin crystal (reviewed in (30)).

As the parasite grows, the ring stage parasite increases in density and develops into a trophozoite (~20 hrs post-invasion) (reviewed in (14, 15)). The trophozoite stage of *P. falciparum* undergoes rapid growth, with the bulk of haemoglobin digestion also occurring during this life stage (31), and begins to sequester to the microvasculature to avoid splenic clearance. Sequestration is mediated by parasite adhesion proteins, such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) of the *var* multigene family (32) (reviewed in (20) and (33)). Briefly, PfEMP1 is exported through Maurer's clefts to the RBC surface membrane, where it forms knob-like protrusions to mediate the adherence of infected RBCs to the walls of blood vessels as well as adherence between uninfected RBCs (rosetting) (reviewed in (33)). After this period of growth, the parasite (40-48 hrs post-invasion) undergoes a phase of intense deoxyribonucleic acid (DNA) replication and multiple nuclear divisions, in addition to producing an assembly of proteins, organelles and molecules required for invasion (34, 35) (reviewed in (15) and (14)). Segregation of these components results in the generation of 16-32 daughter merozoites that separate into distinct compartments within a schizont. Upon maturity, the schizont ruptures both the parasitophorous vacuole and RBC membranes, thus releasing daughter merozoites into the bloodstream to initiate the next cycle of replication (48 hrs post-invasion) (reviewed in (14) and (17)).

A low proportion (<1%) of blood stage parasites travel into the bone marrow (36, 37) and develop into gametocytes (sexual stages) that are taken up by an *Anopheles* mosquito during a blood meal (reviewed in (38)). Gametocytes can be either male or female, with these gametes fusing within the mosquito's mid-gut and forming a diploid zygote (reviewed in (9) and (18)). This zygote undergoes meiosis and transforms into a motile ookinete that traverses the mosquitoes' mid-gut to form an oocyst on the outer mid-gut wall. The oocyst then asexually divides to form a sporoblast, which, upon maturity, releases motile sporozoites that travel to

the mosquito's salivary glands that await the mosquitoes next blood meal to transmit to a new human host.

The lifecycle described here refers specifically to *P. falciparum*, with the other human malaria parasites differing in the length of the blood stage lifecycle and disease burden (section 1.2). *P. vivax* (42-48 hrs) (10), *P. ovale* (~50 hrs) (11) and *P. malariae* (~72 hrs) (16) infections are typically less life threatening compared to *P. falciparum*, attributed to lower parasitemia within the host. *P. vivax* and *P. ovale,* however, form dormant, long-lasting 'hypnozoites' in the liver that re-activate and cause clinical relapse months or years later (11, 39). In contrast, *P. knowlesi,* a zoonotic malaria parasite that normally infects macaque monkeys (4), replicates daily (24 hrs) which can result in high parasitemia and rapid progression into severe malaria (reviewed in (40)).

As all malaria pathology is caused by repeated cycles of replication and growth of blood stage parasites, it is during this stage that clinical symptoms manifest and infection is diagnosed. Indeed, the majority of antimalarial drugs target the blood stages of the malaria parasite's lifecycle in order to clear and treat the disease. Thus, the parasite's blood stages are of great interest as potential drug targets and are the primary focus of this thesis.

1.3 Clinical disease of *P. falciparum.*

Malaria infection can result in a broad spectrum of uncomplicated and severe pathologies. Clinical symptoms of uncomplicated malaria present as a mild, non-specific, irregular febrile-like illness. After a few days the patient may experience periodic episodes of fever that alternates with symptom-free intervals associated with synchronised schizont rupture within the host (reviewed in (41) and (42)). Uncomplicated malaria will often resolve over time, however, sterile immunity is rarely achieved and it is not uncommon for clinically immune adults to be asymptomatic carriers of malaria parasites (43, 44).

Approximately 1-2% (~2,000,000) of *P. falciparum* malaria infections progress into severe disease (complicated malaria) annually (1, 45). Presentations of severe malaria encompasses a range of distinct, but overlapping clinical symptoms including; fever, anaemia, metabolic acidosis, cerebral dysfunction (e.g coma), and pregnancy complications with a high rate of death occurring in severe disease cases if left untreated. A summary of the severe clinical

manifestations and pathogenesis are described below, with further information available in several comprehensive reviews (reviewed in (17, 46) and (47)).

1.3.1 Severe anaemia.

Severe malaria anaemia is caused by the direct and indirect destruction of infected and uninfected RBCs and contributes to an estimated 2.6-10.3% of paediatric inpatient mortality in developing countries (48) (reviewed in (49)). The pathogenesis of severe malarial anaemia is poorly understood, however, and it is proposed that multifactorial combinations of both parasite and host factors contribute to this pathology. Whilst infected RBCs are destroyed via schizont rupture, another major contributor to severe malaria anaemia is the loss of unparasitised RBCs, hypothesised to be a consequence of mis-targeted immune responses (reviewed in (50)). Indeed, estimates from clinical studies suggest that ~8 uninfected RBCs are lost from circulation for every parasitised RBC cleared (51). Studies also suggest that transient suppression or modulation of erythropoietin (EPO) synthesis, an essential growth factor for erythropoiesis, inhibits the proliferation of erythroid precursors and contributes to malarial anaemia (52-54).

1.3.2 Metabolic acidosis and respiratory distress.

Metabolic acidosis and respiratory distress are important indicators of poor patient survival outcomes (55-57). Whilst much of the pathophysiology of metabolic acidosis is unknown, the accumulation of lactic acid within the host's blood and tissues, caused by an increase of anaerobic metabolism, is believed to be a major contributing factor (58). In severe malaria, sequestration of infected and uninfected RBCs within capillaries is thought to impair oxygen delivery to organs, leading to host cell anaerobic glycolysis and lactic acid accumulation (59-61). Lactic acid build up may cause an accumulation of hydrogen (H^+) ions within the host, resulting in a decrease of blood pH (blood acidosis), impairment of oxygen transfer and increasing anaerobic respiration, thus exacerbating the patient's condition (62-64).

1.3.3 Cerebral malaria.

Cerebral malaria is one of the most severe clinical manifestations of *P. falciparum* infection with mortality ranging between 15-25% in affected individuals even with antimalarial treatment (56, 65, 66). Cerebral malaria is characterised by altered consciousness, seizures and the eventual progression into coma and, if left untreated, is almost always fatal with patient death typically occurring within 48 hrs after admission (reviewed in (67) and (68)). Cerebral

malaria is most commonly seen in young children in malaria endemic regions of Africa (2 to 4 years of age), but this pathology has also been observed in young adults in South-East Asia and non-immune individuals (67), suggesting immunity to cerebral malaria develops through repeated exposure to *P. falciparum* (69, 70) (reviewed in (71)).

Cerebral malaria is caused by adherence and sequestration of infected RBCs to the cerebral endothelial cells and microvascular tissues within the brain. Post-mortem studies commonly show sequestered parasites, blocked blood flow within cerebral venules, inflammation of adjacent tissues and disruptions of the blood brain barrier (BBB) in severe cerebral malaria (72-74). It is likely that this pathology contributes to increased anaerobic metabolism and oxidative stress in brain tissue, which is further exacerbated with breakdown of the BBB (75, 76). Children that survive cerebral malaria often exhibit rapid recovery of consciousness, however, these individuals are at an increased risk of long-term neurological and cognitive impairments (77-79).

While here I have focussed on the consequence of parasite sequestration in the brain, *P. falciparum* parasites also sequester in the microvasculature of other organs in infected individuals, including in the placenta during malaria in pregnancy (section 1.3.4) (reviewed in (80) and (81)). Therefore, parasite sequestration is postulated to be a major contributor to malaria pathology throughout the infected individual.

1.3.4 Pregnancy associated malaria.

Pregnancy associated malaria (PAM), defined as either peripheral or placental infection by *Plasmodium* parasites during pregnancy, is a major public health concern with >125 million pregnancies at risk each year (81). PAM arises from sequestration of *P. falciparum* parasitised RBCs within the vascular tissues of the placenta and subsequent infiltration of immune cells that are recruited to fight off infection in the placenta. Accumulation of parasitised RBCs in the placenta is thought to disrupt the exchange of oxygen, nutrients and antibodies to the developing foetus (reviewed in (80) and (82)). PAM results in complications for both mother and baby, including maternal anaemia, low birth weight, premature delivery, spontaneous abortion, intrauterine growth-retardation and still birth delivery if infection remains untreated (83, 84). The risk of morbidity and mortality for mother and baby is most prevalent during the first and second pregnancies, with this risk diminishing with subsequent pregnancies due to the development of maternal immunity (reviewed in (85) and (83)). *P. falciparum* parasites cytoadhering to the placenta display a distinct repertoire of surface proteins that enable

sequestration throughout the maternal vascular spaces (intervillous spaces). Adhesion of infected RBCs to placenta microvascular tissues is proposed to occur via attachment of infected RBCs to ligands such as chondroitin sulfate A (CSA) and hyaluronic acid (86, 87). Notably, a specific PfEMP1 variant (VAR2CSA) has been implicated in binding to CSA and appears to be the key antigen required for parasite sequestration within the placental intervillous spaces (88, 89). With repeated pregnancy and exposure to malaria, women develop specific antibodies against the PfEMP1 VAR2CSA variant that provides protection from disease and improved outcomes for both mother and baby (90-92).

1.4 Malaria control strategies.

In the year 2000, a reduction in the burden of malaria was highlighted as key public health priorities by three of the eight millennium development goals (MDGs) for human health set by the United Nation's Millennium Declaration; these being Goal 4 (reduce child mortality), Goal 5 (improve maternal health) and Goal 6 (combat human immunodeficiency virus (HIV) /AIDS, malaria and other diseases). Between 2000 and 2015, there was a 37% decrease in malaria incidence and 58% decrease in malaria mortality, thereby achieving Goal 4 of the MDG (93, 94). This reduction in global malaria mortality was likely the result of increased international funding that lead to widespread role out of mosquito vector control measures and antimalarial treatments within endemic regions. Under the current guidelines, the WHO aims to eliminate malaria from at least 35 countries as well as reduce malaria incidence and mortality by 90% by the year 2030 (1, 95). In recent years, however, the number of estimated malaria infections and deaths has stabilised (1). This has been attributed to the spread of drug resistant malaria parasites and insecticide resistant mosquito populations in combination with slowing penetration of these control measures into endemic areas (1, 94, 96). An effective vaccine remains a major priority for malaria control efforts that could contribute to further reductions in disease burden, but this has not yet been achieved to date.

1.4.1 Current status for malaria vaccine development.

Vaccine development for malaria has been a key target of research as successful immunisation of people in malaria endemic areas has the potential to reduce the burden and transmission of malaria parasites. However, there is no available vaccine that provides adequate protection against any *Plasmodium* spp.

Over the last 30 years, vaccine development has focused on a number of malaria sub-unit vaccines with the most advanced being RTS,S (Mosquirix), a pre-erythrocytic vaccine comprising the epitopes of *P. falciparum* circumsporozoite protein. Phase III trials of RTS,S conferred partial protection against clinical malaria for 'at risk' age groups, with ~20.3% protection observed in infants (6-12 weeks) and ~35.2% in young children (5-17 months) (97, 98). Concerningly, RTS,S failed to protect children from severe malaria (cerebral malaria, meningitis) (97, 99) and vaccine-induced protection was observed to rapidly decline in these at-risk age-groups (97, 100). RTS,S was approved for use in 2015 (97, 101) and is now undergoing Phase IV implementation trials, however, due to its low efficacy it is likely that a second-generation malaria vaccine will be required.

An alternate strategy that targets the disease-causing parasites are recombinant blood stage vaccines (RBV) that consist of immunogenic antigens from the merozoite's surface or infected RBC membranes (reviewed in (102)). To date, several promising candidates targeting *P. falciparum* merozoite surface antigens have been assessed in Phase I and II human trials (reviewed in (103)), however, the majority demonstrated limited efficacy and failed to progress into Phase III clinical trials. Currently, the major challenges of RBVs are the extensive polymorphisms of the exposed blood stage antigens and insufficient activation of protective immunity in humans (reviewed in (102, 104) and (103)).

Another promising development avenue are live attenuated vaccines consisting of whole blood stage, merozoite or sporozoite stage parasites that are attenuated either genetically (105), chemically (106, 107) or via radiation (108). These vaccines have the added benefit of exposing the entire parasite and a large array of antigens to elicit high levels of protective immunity (reviewed in (109)). The current leading candidate is the radiation-attenuated *P. falciparum* sporozoite vaccine (PfSPZ) that demonstrated 90% protection against blood stage malaria in naïve individuals (110) (reviewed in (111)). PfSPZ is difficult to manufacture, however, requiring *in vivo* extraction and purification of sporozoites from the salivary glands of infected mosquitos and cryopreservation of the vaccine for transport (reviewed in (112)).

Given that an effective malaria vaccine is yet to be developed, vector control and antimalarial drugs will remain the most important arms of malaria control and treatment in the near future.

1.4.2 Mosquito vector control.

Since the year 2000, the global roll-out of insecticide-treated nets (ITNs), long-lasting insecticide-treated nets (LLINS) and/or different classes of indoor residual sprays (IRS) have

contributed to substantial reductions in malaria morbidity and mortality within endemic communities and are currently recommended by the WHO (1, 113). ITNS and IRS at household levels are effective controls of the malaria vector and, when combined with antimalarial drug treatments and adequate health services, remain a successful and cost-effective strategy for reducing the burden of malaria at community levels (1, 114). However, despite almost a decade of global 'roll-out' campaigns, no malaria endemic country has reached the minimum target for universal coverage (80% of households owning one ITN) and only ~61% of the at-risk populations (women and young children) utilise an ITN (1). Resistance to pyrethroids, the only class of insecticides licensed for use on ITNs, as well as other classes of insecticides has spread within malaria endemic regions, limiting the effectiveness of this control strategy (115-117) (reviewed in (118)).

1.5 The past and present of antimalarials.

Vector control remains a key strategy for preventing malaria and the development of a highly effective vaccine is desirable, but treatment of malaria disease still requires access to effective antimalarials to reduce sickness and death (1). The majority of clinically used antimalarials aim to provide fast and effective clearance of disease-causing blood stage parasites from the host, thus relieving disease symptoms and potentially reducing transmission by inhibiting gametocyte development (119). Antimalarials are also used as chemoprophylaxis to protect against infection in travellers (120), intermittent preventive therapies (IPT) to prevent infection in infants and pregnant women (121, 122) and for mass drug administration (123), leading to parasite clearance in endemic communities (124).

As all clinical symptoms of malaria are caused by repeated cycles of growth, replication and lysis of RBCs by parasites, the vast majority of antimalarials have been developed to target the blood stage lifecycle (125-131). However, drug resistance has developed to all clinically used antimalarials that have received widespread use and there are growing concerns that the recent emergence of resistance to frontline artemisinin combination therapies (ACTs) will increase global malaria burdens (132, 133) (reviewed (134)). Given the focus of this thesis on developing new antimalarials with novel mechanisms of action, I will explore the origin, proposed target/mechanism of action and known resistance mechanism(s) of several past and present antimalarials developed for clinical use.

1.5.1 Quinine.

Quinine, a component from the bark of the cinchona (quina-quina) tree, has been one of the most effective treatments for malaria since the isolation of the active agent in 1820 (**Figure 1.4**) (135). Despite the drug's side effects, including 'blackwater fever', where the patients urine darkens and they feel increasingly unwell with treatment, quinine remained a mainstay of malaria treatments until the 1920s when other synthetic 4-aminoquinoline drugs were synthesised and became readily available (43) (reviewed in (135) & (136)).

Quinine, like other 4-aminoquinolines, are highly active against trophozoite and schizont stages of the parasite's blood stage lifecycle (**Figure 1.5**). Although quinine's mechanism of action remains poorly understood, a number of single nucleotide polymorphism (SNPs) mutations identified in various membrane transporters including; the *P. falciparum* chloroquine resistance transporter (*pfcrt*), *P. falciparum* multidrug resistant transporter 1 (*pfmdr1*), *P. falciparum* sodium/proton exchanger 1 (*pfnhe1*) and *P. falciparum* multidrug resistance-associated protein (*pfmrp*), have been shown to influence the parasite's sensitivity to quinine and other aminoquinolines (137-141). Given that a number of these mutations are associated with transporters within the parasite's digestive vacuole, 4-aminoquinolines have been theorised to interfere with haematin detoxification and the crystallization of hemozoin, resulting in the accumulation of toxic haem products within the parasite (142-145). Due to the prevalence of resistance and side-effects, quinine is rarely used as a front-line antimalarial, although this drug is still utilised in some regions for treatment of severe malaria when other antimalarials are unavailable (146, 147). Current WHO guidelines recommend a combination of quinine with an antibiotic, such as doxycycline or clindamycin (section 1.8), as a second-line treatment (146, 147) (reviewed in (135)).

1.5.1 Chloroquine.

Chloroquine, a synthetic 4-aminoquinoline, was introduced in the mid-1940s and quickly became the most extensively used antimalarial (**Figure 1.4**). In 1955, chloroquine was the main antimalarial used for presumed malaria infection during the WHO's Malaria Eradication Program (reviewed in (136)). However, the Malaria Eradication Program was, in part, hampered by chloroquine resistant *P. falciparum* parasites, which first emerged in South-East Asia in the late 1950s and had spread to Africa by the late-1970s. Due to the lack of an effective, low-cost, alternative treatment, global malaria morbidity and mortality spiked, especially among young children in Africa (132, 148).

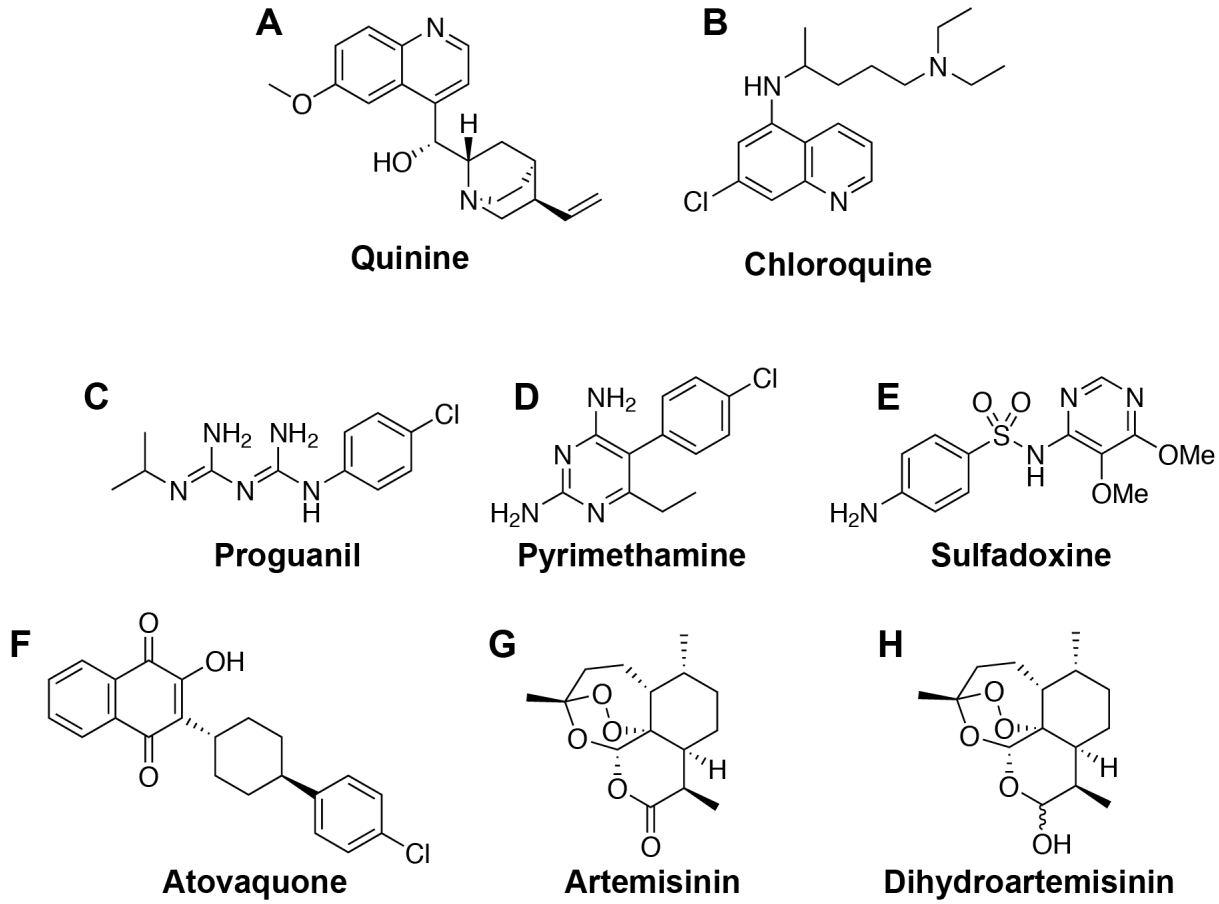

Figure 1.4 Chemical structures of clinically used antimalarials.

4-aminoqunolines **A**) quinine (section 1.5.1) and **B**) chloroquine (section 1.5.2). Folate pathway inhibitors (section 1.5.3) **C**) proguanil, **D**) pyrimethamine and **E**) sulfadoxine. **F**) Atovaquone (section 1.5.4), a structural analogue of ubiquinone. The endoperoxide antimalarial **G**) artemisinin and its semisynthetic derivate, **H**) dihydroartemisinin (section 1.5.5).

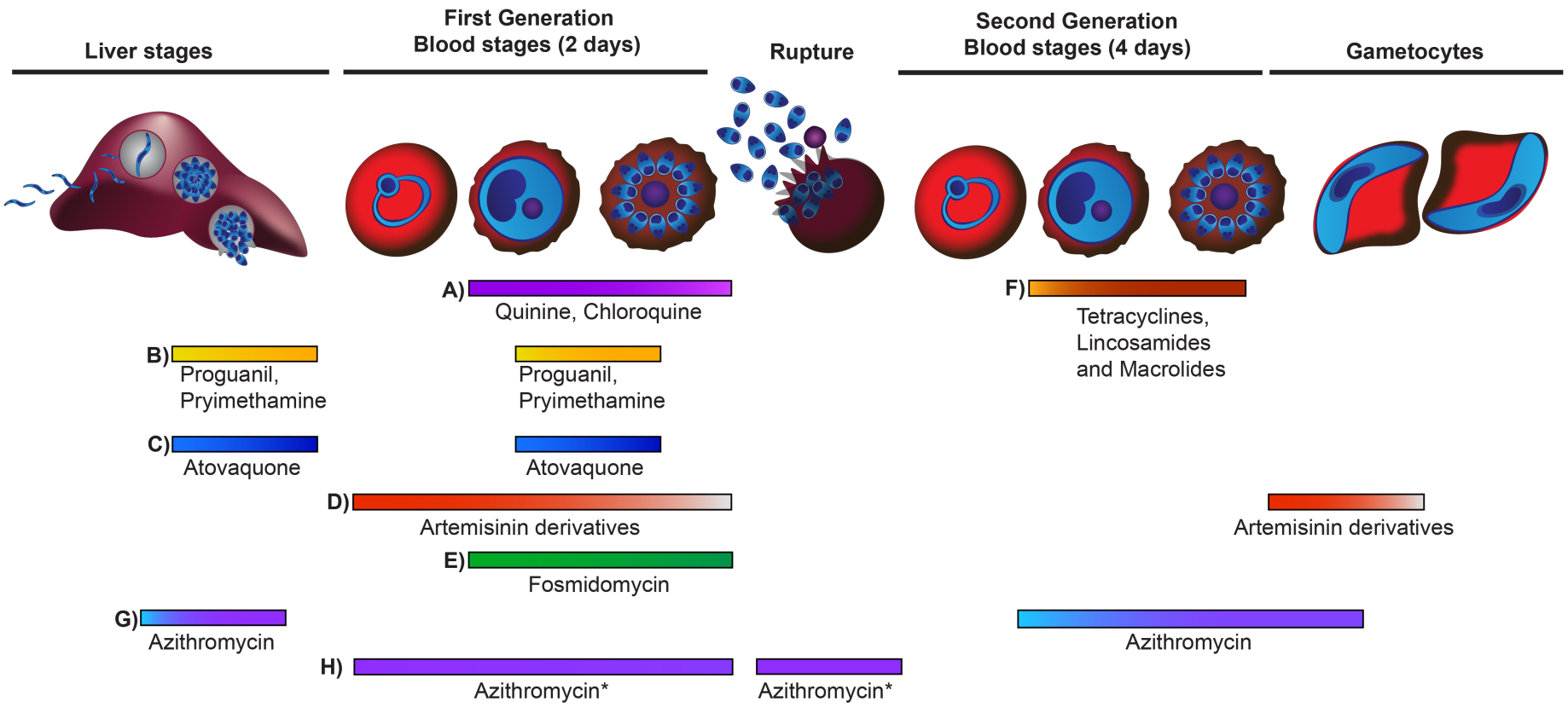
Liver stages
First Generation
Blood stages (2 days)
Rupture
Second Generation
Blood stages (4 days)
Gametocytes
A)
Quinine, Chloroquine
F)
Tetracyclines,
Lincosamides
and Macrolides
B)
Proguanil,
Pryimethamine
Proguanil,
Pryimethamine
C)
Atovaquone
Atovaquone
D)
Artemisinin derivatives
Artemisinin derivatives
E)
Fosmidomycin
G)
Azithromycin
Azithromycin
H)
Azithromycin*
Azithromycin*

Figure 1.5 Stage specificity and timing of antimalarial activity for clinically tested drugs.

The stage specificity of clinically tested antimalarials against *P. falciparum* liver, first- (2 days, ~48 hrs) and second-generation (4 days, ~96 hrs) blood stage parasites and developing gametocytes.

A) 4-aminoquinolines, quinine and chloroquine, target the haeme-detoxication pathway (145, 149) and are active against the trophozoite and schizont blood stage parasites (131, 150, 151). **B**) Antifolates, proguanil and pyrimethamine, target the pyrimidine synthesis pathway and inhibit rapidly replicating liver and blood schizont stage parasites (127, 152). **C**) Atovaquone inhibits the electron transport chain and kills replicating liver and blood schizont stage parasites by inhibiting pyrimidine synthesis (131, 153). **D**) Artemisinin derivatives are highly active against ring stages and inhibit the mature blood stages as well as early stage gametocytes (131, 154, 155). **E**) The antibiotic fosmidomycin is an inhibitor of the isoprenoid biosynthesis pathway and kills first-generation blood stage parasites (156-158). **F**) Tetracycline, lincosamides and macrolide antibiotics that target the apicoplast's bacterial-like ribosome cause 'delayed death' of second-generation progeny parasites due to exhaustion of isoprenoids (130, 157, 159-161). **G**) The antibiotic, azithromycin, is known to have apicoplast-targeted delayed death activity and has been shown to inhibit liver development and transmission of gametocytes to mosquitoes (162-164). **H**) Azithromycin's* proposed quick-killing activity that encompass both inhibition of short-term and first-generation blood stage growth as well as merozoite invasion of the RBC (130, 164, 165).

Similar to quinine, chloroquine's mechanism of action is hypothesised to be related to the haematin detoxification pathway (149) (**Figure 1.5**). During the blood stage lifecycle, the parasite consumes haemoglobin from the RBC within the food vacuole and digests it using proteases into free amino acids, with toxic haem released as a waste product (149, 166). Chloroquine is believed to inhibit haem-detoxification by binding to haem (167, 168) and/or the growing hemozoin crystal(s) (145, 166, 169), thus resulting in the accumulation of toxic haem. As elevated levels of free haem cause cellular damage including protein oxidation, damage or lysis of membranes and/or inhibition of proteases, chloroquine treatment results in parasite death (170, 171) (reviewed in (172)).

Chloroquine-resistance is conferred by SNPs in *pfcrt,* a transporter located within the membrane of the food vacuole that is proposed to efflux chloroquine out of the food vacuole and limit interactions between chloroquine and haem (125, 173). Parasite resistance to chloroquine was widespread by the 1990s resulting in this antimalarial being rarely used for treatment of *P. falciparum* malaria. The WHO does currently recommend chloroquine as a treatment for *P. vivax* malaria (147). However, chloroquine-resistant *P. vivax* parasites were first identified in the late 1980s and are now present throughout many endemic regions, further limiting the utility of this antimalarial for treatment of clinical disease (174, 175).

1.5.2 Antifolates.

1.5.2.1 Proguanil.

Proguanil was developed in the mid-1940s and was one of the first clinically used dihydrofolate-reductase (DHFR) inhibitors for treatment of malaria (**Figure 1.4**). DHFR is an essential enzyme that recycles folates in successive steps for synthesis of nucleic acids including; thymidylate, purines, and methionine. When used alone, proguanil is metabolised within the liver by cytochrome P450 (CYP2C19) into cycloguanil, the active metabolite that disrupts folate synthesis and parasite replication (**Figure 1.5**)(129, 176). Loss of parasite sensitivity to proguanil, however, emerged shortly after clinical use, with mutations within *P. falciparum*'s *dhfr* gene later identified to confer resistance (134, 177, 178). Currently, proguanil is almost exclusively used in combination with atovaquone (under the trade name Malarone) as a first-line prophylaxis for travellers (section 1.5.4).

1.5.2.2 Pyrimethamine.

Pyrimethamine was developed shortly after proguanil in the early 1950s (**Figure 1.4**). Like proguanil, pyrimethamine targets DHFR and interferes with the folate biosynthesis pathway,

causing the arrest of nucleic acid synthesis and the death of replicating late-blood (schizonts) and liver stage (hepatic schizonts) parasites (127, 152) (**Figure 1.5)**. Pyrimethamine has been used in combination with sulfadoxine, a competitive inhibitor of the dihydropteroate synthetase enzyme (DHPS), which is required for a different step in the folate synthesis pathway (179). Consequently, combinations of sulfadoxine-pyrimethamine are synergistic and can also be used in combination with other antimalarials such as chloroquine or artemisinin. However, sulfadoxine-pyrimethamine resistant *P. falciparum* parasites first emerged on the Thailand-Cambodian border in the late-1970s and are now prevalent throughout most malaria endemic regions (180-182). Resistance to sulfadoxine-pyrimethamine is conferred by a sequential series of SNPs within the *dhfr* and *dhps* genes that cause increasing loss of parasite sensitivity to drug treatments (152, 183, 184). Sulfadoxine-pyrimethamine is currently the only antimalarial recommended by the WHO for intermittent preventive treatment of malaria during pregnancy (IPTp) in sub-Saharan Africa, however, the effectiveness of this treatment appears to be declining due to the prevalence of resistance (reviewed in (182)).

1.5.3 Atovaquone.

Atovaquone (566C80), a hydroxy-1,4-naphthoquinone, is a structural analogue of ubiquinone (co-enzyme Q10), a mitochondrial coenzyme directly involved in electron transport (**Figure 1.4**). Thus, atovaquone acts competitively with ubiquinone for the binding of the bc_1 complex (cytochrome b, *cytB*, complex III) within the parasite's mitochondrial electron transport chain (ETC), causing the collapse of membrane potential, loss of mitochondrial function and inhibition of ubiquinone regeneration. Whilst blood stage parasites rely on glycolysis for synthesis of adenosine triphosphate (ATP) (185), maintenance of the mitochondrial ETC is essential for re-oxidation and function of inner-membrane dehydrogenases, including dihydroorotate dehydrogenase, an essential enzyme involved in the pyrimidine biosynthesis pathway (reviewed in (186) and (153)). Thus, collapse of mitochondrial membrane potential and lack of regenerated ubiquinone prevents dihydroorotate dehydrogenase function, resulting in the death of blood stage parasites (reviewed in (153, 187) and (188)) (**Figure 1.5**).

Atovaquone was licenced for treatment of malaria in the mid-1990s, but when used as a monotherapy resistance was observed shortly after clinical evaluation (189). Specific mutations in the *P. falciparum's cytB* gene (in particular Y268S/C/N) (190, 191) have been shown to result in high levels of atovaquone resistance, with parasites tolerant up to a 1000-fold higher concentration of the drug (189). Interestingly, these drug resistant *cytB* mutations have been identified to be lethal to mosquito stages which fail to complete development in rodent models

of malaria (192). Due to the maternal inheritance of the mitochondria, *cytB* mutation(s) are hypothesised to be unable to spread within a population after sexual reproduction in the mosquito vector, thus opening the possibility that atovaquone acts as a 'resistance proofed' drug (192).

Atovaquone is used in combination with proguanil, which works synergistically and increases activity of atovaquone >1000-fold, thereby reducing the impact of resistance mutations. While the mechanism of synergy between atovaquone-proguanil remains elusive, synergism is known to be independent of proguanil's active metabolite (cycloguanil) DHFR inhibitory activity (section 1.5.3) (193, 194). Based on these observations, proguanil is hypothesised to have a secondary mechanism of action that possibly involves the ATP synthase (complex V) in the parasite's mitochondrial ETC, thus sensitising the parasite to atovaquone (187, 195-197). Currently, the atovaquone-proguanil combination, Malarone, is recommended by the WHO as a front-line prophylaxis, as both drugs have activity against liver stages (198, 199), and as a treatment for uncomplicated malaria in travellers or when effective front-line artemisinin combination therapies are not available (147).

1.5.4 Artemisinin and artemisinin combination therapies.

Artemisinin was discovered in the early 1970s and was isolated from the Chinese medicinal herb, *Artemisia annua* (qinghaosu, sweet wormwood), which has a long history of use as a traditional Chinese medicine (200). Due to the low solubility of artemisinin, other semi-synthetic derivatives (artemether, artesunate, arteether, dihydroartemisinin) with improved pharmacological properties are currently used for treatment of malaria (201) (**Figure 1.4)**. Artemisinins are active against early stage gametocytes (transmission stage), mature blood stages, with their potent activity against ring stages being of particular benefit for rapid parasite clearance and fast relief of clinical symptoms (154, 155) (reviewed in (201)) (**Figure 1.5**).

Artemisinins were initially introduced as monotherapies for treatment of uncomplicated malaria. However, a 3-day regime of artemisinin monotherapy regularly resulted in treatment failure and parasite recrudescence (within ~5 days) that was attributed to the drugs short half-life (~1-4 hrs) (202, 203). Therefore, current front-line artemisinin treatments combine artemisinins with a longer-lasting partner in artemisinin combinational therapies (ACTs) (reviewed in (204, 205) and (206)). The rationale of ACTs is that the combination of the fast-acting artemisinin derivative will quickly clear symptoms and parasitemia, after which the slower acting partner drug 'mops up' the remaining parasites, thereby preventing recrudescence and the development of resistance. ACTs were introduced in the mid-1990s in

South-East Asia, when resistance to other antimalarials, such as chloroquine and sulfadoxine-pyrimethamine, had spread through most malaria endemic areas (132, 207-210). In 2005, the WHO recommended ACTs as the front-line treatment for all malaria endemic countries with this move, in combination with ITNs, contributing to the substantial reduction in global malaria morbidity and mortality since the turn of the century (211, 212).

Artemisinin (and its derivates) act as prodrugs and activation appears to involve iron-catalysed scission of the endoperoxide bridge (dihydroartemisinin, DHA) (reviewed in (213) & (214)). While the exact nature of artemisinin's mechanism of action remains somewhat contested, artemisinin induced death has been associated with widespread oxidative damage, promiscuous alkylation, inhibition of protein folding and activity of the parasite's proteasome (215-218).

Currently, worldwide clinical treatment of malaria relies on the efficacy of ACTs. However, after less than a decade of use there was evidence of delayed parasite clearance and treatment failures indicating rising drug resistance emerging from the Greater Mekong sub-region (GMS) in South-East Asia (219-223). SNPs in the *P. falciparum* kelch-13 propeller domain (K13-propeller, *pfk13* gene (PF3D7_1343700)) were found to be associated with artemisinin resistance both *in vitro* and *in vivo* (224, 225). There are several high frequency mutations (C580Y, R539T, Y493H and F4461) correlating with loss of sensitivity to artemisinins and extended parasite clearance times that are typically used as major surveillance tools (225-227). To date, K13-mutations and artemisinin resistance has been identified throughout Southeast-Asia, Eastern-India, and recently Papua New Guinea (224, 225, 228, 229). Thus far, non-synonymous K13-SNPs have been identified in Africa, but were not associated with delayed parasite clearance as the parasites appeared to retain sensitivity to artemisinin (227). Concerns remain that the spread or emergence of artemisinin resistant parasites in Africa would prove disastrous for malaria control efforts and could result in a surge of malaria related morbidity and mortality (133).

1.6 The push for new antimalarials.

While a significant reduction in the global burden of malaria has been observed over the last two decades, in recent years the incidence of malaria disease and deaths has stabilised (1). The emergence of parasites resistant to artemisinins and partner drugs, lack of an effective vaccine and mosquito resistance to insecticides, remain major challenges for the future of malaria

control. Thus, there is a significant need to develop improved control measures in order to reach the next Millennium Development Goal in 2030 (section 1.4) and new antimalarials will play a critical role in future malaria control and eradication strategies. At the frontier of antimalarial development are not-for-profit organisations such as Medicines for Malaria Venture (MMV), the Bill and Melinda Gates foundation and the Malaria Eradication Agenda (malERA) Drugs Consultative Group that aim to develop new antimalarial drugs for future malaria treatments.

In an effort to guide development of new antimalarials, MMV has provided a framework of therapeutic goals that define the life stages to be targeted (target candidate profiles (TCP)) and the final drug product that could be administered (target product profiles (TPP)) (230, 231).

The current TCPs that define the drug properties of most interest for development are:

TCP-1: molecules that clear asexual blood stage parasitaemia.

TCP-3: activity against *Plasmodium* hypnozoites (*P. vivax* and *P. ovale*).

TCP-4: activity against hepatic schizonts.

TCP-5: transmission blocking molecules that target the gametocyte stages.

TCP-6: transmission blocking molecules that targeting the mosquito (endectocides).

(Note: TCP-2 has been retired in the most recent MMV update (230, 231).)

The current TPPs that define the drug products of most interest for development are:

TPP-1 a drug product that controls the case management of clinical symptoms of malaria. Ideally, for uncomplicated malaria this will be a drug combination that is active against blood (TCP-1), transmission stages (TCP-5 and/or TCP-6) and liver stages (TCP-3 and/or TCP-4). For severe malaria, a fast-acting TCP-1 is appropriate.

TPP-2 drugs ideally act as chemoprotection for travellers or during malaria epidemics and will likely include a TCP-4 drug (liver stage) to prevent infection and potentially blood stage activity to prevent establishment of disease-causing blood stage parasites (TCP-1).

The ideal drug is envisaged as a Single Encounter Radical Cure and Prophylaxis (SERCaP) that aims to remove both blood stage and liver stages from the patient, thus acting as radical cure and preventing new infections by blocking transmission.

All new antimalarials should also feature novel modes of action that do not exhibit cross-resistance to current drugs and as single dose drug cures (reviewed in (231) and (230)). Multi-stage activity for a single drug could be particularly advantageous if the inhibitor features favourable pharmacological properties, such as a long half-life and safety profile, in addition to being synergistic with a second antimalarial. Given the success observed with ACTs as well as combination drug therapies used for other pathogens (e.g., tuberculosis and HIV) (reviewed in (232) and (233))), drug development and treatment of malaria going forward will likely consist of combination therapies.

The sections below provide some examples of drugs at an advanced stage of development as antimalarials, several of which are being developed in partnership with MMV (234) (reviewed in (235) and (236)). Given the number of drugs being developed, I provide here only a snapshot of new drugs that have reached pre-clinical (translational to human trials) or clinical (human trials to access) development, with further examples available in published literature reviews (reviewed in (235-237) and (238)).

1.6.1 Antimalarials in clinical development.

1.6.1.1 Endoperoxides inhibitors.

The discovery of artemisinin, featuring a bio-active endoperoxide bridge, has since yielded a class of fully synthetic peroxide antimalarials known as ozonides (reviewed (214)). Ozonides, like artemisinins, target throughout the blood stage lifecycle, inhibit the early stages of gametocyte development (TCP-1 and TCP-5) (**Figure 1.6**), feature a prolonged *in vivo* half-life and maintain activity against artemisinin resistant parasites (239) (reviewed in (240)). Like artemisinin, activation of ozonides requires scission of the endoperoxide bridge, which is likely dependent on a free iron source in the form of haem liberated during parasite digestion of haemoglobin. While the exact mechanism of action of these chemotypes has not been fully elucidated, ozonides likely induce multi-factorial damage to parasites similar to that seen for artemisinin (reviewed in (240) & (214)).

Currently, ozonides are one of the most advanced antimalarials being developed with the synthetic ozonide, Aretfenomel (OZ439), an optimised analogue of Arterolane (OZ277), having progressed into Phase IIb clinical trials (241, 242)

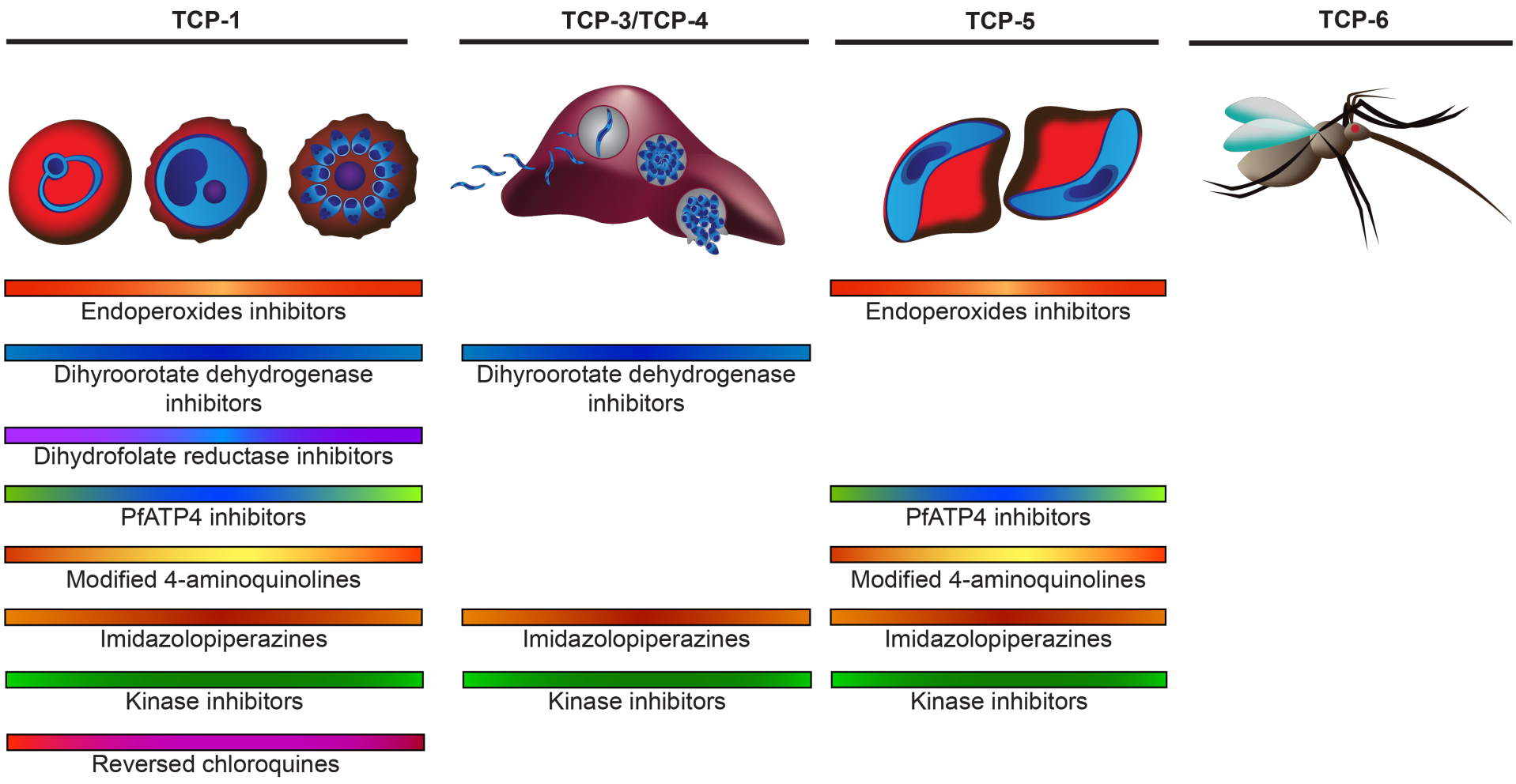
TCP-1
TCP-3/TCP-4
TCP-5
TCP-6
Endoperoxides inhibitors
Dihyroorotate dehydrogenase inhibitors
Dihydrofolate reductase inhibitors
PfATP4 inhibitors
Modified 4-aminoquinolines
Imidazolopiperazines
Kinase inhibitors
Reversed chloroquines
Dihyroorotate dehydrogenase inhibitors
Imidazolopiperazines
Kinase inhibitors
Endoperoxides inhibitors
PfATP4 inhibitors
Modified 4-aminoquinolines
Imidazolopiperazines
Kinase inhibitors

Figure 1.6 Target candidate profiles of antimalarials in development.

MMV's framework of target candidate profiles (TCPs) for antimalarials in clinical and pre-clinical development covered in this thesis (230, 231). TCPs are described by MMV as follows: TCP-1 molecules clear asexual blood stage parasitaemia.TCP-3 drugs have activity against *Plasmodium* hypnozoites (*P. vivax* and *P. ovale*). TCP-4 drugs have activity against hepatic schizonts. TCP-5 are transmission blocking molecules that target gametocyte stages. TCP-6: transmission blocking molecules that target the mosquito (endectocides). Note: TCP-2 has been retired (230, 231).

Endoperoxides (ozonides) target the blood stages and early gametocyte development (TCP-1 and TCP-5) (section 1.6.1.1) (239). The DHODH inhibitor, DSM-265, targets liver and blood stage parasites (TCP-1 and TCP-4) (section 1.6.1.2) (243). The *Pf*DHFR inhibitor, P218, is active against *P. falciparum* blood stages (TCP-1) (section 1.6.1.3) (244, 245). PfATP4 inhibitors, such as cipargamin, target *P. falciparum* blood stages *in vivo* and transmission stages *in vitro* (TCP-1 and TCP-5) (section 1.6.1.4) (246, 247). The modified 4-aminoquinoline, AQ-13, demonstrates potent activity against blood and transmission stage parasites (TCP-1 and TCP-5) (section 1.6.1.5) (248). The imidazolopiperazine, KAF-156, is active against blood, liver and gametocyte stages (TCP-1, TCP-4 and TCP-5) (section 1.6.1.6) (249). The *Plasmodium* kinase inhibitor, MMV390048, has shown activity against blood, liver and transmission stages (TCP-1, TCP-4 and TCP-5) (section 1.6.1.7) (reviewed in (250)). Reversed chloroquines (and other chloroquine analogues) demonstrate activity against asexual blood stage parasites (TCP-1) (section 1.6.2.1) (251).

The pre-clinical evaluation of OZ439 showed blood schizonticide activity against *P. falciparum* and *P. vivax* malaria, *in vivo* curative and prophylactic activity (252) as well as a longer half-life (46-62 hrs) in humans compared to artemisinin (1-4 hrs) (253) (reviewed in (240)).

1.6.1.2 Dihydroorotate dehydrogenase inhibitors.

As covered previously, a number of clinically used antimalarials directly or indirectly kill parasites by inhibiting pyrimidine biosynthesis including pyrimethamine and proguanil (cycloguanil) through targeting DHFR, and atovaquone through effects on dihydroorotate dehydrogenase (DHODH) (reviewed in (186, 187) and (153)).

DSM-265 is the first *Plasmodium* DHODH specific inhibitor to reach clinical development and demonstrates activity against both liver and blood stages (TCP-1 and TCP-4) (**Figure 1.6**), activity against multi-drug resistant parasites, an excellent safety profile, low drug clearance rate and long half-life in humans (243, 254, 255). DSM-265 has completed Phase IIa clinical trials in Peru, with a single dose clearing *P falciparum* parasites, although it was less efficacious against *P. vivax* (243, 256). However, recrudescent *P. falciparum* isolates were observed in clinical trials, with SNPs in the *dhodh* gene found in recrudescent parasites associated with *in vitro* resistance to DSM-265, suggesting a low barrier of resistance selection (256).

1.6.1.3 Dihydrofolate reductase inhibitors.

The second-generation *Pf*DHFR inhibitor, P218, was designed using rational drug design based on co-crystal structures of both inhibitors and substrates for human and *Plasmodium* DHFRs (244). P218, an optimised 2,4-diaminopyrimidine, binds deep within the active site of wildtype and resistant *Pf*DHFRs and overcomes resistance by interacting with conserved amino acid residues within the dihydrofolate binding site. P218 exhibits minimal affinity for human DHFRs, shows nanomolar *in vitro* potency against wildtype (50% Inhibitory Concentration (IC_{50}), 4.6 nanomolar (nM)) and quad-DHFR resistant (IC_{50}, 56 nM) *P. falciparum* blood stage parasites as well as a promising *in vivo* oral efficacy against *P. chabaudi* and *P. falciparum* humanised mice models (TCP-1) (**Figure 1.6**) (244, 245). Currently, P218 has completed Phase I clinical trials (257) and has been proposed as potential replacement of pyrimethamine in combination prophylaxis.

1.6.1.4 ATP4 inhibitors.

The plasma membrane P-type cation translocating ATPase (PfATP4), a sodium (Na^+) efflux pump located on the parasite's plasma membrane, is essential for malaria parasite survival through maintenance of a low cytosolic Na^+ concentrations. There are a growing number of chemotypes that have been identified to target PfATP4, including several compounds in MMV'S Malaria Box (258) (section 1.7.1), spiroindolones, pyrazoles and dihydroisoquinolones (reviewed in (259)) (260). PfATP4 inhibitors are hypothesised to cause accumulation of Na^+ ions within the intracellular parasite, resulting in NPP dependent osmotic swelling and eventual lysis of the infected RBC (260, 261).

Spiroindolones, a novel class of antimalarials identified almost a decade ago in a high-throughput phenotypic screen, remain one of the most advanced classes of PfATP4 inhibitors in development (261, 262). Thus far, the leading spiroindolone analogue, cipargamin (KAE609, NITD609), has shown promising activity against both blood and transmission stages *in vitro* (TCP-1 and TCP-5) (**Figure 1.6**) as well as *in vivo* efficacy for treatment of uncomplicated *P. falciparum* and *P. vivax* malaria (246, 247). Cipargamin is undergoing Phase IIb clinical trials for treatment of uncomplicated malaria in Africa (263). Thus far, resistance to PfATP4 inhibitors (including Cipargamin) has been acquired *in vitro* and are the result of SNPs mutations in the *pfatp4* gene (264). Resistance was shown to develop much slower in *in vivo* murine models, attributed to a high fitness cost from mutations in *pfatp4* (265).

1.6.1.5 Modified 4-aminoquinolines.

Despite the emergence of chloroquine resistant parasites, a number of 4-aminoquinolines have been developed since the mid-1940s that retain activity against chloroquine resistant parasites (266, 267). The current leading 4-aminoquinoline, AQ-13, is a derivative of chloroquine that differs in the amine-side chain and exhibits increased potency against chloroquine resistant lines (268). Like chloroquine and other 4-aminoquinolines, AQ-13's mechanism of action is proposed to be through inhibition of hemozoin biosynthesis (248, 269) (sections 1.5.1 and 1.5.2). AQ-13 shows nanomolar activity against both blood and gametocyte stages (TCP-1 and TCP-5) (**Figure 1.6**) (248) and favourable pharmacokinetic profiles including good oral bioavailability and half-life (3.9 days) (269, 270). At the end of 2017, AQ-13 completed a Phase II clinical trial in Malawi, where it was shown to have equivalent efficacy to the combination of artemether/lumefantrine for treatment of uncomplicated *P falciparum* malaria (270).

1.6.1.6 Imidazolopiperazines.

Imidazolopiperazines are a novel class of antimalarials that were identified though high-throughput screens and feature potent activity against both blood and liver stage parasites (271, 272). Thus far, imidazolopiperazines mechanism of action against parasites is unknown, however, SNPs within the *P. falciparum* cyclic amine resistance locus (PfCARL, PlasmoDB ID: PFC0970w), encoding an uncharacterised protein with seven predicted transmembrane regions, has been associated with drug resistance to this class of compounds *in vitro* (273).

The leading clinical imidazolopiperazine candidate, KAF-156, was optimised based on potent blood, liver and gametocyte stage activity (TCP-1, TCP-4 and TCP-5) (**Figure 1.6**) as well as its favourable pharmacokinetic and safety profiles (249). In humans, KAF-156 rapidly cleared clinical infections of *P falciparum* and *P. vivax.* Although clearance rates were slightly slower than artemisinin, the drug maintained efficacy against artemisinin resistant parasites, showed no major side effects and featured a long half-life (~44 hrs) (274). Currently, KAF-156 is undergoing Phase IIb clinical trials as a combination partner drug with lumefantrine (275).

1.6.1.7 Kinase inhibitors.

Protein kinases are essential in all eukaryotes and effect basic cellular functions through signalling pathways (276). Kinases are regulated by the phosphorylation of key amino acids or the binding of an activator or inhibitor protein, which initiates a conformational change to the kinases' active site, making it accessible to ATP (reviewed in (277) and (278)). An active kinase will then phosphorylate a target protein and ultimately change the target's activity, stability or cellular localisation, thus triggering further signalling events (279) (reviewed in (280)). *Plasmodium* kinases are functionally and structurally divergent from vertebrate hosts and are involved in essential signalling pathways throughout the parasite's lifecycle, making them attractive drug targets (281) (reviewed in (282)).

Several compounds have been identified to inhibit *Plasmodium* kinases, many of which exhibit low micromolar to nanomolar *in vitro* activities against intracellular blood stages (reviewed in (283) and (250)). Among these, the *P. falciparum* phosphatidylinositol-4-OH kinase (PfPI4K), a lipid kinase required for protein trafficking, has been of considerable interest in antimalarial development (284). Thus far, the PfPI4K inhibitor, MMV390048 (inhibitor 37), has demonstrated efficacy against blood, liver and transmission stages (TCP-1, TCP-4 and TCP-5) (**Figure 1.6)**, although it did not show efficacy against *P. vivax* hypnozoites. MMV39004 has

recently progressed into Phase IIa clinical trials in Ethiopia to address the drug's efficacy against *P. falciparum* and *P. vivax* human infections (285, 286) (reviewed in (250)).

1.6.2 Antimalarials in pre-clinical development.

1.6.2.1 Reversed chloroquine drugs.

Reversed chloroquine drugs are hybrids that contain a chloroquine-like moiety linked to a reversal agent, a molecule known to inhibit or circumvent the activity of *Pf*CRT (287, 288). While the mechanism of action of reversed chloroquines has not been completely elucidated, the rationale behind these drug hybrids is that the reversal agent will interfere with PfCRT's ability to efflux chloroquine from the digestive vacuole, thus allowing the chloroquine moiety to inhibit hemozoin formation and cause the build-up of toxic haem (289).

The current lead reversed chloroquine compound, DM1157 (compound 22) (289), has shown low nanomolar potency against both chloroquine sensitive and resistant asexual blood stage parasites (TCP-1) (**Figure 1.6**). Further, DM1157 maintained efficacy against multi-drug resistant field isolates of *P. falciparum* and *P. vivax* from Indonesia, where it showed superior activity in contrast to chloroquine (~3-fold more potent) (251). DM1157 has also demonstrated efficacy against *P. chabaudi*, a rodent malaria model, with no obvious signs of toxicity and is now undergoing Phase I clinical trials for further evaluation of its safety and pharmacokinetic properties in humans (290).

1.7 Emerging antimalarial strategies.

While there has been a dramatic increase of new compounds entering the drug development pipeline over the last decade, it is important to note that the development of next-generation antimalarials is challenging and progression is typically slow. Moreover, even if a lead compound shows excellent efficacy and possesses multiple TCPs it may not reach mainstream clinical use as a result of other challenges, such as emergence of resistance, issues with clinical tolerance, toxicity and/or developmental costs (230, 231). Therefore, a constant pipeline of drug discovery as well as new and novel antimalarials is essential. The sections below provide some examples of drug strategies and/or novel targets that are in the early stages of antimalarial development. Several comprehensive reviews containing further detail on emerging drug development strategies are listed for review (reviewed in (291-294) and (19) (Appendix 1)).

1.7.1 The Malaria Box.

In efforts to accelerate research and discover new drug candidates, MMV publicly released an open-access drug library, termed 'the Malaria Box'. The Malaria Box originated from >6 million compounds that were screened against the blood stages of *P. falciparum* at two pharmaceutical companies (GlaxoSmithKline (GSK) (295) and Novartis (272)) and two academic research centres (St. Jude, Research Hospital, Memphis (296) and Eskitis Institute for Drug Discovery, Griffith University, Australia (297)). These studies yielded >20,000 compounds with low to sub-micromolar activity. From this, ~400 different chemotypes were chosen to make up the Malaria Box based on their favourable drug-like properties, potent antimalarial activity and chemical diversity, with the drug library made freely available to the research community (298, 299)

The activity of the Malaria Box compounds have been tested against liver stages of *P. berghei* (272), transmission stages (300) and the asexual blood stages of a number of *P. falciparum* lines (299). A total of eight Malaria Box compounds have been shown to inhibit all stages of the parasite's lifecycle; blood, liver and/or transmission stages, (reviewed in (299)). To date, the mechanism of action for ~130 (~34%) of the Malaria Box compounds has been proposed. Notably, 28 of the 400 compounds were found to inhibit PfATP4 (258). A further 10 were identified to target the haem detoxification pathway, similar to that of quinine and chloroquine (301). Other targets include, kinesin-5 (302), thioredoxin reductase (303), aminopeptidases (304), DHFR (305), deoxyhypusine hydroxylase (306), inhibitors of isoprenoid biosynthesis (307-309), merozoite invasion of the RBC (310) and autophagy (311, 312) (reviewed in (299)).

Since its publication, the Malaria Box has been extensively studied by >200 research groups with these compounds evaluated against many diseases including other single-cell parasites, fungi, bacteria, helminths, and human cancer cells (299). Surprisingly, a number of Malaria Box compounds showed good efficacy against a number of these pathogens, opening up other avenues for drug development. Following this, MMV released a second open-access compound library, named the Pathogen-Box, which features 376 compounds that target a range of neglected topical diseases and other pathogens. Collectively, the Malaria Box has provided an invaluable starting point for drug development for malaria, as well as other infectious diseases, and has illuminated novel targets, biological processes and chemotypes that are essential for various stages of the parasite's life cycle.

1.7.2 Proteases as antimalarial targets.

Proteases are a highly abundant family of catalytic enzymes present across all domains of life that are primarily involved in protein turnover and regulation of essential biological roles including apoptosis (313), cell cycle progression (314) and cell migration (315). All proteases function by hydrolysing a specific peptide bond of a target protein and are classified on the basis of their preferred catalytic residue with aspartic, cystine, glutamic, asparagine, serine, threonine and metallo proteases constituting the main families (316, 317). Importantly, proteases are validated drug targets with a number of inhibitors used for clinical treatment of both human diseases and infectious pathogens including cancer, diabetes, cardiac disease, HIV and hepatitis C (318-320). Given the dramatic morphological changes that occur throughout *Plasmodium's* lifecycle, proteases play essential roles in regulating various pathways required for parasite viability. Of the ~170 predicted *Plasmodium* proteases, however, few have been extensively characterised or validated as drug targets (reviewed in (321)). The sections below provide an overview of some examples of proteases relevant to this thesis that are in the early stages of antimalarial development. Several comprehensive reviews containing further detail on *Plasmodium* proteases are listed (reviewed in (321-324) and (325)).

1.7.2.1 Falcipains.

Plasmodium falcipains (FPs) are a subgroup of papain-family (Clan CA, family C1) of cysteine proteases that are primarily involved in the degradation of RBC haemoglobin. *P. falciparum* contains four FP homologues, FP-1, FP-2, FP-2' and FP-3, that have attracted considerable interest in antimalarial development (reviewed in (326) and (327)).

The best characterised falcipains, FP-2, FP-2' and FP-3, are known to be essential for haemoglobin catabolism and reside within the parasite's food vacuole (reviewed in (328)). During the blood stage lifecycle, the parasite transports RBC haemoglobin into the acidic food vacuole, where it is hydrolysed and processed by multiple catalytic classes, including cystine, aspartic and metallo proteases, into haem and free amino acids required for growth. Falcipains also indirectly contribute to haemoglobin catabolism by processing and activating aspartic proteases, specifically Plasmepsin I and II (329) (section 1.7.2.2). Indeed, treating parasites with cysteine protease inhibitors, such as E-64 (330), causes enlargement of the food vacuole and accumulation of native haemoglobin, suggesting that falcipains are essential for the initial cleavage of RBC haemoglobin (331) (reviewed in (332) and (328)). Efforts in targeting FPs have involved two major approaches: i) synthetic peptide mimetics and ii) non-peptide small molecular inhibitors (reviewed in (326, 328) and (327)), with various chemotypes showing

nanomolar activity against blood stage *P. falciparum* parasites *in vitro* (333-336). Notably, a number of peptide mimetics, including scaffolds of vinyl sulfones (337, 338) and aldehydes (330, 334), have been shown to specifically target FP-2 and FP-3, demonstrate nanomolar potency against *P. falciparum* and clear parasites in *in vivo* models (333, 334).

Unlike the other falcipains, FP-1 has been linked to playing a role in merozoite invasion of the RBC, with FP-1 specific antibodies and peptidyl epoxides inhibitors shown to block this process (339, 340). However, the biological function of FP-1 during the asexual life cycle remains unclear as genetic disruption did not attenuate blood stage growth, but does affect oocyst development in mosquitos (331, 341).

1.7.2.2 Plasmepsins.

Plasmepsins are a family of aspartic proteases essential for a number of cellular processes in *Plasmodium* including haemoglobin degradation, protein export, merozoite egress and invasion of the RBC (321, 342). *P. falciparum* express a repertoire of 10 plasmepsins (Plm I to X), however, only PlmI, PlmII, histoaspartic protease (HAP, previously known as PlmIII), PlmIV, PlmV, PlmIX and PlmX have been studied in extensive detail (reviewed in (321, 322, 342)).

Similar to falcipains, four aspartic proteases, PlmI, PlmII, HAP/PlmIII and PlmIV, are also localised to the parasites food vacuole and involved in the cleavage and processing of RBC haemoglobin (reviewed in (342)). The temporal expression of plasmepsins differs, with PlmI and PlmII first activated by falcipains (329) at rings to likely initiate haemoglobin digestion while HAP/PlmIII and PlmIV are expressed as of trophozoite stages, with all four persisting until schizogony (343, 344). As plasmepsins share a high degree of functional redundancy (345-347) only a few food vacuole plasmepsin inhibitors have demonstrated nanomolar blood stage potency, with the current leading hydroxyethylamine scaffolds shown to specially target PlmIV (348-350). A more detailed investigation of these leading compounds, however, found them to also inhibit merozoite egress and invasion by interfering with *P. falciparum* subtilisin-like protease 1 (SUB1) (348, 350) (reviewed in (19), Appendix 1).

A plasmepsin of considerable interest in antimalarial development is PlmV, which is directly responsible for processing a number of parasites proteins for export into the host RBC. ~10% of the parasite's proteasome are trafficked beyond the parasitophorous vacuole (351), and many of these exported proteins are essential for parasite virulence and acquisition of nutrients (352) (reviewed in (353) and (354)). PlmV specifically recognises the conserved *Plasmodium* EXport ELement (PEXEL, arginine (R) x leucine (L) x gutamic acid (E) / glutamine (Q) / aspartic acid

(D) (RxLxE/Q/D), with x being any amino acid) export motif and cleaves after the conserved leucine (RxL) residue (355, 356). The protein is then trafficked through the parasite-derived translocon of exported proteins (PTEX) to the RBC surface (352) (reviewed in (353) and (354)). As PlmV is highly conserved across *Plasmodium* parasites and the human host lacks equivalent homologs, it presents an ideal candidate for antimalarial development. Accordingly, several peptide mimetic inhibitors based off the PEXEL motif have since been developed, with many inhibiting PlmV of both *P. falciparum* and *P. vivax* at low nanomolar concentrations (357-360).

Finally, Plm IX and Plm X are unique in that they are expressed during late schizonts/merozoites and are essential for merozoite egress and invasion of the RBC (350, 361, 362). As inhibitors targeting Plm IX and Plm X have been previously covered in my published review (19) (Appendix 1), these plasmepsins will not be discussed further here.

1.7.2.3 Proteasome inhibitors.

The proteasome is a multi-subunit 26S protease complex comprised of a barrel-shaped 20S catalytic core unit and a 19S regulatory cap (reviewed in (363) and (364)) (**Figure 6.1**). Proteasomes degrade ubiquitinated-proteins, an important 'housekeeping' function for all eukaryotes, that is required for the regulation of cellular homeostasis, cell cycle progression and apoptosis (reviewed in (365)).

Plasmodium proteasomes are attractive as therapeutic targets for several reasons. Firstly, given the morphological changes that occur throughout the parasite's lifecycle, constant protein turnover is an essential process and studies have shown that proteasomes are required throughout the parasite's entire lifecycle; liver, blood, transmission and mosquito stages (reviewed in (325) and (366)). Secondly, as almost half of *P. falciparum*'s proteome are proposed as targets for ubiquitination, the 26S proteasome is likely required for the degradation of a significant portion of these proteins (367). Indeed, a number of 26S proteasome inhibitors exhibit sub-micromolar to nanomolar potency against malaria parasites, with a number of peptide vinyl sulfone scaffolds shown to be highly selective against the *P. falciparum* proteasome, have efficacy in clearing blood stage parasites *in vivo* and demonstrate minimal toxicity against host cells and in rodent models (368, 369). Finally, proteasome inhibitors are typically synergistic with artemisinins and exhibit higher potencies against artemisinin-resistant parasites (368, 370, 371). Taken together, the ubiquitin-proteasome system is an attractive avenue for drug development and provides a promising strategy to combat

artemisinin resistance. In Chapter 6, I further explore the 26S proteasome and investigate a panel of analogues developed from the published proteasome inhibitor MG-132 (372, 373).

1.8 The development of antibiotics as antimalarials.

A number of antibiotics have been studied for use as antimalarials with doxycycline regularly used as a prophylactic, (374) and clindamycin (375), azithromycin (376), and fosmidomycin (377) having been tested in clinical trials. The known antimalarial properties of antibiotics are mediated through the apicoplast, a four membrane bound, non-photosynthetic, vestigial plastid organelle that was acquired by apicomplexans ~450 million years ago via secondary endosymbiosis of a photosynthetic red alga (378, 379). The apicoplast is found in most apicomplexan parasites, with the exception of *Cryptosporidium* (380), and maintains functions essential for parasite growth. Present day *Plasmodium* apicoplasts contain a small genome (~35 kb), having transferred the majority of apicoplast-derived genes to the nucleus (381, 382) (reviewed in (383)). ~500 nuclear located genes have protein products targeted to the apicoplast that take part in functions such as lipoic acid biogenesis, type II fatty acid biosynthesis (FASII), Sulphur-ion (Fe-S) cluster assembly as well as haem and isoprenoid biosynthesis pathways (384) (reviewed in (383)).

While these metabolic pathways may be essential at some stage during the parasite's life cycle, the synthesis of isoprenoid pyrophosphate precursors (IPP, isoprenoids) are the sole metabolic product of the apicoplast that is required for blood stage growth (161). As evidence of this, inhibition of the isoprenoid biosynthesis pathway though treatment with the antibiotic, fosmidomycin, results in the immediate death of the parasite (section 1.8.1) (**Figure 1.5**). In contrast, inhibiting apicoplast ribosomal translation with antibiotics that target bacterial-like ribosomes (azithromycin, doxycycline and clindamycin), however, prevents replication of this essential organelle and results in death of the second-generation parasites (delayed death) (sections 1.8.2 and 1.9) (**Figure 1.5**). As exogenous supplementation of IPP has been shown to rescue the growth of blood stage *P. falciparum* parasites, even after complete loss of the apicoplast due to drug treatment, delayed death is hypothesised to be the consequence of exhaustion of all available sources of isoprenoids (161).

In *Plasmodium* spp., isoprenoids are used for a number of biological products, including: i) prenyl modifications of proteins, ii) N-glycosylation and production of

glycosylphosphatidylinositol (GPI) anchors and iii) ubiquinone biosynthesis (reviewed in (385) and (386)). Recently, Kennedy et al., (2019) showed that death is the consequence of disrupted protein prenylation and cellular trafficking defects that impede formation of the digestive vacuole and deprive the parasite of its essential food source, haemoglobin (387). Here, I have detailed the loss of IPP as the key mechanism of action that kills blood stage parasites. Further information regarding the other metabolic functions of the apicoplast at different stages of parasite development can be found in the following reviews (reviewed in (388-391) and (392)).

1.8.1 Inhibitors of the IPP synthesis pathway.

Isoprenoids are present across all domains of life and mediate essential biological functions such as; cell signalling in mammals (e.g., steroids), antioxidation in plants (e.g., carotenoids) and the generation of cell wall precursors in bacteria (reviewed in (393)). While all isoprenoids are synthesised by the sequential condensation of IPP (five-carbon monomer) and dimethylallyl diphosphate (DMAPP), the biosynthetic pathways of IPP synthesis differs between prokaryotic and eukaryotic organisms. In eukaryotes, IPP is synthesised from acetyl-coenzyme A via the mevalonate pathway (MVA pathway). As most prokaryotes lack homologues for the MVA pathway, IPP is synthesised via an alternative route; the mevalonate-independent MEP (2-C-methyl-d-erythritol 4-phosphate)/DOXP (1-deoxy-d-xylulose 5-phosphate) pathway (MEP/DOXP, non-mevalonate pathway), which is completely absent from mammals (158) (reviewed in (393)). In *Plasmodium* spp., all seven enzymes of the MEP/DOXP pathway (*Pf*DOXP synthase, *Pf*IspC, *Pf*IspD, *Pf*IspE, *Pf*IspF, *Pf*IspG, and *Pf*IspH) are encoded within the nuclear genome and transported into the apicoplast by an N-terminal bipartite leader sequence, where they carry out IPP synthesis (394) (reviewed in (386)).

IPP biosynthesis via the MEP/DOXP pathway is essential for development of liver (395), blood (396) and gametocyte stage parasites (397) and, unlike antibiotics that target the apicoplast's ribosome, inhibitors of the MEP/DOXP pathway have been shown to kill parasites during the first-cycle of growth. Several compounds have been identified to target the MEP/DOXP pathway (reviewed in (398)), the most notable being the antibiotic fosmidomycin, an inhibitor of DOXP reductoisomerase, the second enzyme in this pathway (157, 158) (**Figure 1.5**). Recently, fosmidomycin completed Phase II clinical trials in combination with piperaquine, where it demonstrated a 100% cure rate after a 3-day treatment regime and acceptable tolerability for treatment of uncomplicated malaria (377) (reviewed in (399)).

Fosmidomycin, however, faces several limitations for clinical use, namely the drug's low bioavailability, short half-life (1-2 hrs) (400) and side-effects, such as electrocardiogram changes and abnormal heart rhythms (QT prolongation) that were identified in the recent Phase II clinical trial (377). Analogues of fosmidomycin (FR-900098) (158, 401) and other enzymes in the MEP/DOXP pathway such as the IspD inhibitor MMV008138 (307, 309) remain in development (402).

1.8.2 Inhibitors of apicoplast translation.

Most antibiotics with activity against malaria parasite's target the apicoplast's 70S bacterial-like translational machinery (160, 403). Targeting the apicoplast's bacterial-like ribosome prevents replication of this organelle, with the progeny parasites (second generation) inheriting a dysfunctional apicoplast. Apicoplast loss does not result in a growth defect during the first growth cycle (~48 hrs, 2 days) and they will continue to grow and invade new RBCs. During the second cycle of growth (96 hrs, 4 days) the drug-affected parasites lethally arrest even in the absence of an inhibitor due to loss of the apicoplast and its IPP biosynthesis capabilities (**Figure 1.5**) (130, 159, 404). This 'delayed death' activity is evident in the asexual life-stages of both *Plasmodium* spp. parasites (130, 159, 405) and *Toxoplasma gondii*, a closely related apicomplexan parasite (403, 406). Here, I will describe in more detail several antibiotic classes that inhibit apicoplast ribosome translation and cause delayed death of parasites.

1.8.2.1 Tetracyclines.

The tetracyclines are a family of broad-spectrum antibiotics that act by binding to the small ribosomal subunit (30S ribosomal ribonucleic acid (rRNA)) of the bacterial ribosome, preventing the attachment of charged aminoacyl-transfer RNA (tRNA) to inhibit protein synthesis (**Figure 1.7**) (reviewed in (407) and (408)) (409). Doxycycline, a semi-synthetic derivative of tetracycline, is active against both blood and liver stages and is typically well tolerated, but is not recommended for pregnant women or young children (<8 years of age) (reviewed in (374)). Due to doxycycline's delayed onset of action, monotherapy risks rapid progression from uncomplicated malaria into severe disease. Therefore, this antibiotic is typically used in combination with a faster acting partner drug, such as quinine, as an alternative treatment for uncomplicated malaria in regions endemic with chloroquine and multidrug-resistant *P. falciparum* parasites (146, 159, 410) (reviewed in (135)). Doxycycline has also been widely used as a chemoprophylactic for non-immune travellers (146) (reviewed in (135)).

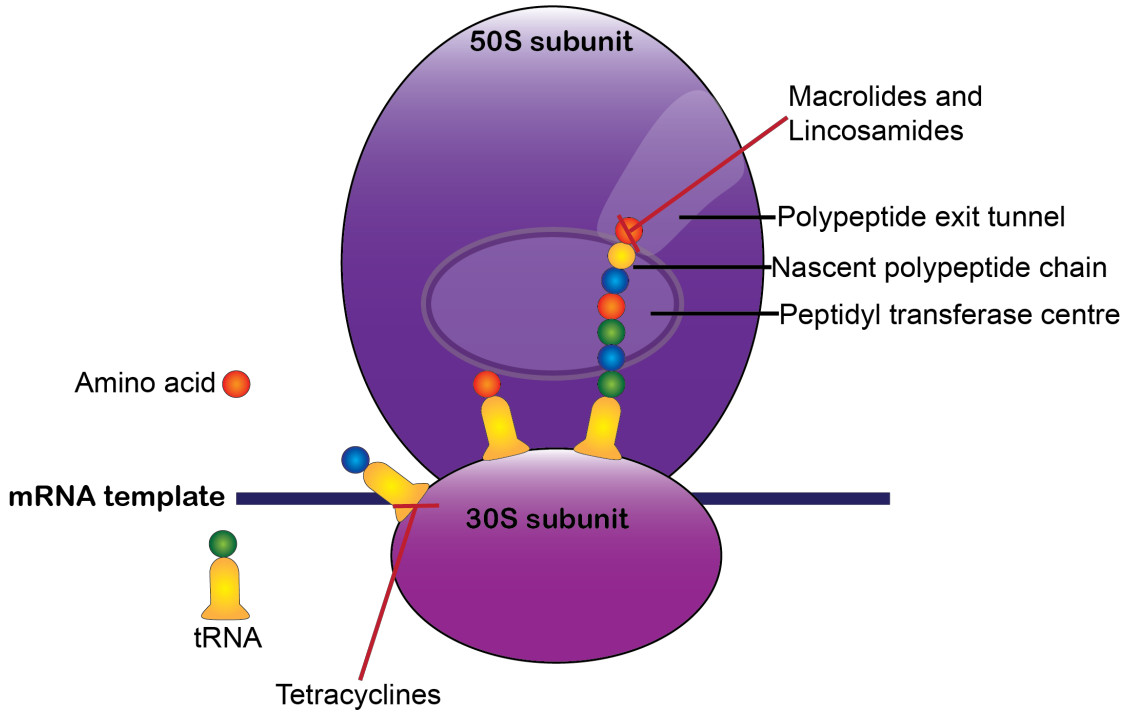

Figure 1.7 Ribosomal binding of antibiotics.

The binding sites of tetracyclines, lincosamines and macrolide antibiotics within the 70S bacterial ribosome. Tetracyclines (e.g. doxycycline) inhibit protein synthesis by binding to the small 30S subunit of the bacterial ribosome and preventing the attachment of tRNA (reviewed in (407) and (408)). Lincosamines (e.g. clindamycin) and macrolide antibiotics (erythromycin and azithromycin) occupy a site within the polypeptide exit tunnel and peptidyl transferase centre of the large 50S ribosomal subunit and prevent protein synthesis by blocking the peptide exit tunnel (reviewed in (411)). Figure adapted from (412).

1.8.2.2 Lincosamides.

Lincosamides constitute a small family of antibiotics featuring amino acid and sugar moieties. Clindamycin, a semi-synthetic variant of lincomycin, is broadly active against both Gram-positive bacterial pathogens and apicomplexan parasites, including *Plasmodium*, *Toxoplasma* and *Babesia* (reviewed in (413)). The mechanism by which clindamycin inhibits these pathogens is via drug binding to the large 50S subunit of the bacterial ribosome, thus blocking the peptide exit tunnel and preventing protein synthesis (**Figure 1.7**) (130, 160, 414). While clindamycin demonstrates a good safety and tolerability profile, this drug features a short half-life (2-4 hrs) and is slow acting with average clearance of *Plasmodium* parasites within the host typically taking 4 to 6 days (375, 415). As a consequence of this slow clearance, clindamycin is rarely used as a monotherapy for treatment of malaria, but is currently recommended in combination with faster acting antimalarials, such as quinine and artesunate, for treatment of uncomplicated malaria in pregnant women if other treatments are unavailable (146, 147).

1.8.2.3 Macrolide antibiotics.

Macrolide antibiotics (14-, 15-, or 16- macrocyclic lactone rings) are a diverse class of antibiotics with broad biological activities including; antibacterial, antifungal, antiparasitic, immunosuppressive/immunomodulatory, anticancer and prokinetic properties (reviewed in (416, 417) and (418)).

Like lincosamides, macrolide antibiotics occupy a binding pocket within between the entrance of the polypeptide exit tunnel (domain II) and peptidyl transferase centre (domain V) of the 50S ribosome (419). Here, the macrolide's desosamine sugar reversibly interacts with specific 23S rRNA nucleotides of ribosomal proteins; L4 (*rpl*4) and L22 (*rpl*22), that form the polypeptide exit tunnel (420-423) (reviewed in (411)). Thus, macrolides arrest cell growth by blocking the elongation of nascent polypeptides exiting the ribosome (**Figure 1.7)**. A major resistance mechanism to macrolides is conferred by various site-specific SNPs or post-translational modifications of L4 and L22, as well as other regions, that induce a structural change within the binding pocket (160) (reviewed in (424, 425) and (411)).

Macrolide inhibition of malaria parasite's bacterial-like apicoplast ribosome results in classic 'delayed death' antimalarial activity (>96 hrs before parasite death is noticeable), presenting a severe limitation for clinical use where rapid parasite clearance is essential for providing a positive treatment outcome (230). It is for this reason that antibiotics with delayed death antimalarial activity, such as the macrolides, lincosamides and tetracyclines are largely used as

prophylactics or in combination with faster acting antimalarials to 'mop-up' parasites that survive the faster acting partner drug. Azithromycin has received the most attention of the macrolides for use as an antimalarial. As repurposing this widely used antibiotic as an antimalarial is the main focus of my research, I will cover azithromycin in more detail here in addition to this drugs history of clinical use against bacterial pathogens and malaria parasites.

1.9 Clinical use of azithromycin.

Azithromycin, a 15-membered ring azalide, is a second-generation macrolide antibiotic that was synthesised in the mid-1980s as a semi-synthetic derivative of erythromycin (**Figure 1.8**) (424, 426). Like other macrolides, azithromycin inhibits protein translation of bacterial ribosomes and features activity against Gram-positive bacteria, *Mycoplasma* spp. and Gram-negative cocci, but has limited activity against other Gram-negative bacteria. Due to azithromycin's broad antibacterial activity, this drug is commonly prescribed for a range of bacterial diseases including infections of the upper and lower respiratory tract, skin, soft tissues and genital tract. Azithromycin also exhibits immunomodulatory effects on the host's innate and adaptive immune responses and has been shown to resolve inflammation by decreasing pro-inflammatory signalling pathways, immune cells and cytokines (reviewed in (417, 427) and (416)). Long-term treatment of azithromycin has beneficial effects for a variety of chronic inflammatory disorders including diffuse panbronchiolitis, bronchiectasis, cystic fibrosis, non-cystic fibrosis and chronic obstructive pulmonary disease (reviewed in (417)).

Azithromycin is typically well tolerated and has favourable pharmacokinetic and pharmacodynamic properties, including a long-half life (68 hrs) (428). This long half-life is partially attributed to the accumulation of azithromycin within acidic compartments (lysosomes) of various cell types as well as the drug's enhanced lipophilicity, that results in its extensive tissue penetration and distribution via entero-hepatic circulation (429, 430, 431, 417).

The use of macrolides, including azithromycin, have been linked to adverse cardiac risks such as QT prolongation and fatal *torsades de pointes* (arrhythmic death) (432). These complications are thought to occur by drug interaction and inhibition of human Ether-a-go-go-related Gene (hERG) potassium (K^+) channels within the heart. Azithromycin has a lower affinity for hERG K^+ channels and appears to cause QT prolongation to a lesser extent than the antibiotics, erythromycin and clindamycin (433) (reviewed in (434) and (435)).

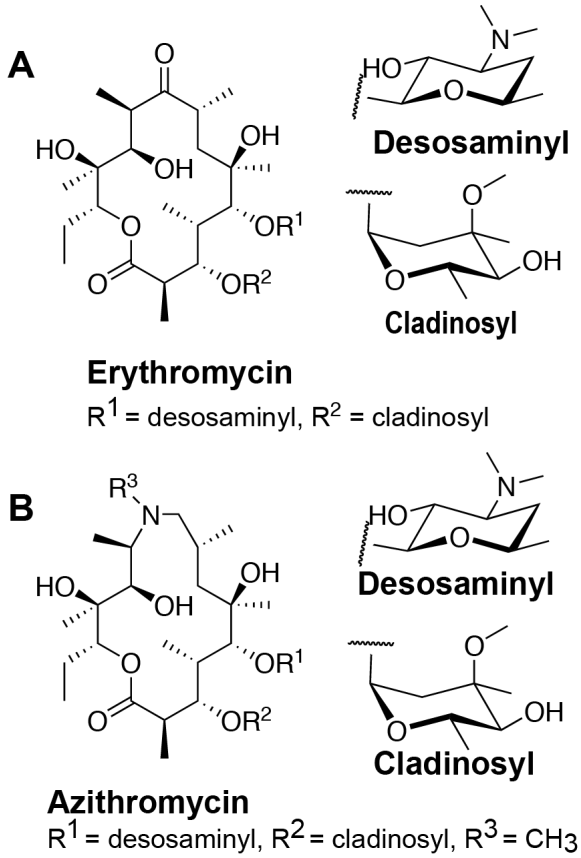

Figure 1.8 Chemical structures of macrolide antibiotics.

A) Structure of erythromycin, a first generation, 14-membered macrolide antibiotic. **B**) Structure of the second-generation macrolide, azithromycin, a semi-synthetic derivative of erythromycin featuring a 15-membered ring.

Moreover, despite the global usage of azithromycin, reports of azithromycin associated *torsades de pointes* are rare and is mostly observed in patients with pre-existing cardiac disease and/or other complicating medical conditions (e.g. older age; acute medical conditions; additional use of other drugs associated with QT prolongation). While the risk of cardiac complications with azithromycin treatments is contested in the literature (436, 437) (reviewed in (434) and (435)), this drug remains one of the most widely used antibiotics in the world (438). Furthermore, azithromycin is commonly used for treatment of paediatric infections and features a well-established safety profile for treatment of young children as well as pregnant and breast feeding women (439-441). Based on continuing clinical usage for a range of bacterial infections and recommendations for use in both pregnant women and children, azithromycin remains one of the most successful and widely prescribed antibiotics used today (438).

1.9.1 Azithromycin's clinical use as an antimalarial.

Azithromycin's antiplasmodial activity was first described in the early 1990s after *in vitro* drug testing of chloroquine sensitive and resistant *P. falciparum* lines (442, 443). Further studies and human trials revealed azithromycin's delayed death mechanism of parasite killing and demonstrated that the drug was most suitable for use as a prophylactic (444-448). As a consequence of azithromycin's unique safety profile and the drug's slow low-nanomolar activity against parasites, azithromycin has been extensively evaluated as a potential treatment for malaria, which is summarised below.

1.9.1.1 Azithromycin and treatment of clinical malaria.

Studies evaluating azithromycin's efficacy as a monotherapy demonstrated that treatment of both *P. falciparum* and *P. vivax* infections (449) failed to clear parasitaemia efficiently and sometimes resulted in treatment failure (376). It was proposed that azithromycin's delayed death mechanism of action contributed to this less than ideal activity and it was hypothesised that combining azithromycin with a more rapidly acting antimalarial agent could improve the clinical applications of azithromycin against malaria. Combinations of azithromycin and artemisinins have shown improved efficacy over artemisinin monotherapies alone. However, the risk of parasite recrudescence was more common in azithromycin/artemisinin regimens compared to other antimalarial combinations (450-452). While recrudescence could in part be attributed to the antagonism observed between azithromycin/artemisinin (453), the slow activity of delayed death was also a contributing factor (376).

More promisingly, combinations of azithromycin/chloroquine (AZCQ) were synergistic in both

in vitro and *in vivo* drug trials (449, 453, 454). In Phase II/III clinical trials, AZCQ was generally well tolerated and superior to the monotherapies tested (455), but was shown to be inferior in comparison to other antimalarial combination therapies and the lower treatment doses tested (azithromycin 0.5 to 1 gram (g); chloroquine 0.6 g) were associated with treatment failure (376, 456, 457). Subsequent studies showed that a higher dose of azithromycin (2 g) with chloroquine had a higher cure rate (>90%), but approached the limits of tolerability (458). Together, these studies demonstrate that azithromycin monotherapies are ineffective and combination therapies are at best equivalent to, but not superior to, available ACTs for malaria treatment (376, 457). Trials of azithromycin as a malaria prophylactic and for use in Intermittent Presumptive Treatment for malaria in pregnancy (IPTp), however, have achieved more positive outcomes.

1.9.1.2 Azithromycin as a malaria prophylactic.

Malaria prophylaxis remains an important strategy for malaria control and prevention, particularly for travellers to endemic areas and during malaria epidemics. When administered daily, azithromycin monotherapy exhibited modest prophylactic activity against *P. falciparum* parasites in Kenyan (447) and Indonesian adults (459) and showed good efficacy against *P. vivax* malaria in Thai adults (98% protection) (460). Azithromycin's protective efficacy against *P. falciparum* was significantly poorer when administered weekly and was inferior to the antibiotic doxycycline that has a similar delayed death mechanism of action (section 1.8.2.1).

More recently, azithromycin's prophylactic activity was assessed in combination with naphthoquine, a 4-aminoquinoline antimalarial (reviewed (461)), in Phase II clinical trials on the China-Myanmar border (462). Low (200 mg of each drug) and high (400 mg of each drug) dose regimes of azithromycin/naphthoquine showed 81% and 90% prophylactic efficacy, respectively, against *P. falciparum* and >90% efficacy against *P. vivax* and *P. ovale* (462).

Interestingly, mass drug administrations of azithromycin during control and treatment campaigns for *Chlamydia trachomatis*, the causative agent of trachoma, observed a significant decrease in childhood deaths in malaria endemic regions (448, 463, 464). The decreased mortality was attributed to a reduction of malaria parasitemia and associated clinical burden through azithromycin's prophylactic activity against malaria (463, 465). In addition, short-term studies in Niger during the low transmission-season observed a significant reduction of malaria parasitemia for ~4-5 months post intervention within communities that were biannually treated with azithromycin for *Chlamydia trachomatis* infection (466, 467). In contrast, other

chemoprevention (468) and longer-term (3 years) (469) studies assessing azithromycin's prophylactic activity in high transmission settings observed no significant reduction in parasitemia's. Although, Chandramohan et al., (2019) noted a reduction in incidences of gastrointestinal (15%), upper respiratory tract infections (15%) as well as non-malarial related illnesses (21%) in children (3 to 59 months of age) following mass drug administration of azithromycin (468). This failure to reduce parasite burdens with long-term treatments and mass administration of azithromycin seen in some studies has raised concerns about *Plasmodium* resistance to azithromycin developing in the field (469, 470). Several resistant mutations in the apicoplast ribosomal genes have been identified in *in vitro* azithromycin treated malaria parasites that correlate in loss of sensitivity to azithromycin's delayed death activity (160, 164, 165). Currently, none of these azithromycin resistance markers have been identified in field samples, suggesting that repeated mass drug-administration is not selecting for malaria parasites resistant to azithromycin within drug treated regions (448, 465, 470).

1.9.1.3 Azithromycin's use in intermittent preventive treatment for malaria in pregnancy.

There is currently a limited selection of antimalarials that can be safely used to regularly treat pregnant women and provide ongoing antimalarial protection from pregnancy associated malaria. The WHO currently recommends sulphadoxine–pyrimethamine as the frontline treatment for IPTp. But in recent years the spread of sulphadoxine–pyrimethamine resistant *P. falciparum* parasites (reviewed in (182)) and the limited efficacy of these drugs against *P. vivax* malaria (471, 472) has prompted further evaluation of alternative drug combinations, including combinations with azithromycin.

As a monotherapy, however, azithromycin is unsuitable for replacing current IPTp treatment regimes. Indeed, investigations of azithromycin as a routine prophylaxis for preterm birth in Southern Malawi observed no significant differences in maternal parasitemia (11.5% vs 10.1%), anaemia (44.1% vs 41.3%) or pregnancy outcomes between azithromycin or the placebo group (473).

Given the efficacy of AZCQ to treat malaria, the safety of the combination and synergism between these drugs (449, 453), AZCQ was tested as a potential IPTp (474). A 3-day fixed-dose regime of AZCQ was assessed for IPTp across five African countries, however, these found no difference in pregnancy outcomes and the drug combination appeared to be poorly tolerated, resulting in the trials early termination (475). Another trial of AZCQ showed better

tolerability but failed to demonstrate superiority to sulfadoxine–pyrimethamine (476), suggesting this combination is not effective nor safe enough to replace current therapies.

Another series of studies tested combinations of azithromycin/sulfadoxine–pyrimethamine for IPTp, with initial trials in Malawi showing 81% fewer recrudescent infections compared to sulfadoxine–pyrimethamine alone (477). Later studies using different dosage regimes of azithromycin have also demonstrated lower risks of premature births, reduced risk of low birthweight and a lower prevalence of *P. falciparum* malaria (478-480). Given azithromycin's broad-spectrum antibacterial and anti-inflammatory properties, it has been proposed that this protection may be independent of the drug's antimalarial activity (477, 480, 481).

1.10 The potential to develop azithromycin as a quick-killing antimalarial.

Azithromycin's known target is the bacterial-like ribosome of the parasites' apicoplast, a remnant plastid organelle. By binding to the 50S ribosomal subunit, azithromycin stops apicoplast replication and the progeny of drug treated parasites die ~4-6 days post treatment (130, 159, 160, 405). Several studies also identified that azithromycin kills asexual blood stage parasites over one cycle of parasite growth (~48 hrs) at higher, micromolar concentrations (IC_{50} ~10 μM) (130, 164, 165).

Studies have also demonstrated that analogues of azithromycin and the related antibiotic, erythromycin (which does not have activity within one cycle of parasite growth), have substantially improved potencies over one cycle of blood stage growth (164, 165, 418, 482-486). In addition, Wilson et al., (2015) found that several clinically used macrolide antibiotics rapidly inhibit merozoite invasion of RBCs in addition to quickly killing intracellular blood stage parasites, with the most potent being azithromycin (165).

To distinguish between apicoplast targeting delayed death activity of azithromycin and activity against merozoite invasion and intracellular parasite growth over 1 cycle, I have named this fast-acting antimalarial activity as 'quick-killing'. Importantly, several quick-killing azithromycin analogues also maintained delayed death activity (165), opening the possibility of developing azithromycin as an antimalarial that: i) maintains prophylactic properties through apicoplast-targeting delayed death activity, ii) rapidly kills intracellular blood stage parasites and iii) has direct merozoite invasion blocking activity, the first example of a clinically used drug that features all these properties. As the main body of my research has explored the quick-

killing activity of azithromycin and azithromycin analogues the current evidence for quick-killing is summarised in detail below.

1.10.1 Targeting merozoite invasion of the RBC as an antimalarial strategy.

The identification of azithromycin's invasion inhibitory properties was a key driver for my investigations into azithromycin analogues as novel quick-killing antimalarials (165). Invasion inhibitory antimalarials have been investigated by a number of research groups across a range of intracellular parasite targets and chemotypes and the strategy of targeting invasion as a key element for clinical use has both potential advantages and disadvantages. In order to summarise this emerging drug development strategy, I have published a comprehensive review on the current status of antimalarial development targeting invasion (19). This review is provided as a component of this thesis in Appendix 1.

1.10.2 Azithromycin's quick-killing activity against intracellular blood stage parasites.

To date, evaluation of azithromycin as an antimalarial for clinical use has been solely based on the drugs nanomolar delayed death activity against the apicoplast's bacterial-like ribosome. There are also several lines of evidence suggesting that azithromycin has a secondary mechanism of action against parasites that is only observed *in vitro* at higher, micromolar concentrations (130, 159, 165). This activity was first described by Dahl et. al (2007), who demonstrated that azithromycin, as well as other macrolides, killed first generation parasites *in vitro* at micromolar concentrations (48 hrs, rings to rings; IC_{50} 8.9 μM) (130). Dahl et al., (2007) also hypothesised that these macrolides interfered with parasite growth via an apicoplast-independent target.

Due to azithromycin's favourable pharmacokinetic properties and safety profile, the pharmaceutical company GlaxoSmithKline (GSK) synthesised a number of azithromycin analogues in order to improve their potency against blood stage parasite growth (418, 482-486) (reviewed in (487)). A broad range of analogues with different chemical modifications were synthesised, with the analogues demonstrating efficacy against both chloroquine sensitive and resistant *P. falciparum* lines, favourable pharmacokinetic properties, as well as low-nanomolar *in vitro* and *in vivo* potency that was superior to that of azithromycin. These studies were discontinued when in vivo studies demonstrated that the leading analogues did not possess superior activity against *P. berghei* rodent malaria parasites compared to azithromycin (418, 484, 486). It should be noted that the *in vivo* studies were performed with

72 hrs of drug treatment and, as *P. berghei* has a 24 hr lifecycle, it is likely that the apicoplast targeting delayed death activity of azithromycin and analogues contributed to parasite clearance. Whether short term treatments of azithromycin and analogues were effective *in vivo* was not explored.

Goodman et al,. (2013) identified analogues of erythromycin and azithromycin that exhibited up-to a 10-fold increase in activity against *P. falciparum* in short-term assays (48 hr) (164). Interestingly, analogues of erythromycin, which have no activity with short-term exposure of malaria parasites, also exhibited improved quick-killing activity which demonstrates that different macrolides can be optimised to improve parasite killing with limited exposure concentrations (164, 165). The leading azithromycin analogue, compound 2, showed improved activity against exoerythrocytic liver-stage parasites with 56 hrs of treatment (IC_{50} 6.9 micromolar (μM)) compared to azithromycin that had no effect up to 40 μM (164). Interestingly, parasites selected for resistance to azithromycin's delayed death activity through a point mutation in the apicoplast ribosomal protein gene (*rp14*) that were up to 57-fold less sensitive to delayed death activity (160, 164), but remained susceptible to short-term intracellular treatments of azithromycin and analogues (164). Together, these studies suggest azithromycin has a secondary mechanism of action that kills parasites with short-term treatments and is independent of apicoplast-targeting activity.

Wilson et al., (2015) investigated this short-term killing activity and demonstrated that azithromycin (IC_{50} 10 μM, ethanol) could stop merozoite invasion of RBCs with a very short treatment time (165). Furthermore, this study presented several lines of evidence suggesting that invasion inhibitory activity is independent of the apicoplast-targeted activity. Firstly, the speed that azithromycin inhibits invasion (30-60 seconds) is much faster than that of delayed death (~4 days), although a 250-fold higher concentration is required. Secondly, the efficacy of azithromycin's merozoite invasion inhibitory activity showed minimal change against an azithromycin delayed death resistant line, suggesting that invasion is independent of apicoplast translation. Finally, analogues that featured >5-fold improvement for invasion (IC_{50} 12e, 15 μM; 1j, 7 μM) over azithromycin (IC_{50} 38 μM, dimethyl sulfoxide (DMSO)), showed no loss of activity in delayed death assays, suggesting that azithromycin's invasion inhibitory activity can be improved independently of delayed death. Azithromycin also quickly killed early ring stage parasites, from post-invasion until schizogony (in cycle, up to 44 hr post-invasion), at a similar concentration to that of invasion inhibition (IC_{50} in ethanol; invasion, 10 μM; 40 hrs, 6 μM). Finally, azithromycin analogues that exhibited improved invasion inhibition also

showed >10 fold improvement during both in cycle (13-fold) and short-term blood stage growth (11-fold, 1 cycle, ~72 hrs post invasion, encompasses an invasion cycle), suggesting that azithromycin and analogues are active well before delayed death is evident.

Taken together, azithromycin appears to feature two different growth inhibiting mechanisms against parasites: 1) delayed death, mediated via inhibition of the apicoplast's bacterial-like ribosome, and 2) a quick-killing mechanism that includes rapid inhibition of merozoite invasion as well as in cycle (40 hrs) and 1 cycle (72 hr) intracellular growth inhibitory activities. Furthermore, it appears that quick-killing is conserved across Apicomplexan parasites as azithromycin also inhibits invasion and early intracellular growth of *T. gondii* (165, 488), merozoite invasion of *P. berghei* parasites (165) and short-term intracellular growth inhibition (1 cycle) of the zoonotic human malaria parasite *P. knowlesi* (405).

Chapter 2. Summary and aims.

2.1 Summary.

Antimalarials have been a mainstay for the treatment, control and cure of malaria. However, for much of the 20-21st centuries antimalarial drug resistance has been, arguably, one of the biggest ongoing public health challenges globally. Historically, parasite resistance to antimalarials has resulted in massive tolls on public health in endemic regions, with malaria-related mortality observed to double in sub-Saharan Africa after the emergence of parasite resistance to chloroquine and sulfadoxine-pyrimethamine (132, 208-210). When ACTs were first adopted for malaria treatment, these drugs were highly effective and contributed to steep reductions in global malaria morbidity and mortality (132, 208, 211, 212). As artemisinins are our last line of defence against multi-drug resistant parasites, successful treatments of malaria are dependent on the clinical efficiency of front-line ACTs. Of major concern, loss of parasite sensitivity and resistance to front-line artemisinins is now prevalent throughout South-East Asia and has recently emerged in Eastern-India and Papua New Guinea (224, 225, 228, 229). Currently, there are no effective alternatives to ACTs and although the drug pipeline is promising (235, 236, 238) (sections 1.6, 1.7 and 1.8) there is now an urgency to identify and explore other novel avenues of antimalarial development.

The macrolide antibiotic, azithromycin, has been proposed for inclusion in ACTs (450, 455), IPTp (477) and as for use as a malaria prophylaxis (163, 465) due to the drug's long half-life (>50 hrs) and unique safety profile (431, 439). In its current form, however, azithromycin has limited clinical efficacy for treatment of uncomplicated malaria, attributed the drug's apicoplast-targeting 'delayed death' phenotype (reviewed in (376)). Over the last decade and a half, a number of studies have proposed that azithromycin has a secondary, unidentified, 'quick-killing' mechanism of action that is independent from apicoplast-targeting delayed death. This quick-killing activity targets the invading merozoite and kills early blood stage parasites at higher, micromolar concentrations (130, 164, 165). Given that 'quick-killing' requires a concentration of drug that is not clinically achievable, current treatment applications of this mechanism are limited (130, 489). Screenings of azithromycin analogues have demonstrated that quick-killing IC_{50}s can be improved with chemical modification, whilst potentially maintaining potency against the apicoplast (164, 165). Thus, exploration of azithromycin analogues presents an attractive development strategy and we hypothesise that azithromycin can be re-developed into an antimalarial with dual-modalities; featuring

improved quick-killing blood stage activity whilst maintaining apicoplast-targeting delayed death prophylaxis.

2.2 Development of azithromycin analogues and proteasome-like inhibitors to combat malaria.

This project primarily focuses on identifying azithromycin analogues with improved quick-killing activities, with the focus of Chapter 4 and 5 being to re-develop azithromycin into a potent, dual-acting antimalarial. To address this, I have obtained >100 azithromycin analogues from two independent sources; 84 that were kindly provided by GlaxoSmithKline (GSK), Tres Cantos facility (418, 482-486) and a smaller panel of 22 analogues, featuring significantly different structures that were originally evaluated as antibacterials (490) kindly provided by Associate Professor Henrietta Venter (University of South Australia) and Shutao Ma (Shandong University, China) (490). In addition, I will use both azithromycin and lead analogues to characterise the quick-killing mechanism of action, with the intention of identifying the secondary target.

For the assessment of azithromycin analogues: The overarching hypothesis is that azithromycin can be engineered into a fast-acting, resistance proofed drug that kills blood stages of *Plasmodium* spp. parasites through dual-mechanisms of action.

The specific aims to these projects are to:

- Characterise azithromycin's quick-killing mechanism across diverse structural analogues, identify features that promote improved quick-killing potency and maintain delayed death activity.
- Identify the secondary mechanism of action that causes quick-killing activity using genetic and metabolomics approaches and determine if the quick-killing mechanism is linked to the apicoplast.

Given the current status of ACTs and emergence of drug resistance observed in some current clinical and pre-clinical candidates under development, constant replenishment of the drug development pipeline with new antimalarials with novel mechanisms of action will be essential for malaria treatment going forward (230, 237, 491). In light of this, my research has also examined the potential to develop tripeptide inhibitors based on the proteasome inhibitor MG-132 that were developed at the University of Adelaide for activity against human malaria

parasites. Further, I investigated whether these compounds could be developed as hypoxia inducible prodrugs to create a compound with reduced activity against mammalian cells.

For the assessment of proteasome inhibitor-like analogues: The hypothesis of this project is that tri-peptide proteasome-like inhibitors based on MG-132 can be engineered into potent blood stage antimalarials and their selectivity against *Plasmodium* spp. parasites can be improved though a hypoxia inducible pro-drug modification.

The specific aim of this project was to:

- Characterise the activity of novel proteasome-like inhibitors based on MG-132 against malaria parasites and explore the efficacy of leads as possible candidates for further development.
- Assess the potential for development of a hypoxia activated prodrug based on the MG-132 like compounds and test their efficacy under *in vitro* hypoxia culture conditions compared to hypoxic culture conditions.

Chapter 4 of this thesis characterises the activity and possible mechanism of action of 84 azithromycin analogues developed and provided by GSK (418, 482-486).

Chapter 5 undertakes a similar characterisation of an additional 22 azithromycin analogues that have greater diversity in their structure which were provided by Associate Professor Henrietta Venter (University of South Australia) and Shutao Ma (Shandong University, China) (490).

Both of these chapters are presented in manuscript format with permission from co-authors.

Chapter 6 of this thesis characterises the activity of the MG-132 analogues synthesised in the laboratory of Prof Andrew Abell, the University of Adelaide. This chapter is presented in a standard chapter format and the drug screening data I produced against malaria parasites is presented with permission of Prof. Andrew Abell and Mr. Aniket Kulkarni who synthesised the compounds.

Chapter 3. Materials and methods.

Here, an in-depth description of all materials and methods relevant to this thesis have been described. Please note that the independent manuscript style chapters (Chapters 4 & 5) have a brief overview of the respective methods used.

3.1 Antimalarial and antibacterial drugs.

All chemicals and reagents were purchased from Sigma-Aldrich (Australia) unless otherwise stated. As a previous study showed that the vehicle of choice can impact the potency of azithromycin *in vitro* (azithromycin IC_{50}, ethanol 10 μM; dimethyl sulfoxide (DMSO) 38 μM) (165), stocks of azithromycin (100 mM), AK-Scientific) were made up in ethanol (Merck) as vehicle to avoid loss of potency. Stock concentrations of the antimalarial quinine (3075 mM, Sigma-Aldrich) was made up in ethanol as vehicle. Chloroquine diphosphate salt (10 mM, Sigma-Aldrich) was dissolved in MilliQ water, which was then filter sterilized (0.22 μm) in a Class II Biosafety Cabinet. Dihydroartemisinin (DHA, 10 mM, Sigma-Aldrich) was dissolved in DMSO (Sigma-Aldrich). The protease inhibitor, MG-132 (10 mM, Sigma-Aldrich), was dissolved in DMSO. WR99210 (Jacobus Pharmaceuticals) was dissolved in complete media and used at a final concentration of 5 nM. All compounds were stored at -20 degrees Celsius (°C) when not in use.

3.1.1 Azithromycin analogues.

Azithromycin analogues used in Chapter 4 were a gift from GlaxoSmithKline (GSK), Tres Cantos, Spain. Synthesis of GSK 1-84 compounds has been described previously (418, 482-486). See **S. Tables 4.1-4.3** in Chapter 4 for further details of chemical structure and origin of each GSK analogue. Group A (A1-13), Group B (B1 & 2), Group C (C1-5) and Group D (D1 & 2) azithromycin analogues used in Chapter 5 were a gift from Associate Professor Henrietta Venter (University of South Australia) and Shutao Ma (Shandong University, China). See **S. Tables 5.1-5.4** in Chapter 5 for the chemical structure and origin of each analogue. Synthesis of these analogues are described previously (490). All azithromycin analogues were dissolved in ethanol at a concentration of 10 mM to avoid loss of potency (165). All stocks in ethanol were sealed with Parafilm 'M' (Bemis, WI, USA) until use, to avoid evaporation, and stored at -20 ^{o}C.

Chapter 3.

3.1.2 Proteasome inhibitor-like compounds.

The proteasome inhibitor-like compounds (PI 1-5) and hypoxia activated pro-drug (PI_{PRO} 1-3) analogues used in Chapter 6 were a gift from Aniket Kulkarni (University of Adelaide, Australia) and Professor Andrew Abell (University of Adelaide, Australia). All proteasome inhibitor-like compounds were dissolved in DMSO to a concentration of 25 mM. See **Table 6.1** and **Table 6.3** in Chapter 6 for details of chemical structure of each proteasome inhibitor-like compound. The synthesis of the compounds will be described in a manuscript being prepared by Aniket Kulkarni and Professor Andrew Abell as part of Mr Kulkarni's PhD completion requirements.

3.2 Culture conditions and parasite lines.

3.2.1 Continuous culture of *P. falciparum* and *P. knowlesi.*

All parasite culture was carried out in a Class II Biosafety cabinet in either 5 mL, 10 mL, or 30 mL dishes, or 175 cm^2 filtered cell culture flasks (Corning). O^+ blood, donated by Red Cross Australia, was used to culture and maintain *P. falciparum* and *P. knowlesi* at 3 % haematocrit. Parasites were grown in RPMI-HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) culture medium maintained at pH 7.2-7.4 (Gibco, Thermo Fisher Scientific), supplemented with 0.5% v/v Albumax II (Gibco), 52 µM gentamycin (Gibco), 367 µM hypoxanthine (Sigma-Aldrich), 2 mM Glutamax, 25 mM sodium bicarbonate ($NaHCO_3$). Standard culture conditions were maintained in airtight boxes at 37 °C in an atmosphere of 1% oxygen (O_2), 4% carbon dioxide (CO_2) and 95% nitrogen (N_2) (492) unless otherwise specified.

3.2.2 *Plasmodium* parasites used in this study.

P. falciparum was the primary malaria parasite species used in this study with all primary drug screens being undertaken with the green fluorescent protein (GFP)-fluorescent *P. falciparum* D10-PfPHG line (493). This line was a genetically modified version of the Papua New Guinean parasite isolate D10 that was adapted in 1983 (493, 494). Stable expression of GFP is maintained through pyrimethamine and blasticidin drug selection (493, 495). As the D10-PfPHG line expresses GFP, accurate quantification of all blood stages via flow cytometry can be achieved using both GFP and ethidium bromide staining (EtBr, Bio-Rad Hercules, CA,

USA) (493) (section 3.7 and **Figure 3.3**). This line also has a reproducible 48 hr blood stage lifecycle (from invasion of merozoites to schizont rupture) with the ~5-fold expansion per cycle facilitating use in merozoite invasion inhibition assays (section 3.6.7) and other assays designed to define the stage of drug activity (section 3.6).

3.2.3 Apicoplast-null D10-PfPHG parasites.

P. falciparum D10-PfPHG parasites lacking the apicoplast (D10-PfPHG$^{apicoplast\text{-}null}$), were used to exclude the possibility of an inhibitor targeting the apicoplast's bacterial-like ribosome (157, 160, 161)Dahl, 2007 #331}. D10-PfPHG$^{apicoplast\text{-}null}$ parasites were generated as previously described (157, 161). Briefly, parasites were treated with 0.35 μM (5x IC_{50}) azithromycin and growth was rescued with daily supplementation of 200 μM isoprenoid pyrophosphate (IPP) (NuChem Therapeutics) into culture medium. After a minimum of 6 days (~3 cycles of growth), successful removal of the apicoplast was confirmed by comparing the sensitivity of D10-PfPHG^{wildtype} and D10-PfPHG$^{apicoplast\text{-}null}$ parasites to reducing concentrations of azithromycin in 2 cycle assays (delayed death, 120 hrs), with parasites measured via flow cytometry (section 3.7). D10-PfPHG$^{apicoplast\text{-}null}$ consistently demonstrated a ~64-fold loss of sensitivity against azithromycin compared to D10-PfPHG^{wildtype} (D10-PfPHG$^{apicoplast\text{-}null}$ IC_{50}, 4.5 μM; D10-PfPHG^{wildtype} IC_{50}, 0.07 μM) (**S. Figures 4.1** and **5.1**), confirming the loss of the apicoplast. After removal of the organelle, D10-PfPHG$^{apicoplast\text{-}null}$ parasites were then used in growth inhibitory assays (section 3.6.5). For standard culture and during all growth assays, D10-PfPHG$^{apicoplast\text{-}null}$ parasites were supplemented with 200 μM IPP.

3.2.4 Drug resistant *P. falciparum* lines.

The *P. falciparum* laboratory line, DD2 is a chloroquine, mefloquine and pyrimethamine resistant clonal isolate originating from the W2-MEF line (496, 497), which was selected from the W2 clone of the Indochina III isolate (498-500). Thus DD2, was used to address the activity of analogues in the context of clinically relevant multidrug resistance. DD2 expands ~6-fold after progression though the blood stage lifecycle (~42 hrs).

Artemisinin resistant (Cam3.II$^{DHA\ resistant(R539T)}$) and sensitive (Cam3.II^{sensitive}) Cambodian isolates (226) were kindly provided by Professor Leanne Tilley (University of Melbourne, Australia) and Professor David Fidock (Colombia University, New York, USA). The artemisinin resistant line, Cam3.II$^{DHA\ resistant(R539T)}$, carries an R539T mutation in the *kelch13* gene (K13) that originated from a clinical isolate in the Pursat province of Western Cambodia

(Professor Rick Fairhurst (National Institute of Allergy and Infectious Diseases, Maryland, USA)). The Cam3.II^{sensitive} line was generated from Cam3.II$^{DHA\ resistant(R539T)}$ by reverting the K13 mutation back into a wildtype allele (T539R) by gene editing with Zinc-finger nucleases (226). Cam3.II^{sensitive} Cam3.II$^{DHA\ resistant(R539T)}$ lines were used concurrently to address the activity of drugs in a clinically relevant *in vitro* model of artemisinin resistance (section 3.6.4). Both Cam3.II$^{DHA\ resistant(R539T)}$ and Cam3.II^{sensitive} expand ~5-fold and their standard blood stage lifecycle is ~44 hrs.

3.2.5 *P. knowlesi.*

The zoonotic malaria parasite *P. knowlesi* line used in this study, PkYH1, is a clonal isolate of *P. knowlesi* H_{hu} that was adapted to *in vitro* culture in human RBCs (501). The PkYH1 line was kindly provided by Dr. Michelle Boyle (QIMR Berghofer Medical Research Institute, Australia) and Professor Manoj Duraisingh (Harvard University, USA). As *P. knowlesi* is more closely related to all other human malaria parasites than *P. falciparum* (502), this species has been proposed as a surrogate system of *P. vivax* to explore the activity and resistance mechanisms of antimalarials (503).The standard blood stage lifecycle of PkYH1 occurs over ~32 hrs with this parasite species expanding ~3-fold per replication cycle.

3.3 Maintenance and determination of parasitemia.

Parasites were maintained in general culture at approximately 3% haematocrit equating to 300 µL of RBCs per 10 mL of culture. Percentage (%) parasitaemia ($\frac{number\ of\ parasitised\ red\ blood\ cells}{total\ number\ of\ red\ blood\ cells} \times 100$) was determined by smearing approximately 2-5 µL of infected RBCs (iRBCs) onto a glass microscopy slide (Paul Marienfeld GmbH & Co.), which was then fixed in 100% methanol (Merck) prior to staining in 10% (v/v) Giemsa's azur eosin methylene blue solution (Merck) for 10 minutes (mins). Parasitaemia on dried slides was determined with a light microscope with 1000x magnification (10x eyepiece, 100x objective) under oil immersion, with >500 cells counted for routine culture. Uninfected RBCs (uRBCs), iRBCs and different intraerythrocytic parasite lifecycle stages (ring, trophozoite or schizont, **Figure 3.1**) can be distinguished and quantitated on the Giemsa stained slides. To maintain parasitaemia, a defined proportion of iRBCs were removed (subbed) and fresh media and fresh uRBCs were added to culture as required.

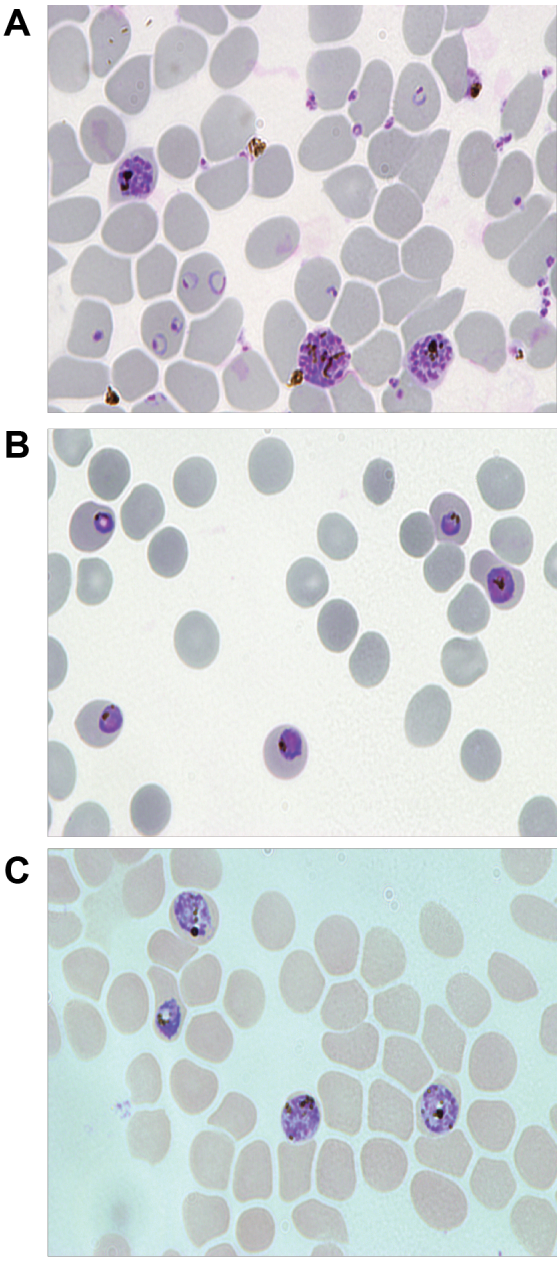

Figure 3.1 Giemsa-stained *P. falciparum* infected RBCs visualised using a light microscope.

As Giemsa stains chromatin, parasites appear blue-purple by light microscopy and as RBCs are a-nucleate, infected RBCs can be differentiated from uninfected RBCs using Giemsa-staining. Within the blood stage, parasites have three distinct phases that can be identified morphologically: A) rupture: early rings (0-6 hrs post invasion) and late schizont stages (40-48 hrs post invasion). B) Trophozoite stage (12-24 hrs post invasion). C) Schizont stages (30-40 hrs post invasion), visualised with 1000X (10X eyepiece 100X objective) magnification.

Culture media was replaced daily (every 24 hrs) and parasitemia was maintained at <5% to ensure health of cultures, unless otherwise stated. For specific parasitaemia and haematocrit values required for assays, iRBCs and uRBCs cells were mixed at a calculated ratio. Firstly, the volume of iRBCs required for the given volume of media was calculated as $\frac{(final\ iRBC\ parasitaemia\ required \times final\ volume\ required)}{Initial\ iRBC\ parasitaemia}$. Secondly, the volume of uRBCs required was calculated as (*final RBC volume required - final iRBC volume*). uRBC and iRBCs were centrifuged for 5 mins at 550 rcf to pellet cells and the volume of packed iRBCs and uRBCs added to make the final haematocrit required in the volume of fresh media required.

3.4 Cryopreservation and thawing of parasites.

3.4.1 Cryopreservation.

Parasite lines that predominantly featured ring stages at a parasitaemia of at least 5% were cryopreserved for future use. Parasite cultures were centrifuged for 5 mins at 550 rcf and the supernatant removed. The resulting pellet was resuspended in 2x packed RBC volume of glycerolyte 47 malaria freezing solution (45% v/v glycerol (Sigma-Aldrich)), 16 mg/mL sodium lactate (Sigma-Aldrich), 0.3 mg/mL potassium chloride, 0.52 mg/mL monosodium phosphate, 1.24 mg/mL disodium phosphate) to ¼ of the culture packed RBC volume added dropwise with agitation. After a 5 min incubation at room temperature, the remaining glycerolyte was added dropwise with agitation until pellet was resuspended in 2x pellet volume. Cell-glycerolyte solutions were then transferred into cryotubes (Thermo Fisher Scientific) in 1 mL aliquots, placed into a foam rack to slow freezing and stored at -80 ºC or in liquid nitrogen for long term preservation.

3.4.2 Thawing parasites.

P. falciparum and *P. knowlesi* cryopreserves were thawed at room temperature prior to gentle resuspension by pipetting and transfer into a 10 mL tube. Parasites were resuspended slowly in 2x pellet volume of 3.5% weight/volume (w/v) sodium chloride solution that was added dropwise with agitation. Cultures were then centrifuged (5 mins at 550 rcf) and the supernatant removed before resuspending the pellet in 2x pellet volume 50/50 mix of the 3.5% w/v sodium chloride solution and Phosphate Buffered Saline (PBS). After re-suspension, the culture was then centrifuged (5 mins at 550 rcf) and the supernatant removed prior to addition of a 2x pellet

volume of 1x PBS. After a final round of centrifugation (5 mins at 550 rcf) the resultant supernatant was removed, and the pellet resuspended into 10 mL of culture medium with up to 300 μL of fresh RBCs prior into a new culture dish.

3.5 Synchronisation of *Plasmodium* parasites *in vitro.*

D10-PfPHG parasites were synchronised predominately with a combination of sorbitol lysis of mature stage parasites and heparin inhibition of merozoite invasion. As DD2, Cam3.II$^{DHA\ resistant(R539T)}$, Cam3.II^{sensitive} and PkYH1 were found to be less tolerant of heparin synchronisation, these lines were synchronised with constant passage over a gradient of 70% Percoll (Sigma-Aldrich) to obtain late stage schizonts that were then allowed to rupture for ~4 hrs prior to ring stage treatment with 5% w/v sorbitol (Sigma-Aldrich), thus enabling effective synchronisation of 0-4 hr old rings. A diagram of each stage; rings, trophozoites and schizonts, can be found in **Figure 3.1**.

3.5.1 Heparin synchronisation.

Heparin is a potent inhibitor of merozoite invasion of RBCs (131) and is used in standard parasite culture to stop and control the majority of merozoite invasion events into the RBC. Thus, an absence of heparin during a short period in of the parasite's lifecycle determines the 'window' of invasion and allows for highly synchronous culture to be achieved. Clinical grade heparin sodium (Pfizer) was added and left in parasite cultures at a concertation of 10 μL/mL immediately after replenishment of culture media (feeding) and left in the culture until late schizonts stage parasites (**Figure 3.1 A** & **C**) were observed. Heparin was then removed from culture via replenishment of fresh media (washing) for the duration of invasion, typically 4-8 hrs (**Figure 3.1 A**). To prevent any further invasion events, heparin was added back into the culture at the end of the invasion window, thus obtaining a tightly synchronised parasite population. To remove late stage parasites and obtain highly synchronous cultures of ring stage parasites, a sorbitol treatment step was introduced to remove late stage parasites and provide a tightly synchronised ring stage culture for further experiments (section 3.6).

3.5.2 Sorbitol synchronisation.

In order to isolate parasites that had recently invaded, sorbitol lysis was used to remove trophozoite or schizont stage parasites (>16 hrs post invasion or older) (**Figure 3.1 B**) and enrich for early ring stage parasites (<16 hrs post invasion or earlier) (504). Briefly, cultures

with ring stage parasites were pelleted via centrifugation for 5 mins at 550 rcf and the supernatant removed. The pellet was then resuspended in pre-warmed (37 ºC) 5% w/v D-sorbitol (Sigma-Aldrich) at 5x pellet volume, before incubation at 37 ºC for 10 or 5 mins for *P. falciparum* and *P. knowlesi*, respectively. Following incubation, cultures were centrifuged for 5 mins at 550 rcf, with the supernatant removed and the cells were resuspended in 10 mL of pre-warmed (37 ºC) culture media. The culture was then either transferred back into a dish/flask or used for other assays after determining parasitaemia.

3.5.3 Magnet activated cell sorting.

As parasites progress though the blood stage lifecycle, haemoglobin is digested and the toxic haem product is converted to inert hemozoin crystals, which contain iron (Fe^{3+}) in the form of magnetic ferriheme molecules (reviewed in (30)). Typically, the hemozoin crystal begins to be visible at >20 hrs post RBC invasion for *P. falciparum* and thus the infected RBC become more magnetic as the parasite progresses though trophozoite stages to schizonts. It is on this basis that late-stage trophozoites and schizonts (**Figure 3.1 A** & **C**) can be purified from whole culture using magnet activated cell sorting (MACS). Magnet purification of late schizont stage parasites was undertaken for the production of purified merozoites. Briefly, two or more 175 cm^2 flasks (Corning) containing high parasitemia culture (>5%) were harvested via resuspension in ~50-60 mL of incomplete media (~30 mLs per flask) (RPMI-HEPES and $NaHCO_3$ only). The culture was then run drop-wise through a MACS size C column (Miltenyi Biotech), prior to washing with 3-5 column volumes of RMPI-HEPES. The column was then removed from the magnet and late-stage parasites were eluted into 60 mL of complete media, prior to separation into two 30 mL culture dishes. The purity of late-stage parasites was confirmed by microscopy analysis of Giemsa stained smears.

3.5.4 Percoll synchronisation.

For obtaining late-stage schizont parasites through Percoll density centrifugation (505, 506), mature schizont stage parasites (**Figure 3.1 A** & **C**) at >3% parasitemia were gently loaded over pre-warmed 70% Percoll (Sigma-Aldrich) and centrifuged for 15 mins at 1200 rcf with slow acceleration and low break. Centrifugation results in two layers; an aqueous top layer and the 70% Percoll layer. Late-stage parasites concentrate at the interface between the aqueous and Percoll layers while the remaining cells (containing rings, mid-trophozoites and uninfected RBCs) form in the pellet. Schizonts at the interface of the aqueous and Percoll gradients were collected and washed in 10 mL of complete media after centrifugation for 5 mins at 550 rcf.

The supernatant was then removed and schizonts were then either added to a set hematocrit volume required to obtain the desired % parasitemia upon rupture or returned into a culture dish at with 2-3% hematocrit RBCs added. Parasites were then allowed to invade for ~3-5 hrs prior to collection and sorbitol treatment of ring stages used for assays.

3.6 Drug susceptibility assays.

To examine the effect of drugs on *Plasmodium* spp. parasite growth, blood stage growth inhibition assays were used, and variations of these assays are outlined in **Figure 3.2.** Modifications to these standard blood stage growth inhibition assays are described below and summarised in the methods section of each respective manuscript chapter (Chapters 4 and 5). All drug dilutions were prepared from stocks on the day of the experiment. The highest concentration of drug was prepared at 10x the starting concentration and serially diluted 2-fold in sterile round-bottom 96-well microtiter plates (Corning), unless otherwise specified. Drugs were then added to the cultures at 10% of the final volume to reach a 1x drug concentration within the 96-well microtiter plate. To minimise non-specific growth inhibitory activity from the vehicle, drugs were added such that starting concentration of the vehicle was diluted >1000-fold for all growth assays. All assays were performed in sterile, round bottom 96-well microtiter plates at a final parasite culture volume of 45 μL. The wells of the outermost rows and columns were filled with 200 μL PBS to stabilize humidity and heat distribution.

3.6.1 Blood stage dose response curves.

All assays were performed with highly synchronous, early ring stage parasites (0-4 hrs post-invasion) (section 3.5) for in cycle, 1 cycle or 2 cycle assays as published previously (131, 165, 493, 507, 508) and described below (**Figure 3.2**). As *P. knowlesi* parasites have a shorter lifecycle (~30 hrs) than *P. falciparum* (~48 hrs) (405, 501, 509, 510), the incubation times for this species were adjusted accordingly.

In cycle growth inhibitory assays were used to address the short-term effects (quick-killing) of an inhibitor from early rings to schizonts (44 hrs post-invasion for *P. falciparum* and 28 hrs post-invasion for *P. knowlesi*, **Figure 3.2 B**). As parasites do not have a chance to multiply during this time period, all in cycle assays were set up at 2-3% parasitemia and 1% haematocrit to enable accurate detection via flow cytometry.

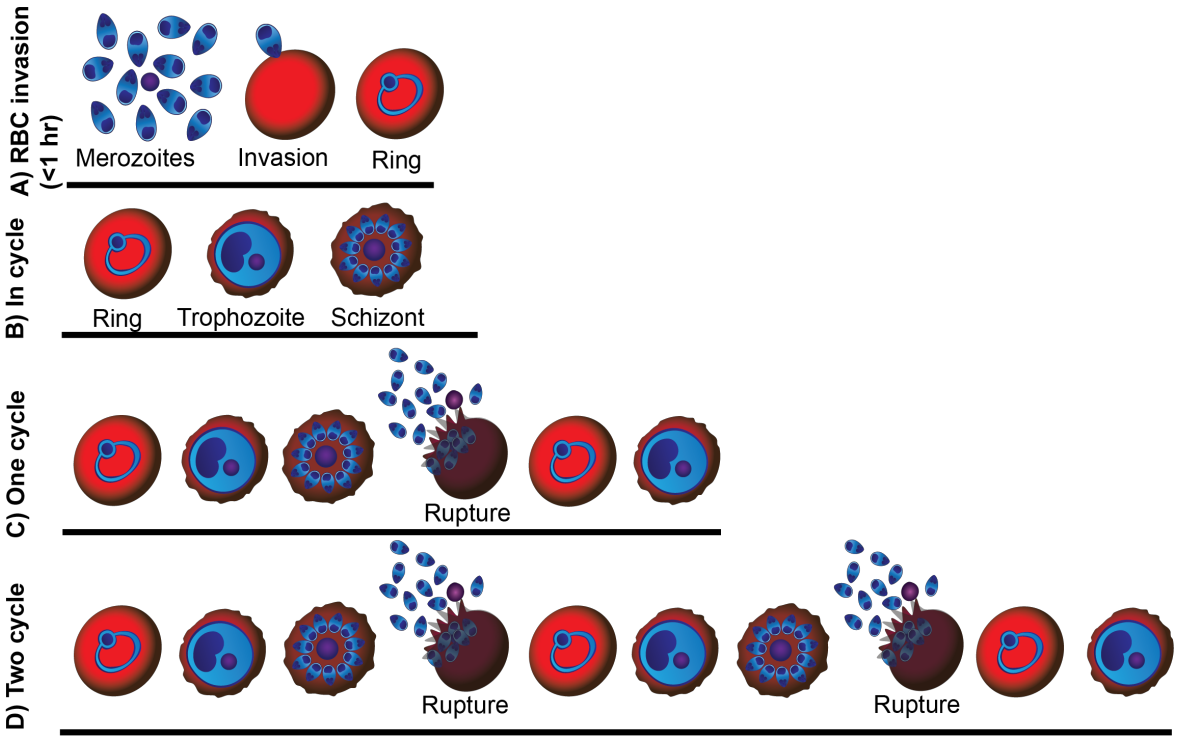

Figure 3.2 Schematic of drug treatment regimens outlining the times of treatment and stage/time of parasitaemia measurement for assays used in this study.

A) Merozoite invasion of RBCs: Merozoites were drug treated prior to addition of RBCs. RBC invasion was measured at early ring stages (<1 hr rings). **B**) In-cycle: highly synchronous, early-ring stage parasites (0-4 hrs post-invasion) were treated with drug, with the resulting growth inhibition analysed at schizont stage (44 hrs post-invasion for *P. falciparum* and 26 hrs for *P. knowlesi*). **C**) 1 cycle: highly synchronous, early ring stage parasites (0-4 hrs post-invasion) were drug-treated and the resulting growth inhibition was measured after one cycle of re-invasion, at schizont stages (72 hrs post-invasion for *P. falciparum* and 58 hrs for *P. knowlesi*). **D**) 2 cycle (delayed death); highly synchronous, early ring stage parasites (0-4 hrs post invasion) were drug-treated and the resulting growth inhibition measured at schizont stage after 2 rupture cycles (120 hrs post-invasion for *P. falciparum* and 78 hrs for *P. knowlesi*).

This figure is adapted from Chapter 4 (**Figure 4.1**).

One cycle (1 cycle) growth assays are a standard assay format to assess growth inhibition for an inhibitor (72 hrs and 58 hrs post-invasion for *P. falciparum* and *P. knowlesi*, respectively, **Figure 3.2 C**) (131, 493, 507, 508). 1 cycle assays were set up at 1% parasitemia and 1% haematocrit to allow parasite multiplication without the risk of exhausting the culture media (crashing) due to too many parasites.

To address the apicoplast-targeting 'delayed death' activity of azithromycin and analogues, two cycle (2 cycle) growth assays (120 hrs and ~78 hrs post-invasion for *P. falciparum* and *P. knowlesi* respectively, **Figure 3.2 D**, (130, 165, 508) were used. As parasites expand up to 30-fold across 2 cycles of growth, these assays were set up at 0.1 to 0.2% parasitemia and 1% haematocrit.

3.6.2 Growth inhibition assays under normoxic conditions.

The antiparasitic activity of hypoxia activatable prodrugs (511) and their parental compounds were assessed in parallel for both standard culture (hypoxic) conditions (1% O_2, 4% CO_2 and 95% N_2) and hyperoxic conditions provided by a standard CO_2 incubator (21% O_2, 5% CO_2 and 74% N_2). Preliminary experiments suggested that growth of parasites under normoxia (512, 513) was slightly slower, but this affect was reduced when using parasites pre-adapted for growth under hyperoxic conditions (normoxia).

3.6.3 Blood stage pulsed drug treatments.

To identify the life-stage specificity of an inhibitor during *P. falciparum*'s blood stage lifecycle, a respective drug was added and/or removed at stringent time points. The inhibitor was first added at the defined life-stage; early rings (0-6 hrs post invasion**)**, late rings (0-12 hrs post invasion), early trophozoites (12-24 hrs post invasion), late trophozoites/early schizogony (24-36 hrs post invasion) and late-schizogony to rupture (36-44 hrs post invasion). To remove drug, parasites were washed by adding 200 µl of complete medium, prior to centrifugation at 300 rcf for 2 mins and removal of supernatant (washing). Plates were then washed 2 more times in 200 µl of complete medium, before 200 µl of complete medium was added to cultures prior to incubation, ensuring dilution of the drug to well below inhibitory levels (131, 165, 493).

3.6.4 Ring-stage survival assays ($RSA_{0\text{-}3h}$) to measure artemisinin resistance.

To test the efficacy of drugs in the context of artemisinin resistance, *in vitro* ring stage survival assays ($RSA_{0\text{-}3h}$) were performed as described previously, with minor modification (222, 226,

514, 515). Briefly, Cam3.II$^{\text{DHA resistant(R539T)}}$ and artemisinin sensitive Cam3.II$^{\text{sensitive}}$ late schizont stage parasites (section 3.2.4) were tightly synchronised over a gradient of 70% Percoll prior to washing in complete medium followed by incubation with fresh RBCs (~1% haematocrit) to allow invasion for 3 hrs (section 3.5.4). Cultures were then treated with sorbitol to obtain 0-3 hrs old rings and to eliminate the remaining schizonts (section 3.5.2). Early-ring stages were then counted and adjusted to 1% parasitemia and 1% haematocrit prior to exposure to a dilution series of the desired drug (e.g. DHA) for 4 hrs. The inhibitor was then washed out using a stringent washing procedure that involved five complete washes with 200 µl of complete media (centrifuged at 300 rcf for 2 mins) and transferred to a new 96-well plate to ensure complete removal of DHA (515). Parasites were then incubated in standard culture conditions for an additional 66 hrs before parasitemia was assessed by flow cytometry at ~70-72 hrs post-invasion (1 cycle, **Figure 3.2 C**).

3.6.5 Apicoplast-null inhibitory assays.

The inhibitory activity of azithromycin or analogues was assessed for D10-PfPHG$^{\text{apicoplast-null}}$ parasites, generated as described in section 3.2.3, were grown in the presence of 200 µM IPP with growth inhibition assessed in parallel with D10-PfPHG$^{\text{wildtype}}$ parasites. Briefly, sorbitol synchronised PfPHG$^{\text{apicoplast-null}}$ and PfPHG$^{\text{wildtype}}$ parasites (4 hr window of synchronisation, section 3.5.1 and 3.5.2) were grown at the respective 90% inhibitory concentrations (IC_{90}) obtained previously for D10-PfPHG$^{\text{wildtype}}$ parasites for both in cycle and delayed death assays. Assays were incubated for either ~44 hrs (in cycle, **Figure 3.2 B**) or 120 hrs (delayed death, **Figure 3.2 D**) with parasitemia determined by flow cytometry. To determine the IC_{50} against PfPHG$^{\text{apicoplast-null}}$ and PfPHG$^{\text{wildtype}}$ parasites, 2-fold reducing concentrations of the desired inhibitor were added as previously described (section 3.6)

3.6.6 *In* vitro synergy experiments.

I*n vitro* synergy experiments were used to address whether two different drugs could act together 'synergistically' and alter the potency of one or both drugs in combinations. For the purpose of this experiment, 'synergistic' activity refers to one agent increasing the potency of the another when used in combination; 'antagonism' refers to when the mixing of two drugs leads to reduced potency of one or both compared to their individual activities; and 'additive' is used to describe when there is no measurable improvement or loss of drug activity in combination. Estimation of drug synergy was performed according to previously established protocols (516). Briefly, serial dilutions were designed so the IC_{50} of each drug fell at

approximately the fourth serial dilution (middle of dilution series). Each drug was tested alone (5:5) and in combination with a respective partner drug in a fixed ratio dilution series (control/test drug ratios; 4:1, 3:2, 2:3, 1:4). Parasites were incubated with drug for 1 cycle (72 hrs, **Figure 3.2 C**) and parasitemia was determined by flow cytometry. The data collected from these assays were used to construct isobolograms to determine the nature of the interaction between two drugs. From this data the fractional inhibitory concentration (FIC) was calculated for each drug combination with the mean sum of the FIC_{50} of each combination with values =1 indicating additive activity, >1 antagonism, <1 synergy. FIC of drug A (FIC_A) and FIC_B was calculated (i.e. for FIC_A (IC_{50} drug A) = IC_{50} (drug A in the presence of x concentration of drug B) / IC_{50} (drug A alone)). The FIC_A and FIC_B were then plotted at each diluted drug combination to create an isobologram. Combinations where data points plot as a straight line indicate an additive drug effect (i.e. FIC = 1), a convex line indicates antagonism (FIC > 1) and concave indicates synergy (FIC < 1).

3.6.7 Merozoite invasion inhibitory assays.

To identify the invasion inhibitory activity of a drug, viable merozoites were purified for use in inhibitory assays as previously described (131, 165, 517). Briefly, 2 or more 175 cm^2 flasks (~300 mL) of D10-PfPHG parasites, 3% haematocrit, 4-5% parasitaemia, were tightly synchronised to a 6 hr window of invasion with heparin (section 3.5.1). At 40-46 hrs post-invasion, schizonts were separated from uninfected RBCs and early-stage parasites via magnet purification though a MACS column (Miltenyi Biotechnology) (section 3.5.3) and eluted into ~60 mL of complete media (2 x ~30 mL dishes per flask) with a final concentration 10 µM of E64 (Sigma-Aldrich) (10 µL of E64 (at 10 mM) per 10 mL), an inhibitor of schizont rupture, was added to each 30 mL dish. Schizonts were then incubated in standard culture conditions for ~5 hrs, the parasites centrifuged at 850 rcf for 10 mins, the supernatant removed, and the pellet gently resuspended in ~1.5-2.5 mL of incomplete media (RPMI-HEPES and $NaHCO_3$, no serum). Resuspended parasites were transferred into a 10 mL syringe prior to filtration through the pre-connected 1.2 µm syringe filter (Minisart, Sartorius stedim). The purified merozoites (22.5 µl) were then aliquoted to a 96-well round-bottom plate that contained 2.5 µl of 10x inhibitor concentration. Invasion was then allowed to proceed through addition of 10 µl uninfected RBCs, bringing the final haematocrit to 0.5%. The plates were agitated at 400 rpm for 10 mins at 37 ºC to allow invasion and newly invaded ring stages were then treated with 5 µg/mL EtBr for 10 min prior to washout and quantitation by flow cytometry (section 3.7).

3.6.7.1 Merozoite washout assays.

Viable D10-PfPHG free merozoites were purified as described above (131, 517). For drug washout, 90 µl of purified merozoites were added into 10 µl of either incomplete media (no serum) or incomplete media plus drug (10 x final concentration) before transfer to a 0.22 µm Ultrafree-MC centrifugal filter (Thermo-Fisher). Filter columns were centrifuged at 750 rcf for 1 minute and washed with 500 µL incomplete media twice. Free merozoites were then resuspended off the filter by pipetting in 45 µl of incomplete media and transferring to 96-well round-bottom plates containing 5 µl of RBCs at 1 % haematocrit (final haematocrit of 0.1 %). In parallel to these washed merozoites, unwashed controls were also set up whereby purified merozoites were simply resuspended in the respective drug and placed directly into the 96-well round-bottom plate without column washing. Plates were agitated at 400 rpm for 10 mins at 37^{o}C to allow invasion and then cultures were incubated at 37 ^{o}C for 30 mins before being washed in 1 x PBS three times to remove uninvaded merozoites. Newly invaded ring stage parasitemia was then measured by flow cytometry (section 3.7) with cells treated with 5 µg/mL EtBr for 10 mins prior to washout.

3.7 Assessment of parasite growth inhibition using flow cytometry.

The protocol for measuring drug inhibition of parasite growth has been described previously (131, 493, 517). Briefly, mature (>36 hrs post-invasion) *P. falciparum* D10-PfPHG parasites were stained with 10 µg/mL of EtBr for 1 hr in a light proof container. The plates were then centrifuged at 300 rcf for 2 mins and the supernatant was removed before final resuspension in 150 µl of 1x PBS. Parasites were then resuspended prior to measurement of parasitaemia on an LSR Fortessa (Becton Dickinson) with an attached 96-well plate reader. All mature (>36 hrs post-invasion) D10-PfPHG parasites were counted with both GFP (GFP; excitation wavelength, 488 nm) and EtBr (Fl-2-high (EtBr; excitation wavelength, 488 nm) gating (**Figure 3.3 B**). D10-PfPHG ring stage parasites (<6 hrs post invasion) from the invasion inhibition assays were stained with 5 µg/mL EtBr for ~10 min prior to washout in 100 µl of 1x PBS and analysis with both Fl-1-high (GFP) and Fl-2-low (EtBr) gates (131, 517) (**Figure 3.3 C**). As the other lines lack GFP, mature stage DD2, Cam3.II$^{\text{DHA resistant(R539T)}}$, Cam3.II$^{\text{sensitive}}$ and PkYH1 parasites were counted with EtBr staining; forward scatter (FSC) and FL-2-high (EtBr) gate (131) (**Figure 3.3 D**). Typically, 20,000-40,000 RBCs were counted in each well and each drug treatment was tested in duplicate wells for each experiment.

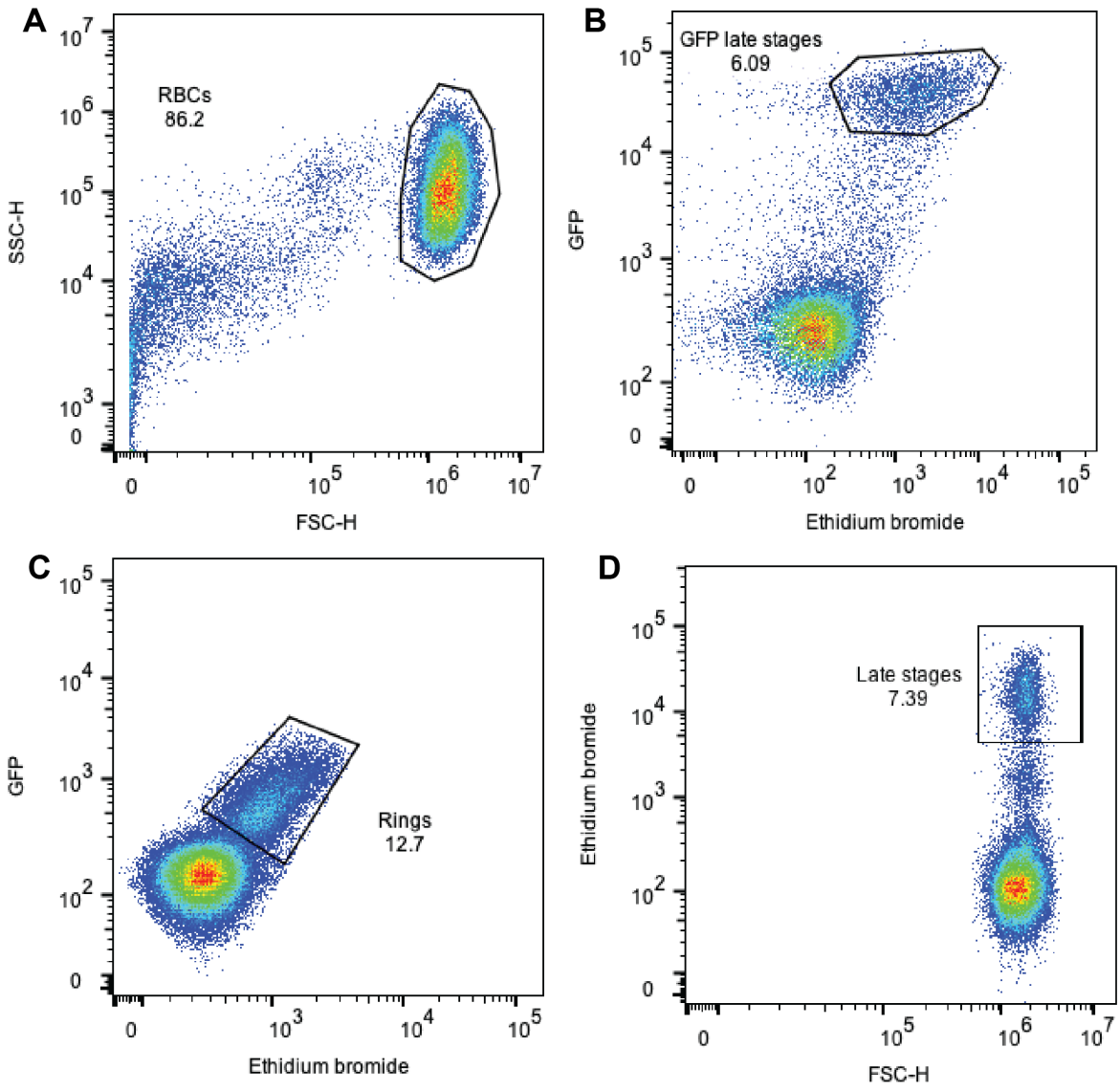

Figure 3.3 Representative gating strategies used to distinguish different life stages of *Plasmodium* parasites.

Representative flow cytometry plots for invasion and growth assays. FlowJo Version 10 was used to analyse all flow cytometry results and all axes were adjusted to log scales. **A**) An initial gate was placed the population of RBCs that were identified in the side scatter (SSC-H) and forward (FSC-H) channels. All parasites were stained with the nuclear stain, ethidium bromide (EtBr), to differentiate between uninfected and infected RBCs. **B**) GFP fluorescent late stage parasites were gated on GFP^{high} and $EtBr^{high}$ populations **C**) while ring stage parasites were gated on GFP^{high} and $EtBr^{low}$ gates. **D**) Non GFP fluorescent parasites were gated on $EtBr^{high}$ and FSC-H populations.

Parasite growth was determined from the parasitaemia counts obtained via flow cytometry. All samples were analysed using FlowJo software Version 10 (TreeStar Inc, Ashland, OR, United States of America) and growth of drug treatments were normalised against growth in >6 non-inhibitory (media) control wells that represented maximal parasite growth in each independent plate.

3.8 Light microscopy analysis of drug susceptibility.

To confirm the growth inhibitory effects of drugs on parasites, phenotypic effects were assessed by light microscopy smears (**Figure 3.1**). Briefly, thin smears fixed with fresh methanol and stained in fresh 10% v/v Giemsa (Merck) for 10 mins. Photos of smears were taken using an Olympus BX51/BX52 light microscope with 1000x magnification (100x lens + 10x eyepiece) under oil immersion (image resolution 300x300). The photos were not altered in any way, but regions of interest were cropped for use.

3.9 Estimation of IC_{50}, IC_{90} and statistical significance.

All 50 and 90 percent (%) inhibitory concentrations (IC_{50} and IC_{90}, respectively) and 50% cytotoxicity concentrations (CC_{50}) were determined for each drug using GraphPad Prism (GraphPad Software Inc) according to the recommended protocol for nonlinear regression (constrained to top= 100 and bottom= 0) of a log-(inhibitor)-versus-response curve. Statistical significance between drug treatments were determined with the GraphPad Prism software using the log-(inhibitor)-versus-response curve with Extra Sum-of-Squares F Test (best-fit LogIC_{50}). *P* values were considered significant if P=<0.05.

3.10 *In vitro* resistance selection.

In efforts to identify the secondary mechanism of action of azithromycin analogues, we attempted to generate a *P. falciparum* resistant line as previously described in (160), with modification. *In vitro* drug resistance selection was carried out using a *P. falciparum* line that had validated resistance to azithromycin's delayed death activity (2 cycle, **Figure 3.2 D**) (D10-AZR^r) (165).

D10-AZR^r features a G91D mutation in the apicoplast ribosomal gene, *rpl4*, which is associated with ~57-fold loss of sensitivity to azithromycin's delayed death activity. I used the potent quick-killing azithromycin analogue, GSK-59, to select resistance as this drug has a modification on the desosamine sugar that blocks delayed death activity. Using both the D10-AZR^r and an analogue incapable of blocking apicoplast ribosomal translation, it was expected that selection of resistance against azithromycin analogues quick-killing mechanism of action could be undertaken in the absence of apicoplast targeting delayed death activity.

D10-AZR^r parasites were exposed to fixed concentrations of the azithromycin analogue, GSK-59 at 3x IC_{50} (GSK-59, 0.13 μM) for three days with daily feeding. Drug concentration was then increased to 5x IC_{50} (GSK-59, 0.4 μM) and maintained at this level for 4 days with daily feeding. The following 2 days, D10-AZR^r parasites were treated at the 3x IC_{50} and fed daily. Parasites were examined every 2 to 3 days to via Giemsa-stained thin blood films to check for viable parasites. After treatment, parasites were fed once every 72 hrs unless rings were seen in smears and once a week 30-40% of culture was replaced with fresh RBCs. Drug treated cultures were maintained for a minimum of 3 months (~90 days) and up-to a maximum of 5 months (~121 days).

3.11 Metabolomics.

3.11.1 Sample extraction for metabolomics analysis.

For all metabolomics experiments, two 150 mL flasks at 6% haematocrit containing tightly synchronised parasites 28-34 hr post-invasion (5-6 hr rupture window), were harvested via magnet purification (section 3.5.3). Infected RBC density was then quantitated as previously described (518). Briefly, the resulting schizonts from magnet purification were diluted by a factor of 50 into a solution of 1x PBS and 1/50 dilution of CountBright Absolute Counting Beads for Flow Cytometry (Invitrogen, Thermo Fisher Scientific) prior to staining with EtBr. The exact count of parasites and beads was counted using flow cytometry and the number of parasites in the volume of parasite material were quantitated using FlowJo Software (TreeStar) as previously described (section 3.7).

For the drug treated parasites, 2 mL of $3x10^7$ parasites were then added into sterile flat bottom, 24 well microtiter plates (Corning) and incubated for 1 hr at 37^{o}C to stabilise the culture. Following this initial incubation, 5x IC_{50} of each drug treatment (DHA, chloroquine,

azithromycin, and azithromycin analogues) and a control (ethanol) of untreated infected RBCs were added in triplicate and incubated for a further 2 hrs. Supernatant was removed and parasites were washed twice with 800 µL ice-cold 1x PBS to avoid changes in parasite metabolites, with cells pelleted via centrifugation at 400 rcf for 5 mins at 4 ºC. Cell pellets were resuspended in 150 µL of ice-cold extraction buffer (methanol containing 1 µM of internal standard compounds, CHAPS (3-(cyclohexylamino)-1-propanesulfonic acid) and PIPES (1,4- piperazinediethanesulfonic acid), was incubated on ice for 1 hr with shaking at 200 rpm. The resulting cell debris was pelleted with centrifugation at 4,000 rcf for 10 mins at 4 ºC. 120 µL of supernatant was transferred to unautoclaved Eppendorf tubes and the remaining ~20 µL were combined to generate a pooled biological quality control (PBQC) sample for analytical quality control (QC). Samples stored at -80 ºC until analysis and shipped on dry ice to the laboratory of Associate Prof. Darren Creek, Monash University. All experiments were performed on two independent occasions.

3.11.2 Metabolomics Liquid chromatography-mass spectrometry analysis.

Liquid chromatography-mass spectrometry (LC-MS) data was acquired on Q-Exactive Orbitrap mass spectrometer (Thermo Scientific) coupled with high-performance liquid chromatography (HPLC) system Dionex Ultimate® 3000 RS (Thermo Scientific) as previously described (312). Briefly, chromatographic separation was performed on ZIC-pHILIC column (5 µm, 4.6 × 150 mm, SeQuant®, Merck) equipped with a guard (ZIC-pHILIC). The mobile phase (A) was 20 mM ammonium carbonate (Sigma Aldrich, MO, USA) and (B) acetonitrile (Burdick and Jackson, Muskegon, MI, USA), needle wash solution was 50% v/v isopropanol. The gradient program started at 80% B and was decreased to 50% B over 15 min then to 5% B until 18 min, kept at 5% B until 21 min, returned to 80% B until 24 min and equilibrated at 80% B until 32 min. The flow rate of 0.3 mL/min and column compartment temperature 25 ºC. The total run time was 32 min with an injection volume of 10 µL. Mass spectrometer operated in full scan mode with positive and negative polarity switching at 35k resolution at 200 m/z with detection range of 85 to 1275 m/z, AGC target was 1e6 ions, maximum injection time 50 ms. Electro-spray ionization source (HESI) was set to 4.0 kV voltage for positive and negative mode, sheath gas was set to 50, aux gas to 20 and sweep gas to 2 arbitrary units, capillary temperature 300 °C, probe heater temperature 120 °C. The samples were analyzed as a single batch to reduce the batch-to-batch variation and randomized to account for LCMS system drift over time.

3.11.3 Data processing using IDEOM.

The acquired LCMS data was processed in an untargeted fashion using open source software IDOEM (519) (http:// mzmatch.sourceforge.net/ideom.php), which initially used *ProteoWizard* to convert raw LC-MS files to *mzXML* format and *XCMS* to pick peaks to convert to *.peakML* files (520). *Mzmatch.R* was subsequently used for the alignment of samples and the filtering of peaks using minimum detectable intensity of 100,000 relative standard deviation (RSD) of <0.5 (reproducibility), and peak shape (codadw) of >0.8. *Mzmatch* was also used to retrieve missing peaks and annotation of related peaks. Default IDEOM parameters were used to eliminate unwanted noise and artefact peaks. Loss or gain of a proton was corrected in negative and positive ESI mode, respectively, followed by putative identification of metabolites by accurate mass within 3 ppm mass error searching against the Kyoto Encyclopedia of Genes and Genomes (KEGG), MetaCyc, and LIPIDMAPS databases and others. To reduce the number of false positive identifications, retention time error was calculated for each putative ID using IDOEM build-in retention time model which uses actual retention time data of authentic standards (~350 standards). Statistical analysis on filtered data was performed using the Matboanalyst web interface (521).

3.12 Haemoglobin fractionation.

The haemoglobin fractionation assay was adapted from (522). Aliquots of 6.5 ml of 30-32 hrs post invasion parasite cultures were adjusted to 8% parasitaemia and 2% haematocrit and then incubated with chloroquine, GSK-66, GSK-71 or ethanol (vehicle control) for 5 h. Treatments were performed in triplicate. Following incubation, the media was aspirated off and the culture was incubated with 2.3 mL of 0.1% saponin in 1 x PBS with protease inhibitors (complete mini protease inhibitor cocktail (Roche)) for 10 min at 4 °C in order to lyse the iRBCs. The parasites were washed three times with PBS and stored at -80°C.

For the haemoglobin fractionation, lysed parasites were resuspended in 50 µL of Milli-Q water and sonicated for 5 min in a water bath sonicator. Following sonication, 50 µL of 0.2 M of HEPES (pH 7.5) was added and the samples were centrifuged at 4,000 rpm for 20 min. The supernatant containing the haemoglobin fraction was carefully transferred to new tubes and 50 µL of 4% v/v sodium dodecyl sulphate (SDS) was added before the samples were incubated at 95 °C for 5 min. Following heating, 50 µL of 0.3 M sodium chloride (NaCl) and 50 µL of 25%

v/v pyridine (Sigma) in 0.2 M HEPES was added, the samples were vortexed and transferred to a 96 well plate. This sample contained the haemoglobin fraction.

The pellets were treated with 50 µL of MilliQ water and 50 µL of 4% v/v of SDS and resuspended before being sonicated for 5 min and incubated at 95°C for 5 min in order to solubilise the free haem. Following incubation, 50 µL of 0.2 M HEPES, 0.3 M NaCl and 25% v/v pyridine were added to the samples. The samples were then subsequently centrifuged at 4,000 rpm for 20 min. The supernatant was transferred to the 96 well plate, corresponding to the free haem fraction.

The remaining pellet containing the hemozoin fraction was solubilised by resuspending with 50 µL of MilliQ water and 50 µL of 0.3 M sodium hydroxide (NaOH). The samples were sonicated for 15 min before 50 µL of 0.2 M HEPES, 0.3 M hydrochloric acid (HCl) and 25% pyridine was added. The samples were then transferred to the 96 well plate, corresponding to the hemozoin fraction. The total amount of haem in each fraction was quantified using a haem standard curve prepared from a 100 µg/mL standard solution of hematin in 0.3 M NaOH. Serial dilution of the standard curve was carried out in a 96 well plate in triplicate and 50 µL of 0.2 M HEPES, 4% v/v SDS, 0.3 M NaCl, 0.3 M HCl and 25% v/v pyridine was added. The absorbance of the standard curve and each fraction was measured at a 405 nm wavelength using a Perkin Elmer Ensight Plate Reader. The samples were normalised via a paired analysis to the ethanol control and graphed as their fold change vs ethanol ± standard error of the mean (SEM). All fractions had >2 replicates from 2 independent experiments

3.13 Assessment of ubiquitinated proteins.

The accumulation of ubiquitinated proteins upon treatment of proteasome inhibitor-like compounds was addressed as described previously (370). Briefly, a 2 mL cultures of late stage D10-PfPHG trophozoites (26-34 hrs post invasion) at >4% parasitaemia, 3% Haematocrit, were incubated for 1.5 hrs in standard culture conditions with 10x IC_{50} concentration of the inhibitor, with this concentration determined previously for MG-132 (0.350 µM), proteasome inhibitor compounds (PI-1, 0.065 µM; PI-2, 0.23 µM; PI-3, 0.44 µM; PI-5, 0.22 µM) for the D10-PfPHG^{wildtype} line. 5 nM of WR99210 was used as a control treatment.

Drug treated cultures were transferred to a 10 mL tube and centrifuged at 440 rcf for 5 mins. The supernatant was removed and the resulting pellet treated with ~2 mL of 0.15% w/v saponin

with CØmplete (Roche) protease inhibitor tablet prior to being transferred to a 2 mL tube and incubated on ice for 10 mins. The pellet was then spun using a benchtop centrifuge at 6000 rcf for 3 mins at 4 ºC, after which the resultant supernatant was removed. A 50/50 mix of the saponin and 1x PBS was used to remove any remaining RBCs and wash the pellet, followed by two additional washes in PBS until the supernatant appeared colourless. The final supernatant was removed and the resulting pellet containing parasites was snap frozen at -80 °C.

Parasite pellets for Western Blot analysis of ubiquitinated parasite proteins were resuspended in reducing sample buffer (0.125 M Tris hydrochloride pH 7, 4% v/v, SDS, 20% v/v glycerol, 10% v/v β-mercaptoethanol (Sigma-Aldrich), 0.002% w/v bromophenol blue (Sigma-Aldrich)) and separated by size using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) 4-12% Bis-Tris Gels (Bolt, Invitrogen) at 110 V for 60 min. Proteins were then transferred to a nitrocellulose membrane (iBlot, Invitrogen) at 20 V for 7 mins, before blocking the membrane for one hour at room temperature in 1% skim milk powder in 0.05% Phosphate buffered saline tween (PBST) blocking buffer. Primary antibodies (mouse PD41 anti-Ubiquitin (1:4000, Santa Cruz Biotech), rabbit anti-GAPDH (a gift from Dr Matthew Dixon, University of Melbourne)) were incubated with the membrane for one hr at room temperature with blocking buffer (1% skim milk powder in 0.05% PBST), before washing three times in 0.05% v/v PBST. Secondary (IRDye ® 800CW goat anti-mouse (1:4000, LI-COR Biosciences), IRDye ® 680RD goat anti-rabbit (1:4000, LI-COR Biosciences)) antibodies were incubated with membranes for 1 hr at room temperature followed by washing twice in 0.05% v/v PBST and finally in PBS to remove unbound secondary fluorescent antibodies. Western Blots were visualised using an Odyssey Infrared imaging system (LI-COR Biosciences). Western blot quantification was performed using Image Studio Lite 5.2.5 (LI-COR Biosciences).

3.14 Antibacterial screen.

Minimum inhibitory concentration (MIC) assays for assessment of *Streptococcus pneumoniae* sensitivity to azithromycin and analogues were performed as described in (523). Antibacterial activity of 2-fold serial dilutions of azithromycin and lead analogues were compared to media controls in the macrolide sensitive *S. pneumoniae* (strain D39) bacteria and grown in Mueller Hinton Broth supplemented with 5% v/v lysed horse blood. All assays were performed in

sterile, round bottom 96-well microtiter plates (Costar), sealed with a breathable seal and incubated overnight for 18-20 hrs at 37 °C in an ambient air incubator prior to manual assessment of drug inhibition. The minimal inhibitory drug concentration, defined as the lowest drug concentration that showed clear inhibition of bacterial growth, was indicated by a media colour change. Minimum inhibitory concentrations (MIC) are expressed as μM.

3.15 Cytotoxicity drug screens.

3.15.1 Standard culture of mammalian cells.

Huh-7D cells ((Sigma-Aldrich), kindly provided by Dr. Nick Eyre, University of Adelaide), were grown in 75 cm^2 culture flasks (Corning) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific) media supplemented with 10% Foetal Bovine Serum (FBS) (Sigma-Aldrich) and non-essential amino acids (Sigma-Aldrich) and were grown in 5% CO_2 within a 37 °C incubator. Media was renewed 2-3 times a week or as needed according to the media's pH indicator. As Huh-7D cells double every 24 hrs, cell cultures were maintained between 30-90% confluency, but did not exceed 90% confluency, in order to minimise exhaustion of culture media. For standard culture, the media was removed, and cells were rinsed with pre-warmed 1x PBS (37 °C) to remove the remining media and FBS. To detach cells, ~3 mLs of trypsin-EDTA (Gibco, Thermo Fisher Scientific) was added to cover the base of the flask and the cells were incubated at 37 °C for ~10 mins. After detachment was observed, trypsin-EDTA was neutralized with the addition of 4x volume of culture media and the cells were centrifuged at 100 rcf for 2 mins. The supernatant was then removed, cells were gently resuspended in a desired volume of growth medium. To quantitate the volume and concentration of cells required for cytotoxicity assays, a 1:1 dilution of Huh-7D starter culture and trypan blue (Thermo Fisher) were mixed and loaded into a Countess cell counting chamber slides (Invitrogen) and counted using an automated haemocytometer, 'Countess' (Invitrogen). After quantitating the total number of cells, number of live cells, number of dead cells and % viability, Huh-7D cells were resuspended into fresh culture medium for further growth or diluted as required for use in cytotoxicity assays.

3.15.2 Cytotoxicity screens of compounds.

To address the toxicity of a drug in mammalian cells, the CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used as per the manufacturer's instructions. Simply, this method

determines the number of viable ATP producing mammalian cells in culture through detection of ATP by chemi-luminescence. As few as ~100 viable cells can be detected using this method. Huh-7D cells were only used for cytotoxicity assays if % viability was >85%. For the assay, 90 µl of culture containing ~10,000 Huh-7D cells were aliquoted into each well of a sterile, flat bottom, 96-well plate (Corning) and incubated for 1 day (24 hrs) in a 37 °C incubator at 5% CO_2 to allow recovery, thus expanding the cell population to ~20,000 cells per well prior to addition of drug. All drug dilutions were prepared from stocks on the day of the experiment and the highest concentration of drug was prepared at 10x the desired starting concentration and serially diluted 2-fold in a sterile, round bottom 96-well microtiter plate (Corning). A 1/10 dilution of drug at the required concentration was added into each well and the plate incubated at 37°C in 5% CO_2 for 24 hrs, expanding to 40,000 cells per well. After incubation, cells were lysed with a 1:1 addition of CellTiter-Glo® Reagent (Promega), thus releasing the ATP that interacts with a recombinant luciferase present in the CellTiter-Glo® Reagent, to produce luminescence. Plates were then shaken for 2 mins and incubated at room temperature for 10 mins to allow stabilization of the luminescent signal (**Figure 3.4**).

As the amount of ATP is directly proportional to the number of metabolically active (living) cells present in each well, measurement of the luminescent signal as relative luminescent units (RLU) provides the viability of the cells in each well. To obtain the luminescent signal, plates were scanned on a Phera Star FS (BMG Labtech, Germany) using the luminescent module (Lum Plus, spectral wavelength 230 nM to 750 nM), according to the manufacturer's instructions. The luminescent output was obtained by normalising the drug treated wells against the non-inhibitory control (media) control wells (100% cells) for each independent plate and calculated as percentage RLU (% viability). The 50 percent (%) cytotoxicity concentration (CC_{50}) was determined for each drug using the GraphPad Prism software as previously described (section 3.9).

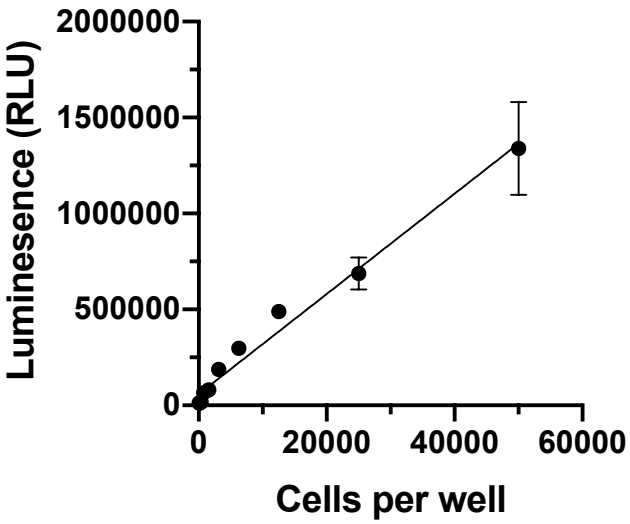

Figure 3.4 Cell number correlates with luminescent output.

There is a direct linear relationship (r^2=0.98) between the luminesence singnal measured and the number of cells (0-40,000 per well). Two-fold serial dilituions of Huh-7D cells within a 96-well plate within DMEM and 10% FBS luminesence recorded 10 mins after a 1:1 additon of CellTiter-Glo® Reagent. Values represent the of SEM of three for each cell number.

Chapter 4. Retargeting the antibiotic azithromycin as an antimalarial with dual modality.

4.1 Statement of authorship.

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Statement of Authorship

Title of Paper	Retargeting the antibiotic azithromycin as an antimalarial with dual modality
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☒ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Amy L. Burns, Brad E. Sleebs, Benjamin Liffner, Richard Harvey, James Paton, James G. Beeson, Christopher D. Goodman, Ghizal Siddiqui, Dovile Anderson, Darren Creek, Geoffrey I. McFadden, Danny W. Wilson.

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Contribution to the Paper	Conceived, designed and performed experiments, analysed results and co-wrote the manuscript.
Overall percentage (%)	62.5
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Determined structural activity relationship for all analogues and co-wrote the manuscript. Contribution 5%
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4.2 Abstract.

Resistance to front-line antimalarial (artemisinin combination therapies) is spreading, and development of new drug treatment strategies to rapidly kill *Plasmodium* parasites that cause malaria are urgently needed. Here, we show that azithromycin—a clinically used macrolide antibiotic that targets the bacterium-like ribosome of the malaria parasites apicoplast organelle and causes a slow-killing 'delayed death' phenotype—can also rapidly kill parasites throughout blood stages of the lifecycle via a 'quick-killing' mechanism of action. Investigation of 84 azithromycin analogues revealed nanomolar quick-killing potency that is directed against the very earliest stage of parasite development within red blood cells. Indeed, the best analogue exhibited ~1600-fold higher potency than azithromycin for a treatment window of less than 48 hours. Analogues were also effective against the zoonotic malaria parasite *P. knowlesi*, and against both multi-drug and artemisinin resistant *P. falciparum* lines. Metabolomic profiles of azithromycin analogue treated parasites had similarities to those of chloroquine treated parasites, suggesting that the quick-killing mechanism of action may in part be localised to the parasite food vacuole. However, metabolomic signatures associated with mitochondrial disruption were also present. In addition, unlike chloroquine azithromycin and analogues were active across all stages of blood stage development, including merozoite invasion, suggesting these macrolides have a multi-factorial mechanism of quick-killing activity. The siting of functional groups added to azithromycin and its quick-killing analogues altered their activity against bacterial-like ribosomes but had minimal change on quick-killing activity, which suggests that apicoplast-targeting, delayed death activity can either be preserved or removed independently of quick-killing. Apicoplast minus parasites remained susceptible to both azithromycin and its analogues, further demonstrating that quick-killing is independent of apicoplast-targeting, delayed death activity. Therefore, development of azithromycin and analogues as antimalarials offers the possibility of targeting parasites through both a quick-killing and delayed death mechanism of action in a single, multifactorial chemotype.

4.3 Introduction.

Malaria is a mosquito-borne disease caused by protozoan parasites of the genus *Plasmodium*. In 2017, there were ~219 million cases of malaria that resulted in ~435,000 deaths (1, 2), with most deaths the result of *P. falciparum* infection in children under 5 years of age within sub-Saharan Africa. Current control strategies include use of insecticide treated bed-nets and artemisinin combination therapies (ACTs), with widespread use of these control measures resulting in significant decreases of malaria mortality (1, 2). However, there is growing concern that the spread of artemisinin resistant *P. falciparum* parasites throughout Southeast-Asia, Papua New Guinea and India will lead to the loss of our most effective drug treatments (224, 225, 228, 229) and new antimalarials with novel mechanisms of action that rapidly clear blood stage parasites are urgently needed (134, 230).

Clinically used macrolide antibiotics, in particular azithromycin, have been proposed as partner drugs for ACTs (470, 524). Macrolide antibiotics target the malaria parasites remnant plastid (apicoplast), which has a bacterial-like ribosomal complex essential for protein translation and organelle biosynthesis (130, 159, 160). The apicoplast is essential for synthesis of isopentenyl pyrophosphate (IPP) precursors required for protein prenylation, ubiquinone biosynthesis and dolichol supply crucial for N-glycosylation and production of GPI anchors (reviewed in (385) and (386)). Indeed, IPP synthesis is the sole function of the apicoplast in blood stages, but apicoplast biogenesis and housekeeping activity is essential for IPP production, making the apicoplast's ribosome an attractive antimalarial target (130, 159, 161). *P. falciparum* parasites treated with clinically relevant (nanomolar) concentrations of macrolide antibiotics exhibit a "delayed death" phenotype that arrests parasite growth during the second replication cycle after treatment (~4 days post-treatment) (130, 159).

Azithromycin exhibits three favourable properties as an antimalarial: a long half-life (>50 hrs) (431), good *in vivo* safety profile (439) and high potency against *P. falciparum in vitro* (455, 475). Azithromycin also shows efficacy as a prophylactic (163)(reviewed in (374)), improved clinical outcomes in combination with pyrimethamine during intermittent preventative treatment for malaria in pregnancy (IPTp) trials (477) and led to a significant decrease in *P. falciparum* infections following mass drug administrations of azithromycin monotherapy for trachoma infection (465). Evidence also suggests that azithromycin inhibits the development of mosquito transmissible parasites and liver stages in rodent models (162-164). Additionally, azithromycin treatment mediates development of protective immunity against blood stage malaria when applied as a single dose in controlled *P. yoelii* mouse models (107). However,

when azithromycin was trialled for treatment of clinical malaria, it exhibited sub-optimal activity as a monotherapy and was generally less effective than the similarly acting antibiotic clindamycin when used in combination with other antimalarials (376). Crucially, the delayed death activity of azithromycin in its current form has limited its use as a treatment for clinical disease.

We previously demonstrated that at higher drug concentrations azithromycin can also cause rapid parasite death (164, 165). Most strikingly, azithromycin rapidly inhibits *P. falciparum* merozoite invasion of RBCs (**Figure 4.1 A**), with longer treatments through one full blood stage growth cycle (from immediately post-invasion to final schizont maturation at 48 hrs, in cycle, **Figure 4.1 B**) inhibitory at the same micromolar concentrations (165). Testing of a small panel of azithromycin analogues showed that these 'quick-killing' IC_{50}s could be enhanced through chemical modification. Importantly, parasites selected for resistance to azithromycin's delayed death activity (120 hrs post-invasion, **Figure 4.1 D**) remained susceptible to both invasion-inhibition and intracellular parasite quick-killing activities (invasion, in cycle and 72 hr inhibition **Figure 4.1 C**), indicating that azithromycin has a secondary, apicoplast independent, mechanism of action (164, 165). Therefore, chemical modification of azithromycin proffered a unique opportunity to develop a dual-acting antimalarial that combines both quick-killing as well as delayed death activities.

In this study, we screen 84 azithromycin analogues and defined their efficacy against different stages of the blood stage lifecycle in fine detail. A high proportion of analogues exhibited improved quick-killing activity over azithromycin against both *P. falciparum* and *P. knowlesi* (a model for *P. vivax*) malaria parasites and were equally effective against parasites lacking an apicoplast. The analogues acted extremely rapidly at inhibitory concentrations with only short treatment times required to kill parasites throughout blood stage development. Given the established safety profile, long-half life, low cost of manufacture, and previous evaluation in ACTs, the re-development of azithromycin into an antimalarial with dual mechanisms of action provides a novel strategy to develop new antimalarials.

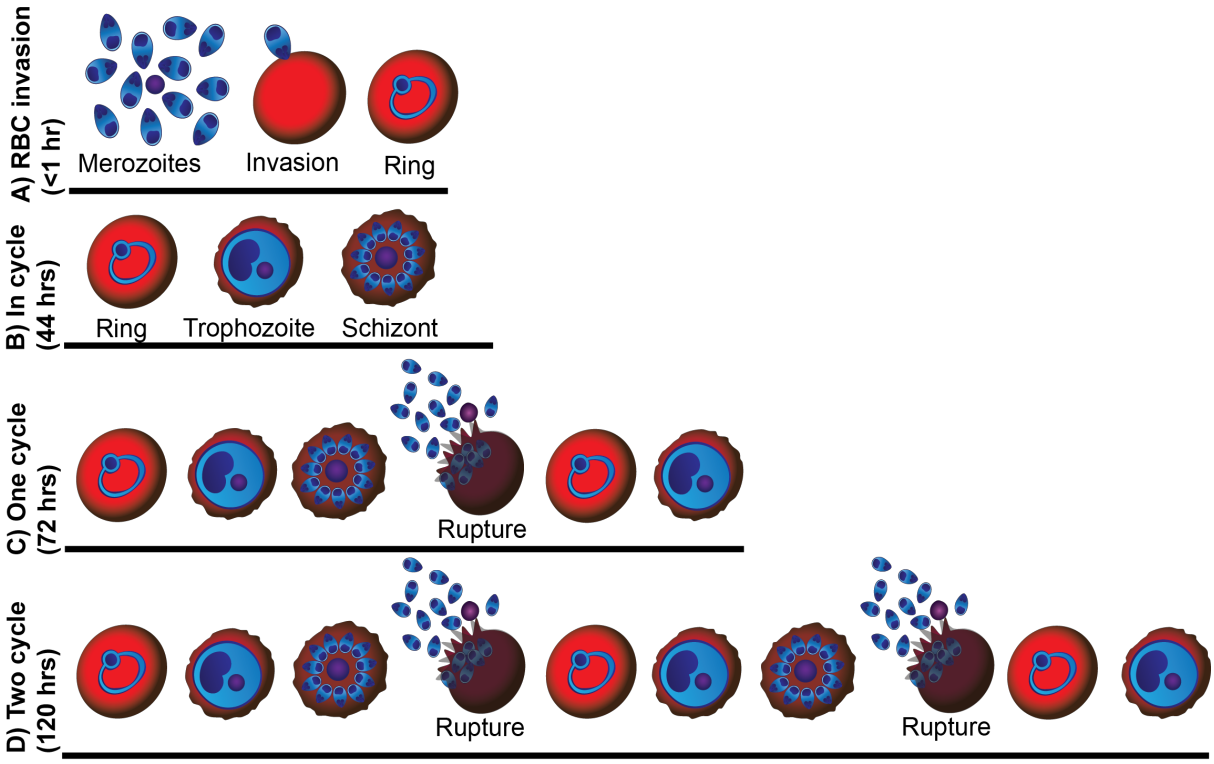


Figure 4.1 Schematic of drug treatment regimens outlining the times of treatment and stage/time of parasitaemia measurement for assays used in this study.

A) Merozoite invasion of RBCs: Merozoites were drug treated prior to addition of RBCs. RBC invasion was measured at early ring stages (<1 hr rings). **B**) In cycle: highly synchronous, early-ring stage parasites (0-4 hrs post-invasion) were treated with drug, with the resulting growth inhibition analysed at schizont stage (0-44 hrs post-invasion for *P. falciparum* and 26 hrs for *P. knowlesi*). **C**) 1 cycle (0-72 hrs): highly synchronous, early ring stage parasites (0-4 hrs post-invasion) were drug-treated and the resulting growth inhibition was measured after ~72 hrs of growth, post one cycle of re-invasion, at schizont stages. **D**) 2 cycle (delayed death); highly synchronous, early ring stage parasites (0-4 hrs post-invasion) were drug-treated and allowed to grow for 92 hrs before washing drug with fresh media (post second invasion cycle). Growth inhibition was assessed approximately 30 hrs later, at schizont stages (0-120 hrs post-invasion for *P. falciparum* and 0-92 hrs for *P. knowlesi*).

4.4 Methods and materials.

4.4.1 Antimalarial drugs.

Azithromycin analogues (GSK 1-84) were a gift from GlaxoSmithKline and were synthesised as described previously (418, 482-486). **S. Tables 4.1-4.3** provide further details of chemical structure and analogue origin. Stock concentrations of quinine (3075 mM Sigma), azithromycin (100 mM, AK-Scientific) and GSK analogues (10 mM, GSK 1-84) were made up in ethanol as vehicle. Chloroquine diphosphate salt (10 mM, Sigma-Aldrich) was dissolved in 10% acetic acid in H_2O. Dihydroartemisinin (10 mM, DHA, Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO). Drugs were added such that the vehicle was diluted >100-fold for merozoite invasion assays and >1000-fold for intracellular growth assays to minimise non-specific inhibition.

4.4.2 Culture and synchronisation of *Plasmodium* spp. parasites.

Green fluorescent protein (GFP) expressing *P. falciparum* D10-PfPHG parasites (493), DD2 (496), artemisinin resistant ($Cam3.II^{DHA\ resistant(R539T)}$) and sensitive ($Cam3.II^{sensitive}$) Cambodian isolates (226) and *P. knowlesi* PkYH1 (501) were cultured in human O^+ erythrocytes (RBCs) (Australian Red Cross Blood Service). Parasites were cultured in RPMI-HEPES culture medium (pH 7.4, 50 μg/ml hypoxanthine, 25 mM $NaHCO_3$, 20 μg/ml gentamicin, 0.5% Albumax II (Thermo Fisher Scientific)) and maintained in an atmosphere of 1% O_2, 4% CO_2 and 95% N_2 according to established protocols (492). Tight synchronisation of D10-PfPHG parasites was achieved using sodium heparin (131, 517). *P. falciparum* DD2, the Cambodian isolates and *P. knowlesi* (PkYH1), were synchronised with continuous passage over a gradient of 70% Percoll (Sigma-Aldrich) for purification of late stage schizonts and 5% w/v sorbitol (Sigma-Aldrich) treatments for ring stages.

4.4.3 Drug inhibition assays.

A diagram outlining the different *Plasmodium* spp. drug inhibition assays used in this study is available in **Figure 4.1** and have been described previously (131, 165). Stage specificity assessment of azithromycin or analogues during blood stage *P. falciparum* development was undertaken through the addition of the drug at the specified time points (0-6 hrs, 0-12 hrs, 12-24 hrs, 24-36 hrs or 36-44 hrs) and the subsequent removal through 3 consecutive washes with 200 μl medium (centrifuged at 300 x g for 2 mins) before resuspending in a final volume of 200 μl. Parasite growth was quantified at late schizont stages (44-48 hrs post invasion) by

flow-cytometry of parasites stained with 10 μg/mL ethidium bromide (EtBr) for 1 hr prior to washing with PBS.

4.4.4 Invasion inhibition assays.

Purification of viable merozoites and merozoite invasion inhibition assays has been described previously (131, 165, 517). Briefly, 300 mL of D10-PfPHG schizont culture, 3% haematocrit, 4-5% parasitaemia tightly synchronised to a 6 hr window of invasion with heparin, were magnet purified (Mitenyi Biotech) away from RBCs at 40-46 hrs post-invasion. Purified schizonts were eluted into 30 mLs, 30 μM of E64 (Sigma-Aldrich) was added and the parasites were left to mature for 5 hrs. Schizonts were filtered through a 1.2 μm syringe filter (Minisart, Sartorius) in incomplete media with $NaHCO_3$ to release merozoites and 22.5 μl of filtrate was added to 2.5 μl of drug prior to addition of RBC (0.5% final haematocrit). Plates were agitated at 400 rpm for 10 mins at 37^{o}C to promote invasion.

For drug washout, 90 μl of purified merozoites was added to 10 μl of either incomplete media (no serum) or incomplete media plus drug before transfer to a 0.22 μm Ultrafree-MC centrifugal filter (Thermo-Fisher). Filter columns were centrifuged at 750 rcf for 1 minute and washed with incomplete media twice. Free merozoites were resuspended off the filter in 45 μl of incomplete media and transferred to 96-well U-bottom plates containing 5 μl of RBCs at 1 % haematocrit (final haematocrit of 0.1 %). Plates were agitated at 400 rpm for 10 mins at 37^{o}C and cultures were incubated at 37^{o}C for 30 minutes. Cells were treated with 5 μg/mL EtBr for 10 min prior to being washed in 1 x PBS and ring stage parasitemia measured by flow cytometry.

4.4.5 Ring-stage survival assays ($RSA_{0\text{-}3h}$).

For ring-stage survival assays (222, 226, 514), tightly synchronised late schizonts of artemisinin resistant Cam3.II$^{\text{DHA resistant(R539T)}}$ and artemisinin sensitive Cam3.II$^{\text{sensitive}}$ parasites were concentrated over a gradient of 70% Percoll (Sigma-Aldrich), washed once in complete medium and incubated for 3 hrs with fresh RBCs to allow invasion. Cultures were sorbitol treated to eliminate the remaining schizonts. 0-3 hrs post-invasion rings were adjusted to 1% parasitemia and 1% haematocrit before exposure to a dilution series of DHA, azithromycin and azithromycin analogues concentrations for 4 hrs. Plates were washed five times with 200 μl of medium before parasites were transferred into a new 96-well plate to ensure the complete removal of drug (515). Parasites were grown for a further 66 hrs, before parasitemia was assessed by flow cytometry.

4.4.6 Apicoplast-null inhibition assays.

Apicoplast-null (D10-PfPHG$^{apicoplast\text{-}null}$) (157, 161) parasites were generated through supplementation of culture media with 200 μM isopentenyl pyrophosphate (IPP) and apicoplast removal through treatment with 0.35 μM (5x IC_{50}) of azithromycin for 6 days, with parasites cultured continuously thereafter with IPP. Removal of the apicoplast was confirmed by growing D10-PfPHG^{wildtype} and D10-PfPHG$^{apicoplast\text{-}null}$ (+IPP) parasites with reducing concentrations of azithromycin for ~120 hrs which identified a ~64 fold-change in the IC_{50} values between the parasite populations (D10-PfPHG$^{apicoplast\text{-}null}$ IC_{50}, 4.5 μM; D10-PfPHG^{wildtype} IC_{50}, 0.07 μM) confirming apicoplast removal (**S. Figure 4.1**). To test for azithromycin analogue activity against the apicoplast, tightly synchronised ring stage D10-PfPHG$^{apicoplast\text{-}null}$ (+IPP) and D10-PfPHG^{wildtype} parasites were treated with the in cycle 90% inhibitory concentration (IC_{90}) of drugs obtained for D10-PfPHG^{wildtype} for ~44 hrs (in cycle) and the resulting growth inhibition determined by flow cytometry.

4.4.7 Flow cytometry and microscopy analysis of inhibition.

Parasitaemia was measured on an LSR Fortessa (Becton Dickinson) with a 96-well plate reader. Mature (>36 hrs post-invasion) *P. falciparum* D10-PfPHG parasites were counted using Fl-1-high (GFP; excitation wavelength, 488 nm) and Fl-2-high (EtBr; excitation wavelength, 488 nm). D10-PfPHG ring stage parasites (<6 hrs post invasion) were counted using a Fl-1-high (GFP) and Fl-2-low (EtBr) gate (131). Mature parasite of the remaining lines were gated with a forward scatter (FSC) and FL-2-high (EtBr) gate (131). Typically, 20,000-40,000 RBCs were counted in each well. Samples were analysed using FlowJo software (TreeStar Inc) with growth of drug treatments normalised against media control wells to calculate the percentage survival. Thin smears for microscopy were fixed with fresh methanol and stained in 10% Giemsa (Merck) for 10 min. IC_{50}s and IC_{90}s were determined for each drug using GraphPad Prism (GraphPad Software) according to the recommended protocol for nonlinear regression (constrained to top= 100 and bottom= 0) of a log-(inhibitor)-versus-response curve. Statistical significance between drug treatments were determined with GraphPad Prism software using the log-(inhibitor)-versus-response curve with Extra Sum-of-Squares F Test (best-fit LogIC_{50}). *P* values were considered significant if P=<0.05.

4.4.8 Selection of azithromycin resistant *P. falciparum* lines.

In vitro selection of quick-killing resistant lines was carried out using a *P. falciparum* (D10-PfPHG) line featuring a G91D mutation in the apicoplast ribosomal gene, *rpl4*, resulting in a

~57-fold loss of sensitivity to azithromycin's delayed death activity (2 cycle, **Figure 4.1 D**) (D10-AZR^r). To select for quick-killing resistance (160), D10-AZR^r parasites were first exposed to 3x IC_{50} of GSK-59 (chloroquinoline moiety) for three days, followed by a 5x IC_{50} concentration for 4 days then 3x IC_{50} for an additional 2 days prior to removal of the drug. After treatment, parasites were fed once every 2 days and once a week 30-40% of culture was replaced with fresh RBCs. Parasites were examined every 2 to 3 days by Giemsa-stained thin blood films for between 3 (90 days) to 5 months (150 days) with no recrudescent parasites observed.

4.4.9 Antibacterial screen.

Antibacterial activity of azithromycin and analogues against *Streptococcus pneumoniae* were determined using 96 well minimum inhibitory concentration (MIC) assays (523). Two-fold serial dilutions were added to macrolide sensitive D39 *S. pneumoniae* in 100 µL Mueller Hinton Broth supplemented with 5% lysed horse blood. Bacterial growth was assessed after 24 hr incubation with drug by estimating the MIC where bacterial growth, as indicated by a media colour change, could be identified **(**MIC expressed as µM).

4.4.10 Sample extraction for metabolomics analysis.

For metabolomics experiments, two 150 mL flasks at 6% haematocrit containing tightly synchronised ~30-34 hr trophozoites were harvested via magnet purification (Miltenyi Biotech). Infected RBC density was quantitated by flow cytometry (518) and 2 mL of 3x 10^7 parasites were added to and incubated in 24 well microtiter plates for 1 hr at 37ºC to stabilise the culture. Drugs (5x IC_{50}) were added and incubated for a further 2 hrs prior to removal of the supernatant, 2x washes with 800 µL ice-cold 1 x PBS with cells pelleted via centrifugation at 400 gs for 5 mins at 4ºC. The remaining cell pellets were resuspended in 150 µL of ice-cold extraction buffer (MeOH) containing 1 µM internal standards; CHAPS and PIPES, and incubated on ice for 1 hr with shaking at 200 rpm. Insoluble material was pelleted with centrifugation at 14,800 gs for 10 mins at 4 ºC and 120 µL of supernatant was collected and stored at -80 ºC until analysis.

4.4.11 Liquid chromatography-mass spectrometry analysis.

Liquid chromatography-mass spectrometry (LC-MS) data was acquired on a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific) coupled with high-performance liquid chromatography system (HPLC, Dionex Ultimate® 3000 RS, Thermo Scientific) as per

previously described (312). Briefly, chromatographic separation was performed on ZIC-pHILIC column equipped with a guard (5 µm, 4.6 × 150 mm, SeQuant®, Merck). The mobile phase (A) was 20 mM ammonium carbonate (Sigma Aldrich), (B) acetonitrile (Burdick and Jackson) and needle wash solution was 50% isopropanol. The column flow rate was maintained at 0.3 mL/min with temperature at 25 ºC and the gradient program was as follows: 80% B decreasing to 50% B over 15 min, then to 5% B at 18 min until 21 min, increasing to 80% B at 24 min until 32 min. Total run time was 32 min with an injection volume of 10 µL. Mass spectrometer operated in full scan mode with positive and negative polarity switching at 35k resolution at 200 m/z with detection range of 85 to 1275 m/z, AGC target was 1e6 ions, maximum injection time 50 ms. Electro-spray ionization source (HESI) was set to 4.0 kV voltage for positive and negative mode, sheath gas was set to 50, aux gas to 20 and sweep gas to 2 arbitrary units, capillary temperature 300 °C, probe heater temperature 120 °C. The samples were analyzed as a single batch to reduce the batch-to-batch variation and randomized to account for LCMS system drift over time.

4.4.12 Data processing using IDEOM.

The acquired LCMS data was processed in untargeted fashion using open source software, IDOEM (519) (http:// mzmatch.sourceforge.net/ideom.php). Initially, *ProteoWizard* was used to convert raw LC-MS files to *mzXML* format and *XCMS* to pick peaks to convert to *peakML* files. *Mzmatch.R* was used to alignment samples and filtering of peaks using minimum detectable intensity of 100,000, relative standard deviation (RSD) of <0.5 (reproducibility), and peak shape (codadw) of >0.8. *Mzmatch* was also used to retrieve missing peaks and annotation of related peaks. Default IDEOM parameters were used to eliminate unwanted noise and artefact peaks. Loss or gain of a proton was corrected in negative and positive ESI mode, respectively, followed by putative identification of metabolites by accurate mass within 3 ppm mass error searching against the Kyoto Encyclopedia of Genes and Genomes (KEGG), MetaCyc, and LIPIDMAPS databases and others. To reduce the number of false positive identifications, retention time error was calculated for each putative ID using IDOEM build-in retention time model which uses actual retention time data of authentic standards (~350 standards). Statistical analysis on filtered data was performed using the Matboanalyst web interface (521).

4.4.13 Haemoglobin fractionation.

The haemoglobin fractionation assay was adapted from (522). Aliquots of 6.5 ml of 30-32 h post invasion parasite cultures were adjusted to 8% parasitaemia and 2% hematocrit and then incubated with chloroquine, GSK-66, GSK-71 or ethanol (vehicle control) for 5 h. Treatments were performed in triplicate. Following incubation, the media was aspirated off and the culture was incubated with 2.3 mL of 0.1% saponin in 1 x PBS with protease inhibitors (complete mini protease inhibitor cocktail (Roche)) for 10 min at 4°C in order to lyse the iRBCs. The parasites were washed three times with PBS and stored at -80°C.

For the haemoglobin fractionation, lysed parasites were resuspended in 50 µL of Milli-Q water and sonicated for 5 min in a water bath sonicator. Following sonication, 50 µL of 0.2 M HEPES (pH 7.5) was added and the samples were centrifuged at 4,000 rpm for 20 min. The supernatant containing the haemoglobin fraction was carefully transferred to new tubes and 50 µL of 4% of SDS was added before the samples were incubated at 95°C for 5 min. Following heating, 50 µL of 0.3 M NaCl and 50 µL of 25% (v/v) pyridine (Sigma) in 0.2 M HEPES was added, the sample containing the haemoglobin fraction were vortexed and transferred to a 96 well plate. The remaining pellets were treated with 50 µL of MilliQ water and 50 µL of 4% SDS and resuspended before being sonicated for 5 min and incubated at 95°C for 5 min in order to solubilise the free heme. Following incubation, 50 µL of 0.2 M HEPES, 0.3 M NaCl and 25% pyridine were added to the samples. The samples were then subsequently centrifuged at 4,000 rpm for 20 min. The supernatant was transferred to the 96 well plate, corresponding to the free haem fraction. The remaining pellet containing the hemozoin fraction was solubilised by resuspending with 50 µL of MilliQ water and 50 µL of 0.3 M NaOH. The samples were sonicated for 15 min before 50 µL of 0.2 M HEPES, 0.3 M HCl and 25% pyridine was added. The samples were then transferred to the 96 well plate, corresponding to the hemozoin fraction. The total amount of haem in each fraction was quantified using a haem standard curve prepared from a 100 µg/mL standard solution of haematin in 0.3 M NaOH. Serial dilution of the standard curve was carried out in a 96 well plate in triplicate and 50 µL of 0.2 M HEPES, 4% SDS, 0.3 M NaCl, 0.3 M HCl and 25% pyridine was added. The absorbance of the standard curve and each fraction was measured at a 405 nm wavelength using a Perkin Elmer Ensight Plate Reader. The samples were normalised via a paired analysis to the ethanol control and graphed as their fold change vs ethanol ± SEM. All fractions had >2 replicates from 2 independent experiments.

4.5 Results.

4.5.1 Azithromycin derivatives show improvement in quick-killing activity against *P. falciparum.*

The IC_{50} values for 72 hr assays with the analogues presented in this study have been published previously (1 cycle assay (**Figure 4.1 C**)) (418, 483-486). Here, we characterise azithromycin analogue quick-killing activity across the malaria parasites blood stage development in fine detail, including their effectiveness against early ring stages and post invasion. We tested for quick-killing activity by treating D10-PfPHG parasites with 10 μM of drug for 44 hr (**Figure 4.1 B** & **S. Tables 4.1-4.3**) and identified 64 of 84 analogues that inhibited growth by >30%. The in cycle IC_{50} values for these 64 analogues were determined (**S. Tables 4.1-4.3**) with all but two analogues showing improved potency over azithromycin (azithromycin IC_{50} with 44 hr in cycle treatment, 11.3 μM) with the most potent compound exhibiting a 1615-fold lower IC_{50} than azithromycin (GSK-66, IC_{50} 0.007 μM) (**S. Table 4.1-4.3** & **Figure 4.1 B**). Notably, 38 analogues showed >10-fold improvement over azithromycin (IC_{50} <1 μM), with 16 exhibiting a >55-fold improvement (IC_{50} <0.2 μM).

The analogues with the low nanomolar 44 hr in cycle activity often featured quinoline or chloroquinoline modifications (**Table 4.1** & **S. Tables 4.1-4.3**). However, there were exceptions including phenyl substituted analogues (GSK-5, 6, 9, 11, 14, 16, 17, 19) and napthalene substituted analogues (GSK-3, 4, 15, 18), which all displayed IC_{50} values <1 μM. There was no structural difference between the most potent analogues and the analogues with activity >1 μM that could explain the observed activity discrepancy. Consistently, chloroquinoline analogues (GSK-1, 2, 56 and 66) were more potent than their respective unsubstituted quinoline counterparts (GSK-7, 10, 58, and 71). Analogues GSK-6 and 9 with thiourea aryl substitution displayed comparable potency (IC_{50} 0.2 and 0.28 μM) to naphthalene analogues GSK-3 and 4. However, a large number of analogues supporting thiourea and urea aryl substitutions were significantly less active, with no clear distinction between the activity and substitution pattern on the aryl ring of thiourea or urea substituted analogues.

Analogues with aliphatic substitution on the urea or thiourea (GSK-31, 35, 38, 45, 47, 51) generally had reduced activity compared to analogues with pendant aryl moieties (**S. Tables 1-3**), suggesting the aryl substituent was important for modulating potency.

Table 4.1 *In vitro* efficacy of antimalarials and azithromycin analogues against *Plasmodium* spp. parasites

Modification	Drug	Intracellular growth D10-PfPHG IC_{50} (μM) [a]	Intracellular growth DD2 IC_{50} (μM) [a]	Invasion inhibition D10-PfPHG IC_{50} (μM) [b]	Intracellular growth PkYH1 IC_{50} (μM) [a]
	Azithromycin	11.31	15.6	10	13
	Chloroquine	0.052	0.311	ND	0.017
	Quinine	0.39	ND	ND	ND
	DHA	0.0583	ND	ND	0.0024
Chloroquinoline	1	0.019	0.082	ND	0.2
	56	0.011	0.093	3.2	0.031
	66	0.007	0.043	ND	0.012
	69	0.031	ND	ND	ND
	70	0.05	ND	ND	ND
	72	0.27	0.065	1.7	0.15
Quinoline	8	0.41	0.52	4.4	0.15
	10	0.48	0.748	ND	0.1
	58	0.048	0.056	ND	0.071
	71	0.053	0.16	ND	0.041
	73	0.31	0.48	ND	0.248
Naphthalene	3	0.183	0.32	1.8	0.095
	4	0.19	ND	2.0	ND
	15	0.67	0.4	3.6	ND
Substituted phenyl	5	0.2	0.4	1.61	0.082
	6	0.28	0.27	ND	0.16
	9	0.53	0.24	ND	0.016
	17	0.7	0.54	ND	0.36

[a] Drug treatment of intracellular growth, from rings to late schizonts, with no rupture cycle (*P. falciparum,* 0-44 hrs; *P. knowlesi,* 0-24 hrs). [b] Drug treatment of merozoites for 10 mins prior to addition of RBCs and a further 50 mins after the addition of RBC prior to washing the drug out and measuring parasitemia by flow cytometry ~1 hr post merozoite treatment. IC_{50}s were performed for *P. falciparum* lines, D10-PfPHG and DD2, or *P. knowlesi* (PkYH1). All assays were measured by flow cytometry. Data represents the means of 3 (or more) experiments expressed as percentage of non-inhibitory control. ND= not done, as activity was <30% in cycle or <20% in invasion inhibitory assays or due to lack of analogue.

Consistent with this observation, analogues that did not terminate with an aromatic substituent and were only decorated with small aliphatic functionality (analogues GSK-34, 44, 46, 50, 52, 53, 54, 55, 62, 64, 83, 84) were either weakly active (>3 μM) or inactive. This data suggested type of functionality and the length of the carbon-chain linking the aromatic group to the macrolactone was not important for activity. However, analogues GSK-56, 57, 58, 66, 67 and 71, with short 3-carbon linkers between the macrolactone and the quinoline group, were among the most potent. Overall, there was no consistent trend between the type of functionality and the length of the carbon-chain linking the aromatic group to the macrolactone.

The position of the pendant quinoline or aromatic system attached to the macrolactone–either N6-, O-desosaminyl, or N-desosaminyl–did not affect the in cycle 44 hr activity of analogues (**S. Tables 4.1-4.3**). For example, analogues with the same quinoline functionality, GSK-1, 56 and 66 attached to either N6-, N-desosaminyl or O-desosaminyl positions, displayed similar IC_{50} values between 7 and 19 nM. This trend was observed amongst other analogues for which there were matched pairs. The cladinosyl group did not affect 44 hr in cycle activity, for example respective analogues with the cladinosyl group, GSK-1, 10, 56 and 66 possessed similar activity compared to analogues without the cladinosyl group, GSK-67, 7 and 57 This observation is consistent with our previous findings on azalide structure activity relationship (165).

4.5.2 Azithromycin analogues show improved activity against merozoite RBC invasion.

We previously showed that azithromycin and analogues inhibit merozoite invasion, with merozoites found to contact, briefly deform the RBC membrane and then detach (165). We investigated whether the 38 analogues that had an in cycle (44 hr) IC_{50} <1 μM could inhibit merozoite invasion at a concentration of 1 μM and identified eight analogues that inhibited invasion by >20% at 1 μM **(Figure 4.1 A** & **S. Tables 4.1-4.3)**. The invasion inhibitory IC_{50} for seven of these analogues with sufficient sample was determined with a 2 to 6-fold reduction in the invasion inhibitory IC_{50} over azithromycin achieved (range GSK-8, 4.4 μM to GSK-5, 1.6 μM) (**Table 4.1** & **S. Figure 4.2**). Importantly, azithromycin analogues with improved in cycle activity also had improved potency against merozoite invasion, confirming previous observation that both invasion and in cycle quick-killing activities can be improved with a single chemical modification (165). We next tested whether azithromycin analogue invasion inhibitory activity was directed against the merozoite or the RBC by treating purified

merozoites with 10 μM of GSK-72 (invasion inhibitory IC_{50} 1.7 μM) prior to washing the drug off the merozoites and mixing merozoites with RBCs (**S. Figure 4.3**). GSK-72 treated merozoites were stopped from invading RBCs after washing off the drug, suggesting that the invasion inhibitory activity of azithromycin analogues is irreversible and direct towards the merozoite.

4.5.3 Quick-killing activity is independent of apicoplast targeting.

We previously showed that quick-killing activity is maintained against delayed death resistant parasites (165), suggesting that quick-killing occurs through a mechanism of action independent of the apicoplast. However, the fact that the apicoplast and apicoplast-ribosome were still present in these drug-treated parasites left open the possibility that quick-killing activity could still be linked to the apicoplast, which is in some instances prone to quick-killing with certain drugs (157). To confirm quick-killing is completely independent of the apicoplast, we generated apicoplast minus (D10-PfPHG$^{apicoplast\text{-}null}$) parasites through prolonged treatment with azithromycin and then rescued with media supplementation with the isoprenoid precursor, isopentenyl pyrophosphate (IPP) (157, 161). D10-PfPHG$^{apicoplast\text{-}null}$ parasites showed a complete loss of sensitivity to azithromycin in 120 hr delayed death assays, confirming that the apicoplast had been removed (**S. Figure 4.1**) (157, 161). In contrast, there was no difference in growth inhibition for the D10-PfPHG$^{apicoplast\text{-}null}$ and D10-PfPHG^{wildtype} parasites when treated with azithromycin and 15 lead analogues at the in cycle D10-PfPHG^{wildtype} IC_{90} concentration for 44 hrs. (**S. Tables 4.1-4.3**) These data confirm that quick-killing activity is independent of the apicoplast, marking rapid parasite clearance as a secondary mechanism of action for azithromycin and analogues.

4.5.4 Azithromycin is a rapid and irreversible inhibitor across blood stage parasite growth.

After confirming that azithromycin analogues have both invasion and intracellular blood stage quick-killing activity that is independent of apicoplast-targeting delayed death, we next determined drug activity across early rings (0-12 hrs post invasion), early trophozoites (12-24 hrs post invasion), late trophozoites (24-36 hrs post invasion), and schizonts (36-44 hrs post invasion) (**Figure 4.2**). Azithromycin demonstrated a similar IC_{50} across each pulsed treatment stage (0-12 hr, IC_{50} 14 μM; 12-24 hr, IC_{50} 16 μM; 24-36 hr, IC_{50} 15 μM) with these values similar to the IC_{50} values obtained for 44 hr (IC_{50} 11.3 μM) and invasion inhibition (IC_{50} 10 μM) treatments (**Figure 4.2 B** & **C**).

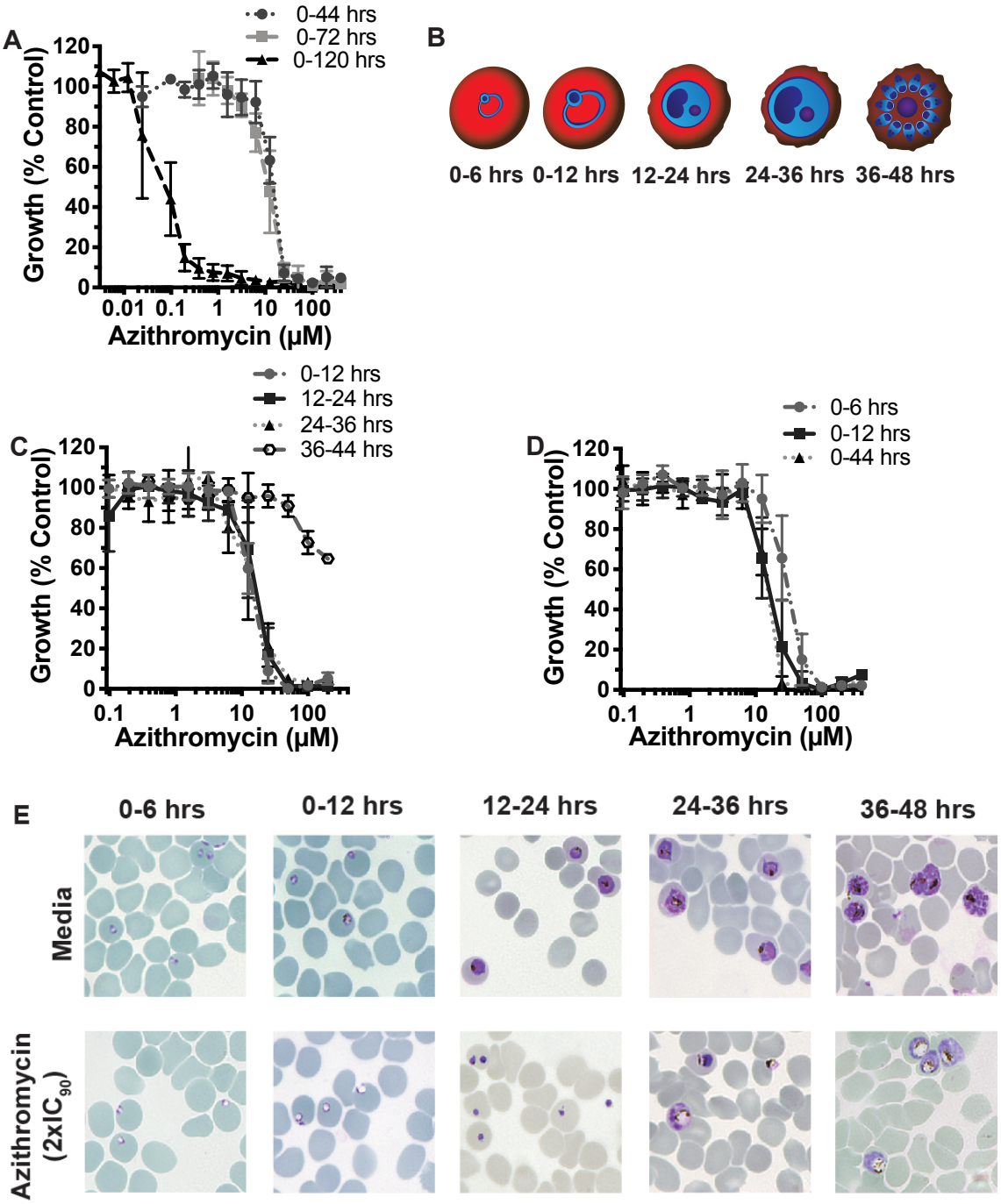

A
0-44 hrs
0-72 hrs
0-120 hrs
Growth (% Control)
Azithromycin (μM)
B
0-6 hrs
0-12 hrs
12-24 hrs
24-36 hrs
36-48 hrs
C
0-12 hrs
12-24 hrs
24-36 hrs
36-44 hrs
Growth (% Control)
Azithromycin (μM)
D
0-6 hrs
0-12 hrs
0-44 hrs
Growth (% Control)
Azithromycin (μM)
E
0-6 hrs
0-12 hrs
12-24 hrs
24-36 hrs
36-48 hrs
Media
Azithromycin (2xIC90)

Figure 4.2 Azithromycin has broad activity against blood stage parasites.

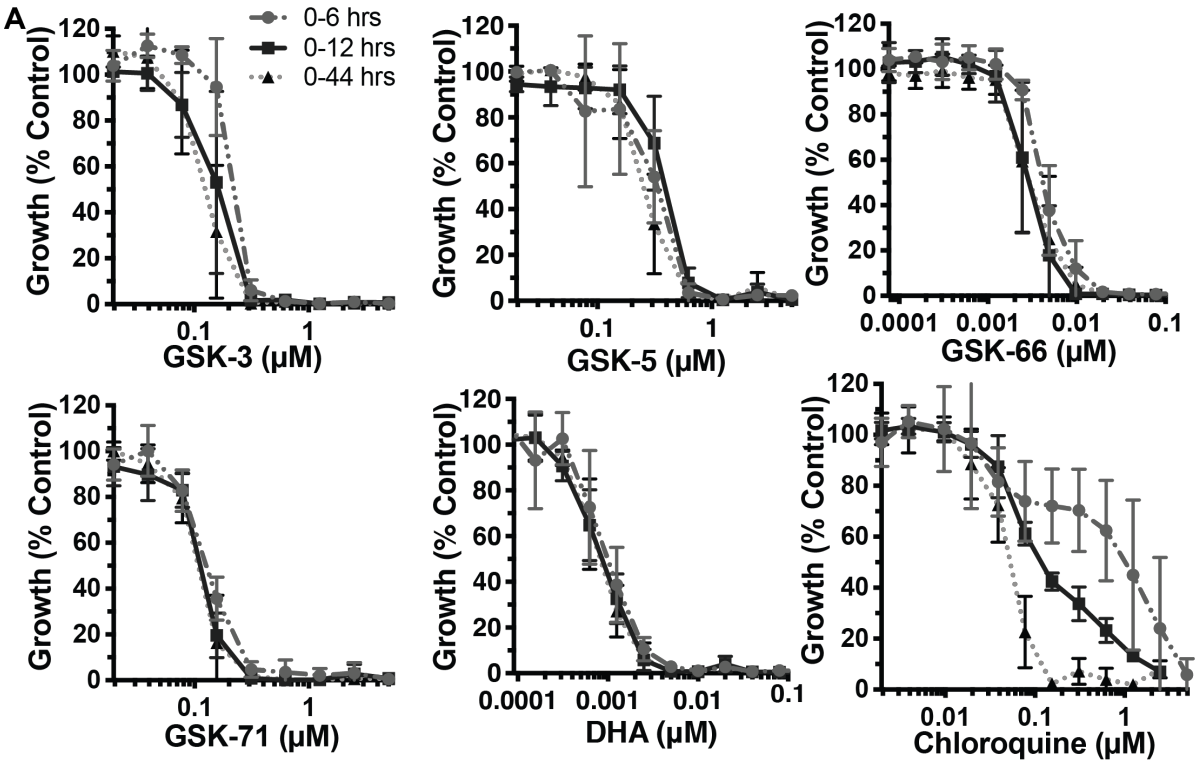
A) Early ring stage *P. falciparum* parasites (0-4 hrs post-invasion) were treated with doubling dilutions of azithromycin and inhibition of growth measured for in cycle (44 hrs, IC_{50}, 11 μM), 1 cycle (72 hrs, IC_{50}, 14 μM) and 2 cycle (delayed death, 120 hrs, IC_{50}, 0.07 μM) assays. (44 hr vs 72 hrs, P=NS; 44 hrs vs 44 hr P=<0.0001; 144 hrs vs 72 hrs <0.0001). **B**) Schematic of drug washout treatment scheme to assess azithromycin's quick-killing stage of activity. Early ring stage parasites (0-4 hrs post-invasion) were aliquoted to a 96-well plate and doubling dilutions of azithromycin added between 0-12 hrs, 12-24 hrs, 24-36 hrs and 36-44 hrs post invasion prior to drug removal by washing with fresh media. **(**C**)** Growth inhibition of azithromycin across 0-12 hrs, 12-24 hrs, 24-36 hrs and 36-44 hrs post invasion prior to drug removal by washing with fresh media. There was no significant difference in IC_{50}s between treatment times, except for 0-12 hrs vs 36-44 hrs (P=0.005), 12-24 hrs vs 36-44 hrs (P=0.01) and 24-36 hrs vs 36-44 hrs (P=0.01). **D**) Growth inhibition of azithromycin with Short-term pulse treatments of very early ring stages across 0-6 hrs and 0-12 hrs post-invasion compared to a full in cycle (0-44 hrs) treatment. There was a significant different in the IC_{50}s between 0-6 hrs vs 0-12 hrs and 0-6 hrs vs 0-44 hrs (P=<0.0001), but not for 0-12 hrs vs 0-44 hrs treatments (P=0.19). **E**) Representative Giemsa stained thin blood smears showing the growth phenotypes seen for non-inhibitory media controls (top panels) and in the presence of 2x IC_{90} concentration of azithromycin (bottom panels) across different stages of intraerythrocytic blood stage development (0-6 hrs, 0-12 hrs, 12-24 hrs, 24-36 hrs and 36-44 hrs). For all growth curves, parasitemia was measured at 44 hrs post invasion at schizont stage via flow cytometry. Data represents the means of 3 (or more) experiments expressed as percentage of non-inhibitory control and error bars represent the ± SEM.

We confirmed that azithromycin's quick-killing activity works rapidly by assessing the morphological effects of pulsed treatment with a 2x IC_{90} drug concentration. Ring stage treatments (0-12 hrs) showed pronounced vacuolation of the cytoplasm, a typical sign of parasite stress. Trophozoite stages (12-24 hrs and 24-36 hrs) appeared either pyknotic or severely vacuolated with only a 12-hr treatment, indicative of rapid cell death (**Figure 4.2 B & E**). Although azithromycin treated schizont stages (36-44 hrs post-invasion) did not show potent growth inhibitory activity when assessed by flow-cytometry, light microscopy smears showed late stage parasites with severe vacuolation and minimal merozoite maturation, indicating this population was indeed killed by azithromycin treatment (**Figure 4.2 E**). These data provide direct evidence that azithromycin acts broadly across invasion and throughout the entire blood stage lifecycle, including early ring stages.

4.5.5 Azithromycin and analogues rapidly kill early ring stage parasites.

Our finding that azithromycin could kill ring stage parasites (0-12 hrs post invasion) with similar efficacy to 44 hrs of drug treatment is of major interest since the majority of clinically used antimalarials, with the notable exception of the artemisinins (370, 525), have relatively poor activity against newly invaded ring stages (127, 128, 151, 526).

To provide further insights into how quickly azithromycin and analogues act against early ring stages, we examined activity of 6 and 12 hr treatments of early ring stages (0-6 hr and 0-12 hrs post-invasion treatments) for azithromycin and a panel of diverse analogues that had nanomolar efficacy. Azithromycin (IC_{50}; 0-6 hrs 30 μM; 0-12 hr 16 μM; 0-44 hr 14 μM) and the analogues tested (GSK-3 IC_{50}; 0-6 hrs, 0.22 μM; 0-12 hrs, 0.15 μM; 0-44 hrs, 0.13 μM; GSK-5 IC_{50}; 0-6 hrs, 0.3 μM; 0-12 hrs, 0.37 μM; 0-44 hrs, 0.26 μM; GSK-66 IC_{50}; 0-6 hrs, 0.004 μM; 0-12 hrs, 0.0028 μM; 0-44 hrs, 0.003 μM; GSK-71 IC_{50}; 0-6 hrs, 0.13 μM; 0-12 hrs, 0.11 μM; 0-44 hrs, 0.11 μM) showed <2-fold reduction in potency with a 6 hr early ring stage treatment compared to a 12 hr ring stage or full 1 cycle (44 hr) treatment, highlighting the drug's efficacy against early ring stages (**Figure 4.2 D & E, Figure 4.3 A & B**). Dihydroartemisinin (DHA) treatment resulted in severe growth retardation with early ring stage treatment (IC_{50}; 0-6 hrs 0.011 μM; 0-12 hrs 0.009 μM; 0-44 hrs, 0.008 μM), consistent with previous publications and this result confirm the rapid action of the azithromycin analogues against early ring stage development (151, 370, 525).


A
0-6 hrs
0-12 hrs
0-44 hrs
Growth (% Control)
GSK-3 (μM)
GSK-5 (μM)
GSK-66 (μM)
GSK-71 (μM)
DHA (μM)
Chloroquine (μM)

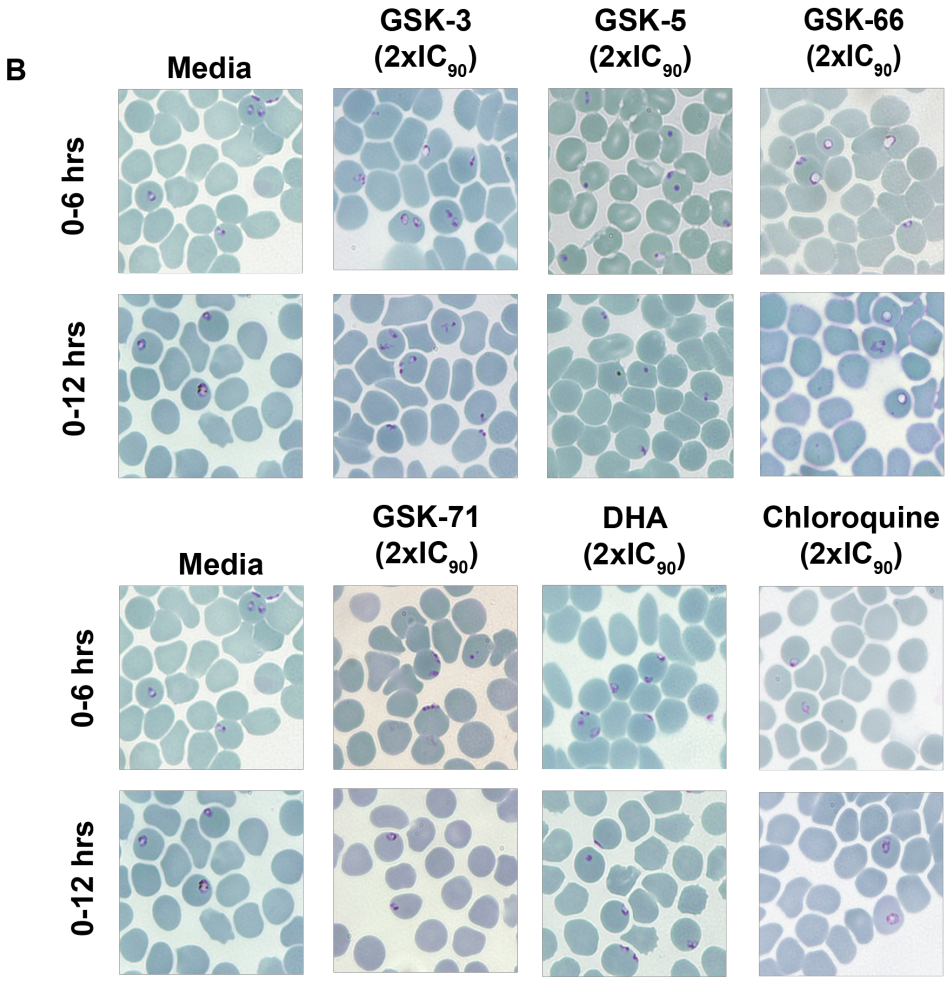

B
Media
GSK-3 (2xIC90)
GSK-5 (2xIC90)
GSK-66 (2xIC90)
0-6 hrs
0-12 hrs
Media
GSK-71 (2xIC90)
DHA (2xIC90)
Chloroquine (2xIC90)
0-6 hrs
0-12 hrs

Figure 4.3 Growth inhibition profiles of azithromycin analogues and control drugs with short term and in cycle drug treatments.

Early ring stage *P. falciparum* parasites (0-4 hrs post-invasion) were treated with doubling dilutions of azithromycin analogues/control drugs for 0-6 hrs and 0-12 hrs prior to washing the drug out of cultures allowing growth to continue until parasites were 44 hrs old. A 0-44 hrs continuous drug control treatment was also included. **A)** Growth inhibition profile of GSK-3 (naphthalene), GSK-5 (substituted phenyl), GSK-66 (chloroquinoline), GSK-71 (quinoline), dihydroartemisinin (DHA) and chloroquine with very early ring stage treatment across 0-6 hrs and 0-12 hrs post-invasion compared to a full in cycle treatment. There was no significant difference drug efficacy between the treatment times of GSK 18 or GSK 20 (P=>0.01). GSK-66 showed a significant difference between 0-6 hr vs 0-12 hr treatments (P= <0.0079) and 0-6 hrs vs 0-44 hrs (P=0.001), but there was no significant difference in drug efficacy between 0-12 hr vs 0-44 hr treatments (P=0.96). GSK 69 and DHA showed no significant difference in efficacy between treatment times (P=>0.01), with the exception of 0-6 hrs vs 0-44 hrs (P=0.005 and P= 0.01, respectively). In contrast, chloroquine demonstrated a significant difference in drug efficacy between all treatment times (0-6 hrs vs 0-12 hrs P= <0.0001; 0-6 hrs vs 0-44 hrs P=<0.0001; 0-12 hrs vs 0-44 hrs P=<0.0001). **B)** Representative Giemsa stained thin blood smears showing the growth phenotypes seen for non-inhibitory media controls, azithromycin analogues GSK-3, GSK-5, GSK-66, GSK-71 and control drugs, DHA and chloroquine (bottom panels) 0-6 hrs post treatment and 0-12 hrs post treatment at 2x IC_{90}. Parasitemia was measured via flow cytometry 44 hrs post-invasion. Data represents the means of 3 or more experiments expressed as a percentage of non-inhibitory control and error bars represent ± SEM.

In contrast, chloroquine had comparatively poor activity for early ring stage treatments as expected (0-6 hrs, 0.73 μM; 0-12 hrs 0.15 μM; 0-44 hrs 0.052 μM), indicating that chloroquine lacks potency against early ring stages. Microscopy analysis was performed for parasites treated with a 2x IC_{90} (0-44 hrs) of azithromycin and analogues to examine the phenotypic changes associated with early ring stage drug treatment (**Figure 4.3 B**). Early (0-6 hrs) ring stages treated with azithromycin, GSK-71, GSK-66 and GSK-3 exhibited vacuolation, with evidence of pyknotic cells developing with extended treatment (0-12 hrs). Notably, GSK-5 resulted in a large number of pyknotic parasites within only 6 hrs of drug treatment, highlighting the speed with which these compounds can act. DHA treatment of early (0-6 hr) ring stages did not lead to a clear change in parasite morphology. However, after extended ring stage treatment (0-12 hrs) pyknotic cells became prominent. No aberrant growth phenotype was observed with chloroquine with treatment of early ring stages (0-6 hr), with evidence of vacuolation only occurring after extended ring stage treatment (0-12 hrs). Short-term pulse treatments confirmed that azithromycin and analogues rapidly kill early ring stage parasites, the growth inhibitory effects are not reversible, and modification of azithromycin can produce analogues with broad and potent efficacy across blood stage parasite growth.

4.5.6 Quick-killing azithromycin analogues maintain activity against drug resistant *P. falciparum* and *P. knowlesi.*

We next investigated whether analogues retained potency against the chloroquine/mefloquine/pyrimethamine resistant *P. falciparum* DD2 line (496), an artemisinin resistant *P. falciparum* Cambodian isolate (222, 226, 514) and the zoonotic human malaria parasite *P. knowlesi* (501) (**Table 4.1**). Relative to the chloroquine sensitive D10-PfPHG line, DD2 parasites exhibited a 0.24 to 8.4-fold loss of sensitivity to azithromycin and analogues. Of note, analogues featuring a chloroquinoline moiety (GSK-1, 56, 66, 72) were 4.77-fold less potent against chloroquine resistant DD2, whereas analogues featuring quinoline, naphthalene and substituted phenyl moieties were on average 1.35-fold less sensitive (n=11 compounds) (**Table 4.1** & **S. Table 4.4)**.

We next tested the efficacy of azithromycin analogues against the *P. falciparum* artemisinin-resistant clinical isolate Cam3.II, which has a mutation within the *Kelch13* propeller gene (R539T, Cam3.II$^{\text{DHA resistant(R539T)}}$) associated with increased early ring stage (0-3 hrs) survival *in vitro* with DHA treatment (222, 226, 514). Early ring stage Cam3.II$^{\text{DHA resistant(R539T)}}$ resistant and a reverted sensitive line (Cam3.II$^{\text{sensitive}}$) were pulsed for 4 hrs before the drug was washed off, with growth determined 66 hrs later via flow cytometry (514, 515). Since comparison of

IC_{50} is of limited relevance in ring stage survival assays, we compared instead the percentage (%) parasite growth of Cam3.II$^{DHA\ resistant(R539T)}$ parasites at the drug concentration that inhibited 95% of growth for the Cam3.II^{sensitive} line. As expected, ~41% Cam3.II$^{DHA\ resistant(R539T)}$ parasites survived DHA treatment at the concentration that killed 95% of Cam3.II^{sensitive} parasites (**Figure 4.4** & **Table 4.2**). In contrast, growth of both the Cam3.II$^{DHA\ resistant(R539T)}$ and the Cam3.II^{sensitive} line lines were equally inhibited at the concentration that killed 95% of DHA sensitive parasites for azithromycin and analogues GSK 56, GSK-71, GSK-3 and GSK-5.

We next tested the activity of azithromycin and analogues against the zoonotic malaria parasite *P. knowlesi*, which is a significant human pathogen in regions of South-East Asia (4) and an *in vitro* culturable model for *P. vivax* (527). We found that azithromycin maintains potency against *P. knowlesi* in both in cycle (28 hrs for *P. knowlesi, Pk*) and delayed death (92 hr) assays compared to *P. falciparum* (*Pf*) (*Pk* in cycle IC_{50}, 13 μM; delayed death IC_{50}, 0.08 μM: *Pf* in cycle IC_{50} 11.3 μM; delayed death IC_{50}, 0.07 μM) (**Table 4.1**) as previously shown (405). We next tested a panel of azithromycin analogues that had potent quick-killing activity against *P. falciparum* for their efficacy against *P. knowlesi* and identified that the majority of analogues had similar quick-killing potency against this divergent parasite species (**S. Table 4.5**). Of interest, the analogue GSK-9 exhibited a significant 33.1-fold improvement in activity against *P. knowlesi* compared to *P. falciparum*, suggesting that some species-specific differences in drug activity can occur. Together, these data support that azithromycin analogues retain efficacy against diverse human malaria parasites and across DHA and multi-drug resistant parasites.

4.5.7 Analogues modified at the macrolactone-ring maintain dual mechanisms of action.

We next sought to define whether the more potent quick-killing azithromycin analogues maintained apicoplast targeting delayed death activity. As quick-killing IC_{50}s for a number of analogues (GSK-1, 4, 5, 29, 57, 66, 71, 78) approached that of the delayed death IC_{50} values of azithromycin (120 hr IC_{50}, 0.07 μM), traditional delayed death assays (i.e. 120 hrs of treatment, **Figure 4.1 D**) could not be undertaken.

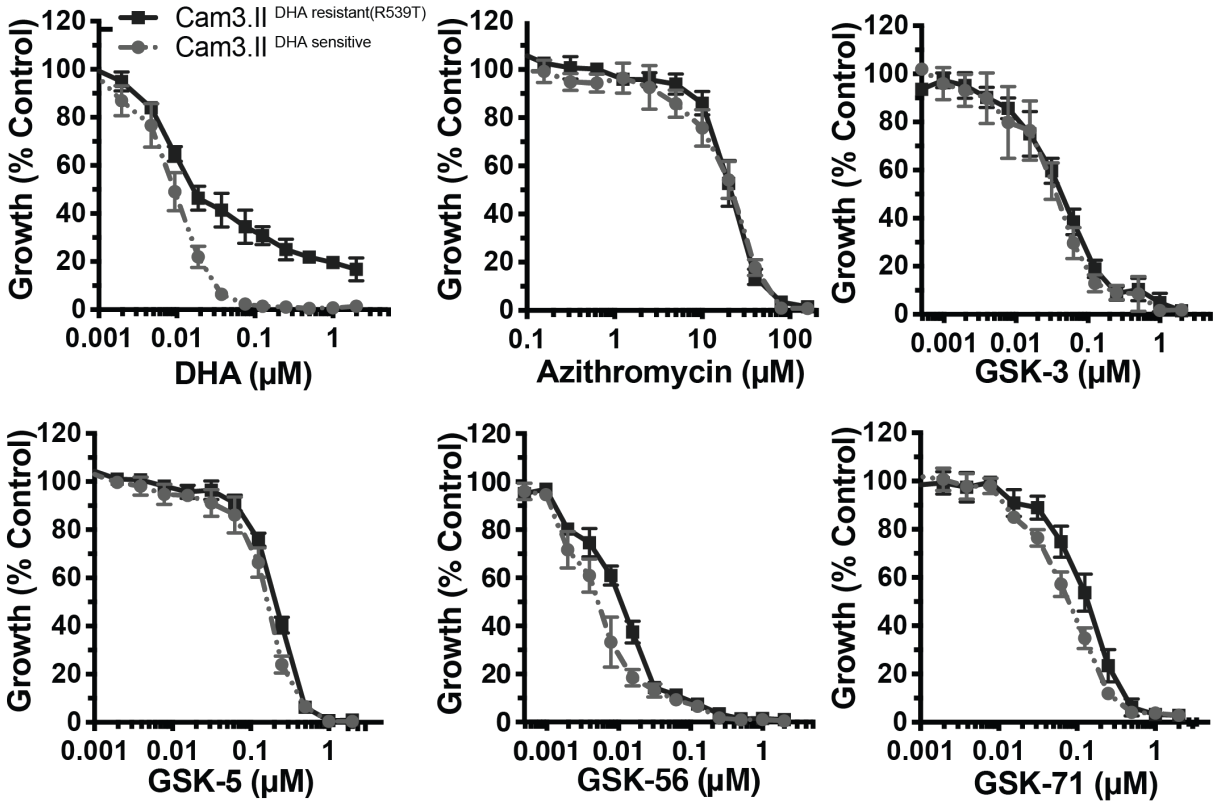


Figure 4.4 Activity of azithromycin analogues against artemisinin resistant parasites.

Lead azithromycin analogues were tested against artemisinin resistant, Cam3.II$^{\text{DHA resistant(R539T)}}$ parasites containing the K13 propeller mutation and reverted, artemisinin sensitive, Cam3.II $^{\text{sensitive}}$ parasites in ring-stage survival assays (4 hr drug pulse of very early rings 0-3 hrs post invasion) prior to washing off drug and assessment of parasitaemia (66 hrs later by flow cytometry). Dihydroartemisinin (DHA), azithromycin, GSK-3 (naphthalene), GSK-5 (substituted phenyl), GSK-56 (chloroquinoline) and GSK-71 (quinoline). Parasitemia was measured via flow cytometry ~72 hrs post-invasion. Data represents the mean of 2 or more experiments expressed as a percentage of non-inhibitory control and error bars represent ± range of 2 experiments.

Table 4.2 Ring-stage survival assay percent survival values from drug treated artemisinin resistant and sensitive parasites.

Modification	**Drug**	**Concentration of drug = 5% growth of Cam3.II^{sensitive} (μM)**	**Growth Cam3.II$^{DHA\ resistant(R539T)}$ (%)**
	DHA	0.05	41
	Azithromycin	100	1
Naphthalene	3	0.4	8
2-chlorophenyl	5	0.5	6
7-chloroquinoline	56	0.055	7
Quinolone	71	0.6	6

Ring stage survival assay the μM concentration of drug (DHA, azithromycin, GSK-3, GSK-5, GSK56 and GSK-71) that resulted in a 5% survival value in artemisinin sensitive Cam3.II^{sensitive}, was then used as the maximal inhibitory concentration of drug sensitive parasites to then assess growth of drug resistant Cam3.II$^{DHA\ resistant(R539T)}$ parasites with the % parasite survival value for the resistant parasites at the maximal inhibitory concentration displayed in the table. Parasites were incubated for one cycle (72 hrs) after pulsed drug treatment and washing prior to measurement of parasitaemia by flow cytometry.

Therefore, we assessed the activity of azithromycin and a panel of quick-killing analogues against the azithromycin sensitive bacteria *Streptococcus pneumoniae* (**S. Table 4.6**) on the basis that this Gram-positive bacteria's ribosome could serve as a proxy for the malaria parasites bacterial-like apicoplast ribosome (160, 414). Consistent with previously published results, limited inhibition of bacterial growth was observed for analogues with an N-substitution on the desosamine sugar moiety (420, 485, 486). Indeed, N-substituted analogues of azithromycin have been deliberately designed to reduce off-target drug activity against bacteria for use in alternative drug applications (420, 485, 486). In contrast, all analogues with N6-substitutions on the macrolactone backbone (GSK-1, 4, 5, 6, 9, 11, 12, 16, 17, 21, 25) had activity against *S. pneumoniae* similar to azithromycin. Thus, selecting the site of azithromycin modification can allow improved quick-killing activity while maintaining apicoplast targeting delayed death activity, or delayed death activity can be removed along with off-target antibacterial effects to produce a quick-killing specific antimalarial.

4.5.8 Analysis of the quick-killing mechanism of action suggests a multi-factorial mechanism of action.

In an attempt to identify the molecular target of quick-killing activity, we selected for *in vitro* drug resistance by subjecting an azithromycin delayed death resistant D10-PfPHG line (D10-AZR^{r}) with a stepwise increase (160) of the quick-killing azithromycin analogue GSK-59 featuring a chloroquinoline substituted desosamine moiety that lacks delayed death activity. After three attempts, we failed to select for resistant parasites >3 months after drug removal, suggesting that the mechanism of quick-killing cannot be readily selected for *in vitro*.

We next undertook an untargeted metabolomics screen to identify changes in the metabolomics signature of azithromycin and the quick-killing analogues GSK-5 (substituted phenyl), GSK-66 (chloroquinoline) and GSK-71 (quinoline) and to compare changes during treatment with these analogues to known antimalarials, such as chloroquine and DHA. Following a 2 hr treatment of trophozoite stage parasites at a 5x IC_{50} (44 hr) concentration, supervised multivariate analysis (partial least squares-discriminate analysis) and heat map showed that the most prominent metabolomic signature shared between azithromycin and analogues was a series of peptides that were increased for all of azithromycin, GSK-71, GSK-5 and chloroquine (**Figure 4.5, S. Figure 4.4** & **S. Table 4.7)**.

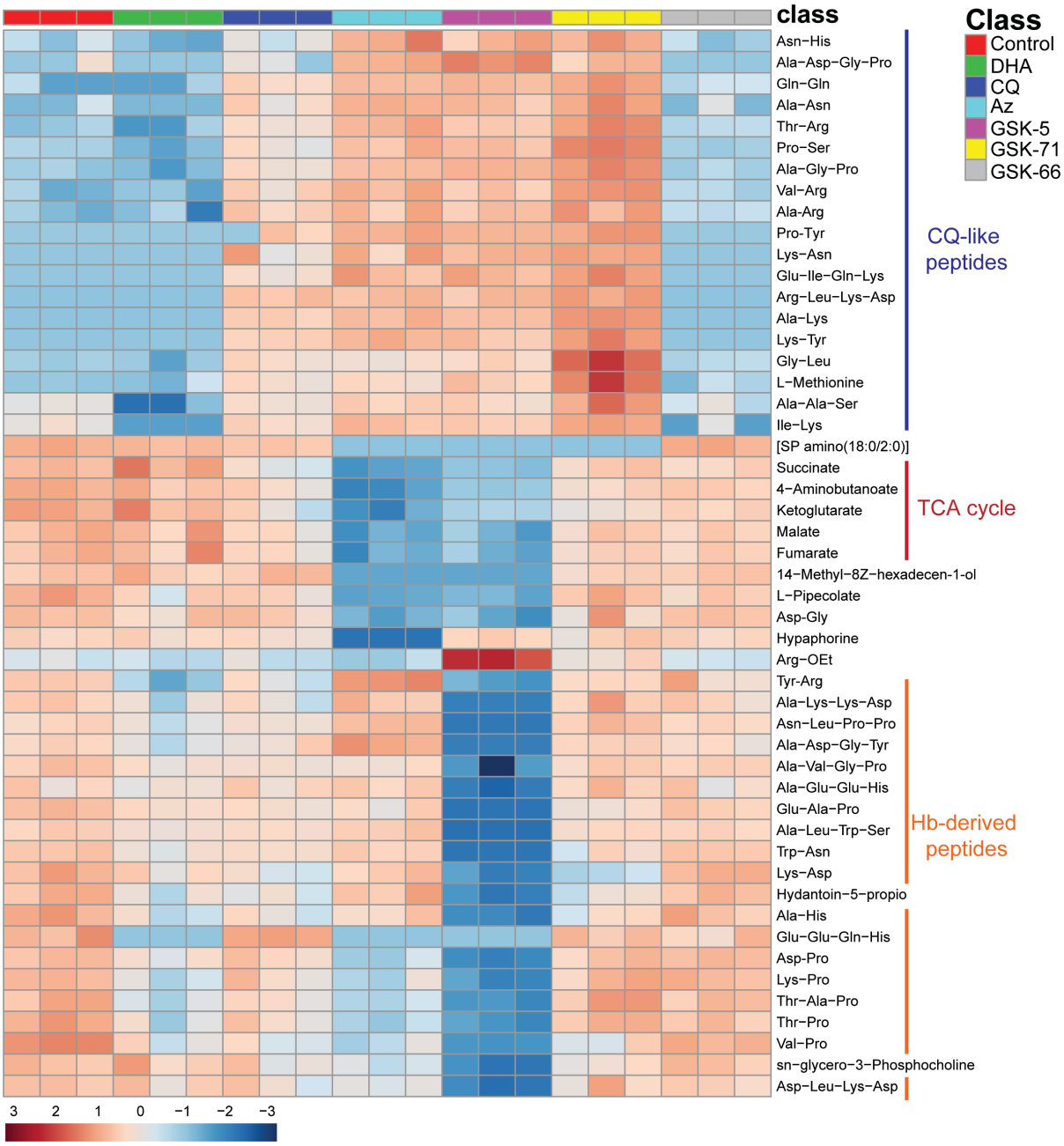

class
Class
Control
DHA
CQ
Az
GSK-5
GSK-71
GSK-66
Asn−His
Ala−Asp−Gly−Pro
Gln−Gln
Ala−Asn
Thr−Arg
Pro−Ser
Ala−Gly−Pro
Val−Arg
Ala−Arg
Pro−Tyr
Lys−Asn
Glu−Ile−Gln−Lys
Arg−Leu−Lys−Asp
Ala−Lys
Lys−Tyr
Gly−Leu
L−Methionine
Ala−Ala−Ser
Ile−Lys
[SP amino(18:0/2:0)]
Succinate
4−Aminobutanoate
Ketoglutarate
Malate
Fumarate
14−Methyl−8Z−hexadecen−1−ol
L−Pipecolate
Asp−Gly
Hypaphorine
Arg−OEt
Tyr−Arg
Ala−Lys−Lys−Asp
Asn−Leu−Pro−Pro
Ala−Asp−Gly−Tyr
Ala−Val−Gly−Pro
Ala−Glu−Glu−His
Glu−Ala−Pro
Ala−Leu−Trp−Ser
Trp−Asn
Lys−Asp
Hydantoin−5−propio
Ala−His
Glu−Glu−Gln−His
Asp−Pro
Lys−Pro
Thr−Ala−Pro
Thr−Pro
Val−Pro
sn−glycero−3−Phosphocholine
Asp−Leu−Lys−Asp
CQ-like peptides
TCA cycle
Hb-derived peptides
3
2
1
0
−1
−2
−3

Figure 4.5 Heat map and hierarchical clustering of metabolites that differed significantly after control drug and azithromycin analogue treatment.

Parasites were treated with chloroquine (CQ) (blue), DHA (green), azithromycin (Az) (light blue), GSK-5 (purple), GSK-71 (yellow), GSK-66 (grey), and ethanol control (red). Vertical clustering displays similarities between sample groups, while horizontal clusters reveal the relative abundances of the 50 most significantly different metabolites. The significantly differentially regulated metabolites are further classified into three different groups, the CQ-like peptides (blue line), TCA cycle (red line) and haemoglobin-derived peptides (orange lines). All compounds were tested with three technical replicates. White indicates no change, while red and blue indicates increased and decreased abundances, respectively. Ward's minimum variance method algorithm was used to generate the hierarchical cluster analysis. Figure supplied by Ghizal Siddiqui (Monash University, Australia).

Increases in these peptides have previously been demonstrated for chloroquine treatment of trophozoites (312), but it was also noted in the study by Creek et al., (2016) that the majority of these peptides could not be directly linked to haemoglobin degradation, the parasites' major food source (**S. Table 4.8**). Paradoxically, GSK-66 which has the most chloroquine like functional group in terms of structure and was the most potent analogue tested in this study, showed little in the way of changed metabolites and gave a profile most similar to untreated control. Since chloroquine is known to inhibit digestion of haemoglobin and formation of hemozoin (145, 166, 169), we next tested analogues GSK-66 (chloroquinoline) and GSK-71 (quinoline) in a haemoglobin fractionation assay (522) (**Figure 4.6**). Trophozoite stage parasites were treated with chloroquine, GSK-71, and GSK-66 at 10x IC_{50} for 5 hrs. There was an increase in measurable haemoglobin, a corresponding small increase in free heme and reduction in hemozoin formation for parasites treated with chloroquine, as expected for this known inhibitor of haemoglobin digestion and hemozoin formation. A similar, build-up in haemoglobin was seen for GSK-71, however, there was no loss in hemozoin, supporting that this drug may have activity in the food vacuole, but this did not translate into measurable inhibition of hemozoin formation. Again, GSK-66 treatment had no effect on haemoglobin or hemozoin levels, supporting the non-targeted metabolomics data which suggests that this drug has limited effects on parasite metabolism at the concentration and duration tested (**Figure 4.6** & **S. Table 4.8**). These data support that azithromycin and analogues have activity in the parasites food vacuole of drug treated trophozoites.

A second shared metabolomic signature was observed for azithromycin and the phenyl substituted analogue, GSK-5, with a major reduction in key metabolites (including succinate, fumarate, malate) of the mitochondrial located tricarboxylic acid (TCA) cycle (**S. Figure 4.5** & **S. Table 4.9**). Although several steps in the *Plasmodium* TCA cycle are considered dispensable in blood stage parasites, the fumarate hydratase conversion of fumarate to malate followed by the malate quinone oxidoreductase (MQO) mediated conversion of malate to oxaloacetate are thought to have important roles in the parasites purine salvage pathway (528, 529). Reduced bioavailability of fumarate and malate, two key metabolites required for efficient purine salvage, would negatively impact on purine production and parasite growth over time and offers a novel drug development strategy. Indeed, a recent paper has identified blood stage inhibitors of MQO in the Pathogen Box (530) suggesting that this pathway is a viable drug target against asexual stage parasites.

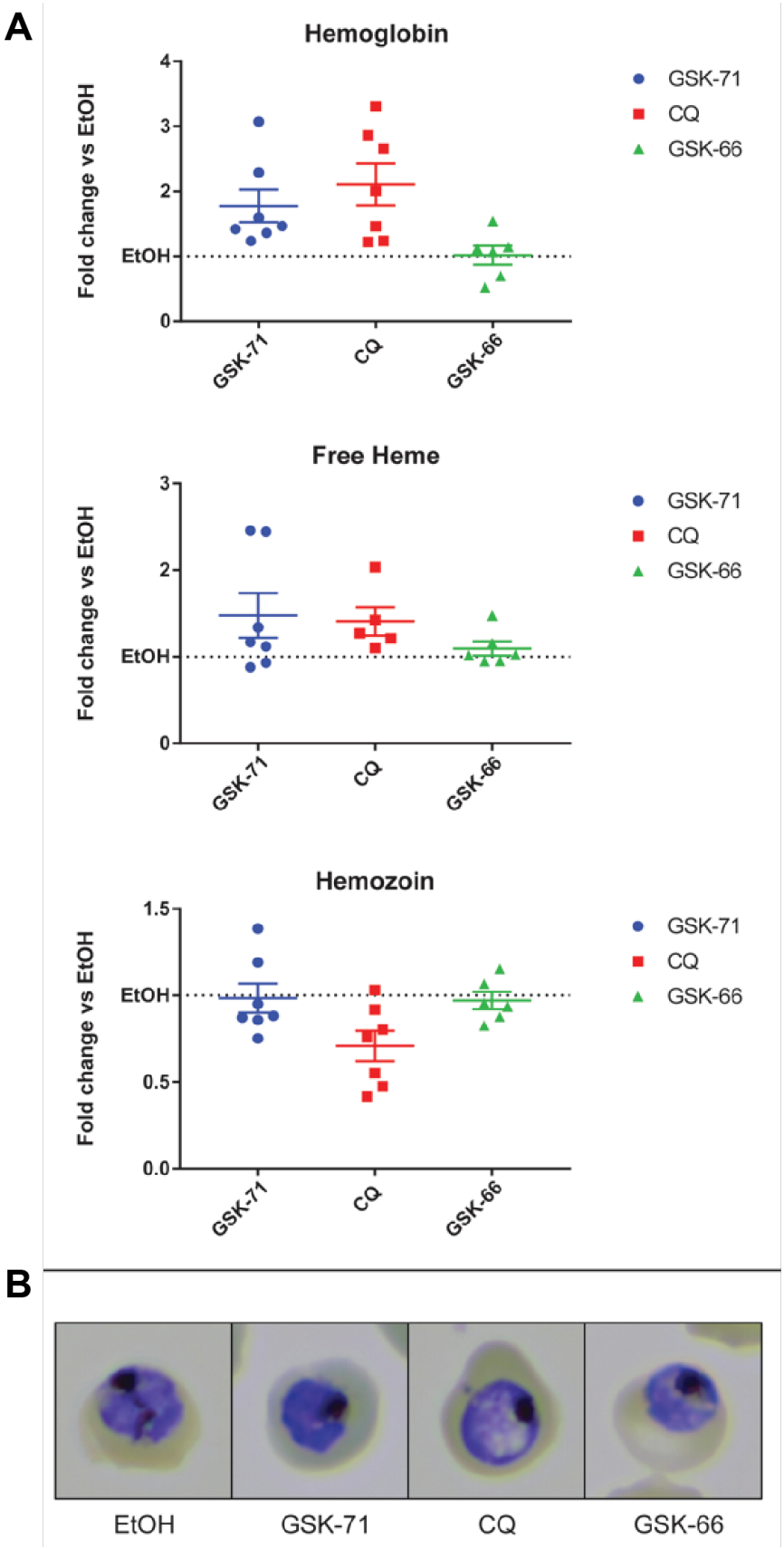

A
Hemoglobin
Fold change vs EtOH
4
3
2
EtOH
0
GSK-71
CQ
GSK-66
GSK-71
CQ
GSK-66
Free Heme
Fold change vs EtOH
3
2
EtOH
0
GSK-71
CQ
GSK-66
GSK-71
CQ
GSK-66
Hemozoin
Fold change vs EtOH
1.5
EtOH
0.5
0.0
GSK-71
CQ
GSK-66
GSK-71
CQ
GSK-66
B
EtOH
GSK-71
CQ
GSK-66

Figure 4.6 Haemoglobin fractionation assay to compare activity of azithromycin analogues to the haemoglobin digestion pathway inhibitor chloroquine.

A) Scatter dot plot representing the relative levels of haemoglobin, free haem and haemozoin in trophozoite stage parasites following a 5 h incubation with 10x IC_{50} (44 hr) concentration of GSK-71, chloroquine (CQ), and GSK-66 expressed as the fold change when compared to an EtOH control. Data is represented as the mean of >3 paired replicates from three independent experiments with the error bars expressed as SEM. **B**) A panel of representative Giemsa stained parasites treated with 10x IC_{50} (44 hr) concentration of GSK-71, chloroquine, GSK-66, and the ethanol negative control after 5 h. Figure supplied by Ghizal Siddiqui (Monash University, Australia).

These data implicate a second membrane bound organelle as a potential target during trophozoite stages of the parasite lifecycle, underlining the potential for multifactorial mechanisms of action. Uniquely in these experiments, GSK-5 also caused a reduction in haemoglobin derived peptides at levels significantly and consistently lower than seen for chloroquine and DHA, two food vacuole targeting drugs (**S. Table 4.8**). Thus, treatment with GSK-5 shared a metabolomics signature with chloroquine that was over-represented with up-regulated non-haemoglobin derived peptides (**S. Table 4.7**), a consistent decrease in haemoglobin derived peptides (unique to GSK-5 in this data set) (**S. Table 4.8**) and a decrease in TCA cycle metabolites (shared with azithromycin) (**S. Table 4.9**). GSK-5 highlights the ability of azithromycin analogues with different structures to interrupt normal metabolic functions of different organelles, even when used at the same fold-IC_{50} and against the same lifecycle stages.

Given the metabolomics evidence suggesting that azithromycin and analogues may target the food vacuole, we investigated whether the rapid ring stage killing activity of the chloroquinoline analogue GSK-66 may be a result of azithromycin pre-sensitising ring stages to the chloroquinoline moiety (**Figure 4.2 A** & **B**, **S. Table 4.1-4.3**). We treated early-ring stage D10-PfPHG parasites (0-6 hrs) with azithromycin at an IC_{10} concentration and added a dilution series of chloroquine. Addition of azithromycin did not potentiate chloroquine's activity against early ring stages, with the IC_{50} of azithromycin+chloroquine remaining well above the activity of GSK-66 (**S. Figure 4.6**). In addition, a range of functional groups were found to potentiate azithromycin's quick-killing activity. These combined data suggest that azithromycin does not pre-sensitise parasites to chloroquinoline-like moieties nor act through disruption of haem-polymerisation *per se* as chloroquine is believed to, but rather may act more broadly within the parasite's food vacuole.

4.6 Discussion.

The spread of parasites resistant to artemisinin combination therapies (ACTs) in South-East Asia, India, and Papua New Guinea highlights the need for novel antimalarial drug treatment strategies to ensure timely and effective treatment of clinical disease (134, 224, 225, 228, 229). Despite limited use against clinical cases of malaria, macrolide antibiotics remain of interest as potential partner antimalarials due to their well-established safety profile in children and pregnant women (470, 477, 524, 531). Recently, we identified that high concentrations of clinically used macrolides inhibit merozoite invasion *in vitro* and showed that this mechanism of action was independent of apicoplast targeting delayed death (165). Here, we demonstrate the potential for the antibiotic azithromycin to be repurposed as an antimalarial with two potent mechanisms of action.

Previous assessment of these analogues *in vitro* identified up to an 800-fold improvement in activity against a drug sensitive 3D7 line in assays that measured growth across one complete blood stage cycle (72 hr treatment time, **Figure 4.1 C**) (418, 482-486). We investigated the activity of a panel of the analogues and identified 64 with improved in cycle activity (44 hr early rings to schizont treatment) compared to azithromycin. Of these, 38 analogues with diverse functional groups including substituted phenyl (GSK-5, IC_{50} 0.02 μM), naphthalene (GSK-3, IC_{50} 0.183 μM), quinoline (GSK-58, IC_{50} 0.048 μM) and chloroquinoline (GSK-66, IC_{50} 0.007 μM) had nanomolar IC_{50}s, providing between an 11 to 1615-fold improvement over azithromycin.

Azithromycin and analogues exhibited equipotent quick-killing activity across intracellular blood stage parasite growth. This included rapid activity against early ring stage development (both 0-6 and 0-12 hrs post invasion) at a similar potency to 0-44 hr (one cycle) treatments. Therefore, azithromycin and analogues have a similar efficacy profile to the artemisinins (370, 525), being effective against early ring stages and across the blood stage lifecycle, but with additional potential to be active against liver and transmission stage parasites (162-164). We found that the azithromycin analogues with the best activity in 44 hr assays (GSK-3, 5, 56 and 72), also exhibited the greatest improvement in invasion inhibitory activity over azithromycin, highlighting that both quick-killing activities can be improved over azithromycin. However, the ability to push potency of merozoite invasion into clinically relevant concentrations below 1 μM may be limited. Importantly, assays where merozoites were treated directly prior to compound removal and addition of RBCs to begin invasion show that the invasion inhibitory

activity of azithromycin and analogues is directed against the merozoite and not against the RBC.

It is interesting to note that improved quick-killing activity is ubiquitous across analogues with phenyl, naphthalene, quinoline and chloroquinoline functional groups (**S. Table 4.1-4.3**). It has previously been hypothesised that the high potency of several analogues featuring quinoline and chloroquinoline moieties was due to these analogues acting like hybrid azithromycin (apicoplast ribosome targeting) and chloroquine (food vacuole target) activity (484, 485) molecules. Interestingly, azithromycin analogues with the four functional groups display properties dissimilar to chloroquine, these being: (i) improved invasion inhibitory activity compared to azithromycin, whereas chloroquine does not inhibit invasion (131, 151); (ii) similar activity against chloroquine resistant and sensitive lines, particularly for analogues featuring substituted phenyl, naphthalene and quinoline moieties (**Table 4.1** & **S. Table 4.5**); and (iii) potent inhibition of very early ring stages (0-6 hrs post invasion), which are largely insensitive to chloroquine. However, additional evidence from this study does support the idea that azithromycin and analogues quick-killing activity may, in part, be acting against the parasite's food-vacuole.

Although our ability to perform comprehensive and detailed SAR comparison was limited by compound availability impacting on matched-pair analysis, some general trends were observed with the analogues available. Analogues with chloroquinoline and quinoline substituents were generally the most potent in one-cycle 44 hr assays. Naphthalene had modest potency and is a close bioisostere of quinoline. In general, analogues with a short carbon linking the amino quinoline to the N6-position of the macrocycle or the *O*- or *N*- position of the desosamine group were the most active (**Table 4.1** & **S. Table 4.1-4.3)**. Appending functional moieties to the N6-position of the macrolactone, or to the desosamine sugar, both conferred significantly improved in cycle activity, with a slight tendency for improved quick-killing activity when the functional group was either attached to the *N*- or the *O*- of the desosamine sugar as opposed to the N6-position of the macrolactone (i.e. chloroquinoline GSK-66^{desos} (IC_{50} 0.007 μM) and GSK 1^{macro} (IC_{50} 0.019 μM); naphthalene GSK 73^{desos} (IC_{50} 0.31 μM) & GSK-20^{macro} (IC_{50} 0.183 μM)). Thus, the position of the functional group on the macrocyclic did not greatly impact activity, suggesting the macrocycle may be acting as a vehicle for transportation of the active functionality.

Within the parasite, it is possible analogues are metabolised and then release the pendant quinoline or aromatic system as the active component of compound. This is possible either by an oxidative mechanism hydrolysing amine linked substituents, or by proteolytic or hydrolytic degradation of the amide and urea functionality linking the pendant quinoline or aromatic group to the macrolactone. In this study, we could not conclusively address whether metabolism was occurring, but this will be an important facet to address in a future mechanistic study of these azalide analogues. The possibility of the macrolactone acting as a delivery vehicle with subsequent metabolic release of the active payload in the parasite raises the prospect for the azithromycin scaffold to be tethered to and act as a delivery vehicle for other antimalarials that act at a similar asexual killing rate to chloroquine, akin to antimalarial candidates undergoing clinical trials such as KAF156 or MMV048 (532). Such a strategy to improve dual target efficacy of azithromycin derivatives, and delay the onset of resistance, are attractive options. Furthermore, while it has been demonstrated that these analogues have efficacy *in vivo* rodent models (418, 484, 486), the effective contribution of quick-killing has not been assessed. In addition, whether these analogues would be stable to first pass metabolism in the liver is another important aspect to consider in future development of the azalide analogue class.

Metabolomic analysis of azithromycin and analogue treated parasites supports that one potential site of drug activity in trophozoite stages is the parasites food-vacuole, with a similar build-up of non-haemoglobin peptides observed for azithromycin, analogues GSK-5 and GSK-71 as seen for chloroquine. However, a number of differences to chloroquine were also observed including the chloroquinoline modified analogue GSK-66 causing minimal change in parasite metabolism, azithromycin and GSK-5 having activity against mitochondrial metabolism and GSK-5 also causing a reduction in haemoglobin derived peptides. While there are limitations in this analysis, including only one lifecycle stage and drug concentration (5x the 44 hr IC_{50}) tested for each analogue, these data clearly demonstrate that azithromycin and analogues likely have multi-factorial mechanisms of action even against a single lifecycle stage. Given the apparent site of activity for azithromycin and analogues includes the membrane bound food-vacuole and mitochondrion, it is possible that additional membrane bound organelles in other lifecycle stages (i.e. the rhoptry in merozoites) could also be the target of these drugs. Additional experimental validation for the site of activity across a range of analogues and lifecycle stages will need to be undertaken in order to detail the potential promiscuity of these drugs in stopping parasite growth.

Previous studies have suggested that azithromycin analogues may act through a chloroquine-like mechanism (484-486) (reviewed in (487)) and evidence presented in this study from metabolomic experiments and haemoglobin fractionation assays supports that one of the sites of activity for azithromycin and analogues is the parasites food-vacuole. If a chloroquine-like targeting of the food-vacuole is an important component of azithromycin and analogues quick-killing activity these modified analogues have two major advantages over chloroquine and quinine for clinical treatment. Firstly, phenyl, naphthalene and quinoline substituted analogues maintained reasonable activity against chloroquine resistant DD2 parasites. The maintenance of potency against chloroquine resistant parasites could be explained by the different properties of the drug limiting the ability of the mutated Chloroquine Resistant Transporter to expel the drug from the developing vacuole (533, 534).

Secondly, azithromycin and analogues have rapid activity against early rings stage parasites. Rapid activity against ring stages is in stark contrast to the poor activity of chloroquine and quinine against these early parasites and it is certainly possible that azithromycin and analogues could access the site of commencing haemoglobin digestion, similar to artemisinin (27, 370, 525), via superior lipophilic properties (484, 485).

Azithromycin and analogues display several other properties of interest. Quick-killing analogues examined in this study maintained activity against chloroquine/pyrimethamine resistant DD2 (**Table 4.1** & **S. Table 4.5**) (496) as well as against artemisinin resistant Cam3.II$^{\text{DHA resistant(R539T)}}$ (222, 226) (**Table 4.2)**, indicating that a number of established resistance mechanisms would not reduce the efficacy of azithromycin analogues in field conditions. Azithromycin and analogues invasion blocking activity is shared across distantly related Apicomplexan parasites such as *Toxoplasma gondii* (165, 488), *P. berghei* (165) and the zoonotic human malaria parasite *P. knowlesi*. Since neither *T. gondii* nor *Plasmodium* spp. merozoites contain a food vacuole, the target of chloroquine, it seems likely that azithromycin and analogues have additional mechanisms of action, with properties such as modulation of intraerythrocytic calcium (Ca^{2+}), interference of kinase signalling pathways, cationic trapping and sequestration within acidic environments, as well as decreasing mobility of phospholipid bilayers demonstrated for azithromycin in other eukaryotic cell systems all potential alternative MOAs contributing to quick-killing (416, 535-538).

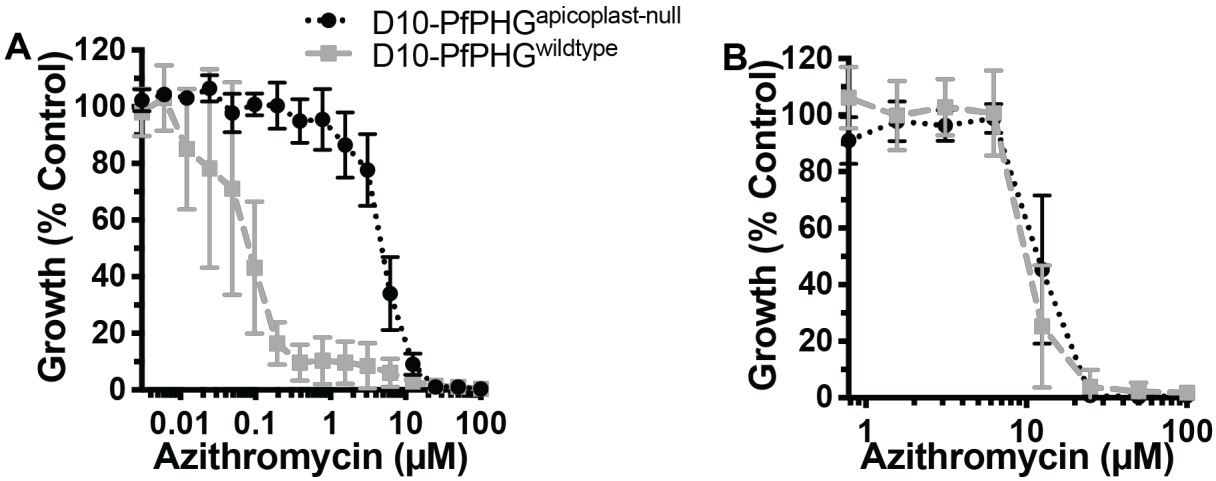
Finally, the influence of the site of modification to azithromycin and the addition of different functional groups was investigated in the context of delayed death activity. Previous studies have demonstrated that the desosamine sugar is critical for binding to bacterial ribosomes, and

we anticipated that modifications to this region would stop apicoplast-targeting delayed death activity (160, 414, 420). However, the potent quick-killing activity of azithromycin analogues (GSK-4, 5, 12, 16, 57, 71 etc. **S. Table 4.6**) precluded assessment of delayed death activity using traditional 120 hr parasite assays. Therefore, we assessed whether a focused set of azithromycin analogues maintained their activity against prokaryotic ribosomes by determining the minimum inhibitory concentration (MIC) activity of the gram-positive bacteria, *S. pneumoniae* (**S. Table 4.6**). Comparison of *P. falciparum* quick-killing IC_{50} and *S. pneumoniae* MIC confirmed that attaching the functional group to the desosamine sugar (GSK-57, 66, 71 and 78) abrogated activity against bacterial ribosomes as expected. In contrast, analogues with the functional group attached to the N6-positon of the macrolactone (GSK-1, 4, 5, 6, 9, 11, 12, 16, 17, 21, 25) maintained activity against *S. pneumoniae*, suggesting that delayed death activity via targeting the bacterium-like ribosome of the apicoplast is maintained in analogues featuring modification to the N6-positon of the macrolactone (GSK-1, 4, 5, 6, 9, 11, 12, 16, 17, 21, 25). Thus, analogues could be modified to act through either single (i.e. quick-killing) or dual (i.e. quick-killing and delayed death) mechanisms of action depending on the properties desired (i.e. quick parasite clearance and/or long-term prophylaxis) and whether removal of non-selective antibiotic activity is preferred over apicoplast-targeting delayed death prophylaxis.

4.7 Conclusion.

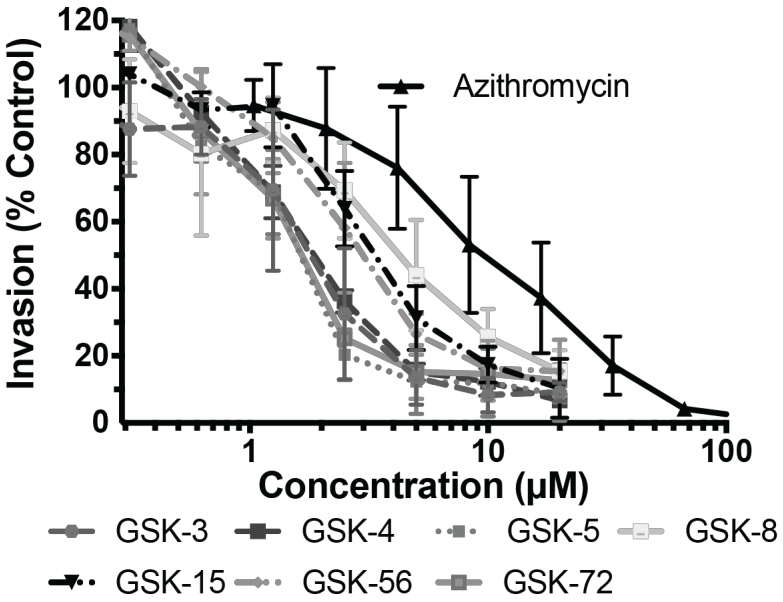
We have shown that azithromycin and analogues have a quick-killing mechanism of action that kills parasites throughout intracellular blood stage development, including inhibition of merozoite invasion of RBCs. Additionally, azithromycin analogues exhibit promising potency against very early ring stage parasites, which is a rare feature amongst antimalarials. Importantly, quick-killing can be improved without losing activity against protein synthesis by the apicoplast ribosome (delayed death). Conversely, the option to engineer azithromycin to remove activity against a bacterium-like ribosome and thereby avoid selection for 'bystander' bacterial resistance is available. Further development of azithromycin analogues offers the prospect of designing compounds with either quick-killing (quick-parasite clearance) mode of action or both quick-killing and slow-killing prophylactic activity. This design strategy should also retard resistance acquisition by hitting two targets. Fine tuning the quick-killing activity of azithromycin analogues significantly broadens its clinical applications and proffers resistance proofing through two independent mechanisms of action.

4.8 Supplementary figures for 'Retargeting the antibiotic azithromycin as an antimalarial with dual modality".



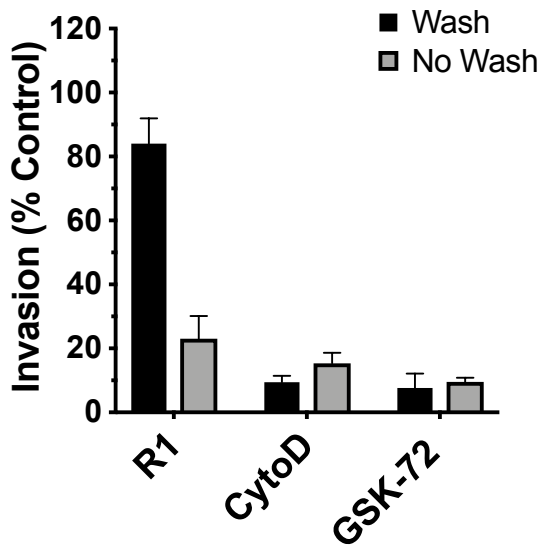
S. Figure 4.1 Growth inhibition profiles of azithromycin in parasites lacking the apicoplast compared to wildtype.

Early ring stage *P. falciparum* parasites (0-4 hrs post-invasion) were treated with doubling dilutions of azithromycin and inhibition of growth measured for **A)** 2 cycle (delayed death, 120 hrs) assays (D10-PfPHG$^{apicoplast\text{-}null}$ IC_{50}, 4.5 μM; D10-PfPHG^{wildtype} IC_{50}, 0.07 μM. P= <0.0001) or **B)** 44 hrs (in cycle) (D10-PfPHG$^{apicoplast\text{-}null}$ IC_{50}, 16 μM; D10-PfPHG^{wildtype} IC_{50}, 11.3 μM. P=0.24) assays. Parasitemia was measured at 120 hrs or 44 hrs post invasion, respectively, at schizont stage via flow cytometry. Data represents the mean of 3 (or more) experiments expressed as percentage of non-inhibitory control and error bars represent ± SEM.



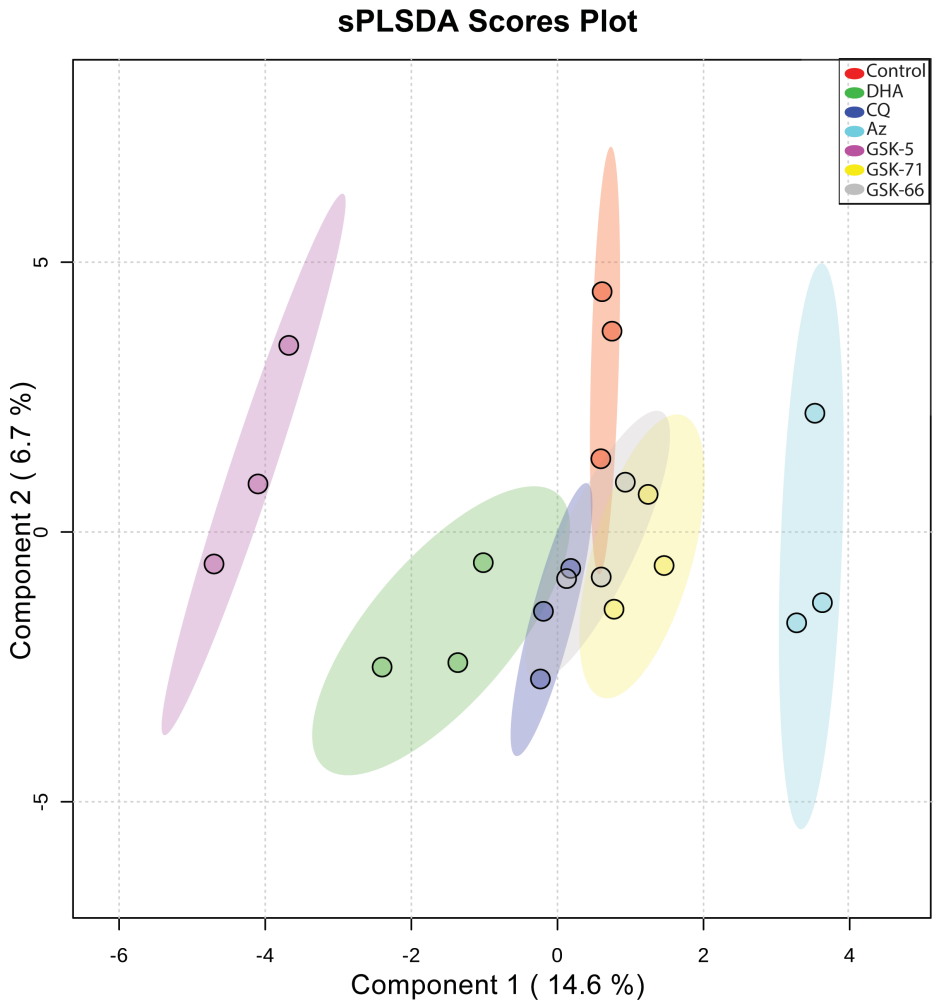
S. Figure 4.2 Azithromycin analogues show improvement in invasion inhibitory activity.

Screening a panel of azithromycin analogues identified 7 with up to 6-fold lower invasion inhibitory IC_{50} activity in contrast to the parental drug azithromycin. IC_{50}s and assessment of invasion inhibitory activity compared to azithromycin are as follows: Azithromycin 10 μM; GSK-4, 2.0 μM (Azithromycin vs GSK-4 P= <0.0001); GSK-5, 1.61 μM (Azithromycin vs GSK-5 P= <0.0001); GSK-56, 3.2 μM (Azithromycin vs GSK-56 P= <0.0001); GSK-8, 4.4 μM (Azithromycin vs GSK-8 P=0.2); GSK-3, 1.8 μM (Azithromycin vs GSK-3 P= <0.0001); GSK-15, 3.6 μM (Azithromycin vs GSK-15 P= <0.001); GSK-72, 1.7 μM (Azithromycin vs GSK-72 P= <0.0001). Newly invaded ring stage parasitemia was measured at 1 hr post invasion at via flow cytometry. Data represents the range or the means of 2 or 3 experiments, respectively, expressed as percentage of non-inhibitory control and error bars represent the range or the ± SEM.



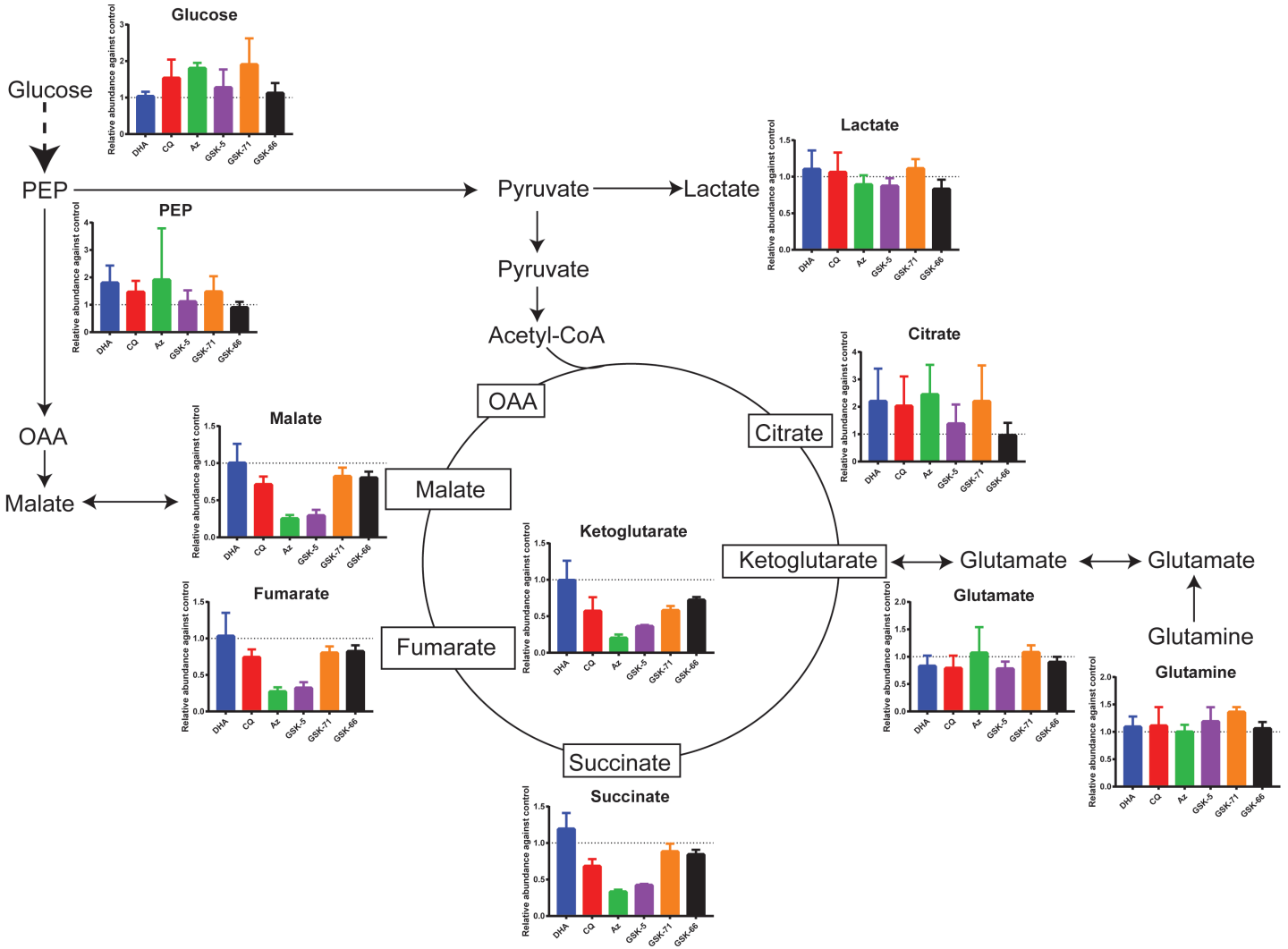
S. Figure 4.3 Azithromycin analogues inhibit merozoite invasion irreversibly.

Purified merozoite washout assay, where the invasion inhibitory effect of azithromycin analogue GSK-72 at 10 μM was compared to other invasion inhibitory drugs. 500 μM Cytochalasin D (cytoD) was included as an irreversible washout control, while 100 μg/mL of R1 peptide was included as a reversible control. Ring stage parasitaemia of newly invaded parasites was determined ~30 minutes post invasion by flow cytometry, with results presented as % parasitaemia relative to a media control. Experiment performed in biological duplicate, with each result representative of the mean of technical duplicates and the error bars represent the ± range of 2 experiments.



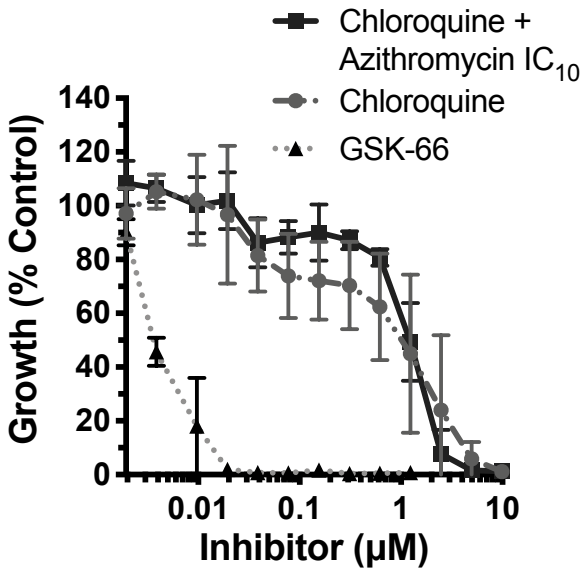
S. Figure 4.4 Sparse partial least square-discriminant analysis (SPLS-DA).

Plasmodium falciparum (D10-PfPHG)-infected red blood cells following treatment with DHA (green), chloroquine (blue), azithromycin (light blue), GSK-5 (purple), GSK-71 (yellow), GSK-66 (grey), and ethanol control (red). sPLS-DA showing scores plot for components one and two, the plots were generated using the top 10 metabolites for each component. Points represent individual sample replicates while the 95% confidence interval is represented by the shaded region. Figure supplied by Ghizal Siddiqui (Monash University, Australia).


Glucose
PEP
Pyruvate
Lactate
Pyruvate
Acetyl-CoA
OAA
Malate
OAA
Malate
Citrate
Ketoglutarate
Glutamate
Glutamate
Glutamine
Fumarate
Succinate
Glucose
PEP
Lactate
Malate
Citrate
Ketoglutarate
Glutamate
Glutamine
Fumarate
Succinate
Relative abundance against control
DHA
CQ
Az
GSK-5
GSK-71
GSK-66

S. Figure 4.5 Model for TCA metabolism following treatment of *Plasmodium falciparum* (D10-PfPHG).

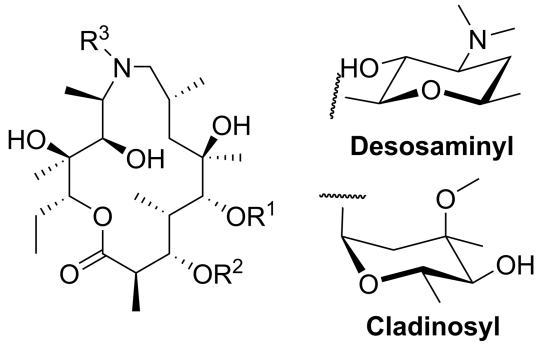
Relative abundance of the TCA metabolites from infected red blood cells treated with DHA (blue), chloroquine (red), azithromycin (green), GSK-5 (purple), GSK-71 (orange), GSK-66 (black), compared with the ethanol control. Data are represented as mean fold change multiplied by corresponding relative standard deviation values. Abbreviations: OAA, oxaloacetate; PEP, phosphoenolpyruvate. Figure supplied by Ghizal Siddiqui (Monash University, Australia).

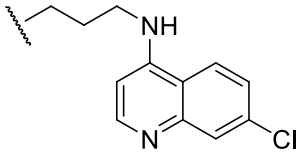
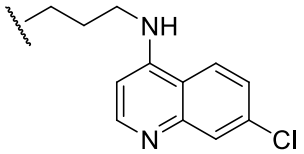


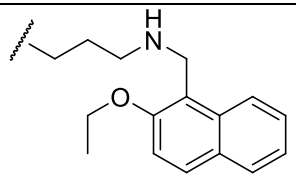
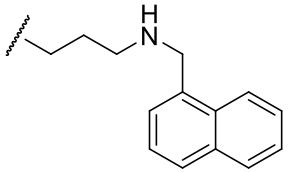
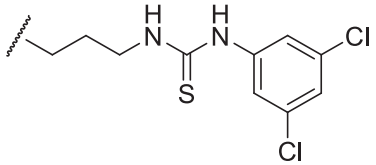
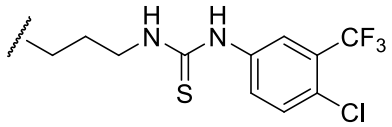
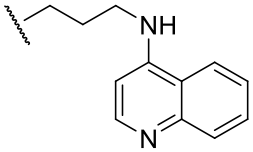
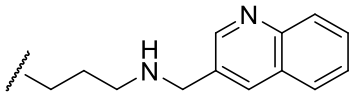
S. Figure 4.6 Azithromycin does not pre-sensitise early-ring stages to chloroquine.

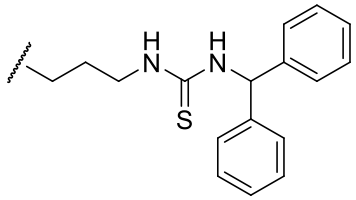
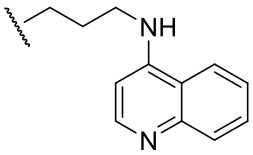
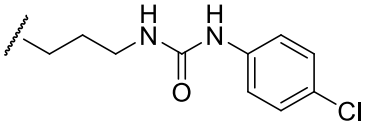
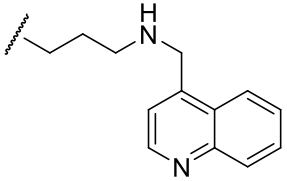
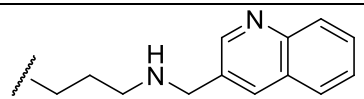
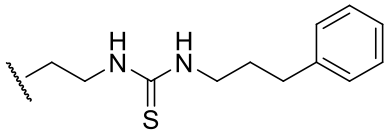
Early ring stage *P. falciparum* parasites (0-4 hrs post-invasion) were treated with doubling dilutions of chloroquine + IC_{10} of azithromycin (IC_{50}; 0-6 hrs, 1.1 μM), chloroquine (IC_{50}; 0-6 hrs, 0.73 μM) or GSK-66 (IC_{50}; 0-6 hrs, 0.004 μM) for 6 hrs, prior to removal of drugs by washing (chloroquine vs chloroquine + azithromycin P=0.0041), (chloroquine vs GSK-66 P= <0.0001) (chloroquine + azithromycin vs GSK-66 P= <0.0001). Parasitemia was measured at 44 hrs post invasion at schizont stage via flow cytometry. Data represents the mean of 3 (or more) experiments expressed as percentage of non-inhibitory control and error bars represent ± SEM.

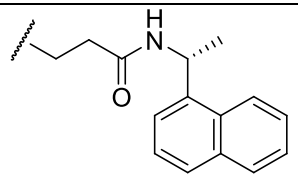
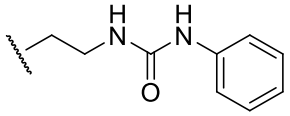
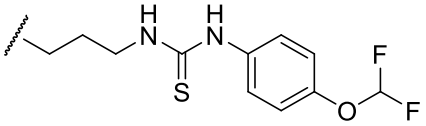
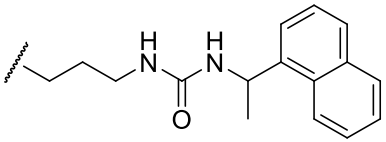
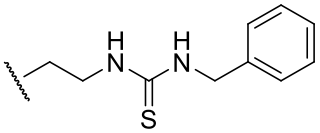
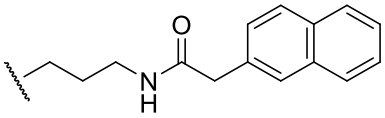
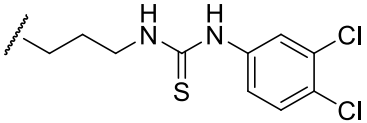
S. Table 4.1 Activities of N6-substituted analogues.

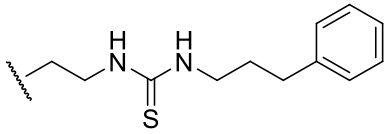
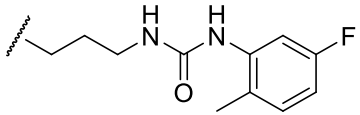
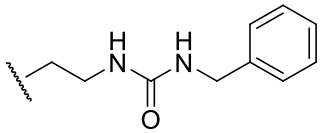
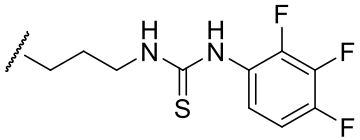
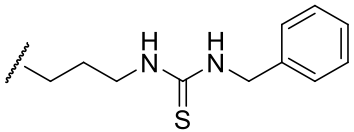
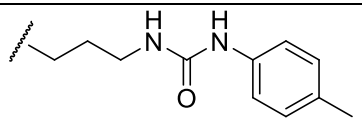
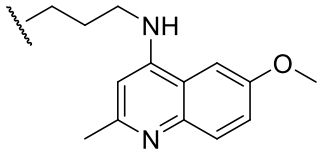


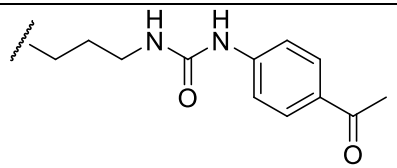
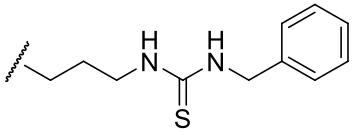
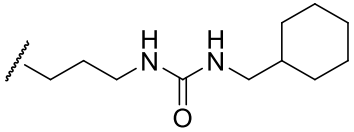
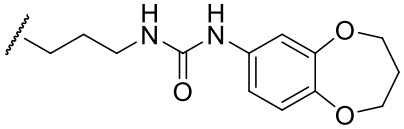
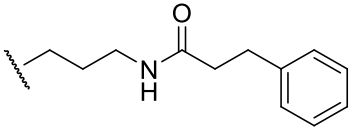
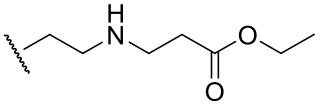
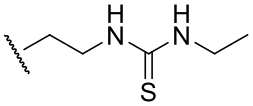
R[1]	R[2]	R[3]	Analogue #	Ref	D10-PfPHG growth (% growth at 10 μM) [a]	D10-PfPHG growth (IC_{50}) μM [a]	Invasion inhibition (% rings at 1 μM) [b]	D10-PfPHG Apicoplast-null growth IC_{90} (% growth) [c]	PkYH1 growth (IC_{50}) μM[a]
desosaminyl	cladinosyl	Me	**Az**	-	63.4	11.3	106		
desosaminyl	cladinosyl		**1**	(485)	10.4	0.019	95.5	28.6	0.2
desosaminyl	H		**2**	(485)	0.31	0.024	104	ND	ND

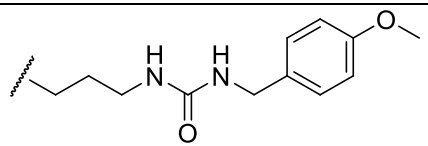
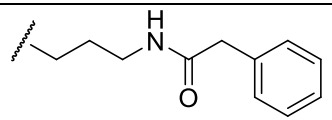
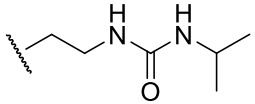
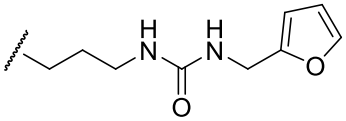
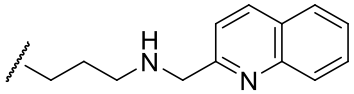
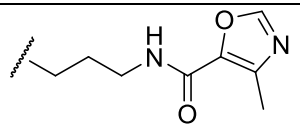
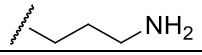
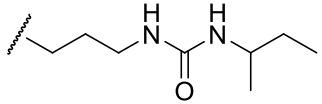
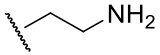
desosaminyl	cladinosyl		**3**	(484)	12	0.18	74	5.0	0.095
desosaminyl	cladinosyl		**4**	(484)	4	0.2	72	ND	ND
desosaminyl	cladinosyl		**5**	(483)	3	0.2	75.4	0.3	0.082
desosaminyl	cladinosyl		**6**	(483)	8	0.28	97.1	1.8	0.16
desosaminyl	H		**7**	(484)	13	0.39	98.7	ND	ND
desosaminyl	cladinosyl		**8**	(484)	0.97	0.88	102	ND	0.15

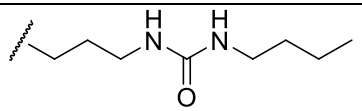
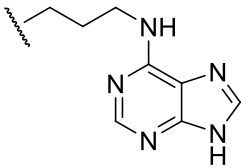
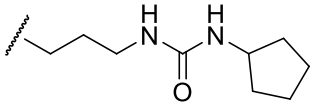
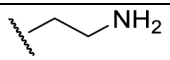
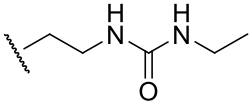
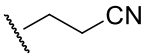
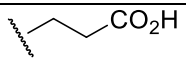
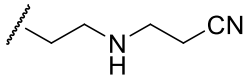
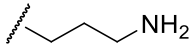
desosaminyl	cladinosyl		**9**	(484)	26	0.44	96.7	4.4	0.016
desosaminyl	cladinosyl		**10**	(484)	10.3	0.48	82.5	2.2	0.1
desosaminyl	cladinosyl		**11**	(483)	1.2	0.53	98.6	ND	ND
desosaminyl	cladinosyl		**12**	(484)	7.8	0.59	108	ND	ND
desosaminyl	H		**13**	(484)	13	0.61	100	ND	ND
desosaminyl	cladinosyl		**14**	(418)	3.1	0.65	93.3	ND	ND

desosaminyl	cladinosyl		**15**	(484)	1.01	0.66	87.3	9.1	ND
desosaminyl	cladinosyl		**16**	(418)	2.4	0.7	114	ND	ND
desosaminyl	cladinosyl		**17**	(483)	14.3	0.7	81.7	7.3	0.36
desosaminyl	cladinosyl		**18**	(418, 482)	1.2	0.84	103	ND	ND
desosaminyl	cladinosyl		**19**	(418)	0.97	0.88	102	ND	ND
desosaminyl	cladinosyl		**20**	(484)	9	1.13	ND	ND	ND
desosaminyl	cladinosyl		**21**	(483)	22	1.2	ND	ND	ND

desosaminyl	cladinosyl		**22**	(418)	15	1.22	ND	ND	ND
desosaminyl	cladinosyl		**23**	(483)	0.33	1.28	ND	ND	ND
desosaminyl	cladinosyl		**24**	(418)	0.44	1.3	115	ND	ND
desosaminyl	cladinosyl		**25**	(483)	0.55	1.4	ND	ND	ND
desosaminyl	cladinosyl		**26**	(418, 482)	0.08	1.71	ND	ND	ND
desosaminyl	cladinosyl		**27**	(483)	11	1.84	ND	ND	ND
desosaminyl	cladinosyl		**28**	(484)	1.8	1.91	ND	ND	ND

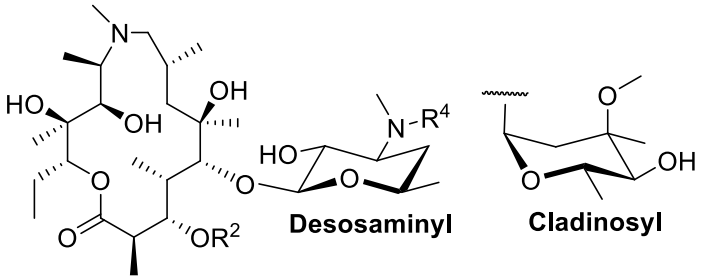
desosaminyl	cladinosyl		**29**	(483)	11.1	2.2	ND	ND	ND
desosaminyl	H		**30**	(418)	0.6	2.32	ND	ND	ND
desosaminyl	cladinosyl		**31**	(483)	0.16	2.92	ND	ND	ND
desosaminyl	cladinosyl		**32**	(483)	0.9	3.07	ND	ND	ND
desosaminyl	cladinosyl		**33**	(484)	7.5	3.41	ND	ND	ND
desosaminyl	cladinosyl		**34**	(418)	28	4.2	ND	ND	ND
desosaminyl	cladinosyl		**35**	(418)	0.54	6.1	ND	ND	ND

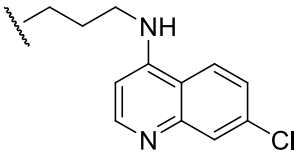
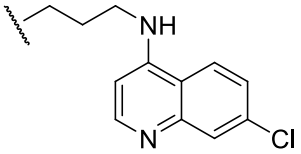
desosaminyl	cladinosyl		**36**	(483)	16.1	6.5	ND	ND	ND
desosaminyl	cladinosyl		**37**	(484)	6	9.4	ND	ND	ND
desosaminyl	cladinosyl		**38**	(418)	22	23.5	ND	ND	ND
desosaminyl	cladinosyl		**39**	(483)	98	ND	ND	ND	ND
H	H		**40**	(484)	89	ND	ND	ND	ND
H	H		**41**	(484)	102	ND	ND	ND	ND
desosaminyl	cladinosyl	H (des-*N*-methyl azithromycin)	**42**	-	105	ND	ND	ND	ND
desosaminyl	cladinosyl	Me (azithromycin)	**43**	-	112	ND	ND	ND	ND
desosaminyl	cladinosyl		**44**	(484)	105	ND	ND	ND	ND
desosaminyl	cladinosyl		**45**	(483)	99.2	ND	ND	ND	ND
desosaminyl	cladinosyl		**46**	(418)	83.1	ND	ND	ND	ND

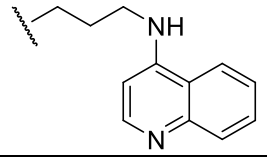
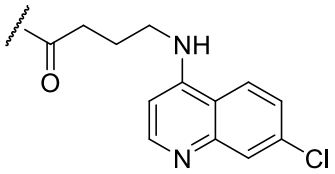
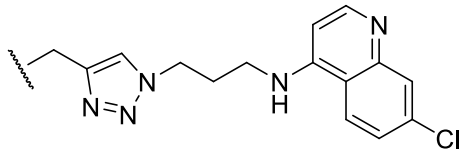
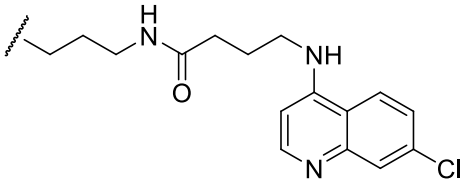
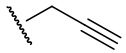
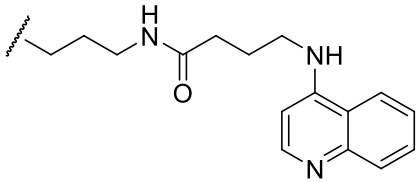
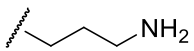
desosaminyl	cladinosyl		**47**	(483)	64	ND	ND	ND	ND
desosaminyl	cladinosyl		**48**	(484)	97.4	ND	ND	ND	ND
desosaminyl	H		**49**	(483)	76.5	ND	ND	ND	ND
desosaminyl	cladinosyl		**50**	(418)	111	ND	ND	ND	ND
desosaminyl	cladinosyl		**51**	(418)	73.1	ND	ND	ND	ND
desosaminyl	cladinosyl		**52**	(418, 483)	100	ND	ND	ND	ND
desosaminyl	cladinosyl		**53**	(484)	101	ND	ND	ND	ND
desosaminyl	cladinosyl		**54**	(418)	84.4	ND	ND	ND	ND
desosaminyl	H		**55**	(484)	98	ND	ND	ND	ND

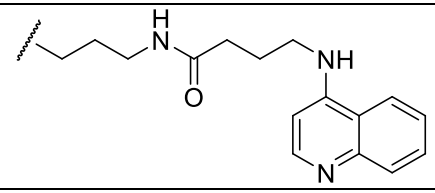
[a] Drug treatment of intracellular growth, from rings to late schizonts, with no rupture cycle (*P. falciparum,* 0-44 hrs or *P. knowlesi,* 0-24 hrs). [b] Drug treatment of merozoites for 10 mins prior to addition of RBCs and a further 50 mins after the addition of RBCs prior to washing the drug out and measuring parasitemia by flow cytometry ~ 1 hr later. [c] Drug treatment of D10-PfPHG$^{apicoplast\text{-}null}$ parasites supplemented with IPP for intracellular growth, from rings to late schizonts, with no rupture cycle (*P. falciparum,* 0-44 hrs). All assays were measured by flow cytometry. Data represents the mean of 3 (or more) experiments expressed as percentage of non-inhibitory control and error bars represent ± SEM. ND= not done, due to <30% activity 0-44 hr in cycle assays, due to lack of analogue or were not prioritised due to more potent alternative structures. Abbreviations: Az, azithromycin.

S. Table 4.2 Activities of desosaminyl *N*-substituted analogues.



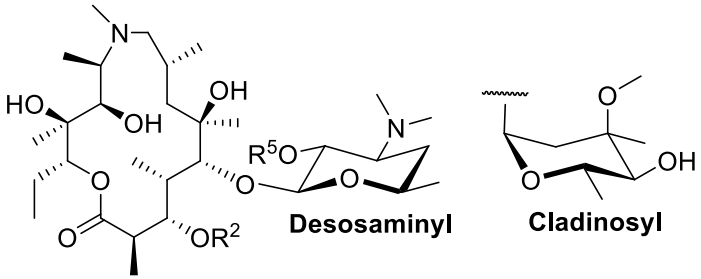
R^2	R^4	Analogue #	Ref	D10-PfPHG growth (% growth at 10 μM) [a]	D10-PfPHG growth (IC_{50}) μM [a]	Invasion inhibition (% rings at 1 μM) [b]	D10-PfPHG Apicoplast-null growth IC_{90} (% growth) [c]	PkYH1 growth (IC_{50}) μM[a]
cladinosyl		**56**	(486)	7	0.011	76.8	17	0.031
H		**57**	(486)	7	0.021	93.9	ND	ND

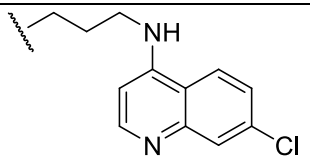
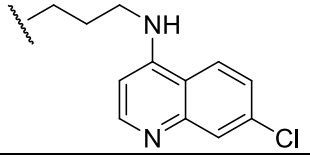
cladinosyl		**58**	(486)	4.2	0.048	94.7	1.6	0.071
cladinosyl		**59**	(486)	8	0.073	108	ND	ND
cladinosyl		**60**	(486)	0.42	0.098	98.5	ND	ND
H		**61**	-	0.4	0.5	102	ND	ND
cladinosyl		**62**	(486)	0.3	3.51	ND	ND	ND
cladinosyl		**63**	(486)	12	3.7	ND	ND	ND
cladinosyl		**64**	(486)	101	ND	ND	ND	ND

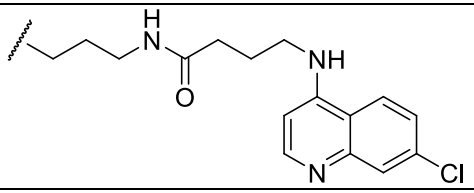
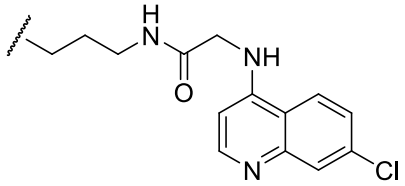
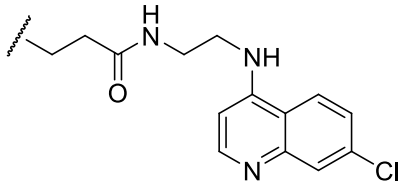
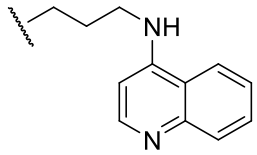
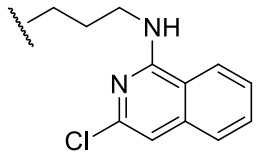
H		**65**	-	101	ND	ND	ND	ND

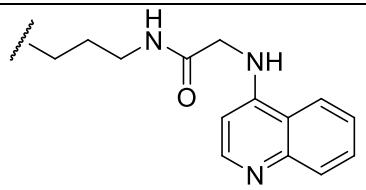
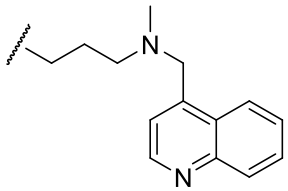
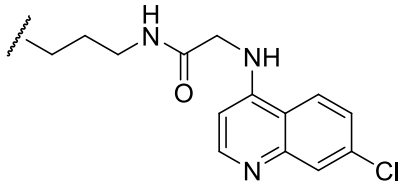
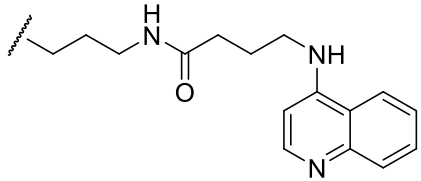
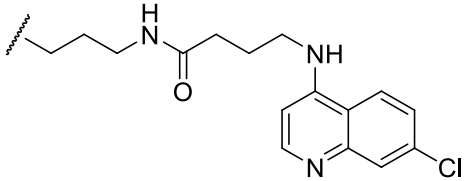
[a] Drug treatment of intracellular growth, from rings to late schizonts, with no rupture cycle (*P. falciparum,* 0-44 hrs or *P. knowlesi,* 0-24 hrs). [b] Drug treatment of merozoites for 10 mins prior to addition of RBCs and a further 50 mins after the addition of RBCs prior to washing the drug out and measuring parasitemia by flow cytometry ~ 1 hr later. [c] Drug treatment of D10-PfPHG$^{apicoplast\text{-}null}$ parasites supplemented with IPP for intracellular growth, from rings to late schizonts, with no rupture cycle (*P. falciparum*, 0-44 hrs). All assays were measured by flow cytometry. Data represents the mean of 3 (or more) experiments expressed as percentage of non-inhibitory control and error bars represent ± SEM. ND= not done, due to <30% activity 0-44 hr in cycle assays, due to lack of analogue or were not prioritised due to more potent alternative structures.

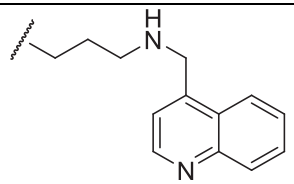
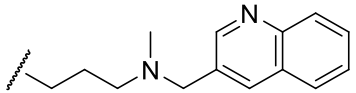
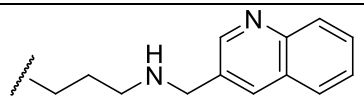
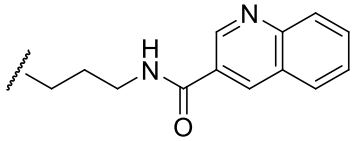
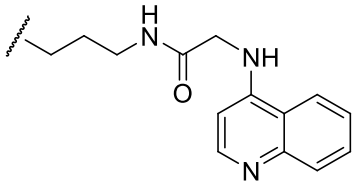
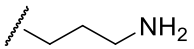
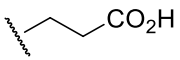
S. Table 4.3 Activities of desosaminyl *O*-substituted analogues.



R^2	R^5	Analogue #	Ref	D10-PfPHG growth (% growth at 10 μM) [a]	D10-PfPHG growth (IC_{50}) μM [a]	Invasion inhibition (% rings at 1 μM) [b]	D10-PfPHG Apicoplast-null growth IC_{90} (% growth) [c]	PkYH1 growth (IC_{50}) μM[a]
cladinosyl		**66**	(485)	0.6	0.007	82.5	12.4	0.012
H		**67**	(485)	0.31	0.024	104	ND	ND

cladinosyl		**68**	(485)	9	0.03	95.4	ND	ND
cladinosyl		**69**	(485)	9	0.03	91.3	ND	ND
cladinosyl		**70**	(485)	10	0.05	78.8	ND	ND
cladinosyl		**71**	(485)	4	0.053	113	9.7	0.041
cladinosyl		**72**	-	6	0.27	79.7	1.6	0.15

cladinosyl		**73**	(485)	0.9	0.31	95.8	1.4	0.248
cladinosyl		**74**	(485)	3	0.34	88	ND	ND
H		**75**	(485)	10	0.35	101	ND	ND
cladinosyl		**76**	(485)	8.5	0.37	89.5	ND	ND
H		**77**	(485)	1.1	0.48	99.4	ND	ND

cladinosyl		**78**	(485)	6	0.51	102	ND	ND
H		**79**	(485)	0.16	1.2	ND	ND	ND
H		**80**	(485)	8	1.98	ND	ND	ND
cladinosyl		**81**	(485)	10	2.76	ND	ND	ND
H		**82**	(485)	1.08	4.8	ND	ND	ND
cladinosyl		**83**	(485)	101	ND	ND	ND	ND
cladinosyl		**84**	(485)	102	ND	ND	ND	ND

[a] Drug treatment of intracellular growth, from rings to late schizonts, with no rupture cycle (*P. falciparum,* 0-44 hrs or *P. knowlesi,* 0-24 hrs). [b] Drug treatment of merozoites for 10 mins prior to addition of RBCs and a further 50 mins after the addition of RBC prior to washing the drug out and measuring parasitemia by flow cytometry ~1 hr later. [c] Drug treatment of D10-PfPHG$^{apicoplast\text{-}null}$ parasites supplemented with IPP for intracellular growth, from rings to late schizonts, with no rupture cycle (*P. falciparum*, 0-44 hrs). All assays were measured by flow cytometry. Data represents the mean of 3 (or more) experiments expressed as percentage of non-inhibitory control and error bars represent ± SEM. ND= not done, due to <30% activity 0-44 hr in cycle assays, due to lack of analogue or were not prioritised due to more potent alternative structures.

S. Table 4.4 In cycle fold-change of analogues in DD2 vs D10-PfPHG.

Modification	Drug	Intracellular growth D10-PfPHG IC_{50} (μM) [a]	Intracellular growth DD2 IC_{50} (μM) [a]	Fold-change DD2 vs D10-PfPHG
	Azithromycin	11.31	15.6	1.4
	Chloroquine	0.052	0.311	5.9
Chloroquinoline	1	0.019	0.082	4.3
	56	0.011	0.093	8.4
	66	0.007	0.043	6.14
	72	0.27	0.065	0.24
Quinoline	8	0.41	0.52	1.3
	10	0.48	0.748	1.5
	58	0.048	0.056	1.1
	71	0.053	0.16	3
	73	0.31	0.48	1.5
Naphthalene	3	0.183	0.32	1.7
	15	0.67	0.4	0.6
Substituted phenyl	5	0.2	0.4	2
	6	0.28	0.27	0.96
	9	0.53	0.24	0.45
	17	0.7	0.54	0.77

[a] Drug treatment of intracellular growth, from rings to late schizonts, with no rupture cycle (*P. falciparum,* 0-44 hrs; D10-PfPHG and DD2). IC_{50}s were determined for chloroquine sensitive D10-PfPHG and multi-drug resistant DD2 lines. The fold change of IC_{50}s of lead analogues for DD2 vs D10-PfPHG is indicated. All assays were measured by flow cytometry. Data represents the mean of 3 (or more) experiments expressed as percentage of non-inhibitory control and error bars represent ± SEM.

S. Table 4.5 In cycle fold-change of analogues for *P. falciparum* (D10-PfPHG) vs *P. knowlesi* (PkYH1).

Modification	Drug	Intracellular growth D10-PfPHG IC_{50} (μM) [a]	Intracellular growth PkYH1 IC_{50} (μM) [a]	Fold-change D10-PfPHG vs PkYH1
	Azithromycin	11.31	13	0.87
	Chloroquine	0.052	0.017	3.1
	DHA	0.00083	0.0024	0.34
Chloroquinoline	1	0.019	0.2	0.095
	56	0.011	0.031	0.35
	66	0.007	0.012	0.58
	72	0.27	0.15	0.18
Quinoline	8	0.41	0.15	2.7
	10	0.48	0.1	4.8
	58	0.048	0.071	0.68
	71	0.053	0.041	1.29
	73	0.31	0.248	1.25
Naphthalene	3	0.183	0.095	1.9
Substituted phenyl	5	0.2	0.082	2.4
	6	0.28	0.16	1.75
	9	0.53	0.016	33.1
	17	0.7	0.36	1.9

[a] Drug treatment of intracellular growth, from rings to late schizonts, with no rupture cycle (*P. falciparum,* 0-44 hrs; *P. knowlesi,* 0-24 hrs). IC_{50}s were determined for *P. falciparum* D10-PfPHG and *P. knowlesi* PkYH1 parasites. The fold change of IC_{50}'s of lead analogues for D10-PfPHG vs PkYH1 is specified. All assays were measured by flow cytometry. Data represents the mean of 3 (or more) experiments expressed as percentage of non-inhibitory control and error bars represent ± SEM.

S. Table 4.6 Activity of analogues against bacterial ribosomes.

Modification site	Drug	MIC average [c]	Intracellular growth D10-PfPHG IC_{50} (μM) [a]	Delayed death D10-PfPHG IC_{50} (μM) [d]
	Azithromycin	0.09	11.3	0.07
Desosaminyl *N*-substituted	57	>10	0.021	ND
	66	>10	0.007	ND
	71	>10	0.053	ND
	78	>10	0.51	ND
N6-substituted	1	0.025	0.019	ND
	4	0.035	0.2	ND
	5	0.09	0.2	ND
	6	0.157	0.28	ND
	9	0.112	0.44	ND
	11	0.075	0.53	ND
	12	0.045	0.59	ND
	16	0.023	0.7	ND
	17	0.023	0.7	ND
	21	0.112	1.2	ND
	25	0.075	1.4	ND

[c] Minimum inhibitory concentrations (MIC) were determined as described in Methods. MIC dilution series were analysed individually. The MIC data represents the means of 3 or more experiments expressed as a percentage of non-inhibitory control. [a] Drug treatment of intracellular growth, from rings to late schizonts, with no rupture cycle (*P. falciparum,* 0-44 hrs; D10-PfPHG). [d] Drug treatment of delayed death, from rings to late schizonts, with two rupture cycles (*P. falciparum,* 0-120 hrs; D10-PfPHG). Both [a] and [d] assays were measured by flow cytometry. Data represents the mean of 3 (or more) experiments expressed as percentage of non-inhibitory control and error bars represent ± SEM. ND= not done due to the potency of in cycle 0-44 hr activity.

S. Table 4.7 Metabolites shared with chloroquine treated parasites.

Putative metabolites	DHA	CQ	Az	GSK-5	GSK-71	GSK-66	Mapped to HB	
							Alpha subunit	Beta subunit
Asn-His	0.62	1.34	3.15	2.59	2.90	0.88	GHG	
Ala-Asp-Gly-Pro	0.00	1.50	7.88	13.93	6.09	0.00		
Gln-Gln	0.60	17.71	36.47	40.37	75.38	3.43		
Ala-Asn	0.00	7.89	18.78	14.22	27.04	1.31	GE	
Thr-Arg	0.69	3.83	8.74	5.92	12.99	1.50		
Pro-Ser	0.62	2.15	3.75	3.56	6.47	0.91	SP	
Ala-Gly-Pro	0.62	3.40	5.94	7.16	11.16	1.11		PE
Val-Arg	0.92	5.66	9.81	6.69	13.99	1.60	RV	
Ala-Arg	0.90	18.29	11.33	13.97	26.58	2.36		
Pro-Tyr	0.00	113.67	35.77	90.67	157.85	0.00		
Lys-Asn	0.00	23.15	33.95	31.32	40.92	0.00		
Glu-Ile-Gln-Lys	0.00	23.25	82.88	80.11	141.82	0.00		
Arg-Leu-Lys-Asp	0.00	87.29	100.65	99.77	194.09	0.00		
Ala-Lys	0.00	42.31	66.83	55.97	130.08	0.00		KA
Lys-Tyr	0.00	30.51	55.00	39.20	101.29	0.00		
Gly-Leu	0.81	2.63	2.35	2.80	9.83	1.22		
Ala-Ala-Ser	0.09	1.28	1.95	1.64	4.86	0.75		
Ile-Lys	0.00	1.56	3.09	2.20	4.96	0.25		LK

List of putative peptides that were significantly perturbed following treatment with DHA, chloroquine (CQ), azithromycin (Az), GSK-5, GSK-71, and GSK-66 in three technical replicates. The order of the amino acids within the proposed peptide sequence has not been confirmed. The red shading denotes small peptides that were increased in abundance following treatment compared to ethanol control, yellow denotes no change, and blue shading denotes peptides that were decreased. Values represent the fold-change relative to ethanol control, and bold denotes changes that were statistically significantly different. The listed peptides (plus any isomeric peptides) were then investigated to determine whether it can be mapped to either the alpha or beta haemoglobin (HB) subunits.

S. Table 4.8 Metabolites mapping to haemoglobin after drug treatment.

Peptide sequence	DHA	CQ	Az	GSK-5	GSK-71	GSK-66	Mapped to HB	
							Alpha subunit	Beta subunit
Tyr-Arg	0.40	0.69	1.48	0.30	0.90	0.98	YR	
Ala-Lys-Lys-Asp	0.47	0.35	1.27	0.00	1.48	0.85	SLDK	
Asn-Leu-Pro-Pro	0.37	0.64	1.90	0.00	1.69	0.98		PPVQ
Ala-Asp-Gly-Tyr	0.48	0.87	2.15	0.00	1.06	0.81		
Ala-Val-Gly-Pro	0.54	0.55	0.58	0.03	0.84	0.82		
Ala-Glu-Glu-His	0.75	1.01	1.14	0.09	1.19	0.94	HVDD	
Glu-Ala-Pro	0.57	0.52	0.59	0.00	0.48	0.78	PAE	
Ala-Leu-Trp-Ser	0.42	0.58	1.19	0.00	0.68	0.80	PVNF	
Trp-Asn	0.44	0.59	0.82	0.00	0.52	0.98		YH
Lys-Asp	0.69	0.51	0.82	0.16	0.37	0.99		
Ala-His	0.40	0.49	0.66	0.11	0.54	0.84	AH	
Glu-Glu-Gln-His	0.00	1.06	0.00	0.00	0.76	0.61		
Asp-pro	0.61	0.81	0.33	0.12	0.93	1.00		VD
Lys-Pro	0.82	0.58	0.56	0.29	0.98	0.99		PK
Thr-Ala-Pro	0.57	0.75	0.52	0.31	1.04	0.91	TPA	
Thr-Pro	0.46	0.65	0.33	0.18	0.88	0.78	PT	
Val-Pro	0.55	0.53	0.44	0.24	0.56	0.82	PV	

List of putative peptides that were significantly perturbed following treatment with DHA, chloroquine (CQ), azithromycin (Az), GSK-5, GSK-71 and GSK-66 in three technical replicates. The order of the amino acids within the proposed peptide sequence has not been confirmed. The red shading denotes small peptides that were increased in abundance following treatment compared to ethanol control, yellow denotes no change, and blue shading denotes peptides that were decreased. Values represent the fold-change relative to ethanol control, and bold denotes changes that were statistically significantly different. The listed peptides (plus any isomeric peptides) were then investigated to determine whether it can be mapped to either the alpha or beta haemoglobin (HB) subunits.

S. Table 4.9 Metabolites associated with the parasite TCA cycle.

Metabolite	DHA	CQ	Az	GSK-5	GSK-71	GSK-66
[SP amino(18:0/2:0)]	0.73	0.55	0.00	0.00	0.00	1.03
Succinate	1.20	0.69	0.34	0.43	0.89	0.85
4-Aminobutanoate	0.86	0.63	0.14	0.25	0.64	0.75
Ketoglutarate	1.00	0.58	0.21	0.37	0.59	0.73
Malate	1.01	0.72	0.26	0.30	0.83	0.81
Fumarate	1.04	0.75	0.28	0.33	0.81	0.83
14-Methyl-8Z-hexadecen-1-ol	1.18	1.26	0.00	0.00	0.72	0.99
L-Pipecolate	0.49	0.53	0.07	0.09	0.81	0.58
Asp-Gly	0.90	0.85	0.27	0.28	0.91	0.97
Hypaphorine	0.95	0.82	0.00	1.03	1.02	0.99

List of putative metabolites that were significantly perturbed following treatment with DHA, chloroquine (CQ), azithromycin (Az), GSK-5, GSK-71, and GSK-66 in three technical replicates. The red shading denotes putative metabolites that were increased in abundance following treatment compared to ethanol control, yellow denotes no change, and blue shading denotes metabolites that were decreased. Values represent the fold-change relative to the ethanol control, and bold denotes changes that were statistically significantly different.

Chapter 5. Targeting malaria parasites with novel derivatives of azithromycin.

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Statement of Authorship

Title of Paper	Targeting malaria parasites with novel azithromycin derivatives
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Amy L. Burns, Brad E. Sleebs, Sonja Frolich, Christopher D. Goodman, Kimberley T. McLean, Ghizal Siddiqui, Dovile Anderson, Darren Creek, James G. Beeson, Geoffrey I. McFadden, Danny W. Wilson. Henrietta Venter, Shutao Ma

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Contribution to the Paper	Conceived, designed and performed experiments, analysed results and co-wrote the manuscript.
Overall percentage (%)	60%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
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5.2 Abstract.

The spread of artemisinin combination therapy resistant *Plasmodium falciparum* parasites remains a global concern and highlights the need to identify new antimalarials for future treatments. Azithromycin, a clinically used macrolide antibiotic, kills parasites via two mechanisms; 'delayed death' by inhibiting the apicoplast's bacterial-like ribosome, and 'quick-killing' that rapidly kills broadly across blood stage development. Here, 22 azithromycin analogues were explored for quick-killing and delayed death activities against *P. falciparum* and *P. knowlesi*. Investigations identified 17 analogues with improved quick-killing against both *Plasmodium* species. Analogues showed 38 to 20-fold higher potency over azithromycin with less than 48 or 28 hours of treatment for *P. falciparum* and *P. knowlesi,* respectively, with leads shown to be >5-fold more selective against parasites than human cells. Quick-killing analogues maintained activity throughout the blood stage lifecycle and against ring stages of *P. falciparum* parasites (<12 hr treatment). Isopentenyl pyrophosphate supplemented parasites that lacked an apicoplast were equally sensitive to quick-killing analogues, indicating that activity of these drugs was not against the apicoplast. Metabolomic profiling of the lead analogue shared a similar profile to chloroquine but also exhibited accumulation of haemoglobin-derived peptides, supporting that the food-vacuole is a likely target of this drugs activity. The azithromycin analogues characterised in this study have additional structure diversity over analogues previously reported to have quick-killing activity and thus provide new starting points to develop azithromycin analogues with quick-killing antimalarial activity.

5.3 Introduction.

Malaria is a mosquito-borne parasitic infection that caused ~219 million clinical cases and ~435,000 deaths in 2017 alone (1). The majority of deaths occurred in children under 5 years of age in sub-Saharan Africa and were the result of *Plasmodium falciparum* infection, the most virulent human malaria parasite (1, 2). A further five malaria parasites (1, 4) that regularly infect humans are also major contributors to the global burden of malaria, thus future control strategies also need to be effective against these species.

Over the last two decades, global distribution of insecticide treated bed nets (ITNs) and artemisinin-based combinational therapies (ACTs) have contributed to a >50% decrease in global malaria mortality (1, 2). However, *P. falciparum* parasites resistant to frontline artemisinin combination therapy (ACTs) have emerged in Southeast-Asia, Eastern-India, South America and Papua New Guinea, increasing the chance that rates of malaria morbidity and mortality will rise (224, 225, 228, 229, 539). The spread of resistance to our most effective class of antimalarials highlights the need to identify new chemotypes with novel mechanisms of action for use in future combination therapies (134, 230).

The clinically used macrolide antibiotic, azithromycin, has been investigated for use as a malaria prophylactic (447, 459) and as a potential partner drug in ACTs (450, 470). Azithromycin's known mechanism of action inhibits protein translation in the parasite's apicoplast, a relic plastid organelle (540), via binding to the peptide exit tunnel of the 50S subunit of the bacterial-like ribosome and blocking the release of peptide chains (160, 420, 421). The apicoplast's sole indispensable role in blood stage parasite growth is the synthesis of isoprenoid's, with these metabolites being essential for protein prenylation, N-glycosylation, production of GPI anchors and ubiquinone biosynthesis (385-387). *In vitro* treatment of malaria parasites with nanomolar concentrations of azithromycin will show no growth defect within the first cycle of growth (~2 days post treatment), however, progeny of treated parasites inherit a defective apicoplast and exhibit 'delayed death' by lethally arresting during the second replication cycle (~4 days post treatment) (130, 159). Disruption of isoprenoid biosynthesis by apicoplast ribosome targeting antibiotics results in the loss of isoprenoid-mediated protein prenylation that is required for vesicular trafficking and food vacuole formation, thereby preventing digestion of RBC haemoglobin - the parasite's major food source (161, 387).

Azithromycin's long half-life (>50 hrs) (431), *in vivo* safety profile for pregnant woman and children (439) and *in vitro* potency against *P. falciparum* has led to its evaluation in Phase II

and III trials as a treatment and prophylactic against malaria (475, 541). Azithromycin demonstrates: i) improved clinical outcomes for intermittent preventative treatment for malaria in pregnancy in combination with sulfadoxine-pyrimethamine (IPTp) (455, 475, 477); ii) efficacy as a prophylactic in combination with naphthoquine, a 4-aminoquinoline, in Phase II clinical trials (462); and iii) reduced rates of *P. falciparum* infection and clinical burden as a monotherapy during mass drug administration for trachoma (465) (reviewed in (374)). Azithromycin has also been shown to inhibit liver stage development in *in vivo* rodent models of malaria and disrupt transmission of gametocytes to mosquitoes *in vitro* (162-164). However, azithromycin is considered inferior as a monotherapy and as a partner drug with artemisinin's compared to the similarly acting antibiotic clindamycin and other antimalarials (reviewed in (376)). Combined with azithromycin's delayed death activity, this has limited the application of this antibiotic as a treatment for clinical malaria (reviewed in (376)).

We have previously investigated a secondary, quick-killing, mechanism of action for azithromycin and analogues (130, 164, 165) (Chapter 4). Azithromycin and analogues were demonstrated to rapidly inhibit *P. falciparum* merozoite invasion of RBCs and effectively kill asexual stages throughout one full blood stage lifecycle (rings to schizonts, in cycle, ~48 hrs). Azithromycin was equipotent throughout the entire blood stage lifecycle and leading analogues were active against ring stage parasites (<6 hr treatments) at nanomolar potencies (165) (Chapter 4). This 'quick-killing' activity was active against parasites selected for resistance to azithromycin's delayed death activity (164, 165) and against parasites that had the apicoplast chemically removed (161), confirming this mechanism to be independent of apicoplast targeted delayed death (Chapter 4). Given concerns that repurposing an antibiotic into an antimalarial could potentially select for azithromycin resistance in pathogenic bacteria (542-544) and cause dysbiosis of the human microbiome (545, 546), medicinal chemistry synthesis efforts have been directed in making non-antibiotic azithromycin analogues (418, 482-486). These studies made use of the fact that modifying azithromycin's desosaminyl sugar typically abrogates binding to bacterial-like ribosomes, thus adding an active functional group to this sugar abolished antibacterial activity.

In efforts to overcome rising antibacterial resistance to azithromycin (547-549) (reviewed in (550)) a number of studies have modified azithromycin in efforts to improve the drugs' activity against macrolide resistant bacteria and broaden its antibacterial spectrum (490, 551, 552) (**S. Table 5.1-5.4**). Recent studies by Yan et al., (2017) have explored the effect of substituting functional groups on the 2'-position of the desosaminyl sugar, the 3-position, as well as

modifying the C11 and C12 sites on descladinosylazithromycin against both macrolide resistant and sensitive bacteria (490). These analogues showed a moderate improvement against bacteria encoding the macrolide resistance genes: i) erythromycin ribosomal methylase (*erm*) that modifies a specific residue within the bacterial ribosome via methylation and interferes with drug binding, and ii) the macrolide efflux (*mef*) gene that transports the drug out from the bacterial cell (490) (reviewed in (553) and (554)). However, most analogues lost potency against sensitive bacteria and were at best equivalent but not superior to azithromycin, suggesting that in general translation blocking activity of these analogues against the bacterial ribosome is reduced with these compounds (490).

Here, we sought to address whether the azithromycin analogues represented in this panel, with their diversity of functional groups and sites of attachment, improved quick-killing and/or maintained delayed death against malaria parasites. The majority of analogues featured superior quick-killing potency over azithromycin against two different human malaria parasites and were effective at killing ring stages with <12 hr treatment. Moreover, these drugs exhibited little in the way of apicoplast-targeting delayed death and activity, suggesting that a broad range of structural modifications to azithromycin can be used to specifically improve antimalarial quick-killing activity, but activity targeting the apicoplast ribosome is not so tolerant.

5.4 Methods and materials.

5.4.1 Antimalarial drugs.

Azithromycin was obtained commercially from AK-scientific (Union City, CA, USA). Synthesis of analogues are described previously (490) with **S. Tables 5.1-5.4** providing further details of chemical structure and the origin of each analogue. Drug stocks of azithromycin (100 mM) (AK Scientific) and all analogues (10 mM) were made up using ethanol as the vehicle. Drugs were added such that the vehicle was diluted >1000-fold for intracellular growth assays to minimise non-specific growth inhibitory activity from the vehicle.

5.4.2 Culture and synchronisation of *Plasmodium* spp. parasites.

Green fluorescent protein (GFP) expressing *P. falciparum* (D10-PfPHG) (493) and *P. knowlesi* (PkYH1) parasites (501) were cultured in human O^+ red blood cells (RBCs) (Australian Red Cross Blood Service) in RPMI-HEPES culture medium (Thermo Fisher Scientific) supplemented with 0.5% AlbuMAX (GibcoBRL), according to established protocols (492). Synchronization of D10-PfPHG parasites for growth inhibition assays was achieved using heparin sodium (Pfizer) as previously described (131, 517). PkYH1 parasites were passaged over a gradient of 70% Percoll (Sigma-Aldrich) to purify late stage schizonts that were then allowed to rupture for ~4 hrs prior to ring stage treatment with 5% w/v sorbitol (Sigma-Aldrich) enabling effective synchronisation of 0-4 hr old rings.

5.4.3 Drug inhibition assays.

A diagram outlining the different drug inhibition assays used in this study to measure *Plasmodium* spp. parasite growth and invasion is available in **Figure 4.2**. Growth assay protocols for measuring drug inhibition of in cycle, ring to schizont stages (approximately 44 hrs post-invasion for *P. falciparum* and 28 hrs post-invasion for *P. knowlesi*) (**Figure 4.2. B**), and 2 cycle (120 hrs post-invasion for *P. falciparum* and 78 hrs post-invasion for *P. knowlesi*, 2 cycles of replication) **(Figure 4.2. D)** have been described previously (131, 165, 510) (Chapter 4). For the assessment of stage specificity for azithromycin or analogues during the blood stage lifecycle of *P. falciparum*, each respective drug was removed at the specified time point (0-6 hrs or 0-12 hrs). To remove drug, a stringent washing procedure, involving 3 consecutive washes with 200 µl medium (centrifuged at 300 x g for 2 mins) was performed with the final resuspension in 100 µl of fresh medium (131). Parasite growth at late trophozoite/schizont stages (44-48 hrs post invasion for *P. falciparum*; 24-30 hrs post invasion

for *P. knowlesi*) was quantified using flow cytometry of parasites stained with ethidium bromide (EtBr) (10 μg/mL for 1 hr) prior to washing with PBS.

5.4.4 Apicoplast-null inhibition assays.

Apicoplast null (D10-PfPHG$^{apicoplast\text{-}null}$) parasites were generated as previously described (157, 161). Briefly, the culture medium was supplemented with 200 μM isopentenyl pyrophosphate (IPP) (NuChem Therapeutics, Canada) and 0.35 μM (5x IC_{50}) of azithromycin for a minimum of 6 days (~3 cycles) and parasites were cultured continuously thereafter with IPP. Successful removal of the apicoplast was assessed by growing D10-PfPHG^{wildtype} and D10-PfPHG$^{apicoplast\text{-}null}$ parasites with reducing concentrations of azithromycin for ~120 hrs (delayed death), which confirmed a loss of sensitivity to azithromycin as observed by a ~64 fold-change in the IC_{50} with apicoplast removal (D10-PfPHG$^{apicoplast\text{-}null}$ IC_{50}, 4.5 μM; D10-PfPHG^{wildtype} IC_{50}, 0.07 μM) (**S. Figure 5.1**). To assess the inhibitory activity of analogues, D10-PfPHG$^{apicoplast\text{-}null}$ and D10-PfPHG^{wildtype} parasites were grown in the presence of the IC_{90} obtained for D10-PfPHG^{wildtype} parasites in in cycle (0-44 hrs) or delayed death (0-120 hrs) assays, or a dilution series of the respective drug. Drugs were added to tightly synchronised ring stage D10-PfPHG$^{apicoplast\text{-}null}$ (+ 200 μM IPP) or D10-PfPHG^{wildtype} (no IPP) parasite lines and assays were incubated for ~44 hrs (in cycle) or 120 hrs (2 cycle, delayed death) as specified with the resulting parasitemia quantitated by flow cytometry.

5.4.5 Flow cytometry and microscopy analysis of growth inhibition.

Parasitaemia was measured on an LSR Fortessa (Becton Dickinson) using a 96-well plate reader. Mature (>36 hrs post-invasion) *P. falciparum* D10-PfPHG parasites were counted using Fl-1-high (GFP; excitation wavelength, 488 nm) and Fl-2-high (EtBr; excitation wavelength, 488 nm) (131). Mature parasites of the PkYH1 line (>24 hrs post-invasion) were gated with a forward scatter (FSC) and FL-2-high (EtBr) gate. Typically, 20,000-40,000 RBCs were counted in each well. All samples were analysed using FlowJo software (TreeStar Inc, Ashland, OR, USA) and growth of drug treatments were normalised against growth of media control wells to calculate the percent survival of drug treated parasites. To address the phenotypic effects of drugs, thin smears were fixed with fresh methanol and stained in fresh 10% Giemsa (Merck) for 10 mins before images of drug treated parasites were taken with an Olympus BX51/BX52 light microscope with immersion oil using 100x magnification.

5.4.6 Mammalian cell cytotoxicity.

Toxicity against mammalian cells was determined using the Huh-7D cell line derived from human hepatocellular carcinoma cells (Sigma-Aldrich). Huh-7D cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific) supplemented with 10% Fetal Bovine Serum (FBS) and non-essential amino acids. Huh-7D cells were grown in an atmosphere of 5% CO_2 in a 37 °C incubator. Cultures were seeded to 40,000 cells in round bottom 96-well microtiter plates (Corning) and incubated with 2-fold serial dilutions of drug for 24 hrs in 5% CO_2 at 37 °C. Post incubation 1:1 addition of CellTiter-Glo Reagent (Promega, USA) was added to each well to lyse Huh-7D cells and release ATP for detection by luminescence (555). Plates were incubated for 10 mins to allow the luminescent signal to stabilise, which was then detected using a Phera Star FS using the luminescent module (Lum Plus, spectral wavelength 230 nM to 750 nM). Cell viability with drug treatment was assessed by comparing cell replication in drug treated wells and normalizing this growth against non-inhibitory control wells (media).

5.4.7 Statistical analysis.

All IC_{50}, IC_{90} and cytotoxicity concentration (CC_{50}) estimates were determined using GraphPad Prism (GraphPad Software) according to the recommended protocol for nonlinear regression (constrained to top= 100 and bottom= 0) of a log-(inhibitor)-versus-response curve. Statistical significance between drug treatments were determined with the GraphPad Prism software using the log-(inhibitor)-versus-response curve with Extra Sum-of-Squares F Test (best-fit LogIC_{50}). *P* values were considered significant if P=<0.05.

5.4.8 Antibacterial screen.

Minimum inhibitory concentration (MIC) assays for assessment of *Streptococcus pneumoniae* sensitivity to azithromycin and analogues were performed as described (523). Briefly, the antibacterial activity of azithromycin and all analogues were assessed with a 2-fold serial dilution in the presence of macrolide sensitive *S. pneumoniae* D39. Cells were inoculated at a final concentration of approximately 10^6 CFU/mL in Mueller Hinton Broth supplemented with 5% lysed horse blood. The MIC was determined to be the concentration of drug that inhibited bacterial growth within a 96-well microtiter tray after 24 hr incubation at 37°C. Drug activity was assessed by determining the minimal inhibitory concentration (MIC) that stopped bacterial growth, as indicated by a media colour change. MICs are expressed as μM.

5.4.9 Sample extraction for metabolomics analysis.

For metabolomics experiments, two 150 mL flasks at 6% haematocrit containing tightly synchronised parasites 28-34 hr post-invasion (5-6 hr rupture window), were harvested via magnet purification (Miltenyi Biotech). Infected RBC density was quantitated by flow cytometry (518) and 2 mL of 3x 10^7 parasites were added into 24 well microtiter plates. Parasites were incubated for 1 hr at 37ºC to stabilise the culture. Following this initial incubation, 5x IC_{50} of the azithromycin analogue C1, chloroquine, dihydroartemisinin (DHA), azithromycin or ethanol (vehicle control) were added and incubated for 2 hrs. Supernatant was removed and parasites washed twice with 800 µL ice-cold 1x PBS, with cells pelleted via centrifugation at 400 gs for 5 mins at 0ºC. The supernatant was discarded and the remaining solid was resuspended in 200 µL of extraction buffer. Blank sample was prepared alongside. Cell pellets were resuspended in 150 µL of ice-cold extraction buffer ($CHCl_3$/MeOH/water (1:3:1 *v/v*)) containing 1 µM internal standards, CHAPS and PIPES, was incubated on ice for 1 hr with shaking at 200 rpm. The resulting cell debris was pelleted with centrifugation at 14,800 gs for 10 mins at 4ºC. 180 µL of supernatant was transferred to Eppendorf tubes and the remaining ~20 µL were combined to make a pooled QC sample. Samples were stored at -80 ºC until analysis.

5.4.10 Liquid chromatography-mass spectrometry analysis.

Liquid chromatography-mass spectrometry (LC-MS) data was acquired on a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific) coupled with high-performance liquid chromatography system (HPLC, Dionex Ultimate® 3000 RS, Thermo Scientific) as per previously described (312). Briefly, chromatographic separation was performed on ZIC-pHILIC column equipped with a guard (5 µm, 4.6 × 150 mm, SeQuant®, Merck). The mobile phase (A) was 20 mM ammonium carbonate (Sigma Aldrich), (B) acetonitrile (Burdick and Jackson) and needle wash solution was 50% isopropanol. The column flow rate was maintained at 0.3 mL/min with temperature at 25 ºC and the gradient program was as follows: 80% B decreasing to 50% B over 15 min, then to 5% B at 18 min until 21 min, increasing to 80% B at 24 min until 32 min. Total run time was 32 min with an injection volume of 10 µL. Mass spectrometer operated in full scan mode with positive and negative polarity switching at 35k resolution at 200 m/z with detection range of 85 to 1275 m/z, AGC target was 1e6 ions, maximum injection time 50 ms. Electro-spray ionization source (HESI) was set to 4.0 kV voltage for positive and negative mode, sheath gas was set to 50, aux gas to 20 and sweep gas

to 2 arbitrary units, capillary temperature 300 °C, probe heater temperature 120 °C. The samples were analyzed as a single batch to reduce the batch-to-batch variation and randomized to account for LCMS system drift over time.

5.4.11 Data processing using IDEOM.

The acquired LCMS data was processed in untargeted fashion using open source software, IDOEM (519) (http:// mzmatch.sourceforge.net/ideom.php). Initially, *ProteoWizard* was used to convert raw LC-MS files to *mzXML* format and *XCMS* to pick peaks to convert to *peakML* files. *Mzmatch.R* was used for alignment of samples and filtering of peaks using minimum detectable intensity of 100,000, relative standard deviation (RSD) of <0.5 (reproducibility), and peak shape (codadw) of >0.8. *Mzmatch* was also used to retrieve missing peaks and annotation of related peaks. Default IDEOM parameters were used to eliminate unwanted noise and artefact peaks. Loss or gain of a proton was corrected in negative and positive ESI mode, respectively, followed by putative identification of metabolites by accurate mass within 3 ppm mass error searching against the Kyoto Encyclopedia of Genes and Genomes (KEGG), MetaCyc, and LIPIDMAPS databases and others. To reduce the number of false positive identifications, retention time error was calculated for each putative ID using IDOEM build-in retention time model which uses actual retention time data of authentic standards (~350 standards). Statistical analysis on filtered data was performed using the Matboanalyst web interface (521).

5.5 Results.

5.5.1 Azithromycin analogues with diverse modifications have improved quick-killing activity against malaria parasites *in vitro.*

All 22 azithromycin analogues were initially assessed for growth inhibition at 10 μM with *in vitro* in cycle assays, from early rings to early schizonts, against *P. falciparum* (D10-PfPHG, 44 hrs) (493) or *P. knowlesi* (PkYH1, 28 hrs) (501) (in cycle (**S. Table 5.1-5.4**)). This primary screen identified 17 of 22 analogues for *P. falciparum* and 18 analogues for *P. knowlesi* that inhibited growth by >40% under these conditions. The 17 analogues inhibitory against *P. falciparum* also inhibited *P. knowlesi* and one analogue (A4) that was active in *P. knowlesi* but not *P. falciparum* were all prioritised for further evaluation.

The in cycle IC_{50} values for the 17 and 18 analogues identified from primary screens were then determined against D10-PfPHG and PkYH1 lines, respectively. All analogues showed improved quick-killing IC_{50} values compared to azithromycin (azithromycin in cycle IC_{50}- *P. falciparum* 11.3 μM; *P. knowlesi* 13 μM). There was a 1.9 to 38-fold improvement in quick-killing activity against *P. falciparum* with four analogues showing >10-fold greater potency than azithromycin (IC_{50}; A13, 0.72 μM; B2 0.67 μM; C1, 0.3 μM; and D1, 0.7 μM) (**Table 5.1** & **S. Table 5.5**). There was a 1.6 to 20-fold improvement in quick-killing potency against *P. knowlesi* (PkYH1) with four analogues demonstrating >10-fold activity compared to azithromycin (IC_{50}; A2, 0.65 μM; A8, 0.78 μM; A13, 0.64 μM; B2, 0.67 μM) (**Table 5.1** & **S. Table 5.5)**.

5.5.2 Diverse azithromycin analogues have improved activity against ring stage parasites.

Recently, we showed that azithromycin and analogues are active against early ring stage development (<12 hrs of treatment) as well as broadly inhibitory throughout the blood stage lifecycle with ~12 hr treatment intervals (Chapter 4). Such broad activity is of interest for clinical treatment as most antimalarials exhibit limited efficacy against early rings (127, 128, 131, 151, 526). To assess whether this held true for the analogues tested in this study, very early ring stage D10-PfPHG parasites (0-4 hrs post invasion) were treated for 6 hrs and 12 hrs with the analogues showing the highest in cycle potency against *P. falciparum* (A13, B2, C1 and D1 (**Table 5.1**)).

Table 5.1 *In vitro* efficacy of azithromycin analogues against *Plasmodium* parasites.

Drug	**Intracellular growth μM (44 hr IC_{50}) D10-PfPHG [a]**	**Intracellular growth μM (28 hr IC_{50}) PkYH1[a]**	**Delayed death μM (120 hr IC_{50}) D10-PfPHG[b]**	**Delayed death μM (96 hr IC_{50}) PkYH1[b]**
Azithromycin	11.3	13	0.07	0.08
A2	1.6	0.65	0.24	0.26
A3	1.5	1.1	0.43	0.39
A4	ND	7.8	ND	ND
A5	1.8	1.5	0.39	0.47
A7	5.2	1.7	ND	ND
A8	1.4	0.78	0.35	0.16
A9	3.3	1.9	0.82	0.77
A10	4.2	1.4	ND	0.71
A11	5.9	4.2	ND	0.95
A12	1.1	1.2	0.3	0.21
A13	0.72	0.64	0.18	0.39
B1	1.4	1.2	0.29	0.11
B2	0.53	0.67	0.14	0.097
C1	0.3	1.4	0.11	0.24
C2	4.7	5.4	ND	ND
C3	3.2	4.0	ND	ND
D1	0.7	1.6	0.11	ND
D2	3.8	2.8	ND	ND

[a] Quick-killing in cycle drug treatment from rings to late schizonts, with no rupture cycle (D10-PfPHG, *P. falciparum,* 0-44 hrs or PkYH1, *P. knowlesi* 0-28 hrs). [b] Delayed death drug treatment, from early-rings to late trophozoites, with two rupture cycle (D10-PfPHG, *P. falciparum,* 0-120 hrs or PkYH1, *P. knowlesi* 0-78 hrs). Data represents the means of 3 (or more) experiments expressed as percentage of non-inhibitory control. ND= not done due to <40% activity 0-44 hr in cycle assays or limited amounts of analogue.

All four analogues exhibited activity against both early (0-6 hr) and late (0-12 hr) ring stage treatments with a higher potency seen for longer 12 hr treatments (A13 (IC_{50}; 0-6 hrs, 2.3 μM; 0-12 hrs, 1.4 μM; 0-44 hrs, 1 μM), B2 (IC_{50}; 0-6 hrs, 2.8 μM; 0-12 hrs, 1.4 μM; 0-44 hrs, 0.9 μM), C1 (IC_{50}; 0-6 hrs, 1.5 μM; 0-12 hrs, 0.7 μM; 0-44 hrs, 0.4 μM) and D1 (IC_{50}; 0-6 hrs, 2.1 μM; 0-12 hrs, 1.5 μM; 0-44 hrs, 0.4 μM)) (**Figure 5.1 A**). For analogues A13, B2, and C1 the IC_{50} of 12 hr treatment differed <2-fold in comparison to IC_{50}s of 44 hr treatment. Shorter 6 hr ring stage treatments exhibited between a 2.3 and 5.25-fold rise in IC_{50} over 44 hr treatments, however, the activity of these analogues with a 6 hr treatment was still significantly better than that recorded for azithromycin (IC_{50}; 0-6 hrs, 30 μM; 0-12 hrs, 16 μM) (Chapter 4).

We next examined the effect of 6 hr and 12 hr ring stage drug treatments on parasite morphology at a 2x IC_{90} concentration (0-44 hrs) for the most potent analogues A13, B1, C1 or D1 using light microscopy (**Figure 5.1 B**). Consistent with flow cytometry data, no aberrant growth phenotype was observed for 6 hrs treatments of early ring stage parasites with any drug. Examination of 12 hr treatments showed evidence of underdeveloped and vacuolated parasites developing for all four drugs, indicative of parasite stress in the face of drug pressure. These data demonstrate that the leading analogues have activity against early ring stage parasite growth, with improved potency evident with longer treatment times.

5.5.3 Analogue toxicity against Huh-7D human cells.

We then investigated the potential of mammalian cell cytotoxicity for a focused group of analogues on the human hepatocellular carcinoma, Huh-7D, cell line (556). Inhibition of Huh-7D cell growth for analogues featuring an IC_{50} of <1 μM in either D10-PfPHG or PkYH1 parasite lines (A2, A8, B2 and C1) was assessed using an ATP-based luminesce detection assay (555). Two analogues, A13 and D1, with IC_{50} values of <1 μM were excluded from this analysis due to limited sample. Compounds A2, A8 and B2 showed relatively low cytotoxicity *in vitro* (CC_{50} >4 μM) with >5-fold and >10-fold higher selectivity index in comparison to the IC_{50}s observed for D10-PfPHG or PkYH1, respectively (**Table 5.2**). The selectivity index of C1 (CC_{50}, 2.3 μM) was 7.6 for *P. falciparum* (IC_{50}, 0.3 μM) but dropped to 1.5 for *P. knowlesi.* However, it remains promising that limited toxicity against mammalian cells is evident with these diverse azithromycin analogues.

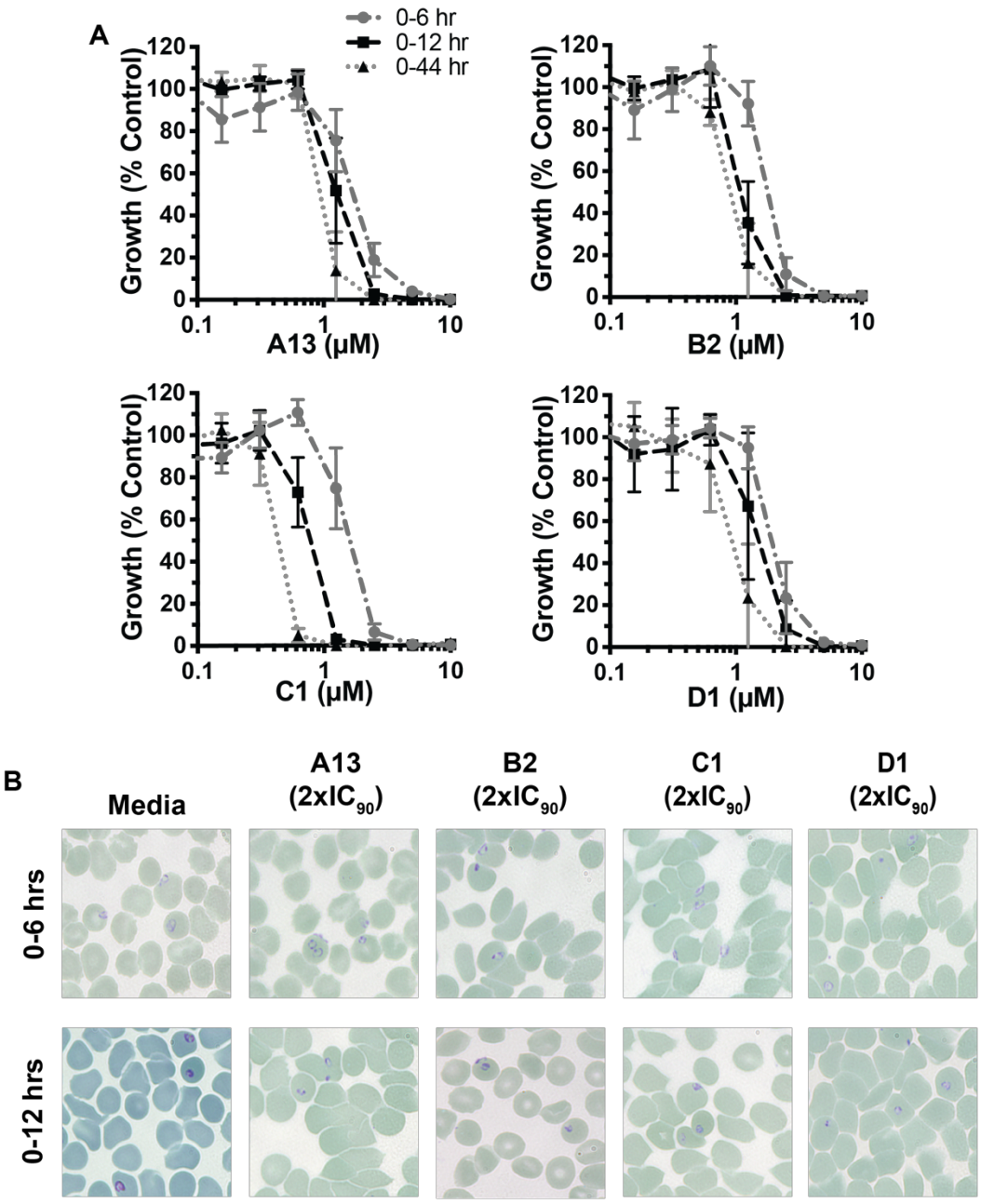

A
0-6 hr
0-12 hr
0-44 hr
Growth (% Control)
A13 (μM)
B2 (μM)
C1 (μM)
D1 (μM)
B
Media
A13 (2xIC90)
B2 (2xIC90)
C1 (2xIC90)
D1 (2xIC90)
0-6 hrs
0-12 hrs

Figure 5.1 Growth inhibition profiles of azithromycin analogues with short term treatment.

A) Growth inhibition profile of A13, B2, C1 and D1 with very early ring stage treatment across 0-6 hrs and 0-12 hrs post-invasion compared to a full in cycle treatment. Early ring stage *P. falciparum* (D10-PfPHG) parasites (<4 hrs post-invasion) were treated with doubling dilutions of azithromycin analogues/control drugs for 0-6 hrs or 0-12 hrs prior to washing the drug out, with cultures allowed to continue growing until parasites were 44 hrs old. A 0-44 hrs control where parasites were maintained on drug was also included. There were significant differences between all 0-6 hrs vs 0-44 hr treatments of A13, B2, C1 and D1 (A13 and B2 P=0.01; C1 and <0.0001). A significant difference was also observed for 0-6 hrs vs 0-12 hrs of A13, B2 and C1(A13 P=0.01; C1 and B2 P=<0.0001) but not D1 (P=NS). Significant difference was observed for 0-12 hrs vs 0-44 hrs for C1 and D1 (P= 0.01), but not A13 or B2 (P=NS). Parasitemia was measured via flow cytometry 44 hrs post-invasion. Data represents the means of 3 or more experiments expressed as a percentage of non-inhibitory control and error bars represent SEM. **B**) Representative Giemsa stained thin blood smears showing the growth phenotypes seen for non-inhibitory media controls, azithromycin analogues A13, B2, C1 and D1 (2x IC_{90}) 0-6 hrs post treatment (top panels) and 0-12 hrs post treatment (bottom panel).

Table 5.2 Cytotoxicity of lead analogues and selectivity against *Plasmodium* parasites.

Analogue	Intracellular growth μM (44 hr IC_{50}) D10-PfPHG [a]	Intracellular growth μM (28 hr IC_{50}) PkYH1[a]	Cytotoxicity μM (CC_{50}) Huh-7D[c]	Selectivity Huh-7D (CC_{50})/ PfPHG IC_{50} [d]	Selectivity Huh-7D (CC_{50})/ PkYH1 IC_{50} [d]
A2	1.6	0.65	8.7	5.4	13.3
A8	1.4	0.78	9.4	6.7	12.1
B2	0.53	0.67	7.9	14.9	11.8
C1	0.3	1.4	2.3	7.6	1.6

[a] Quick-killing in cycle drug treatment from rings to late schizonts, with no rupture cycle (D10-PfPHG, *P. falciparum,* 0-44 hrs or PkYH1, *P. knowlesi* 0-28 hrs). [c] Drug treatment of Huh-7D cells were incubated with the compounds for 24 hrs before the viability was measured. Data represents the means of 3 (or more) experiments expressed as percentage of non-inhibitory control.

5.5.4 Metabolomic investigation of lead compound.

We next used an untargeted metabolomics approach to identify changes in the metabolomic signatures of late trophozoites stages treated for 2 hrs at 5x IC_{50} (44 hr) with the leading analogue in *P. falciparum,* C1. C1 upregulated both haemoglobin-derived and non-haemoglobin derived peptides, with the latter shared with the metabolomic signatures of both azithromycin and chloroquine (312) (Chapter 4) (**Table 5.3**). As noted by Creek et al., (2016), most of these shared, non-haemoglobin derived peptides are associated with specific aspects of protein degradation within the food vacuole (312). However, they are not directly linked to haemoglobin degradation, in contrast to chloroquine's proposed mechanism of haem-detoxification. C1 also upregulated a number of haemoglobin-derived peptides, supporting our earlier proposition that azithromycin has activity against the food vacuole of trophozoite stage parasites (Chapter 4) (**Table 5.4**).

5.5.5 Activity of azithromycin analogues against the *Plasmodium*'s bacterial-like apicoplast ribosome.

After identifying azithromycin analogues with improved quick-killing activities against the blood stages of malaria, we next assessed apicoplast-targeting delayed death activity for each analogue. Given that azithromycin's delayed death IC_{50} is 0.07 μM and 0.08 μM for *P. falciparum* and *P. knowlesi* parasites, respectively, we reasoned that the delayed death activity of analogues would most likely be evident at drug concentrations below 1 μM across 2 cycles of parasite growth. All 22 analogues were screened for potential delayed death activity by treating *P. falciparum* (120 hr treatment) and *P. knowlesi* (78 hr treatment) parasites with 1 μM of drug and quantitating growth inhibition after two rupture cycles. This screen identified 10 of 22 analogues that inhibited growth by >30% at 1 μM across 2 cycles in both *Plasmodium* spp., with two analogues, A10 and A11, only active against *P. knowlesi*. We then evaluated the IC_{50}s for these prioritised drugs across 2 cycles of parasite growth in the respective *Plasmodium* spp. (**S. Table 5.1-5.4**). The majority of analogues featured high-nanomolar activity against *P. falciparum* across 2 cycles of parasite growth and were between 1.5 to 11.7-fold less potent (IC_{50} range; A9, 0.82 μM to B2, 0.097 μM) than azithromycin (0.07 μM) (**Table 5.1** & **S. Table 5.6**). Similarly, the analogues tested against *P. knowlesi* showed 1.2 to 11.8-fold higher IC_{50}s (IC_{50} range; A11, 0.95 μM to B2, 0.097 μM) than azithromycin (0.08 μM) (**Table 5.1** & **S. Table 5.6**).

Table 5.3 Metabolites shared with chloroquine treated parasites.

Putative metabolites	DHA	CQ	Az	C1	Mapped to HB	
					Alpha subunit	Beta subunit
Ala-Asp-Gly-Pro	0.00	1.50	7.88	112.06		
Ala-Asn	0.00	7.89	18.78	17.45	GE	
Thr-Arg	0.69	3.83	8.74	7.14		
Ala-Gly-Pro	0.62	3.40	5.94	5.72		PE
Val-Arg	0.92	5.66	9.81	6.92	RV	
beta-Alanyl-L-arginine	0.90	11.33	18.29	6.67		
Lys-Asn	0.00	23.15	33.95	17.58		
Glu-Ile-Gln-Lys	0.00	23.25	82.88	49.56		
Arg-Leu-Lys-Asp	0.00	87.29	100.65	72.89		
Lys-Tyr	0.00	30.51	55.00	35.40		
Glycyl-leucine	0.81	2.63	2.35	2.50		
L-Methionine	1.20	3.77	4.08	5.03		
Ala-Ala-Ser	0.09	1.28	1.95	2.81		
Ile-Lys	0.00	1.56	3.09	3.05		LK

List of putative peptides that were significantly perturbed following treatment with 5-fold the IC_{50} of DHA, chloroquine (CQ), azithromycin (Az) and C1 in three technical replicates. The order of the amino acids within the proposed peptide sequence has not been confirmed. The red shading denotes small peptides that were increased in abundance following treatment compared to ethanol control, yellow denotes no change, and blue shading denotes peptides that were decreased. Values represent the fold-change relative to ethanol control, and bold denotes changes that were statistically significantly different. The listed peptides (plus any isomeric peptides) were then investigated to determine whether it can be mapped to either the alpha or beta haemoglobin (HB) subunits.

Table 5.4 Metabolites mapping to haemoglobin after drug treatment.

Peptide sequence	DHA	CQ	Az	C1	Mapped to HB	
					Alpha subunit	Beta subunit
L-Tyrosyl-L-arginine	0.40	0.69	1.48	1.52	YR	
Ala-Lys-Lys-Asp	0.35	0.47	1.27	4.27	SLDK	
Asn-Leu-Pro-Pro	0.37	0.64	1.90	6.63		PPVQ
Ala-Glu-Glu-His	0.75	1.01	1.14	4.15	HVDD	
Glu-Ala-Pro	0.57	0.52	0.59	4.11	PAE	
Ala-Leu-Trp-Ser	0.42	0.58	1.19	3.32	PVNF	
Trp-Asn	0.44	0.59	0.82	1.83		YH
Glu-Glu-Gln-His	0.00	1.06	0.00	3.76		
Aspartyl-L-proline	0.61	0.81	0.33	1.04		VD
Lys-Pro	0.58	0.82	0.56	1.82		PK
Thr-Ala-Pro	0.57	0.75	0.52	1.89	TPA	

List of putative peptides that were significantly perturbed following treatment with 5-fold the IC_{50} of DHA, chloroquine (CQ), azithromycin (Az) and C1 in three technical replicates. The order of the amino acids within the proposed peptide sequence has not been confirmed. The red shading denotes small peptides that were increased in abundance following treatment compared to ethanol control, yellow denotes no change, and blue shading denotes peptides that were decreased. Values represent the fold-change relative to ethanol control, and bold denotes changes that were statistically significantly different. The listed peptides (plus any isomeric peptides) were then investigated to determine whether it can be mapped to either the alpha or beta haemoglobin (HB) subunits.

Notably, the analogues showing a 2 cycle IC_{50} similar to azithromycin were also the most potent in 44 hr treatments, opening up the possibility that the improved activity over 120 hr treatments was due to the cumulative activity of quick-killing across two growth cycles.

To address whether the improved activity over 2 cycles of parasite growth was due to quick-killing activity or apicoplast targeted delayed death activity, the 2 cycle growth inhibitory activity for a panel of analogues was tested against parasites lacking the apicoplast (apicoplast-null) (157, 161, 387). First, the apicoplast was chemically removed via prolonged treatments of azithromycin and parasite growth was rescued with IPP supplementation (161). We next examined whether the quick-killing (in cycle, 44 hrs) activity of analogues was affected by removal of the apicoplast by treating D10-PfPHG$^{apicoplast\text{-}null}$ and D10-PfPHG^{wildtype} lines with the in cycle D10-PfPHG^{wildtype} IC_{90} concentration of all quick-killing analogues (**Table 5.1**). As growth inhibition for D10-PfPHG$^{apicoplast\text{-}null}$ parasites was comparable to D10-PfPHG^{wildtype} parasites, this supported our previous observation that the analogues' quick-killing mechanism is unrelated to the apicoplast ribosome (Chapter 4) (**Figure 5.2 A** & **S. Table 5.7**).

To determine whether analogues also killed parasites through targeting the apicoplast's bacterial-like ribosome, we compared growth of D10-PfPHG^{wildtype} and D10-PfPHG$^{apicoplast\text{-}null}$ lines across 2 cycle assays with the delayed death D10-PfPHG^{wildtype} IC_{90} concentration of azithromycin and analogues (**Table 5.1**). D10-PfPHG^{wildtype} parasite growth was abolished when treated with azithromycin, whereas D10-PfPHG$^{apicoplast\text{-}null}$ parasites grew normally, consistent with the 'rescue' phenotype expected with an apicoplast ribosome targeting drug (157, 161) (**Figure 5.2 B**). However, no rescue was observed for the D10-PfPHG$^{apicoplast\text{-}null}$ line with any analogue, results that are consistent with the activity of the non-apicoplast targeting control drug, chloroquine (targets haem-detoxification) (**Figure 5.2 B** & **S. Table 5.7**). To further confirm this observation, we determined the IC_{50}s for analogues that exhibited micromolar quick-killing activities but nanomolar delayed death IC_{50} values (A5, A7, A8, A9, A11 and B2), a pattern similar to that of azithromycin, against D10-PfPHG^{wildtype} and D10-PfPHG$^{apicoplast\text{-}null}$ parasites (**Table 1** & **S. Figure 5.2**). The IC_{50}s of these 6 analogues against the D10-PfPHG^{wildtype} and D10-PfPHG$^{apicoplast\text{-}null}$ lines were almost identical (**S. Figure 5.2**), confirming that apicoplast targeting delayed death activity does not contribute to the activity of these compounds.

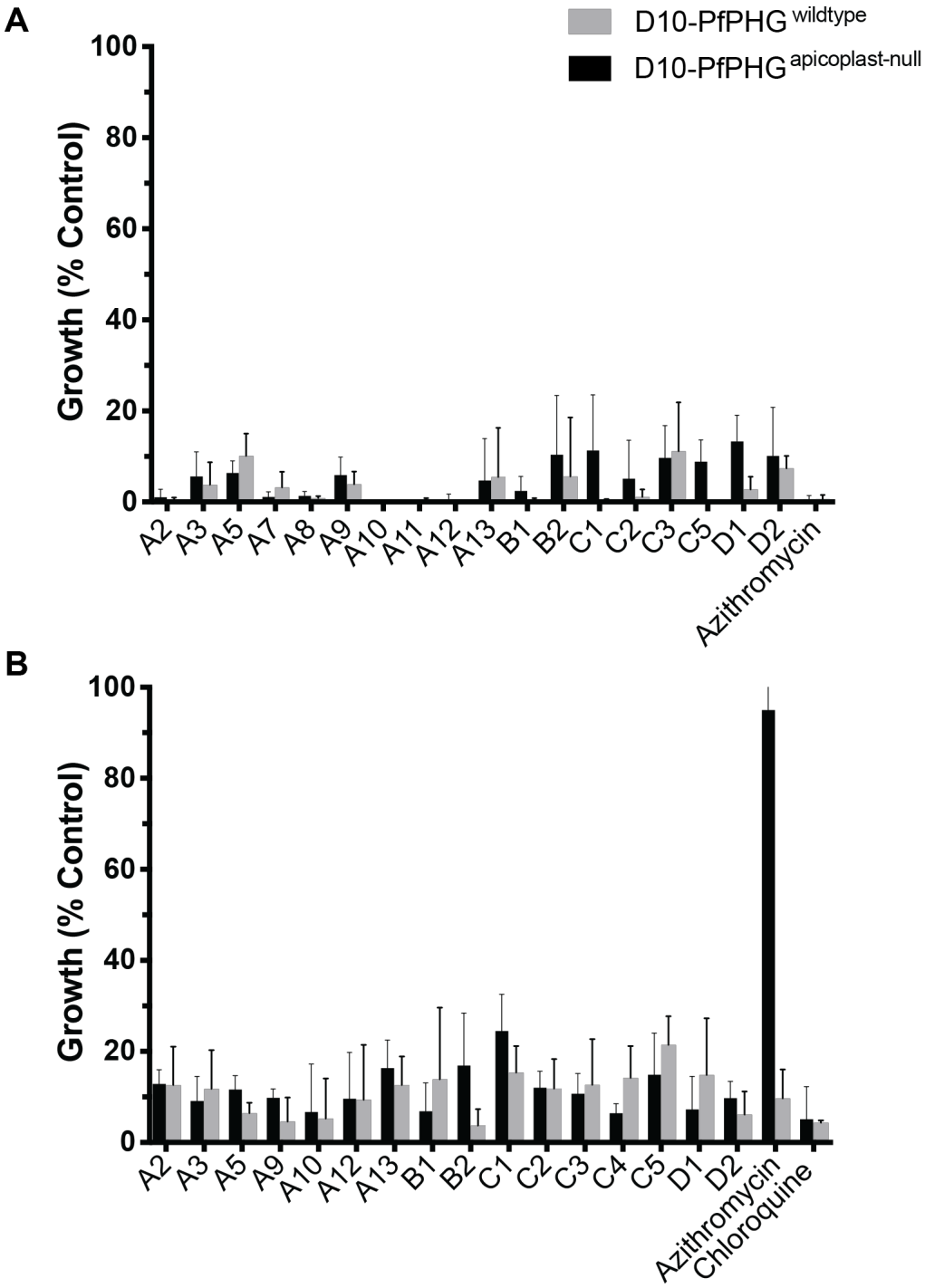

A
D10-PfPHG wildtype
D10-PfPHG apicoplast-null
Growth (% Control)
100
80
60
40
20
0
A2
A3
A5
A7
A8
A9
A10
A11
A12
A13
B1
B2
C1
C2
C3
C5
D1
D2
Azithromycin
B
Growth (% Control)
100
80
60
40
20
0
A2
A3
A5
A9
A10
A12
A13
B1
B2
C1
C2
C3
C4
C5
D1
D2
Azithromycin
Chloroquine

Figure 5.2 Activity of azithromycin analogues in the presence or absence of the apicoplast.

Early ring stage D10-PfPHG^{wildtype} (no IPP) (grey bars) or D10-PfPHG$^{apicoplast\text{-}null}$ (+IPP) (black bars) *P. falciparum* parasites (0-4 hrs post-invasion) were treated with: **A)** the IC_{90} of in cycle growth inhibition, addition of IPP did not rescue parasite growth from quick-killing activity of azithromycin or analogues, and **B)** the IC_{90} of 2 cycle (delayed death) growth inhibition, IPP rescued parasite growth from azithromycin's delayed death activity (inhibitor of the apicoplast's bacterial-like ribosome) but did not rescue parasite growth from chloroquine (targets the digestive vacuole) or analogues. Parasitemia was measured at 44 hr or 120 hrs post invasion at schizont stage via flow cytometry for in cycle and delayed death assays, respectively. Data represents the means of 3 (or more) experiments expressed as percentage of non-inhibitory control and error bars represent the ± SEM.

5.5.6 Activity of azithromycin analogues against bacterial ribosomes.

The azithromycin analogues examined in this study featured a range of modifications that could contribute to improved quick-killing potency, however, we identified that these analogues also lost delayed death activity. While the activity of these analogues have been addressed previously using a number of macrolide resistant *Streptococcus pneumoniae* strains, these studies did not test the analogues activity against an erythromycin sensitive *S. pneumoniae* (490), thus precluding direct comparison to our earlier study that assessed quick-killing analogue activity against an macrolide sensitive strain of *S. pneumoniae* (Chapter 4). Therefore, to allow clearer comparison of whether analogues with quick-killing antimalarial activity had lost or reduced activity against a bacterial ribosome we tested the potency of all 22 analogues against the Gram-positive, azithromycin sensitive, bacteria *S. pneumoniae* (D39) (**S. Table 5.7**). However, none of the analogues showed growth inhibitory activity against *S. pneumoniae* that was equivalent to azithromycin (MIC 0.125 μM) (**S. Table 5.7**). As the intact desosamine sugar is required for binding to the bacterial-like ribosome, it was no surprise that Group C and D compounds (**S. Table 5.3-5.4**), featuring a desosamine modification, showed limited evidence of targeting bacterial-like ribosomes in this bacterial strain (MICs >4 μM) (414, 421, 557). Group A, featured a cyclic carbonate in the 11, 12 positions and aryl or alkyl carbamoyl substitution in the 3-position of descladinosylazithromycin, and B analogues, featuring aryl or alkyl carbamoyl substitution of both the 3- and the 11- position of descladinosylazithromycin, also showed minimal growth inhibition after treating *S. pneumoniae* with MICs >1 μM (**S. Table 5.1-5.2**). The only exception in this series was B1 that featured high-nanomolar activity (MIC, 0.25 μM), suggesting this analogue may retain some activity against bacterial ribosomes, however, it was not active against the apicoplast's ribosome (**Figure 5.2 B** & **S. Table 7**).

In general, there was little evidence that the azithromycin analogues tested in this study had significant apicoplast ribosome targeting delayed death activity. The evidence from screens using erythromycin sensitive *S. pneumoniae* and published studies further suggest that these analogues likely have minimal antibacterial activity at concentrations where quick-killing antimalarial activity is evident (490). Thus, the panel of azithromycin analogues described in this paper could provide additional starting points to develop quick-killing azithromycin analogues with minimal activity against bacteria.

5.6 Discussion.

In malaria parasites, the antibiotic azithromycin is known to inhibit translation of the apicoplast's bacterial-like ribosome and cause 'delayed death' of the second-generation of daughter parasites (130, 159, 160). Whilst azithromycin exhibits a long half-life and favourable safety profile (431, 439), its clinical application for treatment of malaria is compromised by the 4-day delay in parasite clearance (130, 376). Recently, we demonstrated that azithromycin also kills blood stage parasites through 'quick-killing' and that chemical modification improves this activity (165) (Chapter 4).

Here, we characterised a panel of azithromycin analogues, previously demonstrated to have moderate efficacy against bacteria (490), for their inhibitory activity against malaria parasites. Twenty-two azithromycin analogues were assessed for quick-killing and delayed death activities against *P. falciparum* and *P. knowlesi*. We identified 17 of 22 analogues with improved in cycle potency against *P. falciparum* and 18 showing improved quick-killing (28 hr treatment) for *P. knowlesi,* in comparison to azithromycin. Improvements in quick-killing was evident across a range of chemotypes and the activity of analogues investigated in this study was similar for both *Plasmodium* species, although minor differences in susceptibility were observed. Descladinosylazithromycin analogue C1 (3-*O*-(3-chlorophenyl) carbamoyl) was found to have the most potent quick-killing activity against *P. falciparum*. Whereas descladinosylazithromycin analogue A2 (3-*O*-(3-methylphenyl) carbamoyl), A13 (3-*O*-(phenyl) carbamoyl) and B2 (11-O-(hexyl) carbamoyl, 3-O-(3-methoxyphenyl) carbamoyl) were the most potent quick-killing analogues against *P. knowlesi.* We have previously identified that loss of azithromycin's cladinose sugar results in a 6.5-fold reduction in potency against malaria parasites (165). However, in this study improved quick-killing activity of analogues was evident even for those that had functionality substituted in place of the cladinose sugar. In general, there was also no functional group commonality between the most active compounds that can be exploited to further develop their quick-killing activity.

Previously, we showed azithromycin analogues to have remarkably rapid potency against early ring stage parasites with 6 and 12 hr treatments compared to treatment across one full cycle of intracellular growth (0-44 hrs) (Chapter 4). While the compounds described in this study did not reach the potency of several analogues we described previously (Chapter 4), the most potent analogues showed a clear growth inhibitory phenotype against early ring stage parasites, with vacuolated parasites visible with 12 hrs of treatment. As most antimalarials inhibit trophozoite or schizont stage parasites (127, 128, 151, 370, 525, 526), the fact these diverse analogues

remained active with <12 hrs of treatment at concentrations similar to that required to kill parasites across 44 hrs is promising for azithromycin's re-development as a quick-killing antimalarial.

To date, the mechanism of quick-killing for azithromycin and analogues has not been completely elucidated. Evidence from metabolomics studies using C1 from this study and analogues in a preceding study (Chapter 4) indicate that alterations in parasite metabolites with treatment of azithromycin and analogues resembled that observed for chloroquine, wherein a pattern of non-haemoglobin peptide is enriched (312). These data suggest that quick-killing activity of azithromycin analogues could be mediated though damage to the parasite's food vacuole and ultimately disrupt haemoglobin digestion and hemozoin formation. Indeed, azithromycin is known to accumulate within acidic compartments of various cells (416, 537) and the improvement of azithromycin's cationic profile and lipophilic properties through modification (165) may potentiate the accumulation and damage caused by the drug within acidic compartments. Further supporting this possibility, compound C1 also caused a build-up of peptides that could be linked back to haemoglobin, a signature shared with dihydroartemisinin in these experiments that highlights that activity against the food vacuole may be an important mechanism for azithromycin analogues, in addition to other multi-factorial actions.

Interestingly, azithromycin and analogues do not completely mimic the activity of chloroquine. Firstly, azithromycin and a range of analogues tested with different modifications have activity against very early ring stage parasites, a stage of the lifecycle that chloroquine has poor activity against (131, 151) (Chapter 4). The food vacuole itself is only fully formed by mid-ring stages (~12-18 hrs post invasion), removing the predominant site of chloroquine-like activity as a logical target during early ring stage development (27, 28). However, there is evidence that haemoglobin digestion occurs earlier in the lifecycle and therefore, azithromycin and analogues could disrupt digestion at this early stage of ring stage development (27, 558, 559). Secondly, the quick-killing activity of azithromycin and analogues is known to disrupt *Plasmodium* spp. merozoite invasion of RBCs as well as short term intracellular development and host-cell invasion of a related apicomplexan parasite, *Toxoplasma gondii* (165, 488). Neither *Plasmodium* merozoites nor *T. gondii* digest haemoglobin nor create hemozoin that could be capped by a chloroquine-like activity. Therefore, the quick-killing activity of azithromycin and analogues may encompass a number of mechanisms of action against the malaria parasite, but

activity against the parasite's food vacuole appears to be an important target during trophozoite stages.

Finally, we found the lead analogues had moderate activity against human cells and maintained >5-fold selectivity against *P. falciparum* parasites over human Huh-7D cells. Whilst this suggests that these analogues offer acceptable toxicity and provide a reasonable starting point to warrant further evaluation and development, ideally a higher selectively window (of up to 100-fold) and further evaluation in other cell types would be required.

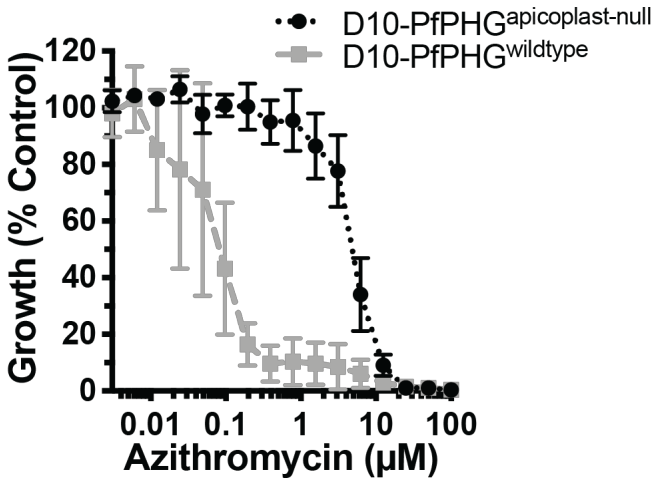
An important consideration in developing azithromycin analogues as quick-killing antimalarials includes the safety and non-specific activity against the gut microbiome and other bacteria (543-546). Azithromycin's known mechanism of action against malaria parasites is the result of its antibacterial activity and targeting of the bacterial-like ribosome of the parasites apicoplast (130, 160, 420, 421). Whilst combinations of azithromycin's delayed death prophylaxis and quick-killing activities could prevent parasite recrudescence and reduce emergence of drug resistant parasites during treatment, it is also possible that the remaining antibacterial activity could have off-target effects on the human microbiome and enrich for macrolide resistant pathogenic bacteria (543-546). Thus, it may be desirable to develop an azithromycin analogue featuring improved quick-killing for treatment of clinical malaria, but no activity against the bacterial-like ribosome.

Azithromycin analogues tested in this study showed no significant activity against the parasites' apicoplast ribosome, with parasites lacking an apicoplast showing equal susceptibility as wildtype parasites (157, 161). Moreover, analogues lost potency relative to azithromycin against the azithromycin sensitive *S. pneumoniae* (strain D39). Together, these data suggest that modifications of the diverse azithromycin analogues tested in this study have largely removed activity against bacterial-like ribosomes, providing a number of new azithromycin-based structures to develop quick-killing antimalarials.

5.7 Conclusions.

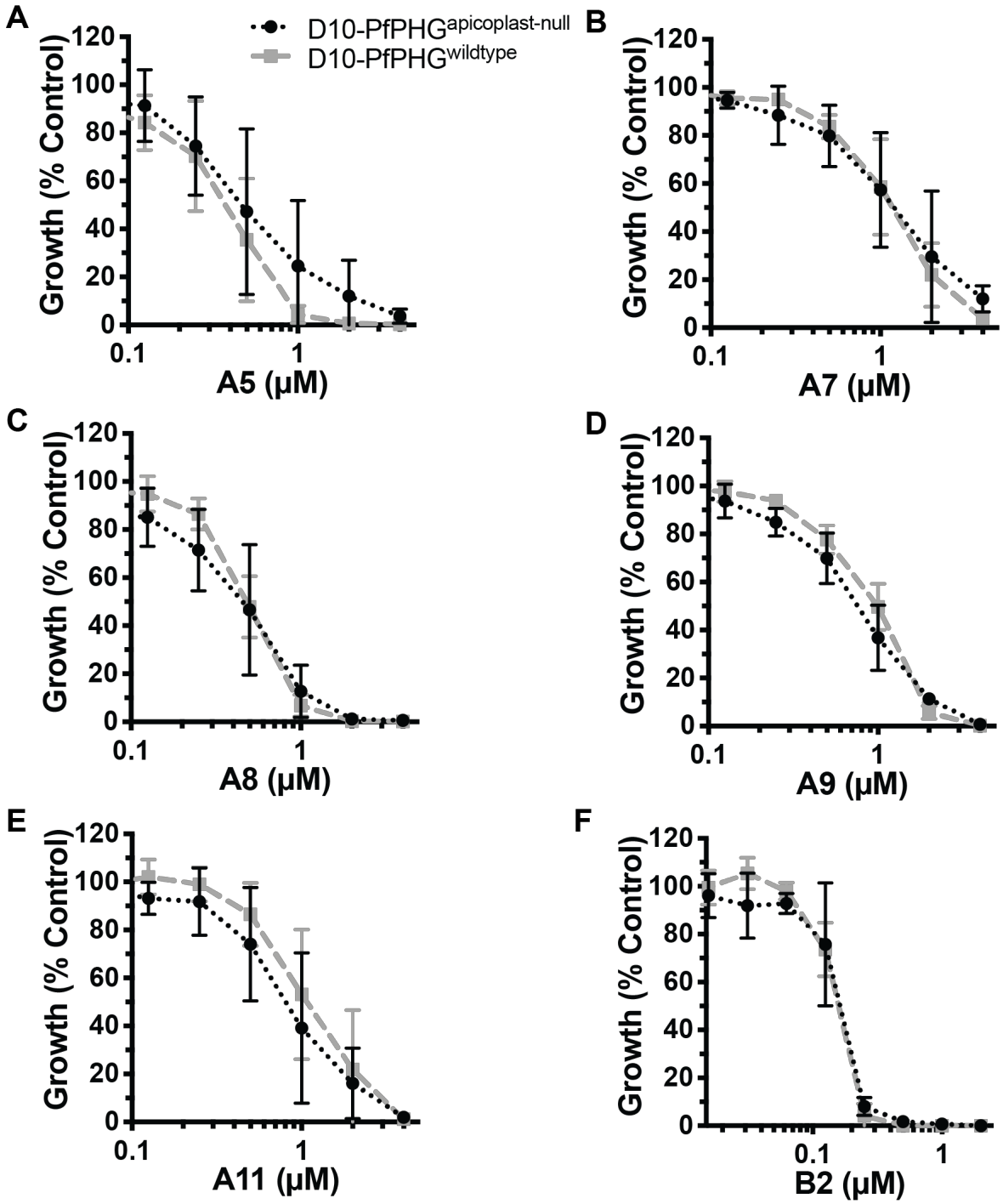
We have shown that azithromycin analogues developed to have modest and variable efficacy against bacterial pathogens have improved quick-killing activity against blood stage malaria parasites. Leading analogues showed cross-species efficacy against both human and zoonotic *Plasmodium* species and an acceptable selectivity index against parasites over mammalian cells during early stage development. Metabolomics analysis of drug treated late stage parasites supported the idea that azithromycin analogues can target the parasites food vacuole during trophozoite stage development, but the activity against early ring stage parasites indicated that they provide a broader spectrum of activity against blood stage parasite development than the clinically used food-vacuole targeting antimalarial chloroquine. Analogues showed limited efficacy in assays that assessed activity against bacterial-like ribosomes, suggesting that quick-killing antiparasitic activity can be selected over antibiotic activity over this diverse range of chemotypes.

5.8 Supplementary figures for 'Targeting malaria parasites with novel derivatives of azithromycin'.



S. Figure 5.1 Growth inhibition profiles of azithromycin in parasites lacking the apicoplast.

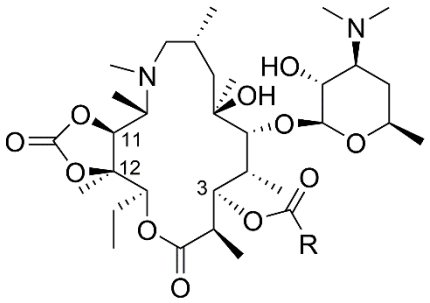
Early ring stage *P. falciparum* parasites (0-4 hrs post-invasion) were treated with doubling dilutions of azithromycin and inhibition of growth measured for 2 cycle (delayed death, 120 hrs) assays (D10-PfPHG$^{apicoplast\text{-}null}$ IC_{50}, 4.5 μM; D10-PfPHG^{wildtype} IC_{50}, 0.07 μM. P= <0.0001). Parasitemia was measured at 120 hrs post invasion at schizont stage via flow cytometry. Data represents the means of 3 (or more) experiments expressed as percentage of non-inhibitory control and error bars represent ± SEM.

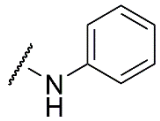
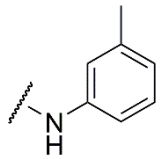

A
D10-PfPHG^apicoplast-null
D10-PfPHG^wildtype
Growth (% Control)
A5 (μM)
B
A7 (μM)
C
A8 (μM)
D
A9 (μM)
E
A11 (μM)
F
B2 (μM)

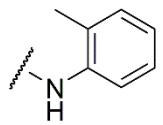
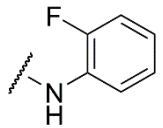
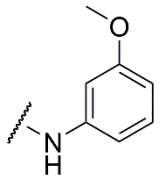
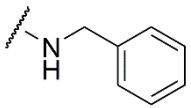
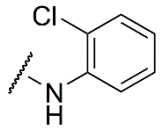
S. Figure 5.2 Removal of the apicoplast does not rescue parasites from azithromycin analogue activity.

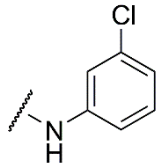
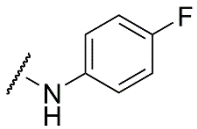
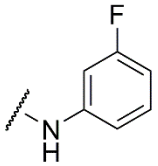
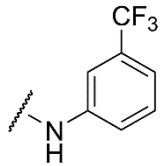
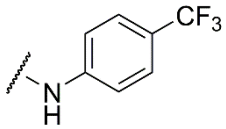
Early ring stage *P. falciparum* D10-PfPHG^{wildtype} and D10-PfPHG$^{apicoplast\text{-}null}$ parasites (<4 hrs post-invasion) were treated with doubling dilutions of analogues and inhibition of growth measured 2 cycles latter (delayed death, 120 hrs) for compounds: **A**) A5 (D10-PfPHG$^{apicoplast\text{-}null}$ IC_{50}, 0.5 μM; D10-PfPHG^{wildtype} IC_{50}, 0.4 μM. P=NS). **B**) A7 (D10-PfPHG$^{apicoplast\text{-}null}$ IC_{50}, 0.37 μM; D10-PfPHG^{wildtype} IC_{50}, 0.49 μM. P=NS). **C**) A8 (D10-PfPHG$^{apicoplast\text{-}null}$ IC_{50}, 0.63 μM; D10-PfPHG^{wildtype} IC_{50}, 0.62 μM. P=NS). **D**) A9 (D10-PfPHG$^{apicoplast\text{-}null}$ IC_{50}, 1.4 μM; D10-PfPHG^{wildtype} IC_{50}, 1.8 μM. P=NS). **E**) A11 (D10-PfPHG$^{apicoplast\text{-}null}$ IC_{50}, 1.6 μM; D10-PfPHG^{wildtype} IC_{50}, 2.9 μM. P=NS). **F**) B2 (D10-PfPHG$^{apicoplast\text{-}null}$ IC_{50}, 0.17 μM; D10-PfPHG^{wildtype} IC_{50}, 0.16 μM. P=NS). Data represents the means of 2 (or more) experiments expressed as percentage of non-inhibitory control and error bars represent the SEM.

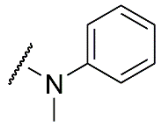
S. Table 5.1 Activities of A-group azithromycin analogues.



Analogue ID	R-group	Carbamoyl substituent (R-group) name	Intracellular growth D10-PfPHG at 10 μM (% growth) [a]	Intracellular growth D10-PfPHG at 1 μM (% growth) [b]	Intracellular growth PkYH1 at 10 μM (% growth) [a]	Intracellular growth of PkYH1 at 1 μM (% growth) [b]	Original reference
A1		phenyl	93	92	79	95	(490)
A2		3-methylphenyl	3	16	3	16	(490)

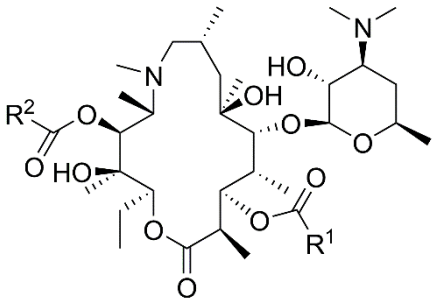
A3		2-methylphenyl	3	7	2	11	(490)
A4		2-fluorophenyl	95	86	24	93	(490)
A5		3-methyloxyphenyl	4	7	3	8	(490)
A6		benzyl	93	94	70	97	(490)
A7		2-cholophenyl	9	32	4	33	(490)

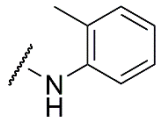
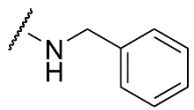
A8		3-cholophenyl	3	3	5	4	(490)
A9		4-fluorophenyl	8	76	9	78	(490)
A10		3-fluorophenyl	3	63	2	24	(490)
A11		3-trifluorophenyl	11	90	24	21	(490)
A12		4-trifluorophenyl	3	13	6	1	(490)

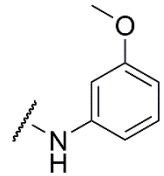
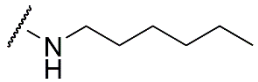
A13		N-methyl-phenyl	3	12	1	2	(490)

[a] Drug treatment of intracellular growth, from rings to late schizonts, with no rupture cycle (D10-PfPHG, *P. falciparum,* 0-44 hrs or *PkYH1*, *P. knowlesi* 0-28 hrs). [b] Drug treatment of delayed death, from early-rings to late trophozoites, with two rupture cycle (D10-PfPHG, *P. falciparum,* 0-120 hrs or *PkYH1*, *P. knowlesi* 0-78 hrs). Data represents the means of 3 (or more) experiments expressed as percentage of non-inhibitory control.

S. Table 5.2 Activities of B-group azithromycin analogues.

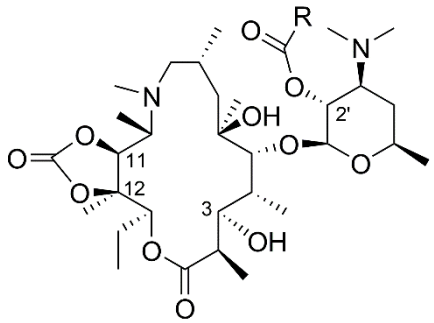


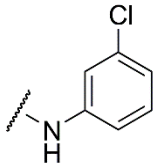
Analogue ID	R-groups	Carbamoyl substituent (R-group) name	Intracellular growth D10-PfPHG at 10 μM (% growth) [a]	Intracellular growth D10-PfPHG at 1 μM (% growth) [b]	Intracellular growth PkYH1 at 10 μM (% growth) [a]	Intracellular growth of PkYH1 at 1 μM (growth %) [b]	Original reference
B1	R^1 	2-methylphenyl	3	18	4	3	(490)
	R^2 	benzyl					

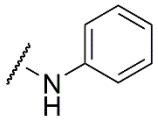
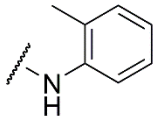
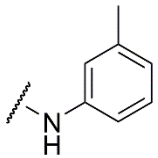
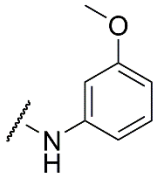
B2	R^1 	4-methyloxyphenyl	2	6	4	2	(490)
	R^2 	hexyl					

[a] Drug treatment of intracellular growth, from rings to late schizonts, with no rupture cycle (D10-PfPHG, *P. falciparum,* 0-44 hrs or *PkYH1*, *P. knowlesi* 0-28 hrs). [b] Drug treatment of delayed death, from early-rings to late trophozoites, with two rupture cycle (D10-PfPHG, *P. falciparum,* 0-120 hrs or *PkYH1*, *P. knowlesi* 0-78 hrs). Data represents the means of 3 (or more) experiments expressed as percentage of non-inhibitory control.

S. Table 5.3 Activities of C-group azithromycin analogues.

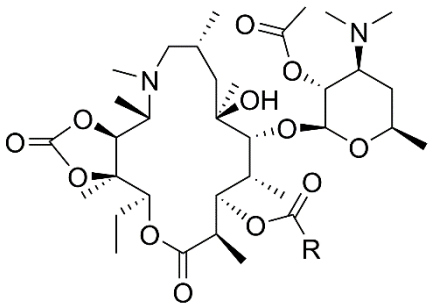


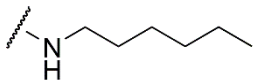
Analogue ID	R-group	Carbamoyl substituent (R group) name	Intracellular growth D10-PfPHG at 10 μM (% growth) [a]	Intracellular growth D10-PfPHG at 1 μM (% growth) [b]	Intracellular growth PkYH1 at 10 μM (% growth) [a]	Intracellular growth of PkYH1 at 1 μM (growth %) [b]	Original reference
C1		3-chlorophenyl	3	0.7	0.4	2	(490)

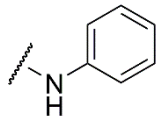
C2		Phenyl	7	73	12	74	(490)
C3		2-methylphenyl	6	67	2	73	(490)
C4		3-methylphenyl	85	89	74	96	(490)
C5		3-methyloxyphenyl	85	83	72	95	(490)

[a] Drug treatment of intracellular growth, from rings to late schizonts, with no rupture cycle (D10-PfPHG, *P. falciparum,* 0-44 hrs or *PkYH1*, *P. knowlesi* 0-28 hrs). [b] Drug treatment of delayed death, from early-rings to late trophozoites, with two rupture cycle (D10-PfPHG, *P. falciparum,* 0-120 hrs or *PkYH1*, *P. knowlesi* 0-78 hrs). Data represents the means of 3 (or more) experiments expressed as percentage of non-inhibitory control.

S. Table 5.4 Activities of D-group azithromycin analogues.



Analogue ID	R-group	Carbamoyl substituent (R group) name	Intracellular growth D10-PfPHG at 10 μM (% growth) [a]	Intracellular growth D10-PfPHG at 1 μM (% growth) [b]	Intracellular growth PkYH1 at 10 μM (% growth) [a]	Intracellular growth of PkYH1 at 1 μM (growth %) [b]	Original reference
D1		hexyl	3	3	1	85	(490)

D2		phenyl	31	77	12	84	(490)

[a] Drug treatment of intracellular growth, from rings to late schizonts, with no rupture cycle (D10-PfPHG, *P. falciparum,* 0-44 hrs or *PkYH1*, *P. knowlesi* 0-28 hrs). [b] Drug treatment of delayed death, from early-rings to late trophozoites, with two rupture cycle (D10-PfPHG, *P. falciparum,* 0-120 hrs or *PkYH1*, *P. knowlesi* 0-78 hrs). Data represents the means of 3 (or more) experiments expressed as percentage of non-inhibitory control.

S. Table 5.5 In cycle fold-change of analogues vs azithromycin.

Analogue	Intracellular growth D10-PfPHG IC_{50} (μM) [a]	Intracellular growth PkYH1 IC_{50} (μM) [a]	Azithromycin / D10-PfPHG IC_{50} (μM) [a]	Azithromycin / PkYH1 IC_{50} (μM) [a]
Azithromycin	11.3	13		
A2	1.6	0.65	7.1	20
A3	1.5	1.1	7.5	11.8
A4	ND	7.8	ND	1.6
A5	1.8	1.5	6.2	8.7
A7	5.2	1.7	2.2	7.6
A8	1.4	0.78	8.1	16.7
A9	3.3	1.9	3.4	6.8
A10	4.2	1.4	2.7	9.3
A11	5.9	4.2	1.9	3.1
A12	1.1	1.2	10.2	10.8
A13	0.72	0.64	15.7	20.3
B1	1.4	1.2	8.1	10.8
B2	0.53	0.67	21.3	19.4
C1	0.3	1.4	38	9.2
C2	4.7	5.4	2.4	2.4
C3	3.2	4.0	3.5	3.3
D1	0.7	1.6	16.1	8.1
D2	3.8	2.8	3	4.6

[a] Drug treatment of intracellular growth, from rings to late schizonts, with no rupture cycle (D10-PfPHG, *P. falciparum,* 0-44 hrs or PkYH1, *P. knowlesi* 0-28 hrs). Data represents the means of 3 (or more) experiments expressed as percentage of non-inhibitory control. ND= not done due to <40% activity 0-44 hr in cycle assays or limited amounts of analogue.

S. Table 5.6 Delayed death fold-change of analogues vs azithromycin.

Analogue	**Intracellular growth D10-PfPHG IC_{50} (μM) [b]**	**Intracellular growth *Pk*YH1 IC_{50} (μM) [b]**	**D10-PfPHG / azithromycin IC_{50} (μM) [b]**	***Pk*YH1/ azithromycin IC_{50} (μM) [b]**
Azithromycin	0.07	0.08		
A2	0.24	0.26	3.4	3.25
A3	0.43	0.39	6.1	4.8
A5	0.39	0.47	5.5	5.8
A7	0.76	0.59	10.8	7.3
A8	0.35	0.16	5	2
A9	0.82	0.77	11.7	9.6
A10	1.1	0.71	15	8.8
A11	0.9	0.95	12.8	11.8
A12	0.3	0.21	4.3	2.6
A13	0.18	0.39	2.5	4.8
B1	0.29	0.11	4.1	1.3
B2	0.14	0.097	2	1.2
C1	0.11	0.24	1.5	3
C2	1.1	ND	15.6	ND
C3	1.0	ND	14.7	ND
C4	2.7	ND	25.7	ND
C5	1.8	ND	18.5	ND
D1	0.11	ND	1.5	ND
D2	1.3	ND	18.5	ND

[b] Drug treatment of delayed death, from early-rings to late trophozoites, with two rupture cycle (D10-PfPHG, *P. falciparum,* 0-120 hrs or PkYH1, *P. knowlesi* 0-78 hrs). Data represents the means of 3 (or more) experiments expressed as percentage of non-inhibitory control. ND= not done due to <40% activity 0-44 hr in cycle assays or limited amounts of analogue.

S. Table 5.7 Activity of analogues against bacterial-like ribosomes.

Analogue	MIC average [d]	Intracellular growth D10-PfPHG $^{Apicoplast-null}$ IC_{90} (% growth) [a]	Intracellular growth D10-PfPHG $^{Apicoplast-null}$ IC_{90} (% growth) [b]
Azithromycin	0.125	0	95
A1	>16	ND	ND
A2	4	0	12
A3	2	5	9
A4	4	ND	ND
A5	8	6	11
A6	>16	ND	ND
A7	4	1	ND
A8	2	1	ND
A9	4	6	10
A10	8	0	5
A11	>16	0	ND
A12	4	0	13
A13	1	4	16
B1	0.25	0	13
B2	8	10	16
C1	16	11	15
C2	4	5	12
C3	8	9	10
C4	>16	ND	6
C5	8	8	15
D1	8	13	7
D2	8	10	9

[d] Minimum inhibitory concentration (MIC) were determined as described in Methods. MIC dilution series were analysed individually, and values are represented as (μM). The MIC data represents the means of 3 or more experiments expressed as a percentage of non-inhibitory control. [a] Drug treatment of D10-PfPHG $^{apicoplast-null}$ parasites supplemented with IPP for intracellular growth, from rings to late schizonts, with no rupture cycle (*P. falciparum,* 0-44 hrs). [b] Drug treatment of D10-PfPHG $^{apicoplast-null}$ supplemented with IPP for delayed death assays, from early-rings to late trophozoites, with two rupture cycle (*P. falciparum,* 0-120 hrs). Data represents the means of 3 (or more) experiments expressed as percentage of non-inhibitory control. ND= not done due to <40% activity 0-44 hr in cycle assays or limited amounts of analogue.

Chapter 6. Characterising the antimalarial activity of novel proteasome-like inhibitors.

6.1 Preface and statement of contribution.

In the interest of clearly representing my contribution, the proteasome-like inhibitors and proteasome-like pro-drugs used in this chapter were synthesised in-house by Aniket Kulkarni and Prof. Andrew Abell (University of Adelaide, Australia). The chemical synthesis of these compounds is not covered in this thesis as this will form a large part of Aniket Kulkarni's thesis due for submission late 2019. Both the biological assays and the chemical synthesis are expected to be combined in a joint publication with the Abell group in 2020 subject to the characterisation of some additional analogues outside the scope of my involvement in this study.

Statement of contribution

Title of Chapter	Characterising the antimalarial activity of novel proteasome inhibitors
Publication Status	**Thesis chapter subject to publication**
Publication Details	Amy Lee Burns, Aniket Kulkarni, Andrew Abell, Danny Wilson

Principal Authors

Name of Principal Author (Candidate)	Amy Lee Burns		
Contribution to the Paper	Conceived, designed, performed experiments and analysed the results represented in this Chapter. Wrote the chapter. This chapter deals specifically with the characterisation of compound activity against malaria parasites *in vitro* by Ms Burns. The chemical synthesis of these compounds undertaken by Mr Kulkarni is outside the scope of this chapter.		
Overall percentage (%)	60%		
Certification:	This chapter reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary Author of this chapter.		
Signature		Date	28/10/2019

Name of second Principal Author (Candidate)	Aniket Kulkarni		
Contribution to the Paper	Conceived, designed and made the compounds used in this chapter, assisted in analysing results and provided advise. The synthesis of these compounds undertaken by A. Kulkarni will be presented separately in his thesis as the major contributor to that work and in a co-author publication between the collaborators represented here.		
Overall percentage (%)	20%		
Signature		Date	27/10/2019

Co-Author Contributions

Name of Co-Author	Andre Abell		
Contribution to the Paper	Conceived the compounds used in this Chapter, assisted in analysing results and provided advise.		
Overall percentage (%)	15%		
Signature		Date	28/10/2019

Name of Co-Author	Danny W. Wilson		
Contribution to the Paper	Conceived experiments, performed experiments assisted in analysing results, provided advise and edited chapter.		
Overall percentage (%)	15%		
Signature		Date	18/10/2019

6.2 Introduction.

6.2.1 Combating resistance to front-line artemisinin combination therapies by targeting the malaria proteasome.

Currently, the front-line treatments for *P. falciparum* malaria are artemisinin combination therapies (ACTs), which are composed of a rapid-acting artemisinin derivative (artemether, artesunate, arteether and DHA) and a longer-lasting partner drug (202, 203). There are major concerns, however, that spread of artemisinin resistance (224, 225, 228, 229, 539) and the loss of our most effective class of antimalarials will increase malaria related morbidity and mortality (133). New drug treatments are therefore urgently needed, and one of the most promising strategies being developed involves targeting the malaria parasite's 26S proteasome (230, 231).

Proteasomes are multi-catalytic protease protein complexes that are responsible for essential 'housekeeping' roles in all eukaryotes by selectively degrading and recycling proteins through the ubiquitin-proteasome system (UPS). The UPS covalently links a polyubiquitin chain to damaged, unfolded, misfolded or redundant target proteins, with this ubiquitinated protein then recognised and degraded into small peptides by the proteasome (560). As accumulation of defective and redundant proteins is lethal to cells, the UPS is essential for a range of processes including cellular homeostasis, cell cycle progression, transcriptional regulation and apoptosis (365, 561).

Plasmodium parasites contain a typical eukaryotic (26S) proteasome that is likely responsible for degrading ubiquitinated proteins within the cytoplasm (562, 563) (reviewed in (325)). This complex consists of a 20S catalytic core subunit and two 19S regulatory cap complexes (325, 564) (**Figure 6.1**). The 20S catalytic core is comprised of two external α-subunits (α1 to α7) and two flanking β-subunit rings (β1 to β7). Three β-subunits; β1, a caspase-like (cleaves after acidic residues); β2, a trypsin-like (cleaves after basic residues); and β5, a chymotrypsin-like (cleaves after hydrophobic residues), are essential and mediate the catalytic activity of the proteasome (reviewed in (363)). The two 19S regulatory caps located on the outside of the complex recognise, de-ubiquitinates, unfolds and controls the entry of target proteins into the 20S catalytic core (565) (reviewed in (364)). The protein is then linearly translocated into the central chamber of 20S catalytic core, where it is degraded into small peptide fragments by the multi-catalytic action of β1, β2 and β5 proteases (reviewed in (564) and (363)).

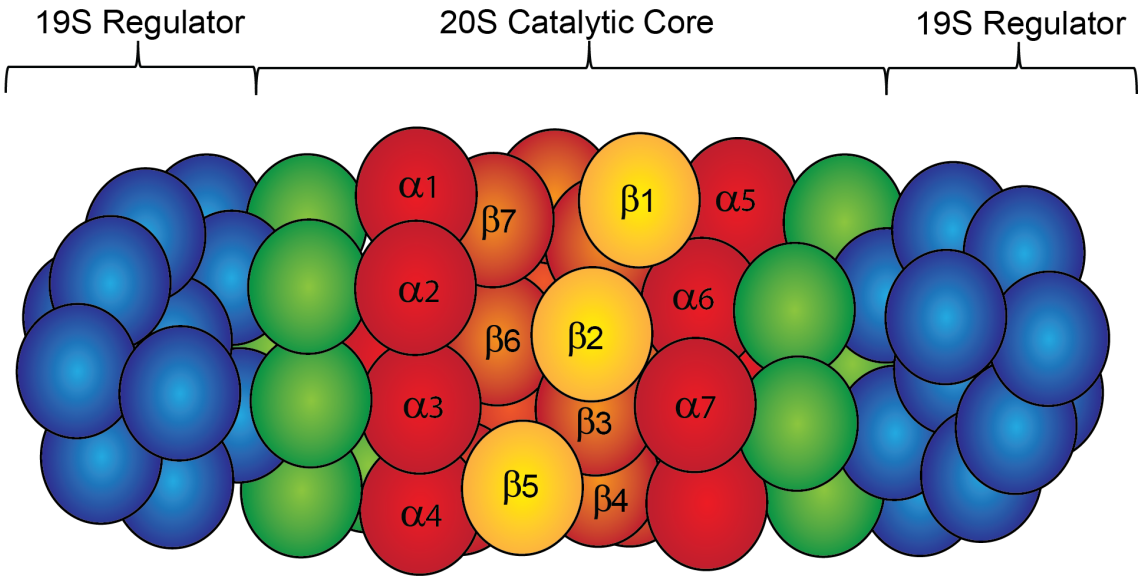


Figure 6.1 Schematic model of the 26S proteasome.

The 26S proteasome is comprised of two 19S regulatory cap subunits and a 20S catalytic core subunit. The 20S catalytic core of the proteasome (red, orange and yellow) is arranged as a stack of four rings, two α-subunits (red) (α1 to α7) and two β-subunits (orange and red) (β1 to β7). The β-subunits, β1, β2 and β5 (yellow), mediate proteolytic cleavage. The 19S regulatory cap (blue and green) subunits control the entry of target proteins and substrates into the complex. Figure is adapted from (http://www.bostonbiochem.com/products/proteasome) (566).

In *Plasmodium,* the 26S proteasome is expressed throughout every life-stage (liver, blood, transmission and mosquito stages) (563, 567-569) and as much as half of the malaria parasite's proteome is thought to be regulated through ubiquitination and degradation (367). Therefore, as the proteasome is essential for parasite survival, it is considered an attractive avenue for drug development.

6.2.2 Inhibitors of *Plasmodium* proteasomes.

A number of peptide-based proteasome inhibitors have previously been explored in the literature (570-573), with many studies specifically investigating the antimalarial activity of tri-peptide inhibitors (reviewed in (366) and (574)). Briefly, tri-peptide inhibitors are typically comprised of a common β-strand scaffold, key amino acids that impact the target specificity (P1 to P3). The N-terminal cap of the molecules, in the case of MG-132, is capped by a benzyloxycarbonyl group, that confers a degree of specificity to the protease target. The C-terminal electrophile 'warhead' covalently binds to the target and deactivates the proteasome (316, 373, 575). The 'first-generation' tri-peptide inhibitors, such as the aldehyde MG-132 (330) (**Figure 6.2**), α,β-epoxyketones including carmaphycin B (576), and vinyl sulfones (368, 369) were found to have low-nanomolar activity against blood stage parasite growth (reviewed in (366)).

As some of these early inhibitors and their derivatives were originally developed as anti-cancer drugs, they also had activity against the host proteasome leading to toxicity against mammalian cells (reviewed in (577) and (366)). Subsequently, a variety of synthetic tri-peptides have been developed that increased the *Plasmodium*-specific activity (368, 369, 373, 576). Similar to first generation tri-peptides, these second generation parasite proteasome specific inhibitors are comprised of a chemically reactive C-terminal 'warhead' and a β-strand with key amino acids at the P1 to P3 sites (316, 373, 575). Malaria parasite proteasome specific inhibitors are designed to take advantage of structural differences in the catalytic binding pockets between *Plasmodium* and human proteasome active subunits. Malaria specific proteasome inhibitors contain 'parasite preferred' amino acids at the P1 to P3 sites that improve the specificity of binding to the active β-subunits in the 20S core and are less 'preferred' by the human proteasome (368) (**Figure 6.2**).

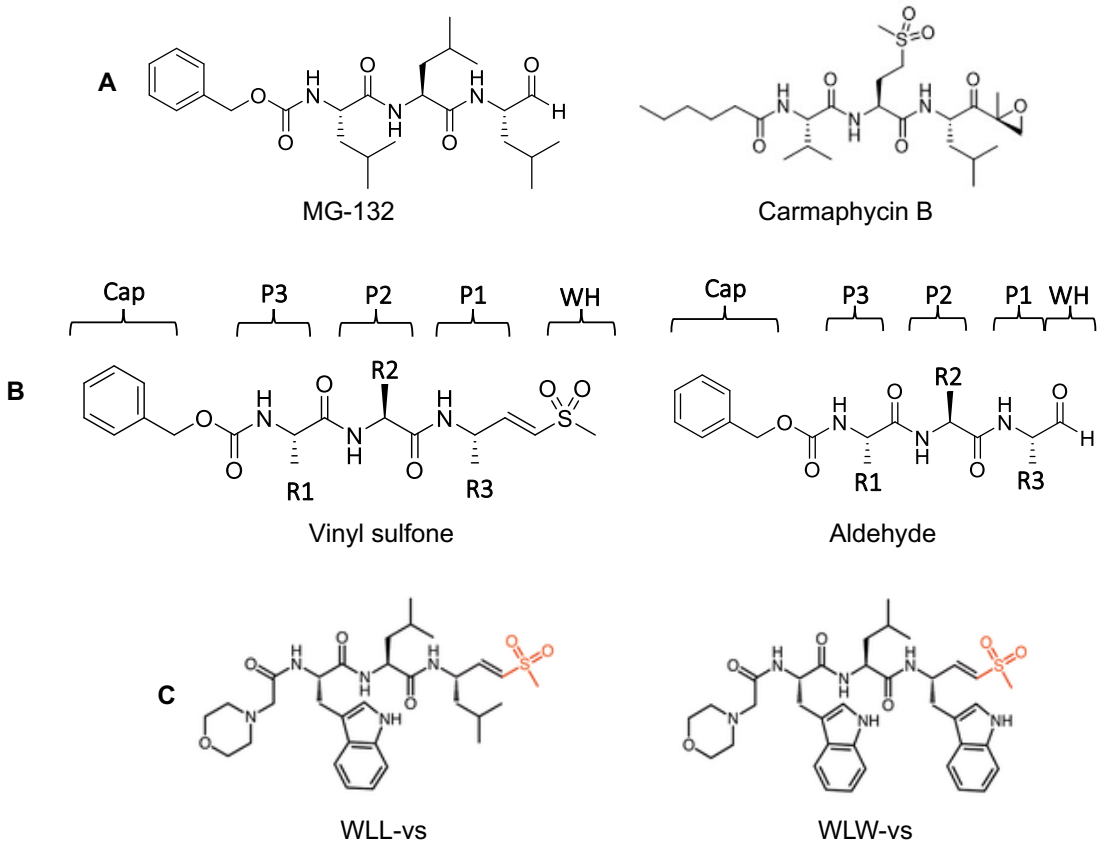


Figure 6.2 Structure of tripeptide proteasome-like inhibitors with antimalarial activity.

A) First-generation tri-peptide proteasome inhibitors, MG-132 (Carboxybenzyl (Cbz)-leucine-leucine-leucine-aldehyde) and caramphycin B (hexanoyl-valine-methionine sulfone-leucine-α,β-epoxyketone). Adapted from (373, 576). **B**) Representations of the peptide (P) and corresponding modification (R) sites that define the amino acids making up the tri-peptide structures used in this study. The majority of tri-peptides tested in this study contain a carboxybenzyl cap and either a C-terminal vinyl sulfone or aldehyde 'warhead' (WH). Adapted from (368) and (373). **C**) Leading scaffolds of peptide vinyl sulfones (vs) with potent antimalarial activity, Tryptophan-Leucine-Leucine (WLL-vs) and Tryptophan-Leucine-Tryptophan (WLW-vs.) The vinyl sulfone warhead is coloured red. Adapted from (578).

Current leading tri-peptide scaffolds vary between peptide and warhead compositions with covalent tri-peptide scaffolds containing a vinyl sulfone (vs) warhead (tryptophan-leucine-leucine (WLL-vs) and tryptophan-leucine-tryptophan (WLW-vs.)) (368, 369) (**Figure 6.2**) having highly potent activity against blood stage parasites both *in vitro* and *in vivo*. Notably, vinyl sulfones (334, 338) as well as one of the early proteasome inhibitors to be tested against malaria, MG-132 (330, 373), can also have activity against cysteine proteases including enzymes involved in haemoglobin digestion within the parasite's food vacuole (reviewed in (328)). However, the leading scaffold (WLL-vs) appears to selectively target the catalytic subunits (β1, β2, β5) of *P. falciparum*'s 20S core and has been demonstrated to have >200-fold more selectivity against malaria parasites over mammalian cells and was well tolerated in rodent models (368, 369). The activity of vinyl sulfones outside of the asexual lifecycle, however, is yet to be tested (368, 369, 371).

6.2.1 Synergy of proteasome inhibitors with DHA.

A number of first-generation and second-generation (i.e. WLW-vs) antimalarial proteasome inhibitors synergise with artemisinins both *in vitro* and *in vivo* (368, 370, 371). As the antimalarial mechanism of action of artemisinin promotes widespread protein damage, the parasite is reliant on the proteasome to resolve the build-up of defective proteins (reviewed in (325)). Therefore, inhibiting proteasome function in the face of increasing cellular damage synergises the activity of artemisinin and proteasome inhibitors, thus increases their potency (218, 371). Furthermore, artemisinin resistant parasites are more reliant on the UPS even in the absence of drug treatment, which in turn makes them more sensitive to the activity of proteasome inhibitors (370, 579). These findings have opened up the possibility of overcoming artemisinin resistance and prolonging our ability to use these front-line drugs through use of artemisinin/proteasome inhibitor combinations (368).

6.2.2 Targeting drug site of action using hypoxia activated pro-drugs.

In an effort to reduce drug activity against non-target cells, pro-drug strategies wherein the drug administered has little activity *in vitro* and *in vivo* until chemical and/or enzymatic conversion releases the active drug, have been developed (580). Reasons to design a pro-drug that selectively releases the active compound to a specific biological niche include masking off-target activity or improving solubility, metabolic stability, oral bioavailability, half-life and lipophilicity (581). Currently, ~10% of all marketed drugs approved by the US Food & Drug Administration (FDA) can be classified as pro-drugs (reviewed in (581)) and many are used as

effective medicines for a variety of illnesses including cancer, multiple sclerosis, neurological disorders, cardiovascular and infectious diseases (reviewed in (581) and (582)).

In malaria, peroxide antimalarials, such as artemisinin and ozonides, are classed as pro-drugs as their activity is dependent on scission of the endoperoxide bridge by an iron source, such as the haem that is released during haemoglobin digestion (reviewed (214) and (240)). Likewise, proguanil, an inhibitor of *Plasmodium*'s DHFR and folate synthesis pathway, is metabolised into the active form, cycloguanil, by liver cytochrome P450 (CYP2C19) (129, 176). Malaria parasites typically infect only a relatively small proportion of RBCs that are circulating in the bloodstream. Deliberate modification of antimalarials into a prodrug has been explored using infected RBC haem, a by-product produced from haemoglobin digestion, as the catalysing factor (583-585). These studies modified drugs, such as DPAP3 protease inhibitors, that have potential off-target effects on similar host-cell enzymes and found that transformation to a haem activatable pro-drug reduced toxicity in mammalian cells, improved activity against the parasites as well improving efficacy and reducing off-target effects in an *in vivo* rodent malaria model (584).

Our collaborators, Prof Andrew Abell and Mr Aniket Kulkarni (School of Physical Sciences, The University of Adelaide), have been investigating the development of hypoxia-activated pro-drugs (HAP, bio-reductive pro-drugs), with a specific interest in developing hypoxia-activated proteasome inhibitors. HAPs are inactive in normal oxygen environments (normoxia) but undergo enzymatic reduction in low oxygen conditions (hypoxia) through the action of reductase enzymes (511, 586).

The development of HAPs is being driven by the search for new, safe, cancer treatments since a tumour can often be a highly hypoxic environment (reviewed in (587)). While a RBC, whose main function is to transport oxygen, may not immediately resemble a hypoxic environment, we reasoned that several factors open the possibility that infecting malaria parasites may reside in a hypoxic environment. Firstly, oxygen in RBCs is typically bound to haemoglobin. However, in the infected RBC, up to 80% of haemoglobin (588) is digested by the malaria parasites with the infected RBC demonstrating ~70% reduction in oxygen transport in comparison to uninfected RBCs (589) (reviewed in (590)). Secondly, although blood oxygen levels vary (from 5% within the venous and arterial blood to 13% in the lungs (591)), the blood stage parasites utilize anaerobic glycolysis for energy production rather than oxidative phosphorylation, thus access to oxygen may not be inherent in the infected RBC (185). Finally, blood stage parasites are typically grown in near-hypoxic environments *in vitro* (0.5% to 5%

O2) (492, 586), and it might be possible that these low oxygen levels could be sufficient to activate the inhibitor (511, 586).

We investigated two HAPs strategies in this study; a i) bio-reductive 4-nitrobenzyl moiety and ii) an azo linker group, which were based on the most active proteasome inhibitor analogues we tested. Briefly, the 4-nitrobenzyl groups (PIpro-4 and PIpro-5) are sensitive to a reductive environment. In cells, sufficient hypoxia will initiate the overexpression of reductase enzymes that convert the nitro group (4-nitrobenzyl) to its corresponding 4-aminobenzyl group. This 4-aminobenzyl group then breaks off from the molecule, thus liberating the active inhibitor. Likewise, the azo linker group of PIpro-1 is also sensitive to reduction and in hypoxia, is reduced to produce the corresponding anilines to release the active inhibitor.

6.3 Investigation of novel proteasome-like inhibitors and the potential for hypoxia inducible prodrug activity against blood stage malaria.

In this chapter, I investigated a series of tri-peptide proteasome inhibitors (PI) broadly related to known proteasome inhibitors featuring two different C-terminal electrophilic 'warhead' groups: (i) peptide vinyl sulfone warhead engineered from published proteasome inhibitors (PI-1 to PI-3) (372); or (ii) an aldehyde warhead similar to the commercial proteasome inhibitor, MG-132 (PI-4 and PI-5) (330, 373) (**Figure 6.2**). Using these existing compounds developed for studies in the laboratory of Prof. Andrew Abell (School of Physical Sciences, The University of Adelaide), I assessed compound activity against the blood stages of *P. falciparum* and *P. knowlesi* and examined their cytotoxicity against human cells. As we anticipated that any inhibition of the proteasome would enhance the activity of artemisinin, drug-drug interactions between combinations of DHA and the leading compound (PI-1) were also investigated. Additionally, I investigated whether HAPs inhibitors, featuring a bio-reductive 4-nitrobenzyl moiety or an azo linker group, that were based around the three most potent analogues tested, could work for treatment of malaria infected RBCs as a strategy to reduce host-toxicity of existing proteasome inhibitors (511, 586).

6.4 Results.

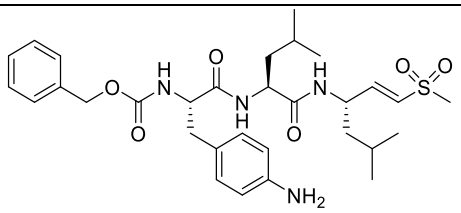
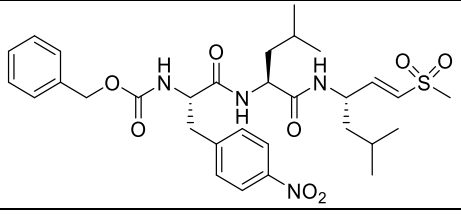
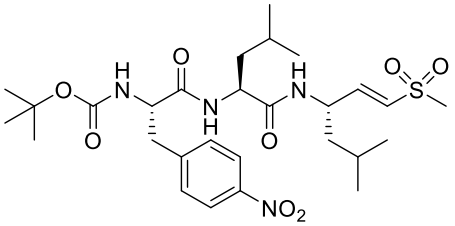
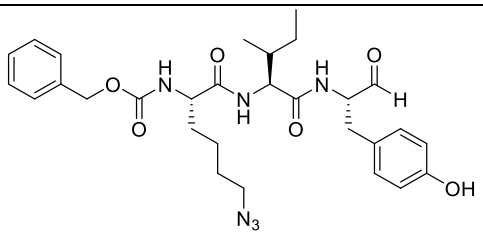
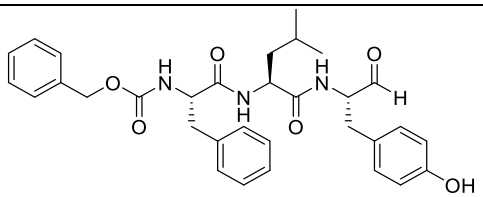
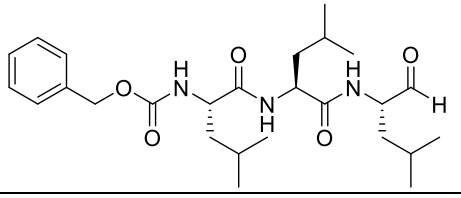
6.4.1 Activity of proteasome-like inhibitors against *P. falciparum.*

Here, the blood stage IC_{50} values of MG-132-like (N-terminal carboxybenzyl (Cbz) cap) tri-peptide vinyl sulfone (PI-1, PI-2 and PI-3) and aldehyde tri-peptide analogues (PI-4 and PI-5 (based on molecules developed by Geurink et al., (2013) (372)) were determined using 1 cycle *in vitro* assays (**Table 6.1** & **Figure 6.3 A**) against a synchronous culture of *P. falciparum* parasites (D10-PfPHG, 72 hrs of treatment) (131, 372, 373, 493). The compounds published by Geurink et al., (2013) (372) contained an azido-phenylalanine (azido-F) cap and were previously tested against purified 26S mammalian proteasomes, but not *in vitro* against malaria parasites. Therefore, the activity and specificity of the analogues used in this study against malaria parasites is unknown (372).

All proteasome inhibitors were found to be highly potent in 1 cycle assays and exhibited low-nanomolar IC_{50} values. The most potent analogue was the peptide vinyl sulfone PI-1 (Cbz-4-amino-L-L-vs) (IC_{50}, PI-1 0.0065 μM) that demonstrated activity against blood stage parasites, similar to the published activity of chloroquine (373, 592) (**Table 6.1** & **Figure 6.3 A**). It is interesting to note that abolishing the Cbz moiety on the vinyl sulfones, PI-1 (Cbz-4-amino-L-L-vs) and PI-2 (Cbz-4-nitro-L-L-vs) (IC_{50}, PI-2, 0.023 μM), resulted in a 2 to 6.5-fold loss of activity against parasites compared to PI-3 (*tert*-butyloxycarbonyl (Boc)-4-nitro-L-L-vs) (IC_{50}, PI-3, 0.044 μM). Moreover, replacement of the 4-amino in PI-1 with a 4-nitro (PI-2) at the P3 position led to the 3.5-fold loss of potency against *P. falciparum.*

The aldehyde (Al) analogues, PI-4 and PI-5, demonstrated superior activity to the parent molecule MG-132 (Cbz-L-L-L-Al) (IC_{50}, 0.035), with replacement of the P3, P2 and P1 leucine substituents in MG-132 with either non-natural (P1-4 (Cbz-azidonorleucine-isoleucine-tyrosine (Y)-Al), IC_{50}, 0.018 μM) or natural amino acids substituents (PI-5 (Cbz–F-L-Y-Al), IC_{50}, 0.022 μM), resulting in ~2-fold increase in potency. The blood stage activity between P1-4 and PI-5, however, was almost identical with <1-fold change observed. Promisingly, these MG-132 aldehyde analogues were more potent against malaria parasites than similar tri-peptide aldehydes developed in a previous study (373).

Table 6.1 Antimalarial activity of proteasome-like inhibitors.

Compound	Chemical structure	Intracellular growth D10-PfPHG IC_{50} (μM) [a]	Intracellular growth PkYH1 IC_{50} (μM) [a]
PI-1 (Cbz-4-amino-leucine-leucine-vs)		0.0065	0.0076
P1-2 (Cbz-4-nitro-leucine-leucine-vs)		0.023	ND
PI-3 (Boc-4-nitro-leucine-leucine-vs)		0.044	ND
P1-4 (Cbz-azidonor leucine-isoleucine-tyrosine -Al)		0.018	0.036
P1-5 (Cbz-phenylalanine - leucine - tyrosine -Al)		0.022	0.057
MG-132 (Cbz-leucine-leucine-leucine -Al)		0.035	ND

[a] All drug treatments were performed in standard growth conditions, from rings to late schizonts, with 1 rupture cycle (*P. falciparum* D10-PfPHG, 72 hrs; *P. knowlesi* PkYH1, 58 hrs). All assays were measured by flow cytometry. Data represents the mean of 3 (or more) experiments expressed as percentage of non-inhibitory control. ND= not done due to limited amount of compound.

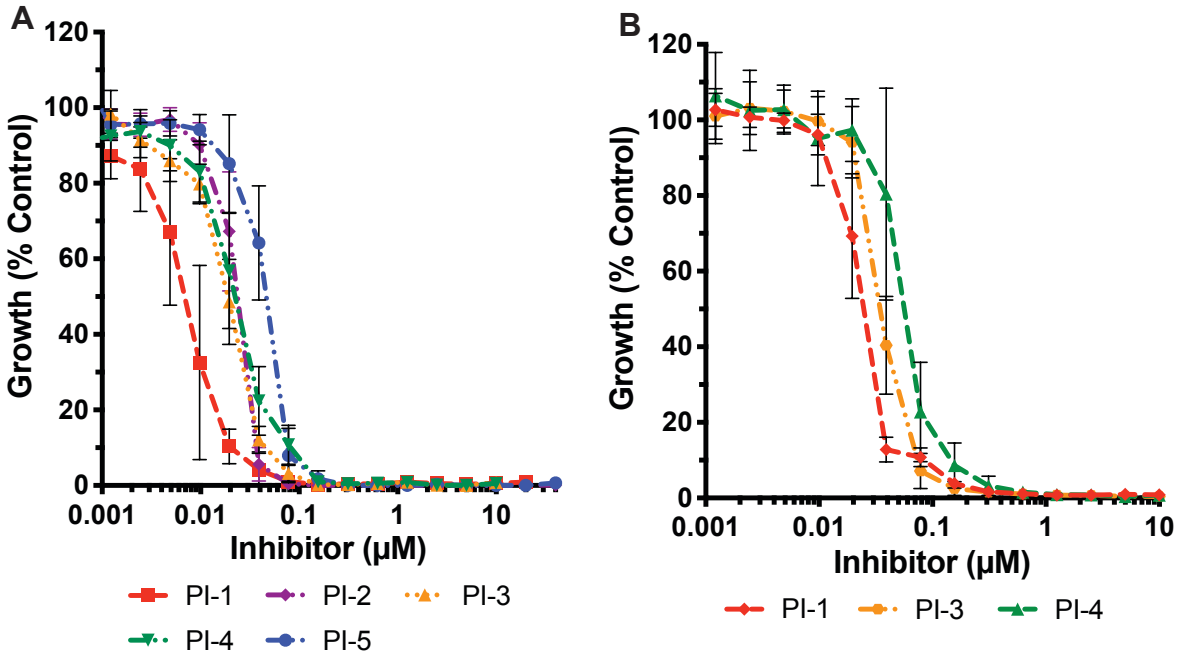


Figure 6.3 Antimalarial activity of proteasome-like inhibitors against *Plasmodium* spp.

Early ring stage *P. falciparum* (**A**) or *P. knowlesi* (**B**) parasites (0-4 hrs post-invasion) were treated with doubling dilutions of peptide vinyl sulfones (PI-1, PI-2, and PI-3) or tri-peptide aldehyde (PI-4 & PI-5) proteasome-like inhibitors for 1 cycle of growth (72 hrs and 58 hrs, respectively). For all growth curves, parasitemia was measured via flow cytometry. Data represents the mean of 3 (or more) experiments expressed as percentage of non-inhibitory control and error bars represent ± SEM.

We next investigated the activity of the proteasome-like inhibitors against a *P. knowlesi* line (PkYH1) using standard 1 cycle assays (~58 hrs of treatment) for this line, which features a shorter lifecycle than *P. falciparum* (D10-PfPHG, 48 hrs; PkYH1, ~32 hrs). Two inhibitors, PI-2 and PI-3 were excluded from this analysis due to limited availability. The IC_{50} values for the peptide vinyl sulfone, P1-1 (IC_{50}, 0.0076 μM), and aldehyde analogues, PI-4 (IC_{50}, 0.036 μM) and PI-5 (IC_{50}, 0.057 μM) in *P. knowlesi* were up-to 3-fold higher than in *P. falciparum* (**Table 6.1** & **Figure 6.3 B**), potentially reflecting the shorter lifecycle of *P. knowlesi* (509, 593). Similar to *P. falciparum,* the vinyl sulfone, PI-1, demonstrated the highest potency. Likewise, the aldehyde analogues were 4.7 and 7.5 less potent than PI-1, for PI-4 and PI-5, respectively, and shared a similar blood stage activity in both *P. knowlesi* and *P. falciparum* (~1.5-fold change).

6.4.2 Toxicity of proteasome-like inhibitors against Huh-7D cells.

We next sought to investigate the potential of drug-induced cytotoxicity of these compounds against the human hepatocellular carcinoma cell line, Huh-7D (556, 594). Growth of Huh-7D cells was assessed for four compounds (PI-1, PI-2, PI-3 and PI-4) after one full cycle of growth and division (24 hrs) using an ATP-based luminesce detection assay. One compound, PI-5 was excluded from this analysis due to limited availability. Both inhibitor subtypes showed low cytotoxicity profiles *in vitro* (CC_{50}, PI-1, 7.1 μM; PI-2, 15.4 μM; PI-3, 5.1 μM; PI-4, 9.6 μM) with all demonstrating >100-fold selectivity against both D10-PfPHG and PkYH1 over human Huh-7D cells (**Table 6.2**). Notably, the peptide vinyl sulfones, PI-1 and PI-2 were >900-fold more selective for D10-PfPHG over Huh-7D cells. Replacement of Cbz cap (PI-1 and PI-2) with Boc, a non-natural protective group, resulted in some loss of parasite specificity, with PI-3 exhibiting only 116-fold higher selectivity against malaria parasites over human cells, in contrast to PI-2.

6.4.3 PI-1 synergy with dihydroartemisinin.

Since a number of studies have demonstrated synergy between proteasome inhibitors and DHA, we tested for synergy between the most potent compound PI-1 and DHA using a fixed ratio isobolagram analysis (516) (**Figure 6.4**). There was, however, no evidence of synergy between PI-1 and DHA. Instead, there was a trend towards antagonism with the drugs *in vitro*. This result was surprising as the parasite would be reliant on the proteasome to resolve the artemisinin induced protein damage (218, 370), with published proteasome inhibitors previously shown to strongly synergise with DHA (368, 370, 371, 572).

Table 6.2 Activity of selected compounds against parasites and human cells.

Compound	Intracellular growth D10-*Pf*PHG IC_{50} (μM) [a]	Intracellular growth *Pk*YH1 IC_{50} (μM) [a]	Cytotoxicity of Huh-7D cells (μM) [b]	Selectivity Huh-7D (CC_{50})/ D10-PfPHG IC_{50}	Selectivity Huh-7D (CC_{50})/PkYH1 IC_{50}
PI-1	0.0065	0.024	7.1	1092	295
PI-2	0.018	0.036	15.4	855	428
PI-3	0.044	ND	5.1	116	ND
PI-4	0.022	0.057	9.6	437	168.4

[a] Drug treatment of intracellular growth, from rings to late schizonts, with 1 rupture cycle (D10-PfPHG, *P. falciparum,* 72 hrs or PkYH1, *P. knowlesi,* 58 hrs). All assays were measured by flow cytometry. [b]Huh-7D cells were incubated with the compounds for 24 h before cell viability was measured via luminescence. All data represents the mean of 3 (or more) experiments expressed as percentage of non-inhibitory control. ND= not done due to limited amount of compound.

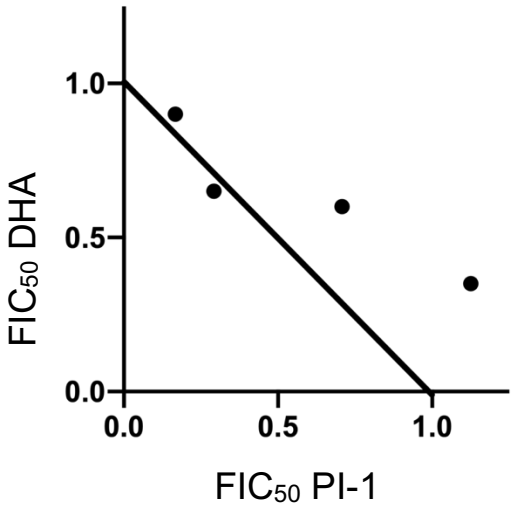


Figure 6.4 PI-1 does not synergise with DHA.

Isobologram of PI-1 and DHA tested on synchronous D10-PfPHG parasites. Early-ring stage parasites (0-4 hrs post invasion) were exposed to compounds mixed at fixed ratios of their individual IC_{50} values (control/test drug ratios; 0:5, 4:1, 3:2, 2:3, 1:4, 5:0) and exposed for 72 hrs with parasitemia measured via flow cytometry. Fractional IC_{50} (FIC_{50}) values were plotted for each drug with results compared against a hypothetical isobole line demonstrating a perfect additive interaction (straight line). Antagonism is evident if the induvial FIC_{50} values fall above the line of additive interaction. Data represents the mean of 3 (or more) experiments expressed as percentage of non-inhibitory control.

Given this finding, we next sought to test whether the proteasome inhibitors in this panel directly inhibited proteasome activity.

6.4.4 Proteasome-like inhibitors do not cause a build-up of ubiquitinated proteins.

The activity of drugs that inhibit the malaria parasite's proteasome can be demonstrated by assessing the build-up of ubiquitinated protein that is marked for destruction over a short time period. In the presence of a true proteasome inhibitor, a build-up of ubiquitinated protein can be detected on a western blot with as little as 1.5 hours of drug treatment using an anti-ubiquitin antibody (**Figure 6.5**).

Treatment of late trophozoites with a 10x IC_{50} of peptide vinyl sulfones (PI-1, PI-2 and PI-3) or aldehyde analogues, PI-5, for 1.5 hrs did not result in a significant build-up in ubiquitinated protein above that seen for untreated parasite controls (**Figure 6.5 A** & **B**). In contrast, treatment with the validated *P. falciparum* proteasome inhibitor MG-132 caused an increase of ubiquitinated proteins (>100% of ubiquitinated protein) compared to the untreated parasite control. These data suggest that despite these compounds being engineered from known proteasome inhibitors (372, 373), they have limited activity against the *P. falciparum* proteasome. For this reason, I will refer to these drugs as proteasome-like inhibitors.

6.4.5 Assessment of proteasome-like hypoxia activated prodrug inhibitors.

In order to limit the potential of cross-reactivity against human enzymes for the proteasome-like inhibitors, we investigated whether we could redevelop these analogues into hypoxia-activated pro-drugs that would enable specific targeting and activation within infected RBCs (511, 586). The *in vitro* activity of the HAP proteasome inhibitor-like analogues, mono (PI_{PRO}-1), an azo homodimer of PI-1, and dual tri-peptide aldehydes (PI_{PRO}-4, PI_{PRO}-5) featuring a bio-reductive 4-nitrobenzyl moiety, were investigated (**Table 6.1** & **Table 6.3**).

I compared the *in vitro* activity of HAPs (PI_{PRO}-1, PI_{PRO}-4 and PI-$_{PRO}$5) and parental compounds (PI-1, PI-4 and PI-5) concurrently against D10-PfPHG parasites in standard hypoxic malaria tissue culture conditions (1% O_2) and hyperoxic (21% O_2) environments using 1 cycle growth assays (72 hrs) (**Table 6.3**) (586). We anticipated that HAP analogues would not activate in hyperoxia which would reduce their *in vitro* antimalarial activity relative to the parental compound. However, if the bio-reductive sidechain is successfully cleaved under hypoxia the pro-drugs should show improved activity compared to the parental compounds.

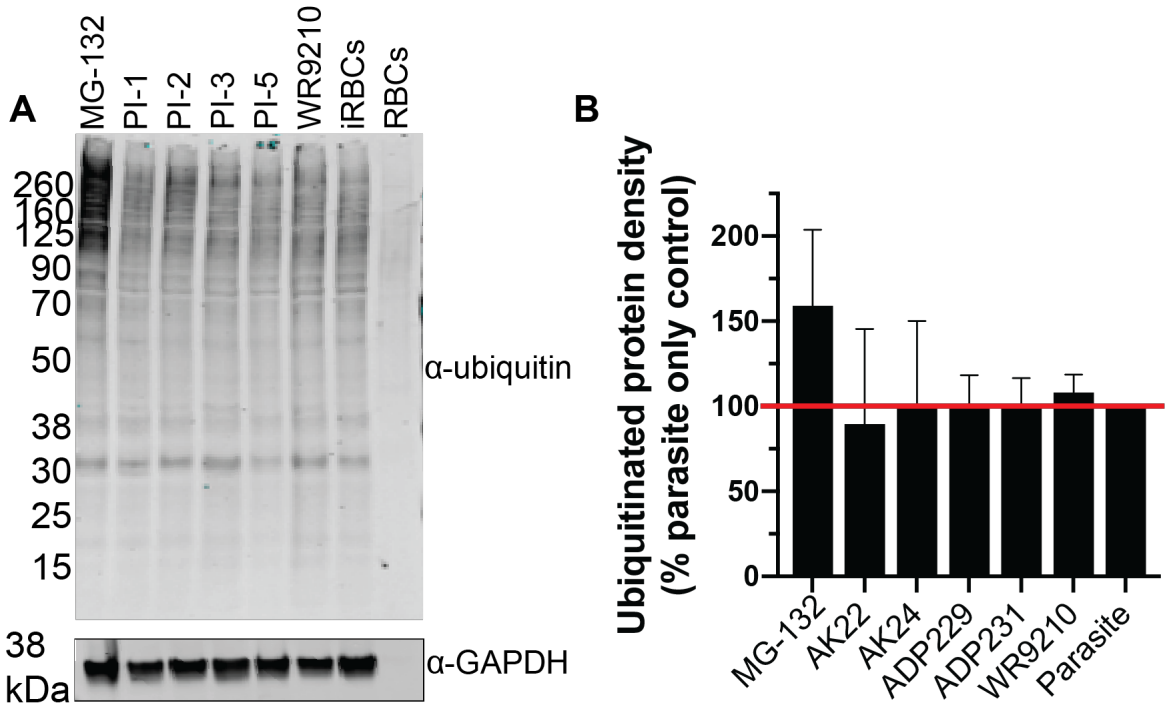
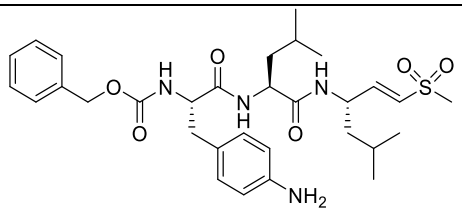
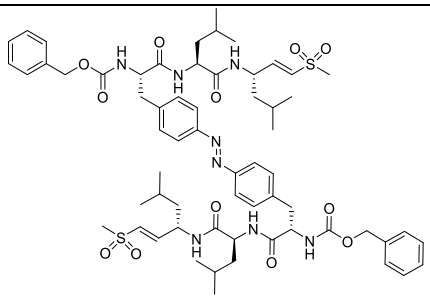
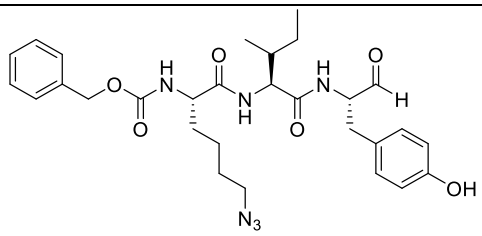
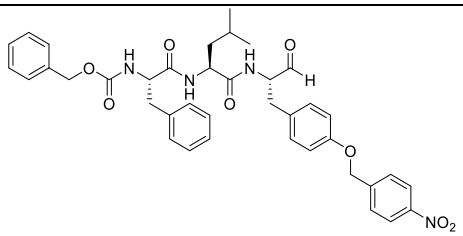
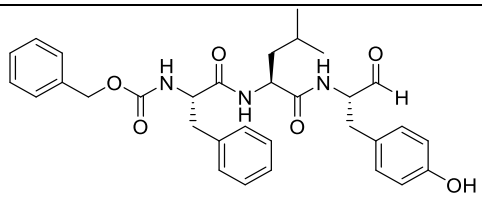
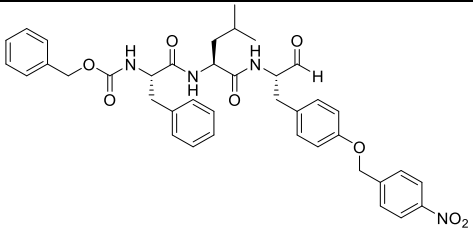


Figure 6.5 Ubiquitination of *P. falciparum* proteins following treatment with proteasome-like inhibitors.

Uninfected RBCs or late trophozoites-infected RBCs (26-34 hrs post-invasion) (>4% parasitaemia) were incubated with 10x the IC_{50} concentration of MG-132, PI-1, PI-2, PI-3, PI-5 and 5nM of WR99210 as the control for 1.5 hrs at 37 °C. Parasite pellets were analysed by SDS-PAGE and western blot, prior to being probed with anti-ubiquitin mouse PD41 and control rabbit anti-GAPDH antibodies. **A**) Representative western blot. **B**) Densitometry quantitation of ubiquitinated protein levels with drug treatment, mean of 3 replicates. The red line indicates the levels of ubiquitinated protein in DMSO control treated cells, values above significantly above this level would indicate a build-up of ubiquitinated protein due to inhibition of the parasite proteasome.

Table 6.3 Antimalarial activity of hypoxia activated pro-drug proteasome-like inhibitors

Compound	Chemical structure	Intracellular growth D10-*Pf*PHG IC_{50} (μM) hypoxia[a]	Intracellular growth D10-*Pf*PHG IC_{50} (μM) hyperoxia[b]
PI-1		0.0064	0.0076
PI_{PRO}-1		0.75	0.72
PI-4		0.018	0.012
PI_{PRO}-4		0.26	0.29
PI-5		0.022	0.011
PI_{PRO}-5		0.3	0.27

[a] Drug IC_{50} in standard 'hypoxic' (1% O_2, 4% CO_2 & 95% N_2) culture conditions, from rings to late schizonts, with 1 rupture cycle (D10-PfPHG, *P. falciparum,* 0-72 hrs). [b] Drug IC_{50} in 'hyperoxic' (21% O_2, 5% CO_2, 74% N_2) culture conditions, from rings to late schizonts, with 1 rupture cycle (D10-PfPHG, *P. falciparum,* 72 hrs). All assays were measured by flow cytometry. Data represents the means of 3 (or more) experiments expressed as percentage of non-inhibitory control.

The parental compounds (PI-1, PI-4 and PI-5) showed minimal change in IC_{50} values between hypoxic and hyperoxic treatments (hypoxia IC_{50}, PI-1 0.0065; PI-4, 0.018 μM; PI-5 0.022) (hyperoxia IC_{50}, PI-1 0.0076; PI-4, 0.013 μM; PI-5 0.012) (**Figure 6.6, Tables 6.1** & **6.3**). Likewise, no significant change was observed between HAPs across the conditions (hypoxia IC_{50}, PI_{PRO}-1, 0.75 μM; PI_{PRO}-4, 0.26 μM; PI-$_{PRO}$5, 0.3 μM) (hyperoxia IC_{50}, PI_{PRO}-1, 0.73 μM PI_{PRO}-4, 0.29 μM; PI-$_{PRO}$5, 0.3 μM). Since the reduced potency of the pro-drugs relative to the parent drug did not improve under hypoxic conditions, these data suggest that standard malaria culture conditions are insufficient to cleave the bio-reductive sidechain and improve the potency of these drugs (**Figure 6.6** & **Table 6.3**).

6.5 Discussion.

6.5.1 Activity and possible mechanism of action of proteasome-like inhibitor compounds.

Plasmodium proteasomes have emerged as a viable drug target due to their essential role throughout the parasite's entire lifecycle (325, 366) and contribution to artemisinin resistance (218, 370, 579). Proteasome inhibitor selectivity against the parasite rather than human proteasome, however, remains a major consideration for their development. While new *Plasmodium*-specific chemical scaffolds have been reported, drug-induced mammalian cell cytotoxicity (368, 369) and *in vitro* activity against human β5 or β2 subunits within the 20S core (368, 369, 595, 596) is still present for some compounds (**Figure 6.1**).

Here, the *in vitro* blood stage activity of two different chemical subtypes of proteasome-like inhibitors were investigated against *Plasmodium* parasites. Both the peptide vinyl sulfones and aldehyde-based inhibitors featured low-nanomolar activity against blood stage parasites, with the most potent compound, PI-1, featuring a vinyl sulfone 'warhead'. Here, I found that maintenance of a bulky, Cbz group at the N-terminal cap (R3) and the presence of a protonated, non-natural 4-amino substituent P3 (compound PI-1) resulted in improved activity over analogues with an oxidized, 4-nitro group (PI-2 and PI-3) or those that had Cbz replaced with a Boc capping group (PI-3). With the limited number of compounds tested, however, it was not possible to infer greater insights into how the SAR impacts on parasite growth inhibition. These results are consistent with prior studies demonstrating that modification at the N-terminal cap, P1 and P3 substituents can be exploited to enhance antiparasitic activity (368, 369). Indeed, the leading analogue, PI-1 (IC_{50} 0.0065 μM), demonstrated similar activity to the leading *P. falciparum*-specific vinyl sulfone scaffold WLL-vs (IC_{50} 0.006 μM) (368, 369) (**Figure 6.2**). Promisingly, the activity of PI-1 was also compatible to recently optimised *P. falciparum* proteasome vinyl sulfone inhibitors that featured other non-natural amino acids (IC_{50} 0.022 μM) as well as pharmacologically optimised derivatives (IC_{50} 0.007 μM) (369).

In a previous study, modification of MG-132 increased the selectivity of analogues for calpains, a cystine protease, over the proteasome (373), but these analogues exhibited a significant loss of potency against parasites relative to MG-132 (Cbz-L-L-L-Al). Here, two tri-peptide aldehyde analogues (PI-4 (Cbz-azidonorleucine-isoleucine-Y-Al) and PI-5 (Cbz-F-L-Y-Al)) demonstrated a small increase of potency over MG-132 and were >100-fold more potent than the MG-132 analogues that were previously tested in collaboration with the Abell Laboratory

(373). The structural differences between these analogue sets featured substitution of leucine at P1 site of MG-132 with a tyrosine (PI-4 and PI-5) and replacement of the P3 leucine with a leucine isomer (azido-norleucine) (PI-4) or phenylalanine (PI-5). While a similar activity was observed between the two analogues tested in this study, the fact that these modifications increased the potency against *P. falciparum* parasites suggests that the P1 and P3 sites of MG-132 can be further exploited.

While the activity of leading second-generation proteasome inhibitors have been applied in rodent models of malaria (369, 572), the susceptibility of other human malaria parasites has not been investigated (405, 509). Here, we showed the activity of a focused group of compounds was similar between both *P. falciparum* and *P. knowlesi,* with a very minor difference in parasite susceptibility for the peptide vinyl sulfone (PI-1) observed. Taken together, this data suggests that both these chemical sub-classes maintain a similar activity against different human malaria parasites and that the tripeptide inhibitors tested in this study work broadly against malaria (405, 503, 509).

An important consideration for developing proteasome-like inhibitors the assessment of potential non-target activity against the human proteasome. We found that both inhibitor subtypes were more selective against *P. falciparum* over the hepatocellular carcinoma cell line, Huh-7D, with a selectivity index of >100-fold for all compounds tested, a level well above that used previously to select compounds for inclusion as chemical starting points in the Pathogen Box (Medicines for Malaria) (597, 598). These results suggest that the compounds have limited off-target activity against mammalian cells and are a viable starting point to develop new antimalarial tri-peptide inhibitors. The initial aim of this study was to develop these compounds as antimalarial proteasome inhibitors based on the proteasome inhibiting properties of the parent molecule they were modified from (372, 373). As discussed below, the experimental evidence achieved to date suggests that these compounds may be acting through a different mechanism of action.

The tri-peptide vinyl sulfones (PI-1, PI-2, PI-3) and tri-peptide aldehydes (PI-5) did not cause a build-up of ubiquitinated proteins in drug treated cells that would indicate inhibitory activity against the malaria proteasome (218, 367, 370). Moreover, the lack of synergy observed between PI-1 and DHA, in contrast to the strong synergy reported for published peptide vinyl sulfones and DHA (368, 370, 371), also suggests limited activity against the P. *falciparum* proteasome. Based on this evidence, it appears unlikely that these inhibitors target the parasite proteasome. It has been shown that tri-peptide vinyl sulfones also target a spectrum of cysteine

proteases (599). In *P. falciparum,* cysteine proteases are known to play critical roles in haemoglobin digestion (331), invasion (340) and egress (600, 601) (reviewed in (321)). Indeed, similarly structured vinyl sulfones have been shown to target *Plasmodium* papain-family falcipains, essential cysteine proteases that play a major role in haemoglobin digestion (337, 338). Similarly, MG-132, the parent drug of PI-4 and PI-5, is also known to inhibit cysteine proteases and haemoglobin digestion (330), opening up the possibility that haemoglobin digestion could be an additional target of these compound. Moreover, we can speculate that inhibiting falcipains and blocking haemoglobin digestion and release of free haem may antagonise artemisinin, as a free haem is required to excise the endoperoxide bridge in order to active its antimalarial activity (126, 559). Thus, there is the possibility that preventing the initial cleavage of RBC haemoglobin through inhibiting cysteine proteases, such as falcipains, could have contributed to the trend towards antagonism observed for the mix of PI-1 and DHA. Taken together, this opens up the possibility that both tri-peptide vinyl sulfones and aldehydes share a non-proteasome targeting mechanism of action such as against cysteine proteases. Finally, whilst the low mammalian cell toxicity of these drugs suggest specific activity against parasite proteins, other off target effects on human caspases involved in the immune response could not be addressed in this assay.

6.5.1.1 Future directions to determine the mode of action for proteasome-like inhibitors.

Further investigations looking at the activity of these drugs against haemoglobin digestion will be a priority in future work. To investigate whether these compounds specifically inhibit haemoglobin degradation, the accumulation of undegraded RBC haemoglobin post-treatment could be resolved using Coomassie-stained SDS-PAGE (330) or by following the course of parasite development and food-vacuole morphology using light microscopy of Giemsa-stained thin smears (330, 331, 368). Inhibitors of cystine proteases required for haemoglobin digestion and aspartic proteases, enzymes also involved in haemoglobin degradation, have been shown to strongly synergise *in vitro* (602). Future studies could address whether the inhibitors presented here also have synergistic activity with the aspartic protease inhibitor, pepstatin. If activity against falcipains is implicated for these drugs, activity against specific falcipains could be addressed via SDS-PAGE gel assays using a fluorescent-based papain-family probe (BODIPY-TMR-DCG04) (368, 603) or through testing against falcipain knock-out lines (331, 604).

As development of these compounds progresses, they should undergo testing against human and mouse microsomes for assessment of absorption, distribution, metabolism and excretions studies to inform on host tolerability, drug half-life and metabolomic clearance rates for further chemical optimisation or assessment in *in vivo* mouse models of malaria (369, 573). These studies will be ongoing between the Wilson and Abell laboratories at the University of Adelaide.

6.5.2 Development of hypoxia activated tri-peptide pro-drugs.

One strategy for reducing non-specific activity of drugs for treatment of human disease is to administer a prodrug that activates directly where it is needed. We investigated whether a hypoxia-activated pro-drug strategy that has been developed to target cancers could also work against malaria parasites using the proteasome inhibitor-like compounds developed in this study (PI_{PRO}) (**Table 6.3**). We hypothesised that the parasite's utilisation of anaerobic glycolysis (185), reduction in haem-bound oxygen from the RBC during haemoglobin digestion (149, 166) and standard 'hypoxic' culture conditions (1% O_2) (492) may be able to activate the pro-drug *in vitro* (586). No change in activity, however, was observed for HAPs featuring either the azo linker group or bio-reductive 4-nitrobenzyl moiety in hypoxia (1% O_2) or hyperoxia (21% O_2) environments, suggesting this level of oxygen in standard 'hypoxic' culture conditions are insufficient to initiate cleavage and active the pro-drug.

Previous publications have reported activation of similar HAPs under lower oxygen conditions (<1% O_2) (511, 586). To overcome this, *in vitro* culture using a hypoxia chamber or a specialised gas mix featuring a lower oxygen could be trailed to activate the HAPs (492, 512). Alternatively, it is also feasible that the azo linker group and bio-reductive 4-nitrobenzyl moieties are too stable and thus as a proof-of-principle to prove that HAPs can be activated in *in vitro* malaria culture the addition of a reducing agent, such as 1, 4-dithiothreitol (DTT) or glutathione, could be added into cultures to forcibly active the drug (605, 606).

While at first glance the results of this study suggest that HAPs are difficult to activate in malaria infected RBCs, there are several lines of evidence implicating hypoxia as a contributing factor in malaria pathogenesis that could drive such a strategy going forward. Firstly, the sequestration of infected RBCs to uninfected RBCs and within the host's microvascular reduces blood oxygen levels and creates a localised hypoxic environment (607, 608) (reviewed in (609)). Secondly, hypoxia is an important factor in pregnancy associated malaria (PAM) (610-612), where infected and uninfected RBCs and immune cells adhere to the intervillous spaces

of the placenta and cause placental or foetal hypoxia (613). Finally, the progression of cerebral malaria in *in vivo* mouse models is associated with hypoxia and blood acidosis within the host (614, 615).

The avenues explored *in vitro* could provide a proof-of-principal for HAPs, however, it is also possible that these approaches will not be feasible in *in vivo* models, especially if a high concentration of a reducing agent is required. Moreover, the implementation of leading HAP strategies in cancer treatment has not been successful to date, with three HAPs failing Phase II/III clinical trials due to insufficient drug activity at the site of cancer growth (reviewed in (587)). This suggests that the level of hypoxia within a human tumour is not sufficient to activate current HAPs, highlighting one of the problems that needs to be overcome if HAP strategies are to be useable for drug treatment. Finally, while additional studies are needed to determine if infected RBCs could possibly provide a reductive force strong enough to activate HAPs to target the parasite, there is no clear indication in this preliminary data to suggest that the infected RBC environment could activate HAPs. As further classes of HAPs are developed (reviewed in (616) and (617)), it is feasible that different sidechains that are more sensitive to cleavage in standard culture conditions (1% O_2) or other parasite-derived activities may be developed that could prove easier to activate and target infected RBCs (618, 619).

6.6 Conclusion.

Here, we have investigated the *in vitro* activity of a small panel of compounds derived from known proteasome inhibitors that have nanomolar potency against the blood stages of *P. falciparum* and *P. knowlesi*. The mechanism of action of these compounds is not clear at this stage as they were not found to inhibit proteasome activity in traditional protein ubiquitination assays. Importantly, the majority of proteasome inhibitors demonstrated an exceptional selectivity profile against parasites compared to human Huh-7D cells. Although we were unable to show that hypoxia activated pro-drug versions of these compounds could be activated under *in vitro* culture conditions, the potency of the tri-peptide vinyl sulfones and aldehyde inhibitors combined with their limited toxicity against mammalian cells makes them a promising starting point for further development as new antimalarial chemotypes.

Chapter 7. Final discussion.

7.1 Introduction.

Malaria remains a major health concern throughout the world and is directly associated with the deaths of >400,000 people every year (1). Consistent emergence and spread of drug resistant parasites has severely compromised the efficacy of past and present front-line antimalarials (reviewed in (134)), resulting in significant increases in malaria related mortality (132, 208). Of current concern is the spread of artemisinin resistant parasites that have severely undermined the effectiveness of front-line artemisinin combination therapies (ACTs) (134, 224, 225). Over the last two decades, substantial progress has been made in developing new, effective, antimalarial chemotypes with novel mechanisms of action (Chapter 1) (reviewed in (235) and (236)). Parasite resistance against some of these emerging antimalarials, however, has been readily selected for *in vitro, in vivo* and/or has been observed in clinical trials (256, 265, 273). Therefore, the need to develop new antimalarial treatment strategies remains and a constant pipeline of new chemotypes featuring novel mechanisms of action would be of major benefit for ongoing treatment, control and eradication strategies against malaria (230).

Considering this, the overarching aim of this thesis was to identify novel antimalarial development avenues that could provide useful partner drugs in future combination therapies. The work described here explored three potential drug development strategies. The first focused on developing azithromycin's quick-killing secondary mechanism of action and whether this novel mechanism is a viable antimalarial development strategy (Chapters 4 and 5). The second was to investigate whether azithromycin could be re-developed into an antimalarial featuring both quick-killing and delayed death activities against malaria parasites (Chapters 4 and 5). Finally, the third investigated a panel of analogues, developed from known proteasome inhibitors, to assess whether these chemotypes offered improved activity against malaria and whether they could be successfully modified into pro-drugs with hypoxia-inducible activity (Chapter 6). Here, in this final chapter, I present a summary of my findings and elaborate on the benefits, shortcomings and implications of these development avenues. I also provide some direction as to where this work could lead in the future and what investigations should be prioritised to validate and develop these drugs into potential next generation antimalarials.

7.2 Azithromycin's quick-killing activity.

Azithromycin's known mechanism of action against *Plasmodium* spp. malaria parasites is through inhibiting protein translation of the apicoplast's bacterial-like ribosome, which causes 'delayed death' of the parasite ~4 days after treatment (130, 159, 160). Despite azithromycin's long half-life and established safety profile (431, 439), the slow nature of delayed death has limited this drug's efficacy in the treatment of malaria (reviewed in (376)). Prior to this thesis, azithromycin was shown to kill blood stage parasites and inhibit merozoite invasion of the RBC though an unidentified mechanism of 'quick-killing' that was proposed to be independent of delayed death (130, 164, 165). The evidence for quick-killing being a secondary mechanism of action included the drugs ability to inhibit merozoite invasion of the RBC with <1 minute treatments and the fact that delayed death resistant parasites were not cross-resistant against the quick-killing activities of invasion inhibition and blood stage growth (164, 165).

In the first part of this study, I characterised and verified azithromycin's quick-killing mechanism and explored two different panels of azithromycin analogues for their blood stage activity. Firstly, I confirmed that quick-killing was completely independent of delayed death, as blood stage parasites lacking the apicoplast, and thus the ribosomal target, remained susceptible to the quick-killing activity of azithromycin and analogues. Moreover, stage-specificity assays revealed azithromycin and analogues rapidly and irreversibly inhibited parasite growth throughout the entire blood stage lifecycle, including ring stages, an activity profile that differs greatly to delayed death activity where parasite growth is not noticeably inhibited for the first 96 hours post ring stage treatment (130, 165). Promisingly, the majority of analogues demonstrated superior blood stage (44 hrs, in cycle) and invasion inhibitory activity over azithromycin, suggesting that both quick-killing mechanisms can be improved simultaneously. A number of analogues displayed low-nanomolar quick-killing IC_{50} values, with values approaching the clinically relevant concentration seen for delayed death activity (130) (Chapter 4). Azithromycin and analogues maintained their quick-killing potency against the zoonotic malaria parasite, *P. knowlesi,* suggesting that the mechanism of quick-killing is active against evolutionarily diverse *Plasmodium* parasites (503) (Chapters 4 and 5). The majority of analogue chemotypes were also active against both artemisinin-resistant parasites and the multi-drug resistant *P. falciparum* parasite line, DD2, suggesting the activity of most analogues was not influenced by existing resistance mechanisms. Some analogues, featuring a chloroquinoline modification, lost potency (~4-fold) against DD2, relative to the drug-susceptible D10-PfPHG line. As the chloroquine, mefloquine and pyrimethamine resistant DD2

(496, 497) is not of the same clonal lineage as D10-PfPHG, we could not verify if this loss of activity was a consequence of specific drug resistance mechanisms in DD2 or pre-existing genetic variation between lines. To overcome this, future studies should examine the activity of analogues between identical clonal lineages with singular resistance phenotypes.

7.2.1 Investigation of azithromycin's quick-killing mechanism of action.

In an effort to dissect azithromycin's secondary mechanism of action, I assessed the metabolomic profile of parasites upon treatment with azithromycin and selected analogues in collaboration with Assoc. Prof Darren Creek, Dr. Ghizal Sidiqui, Dr. Dovile Anderson and Miss Amanda De Paoli (Monash University, Australia). At the concentrations and life-stage tested, metabolomic signatures of pulsed azithromycin, GSK-5, GSK-71 and C1 treatments were similar to that observed for chloroquine (312) (Chapters 4 & 5). Therefore, these data support the possibility that the quick-killing mechanism of azithromycin and analogues may be associated with the parasite's food vacuole of trophozoites (588). It also should be noted that the majority of these shared peptides could not be directly linked to haemoglobin degradation (312). Therefore, while the inhibition of haematin formation is considered a major mechanism of chloroquine's activity (167, 168, 620), this activity was not immediately evident for the metabolomic data of chloroquine, azithromycin or the analogues tested here. Haemoglobin fractionation assays, however, did show chloroquine and GSK-71 (quinoline moiety) to inhibit haemoglobin digestion and hemozoin formation, consistent with chloroquine's accepted mechanism, further supporting quick-killing's activity against the food vacuole. Surprisingly then treatment with GSK-66, the most potent quick-killing analogue tested and features a chloroquinoline moiety, did not cause a metabolomic signature similar to chloroquine and was not associated with changes in haemoglobin digestion using either metabolomics or haemoglobin fractionation assays. Since these treatments were conducted on mid-trophozoite stage parasites, it may be that this specific stage of development or the short treatment time led to minimal efficacy of GSK-66. GSK-66, however, was highly effective in 6 and 12 hr pulsed treatment assays against early and late ring stages, respectively, suggesting that this analogue is active against parasites with only short-term treatment. Therefore, it remains to be resolved if GSK-66 has an alternative mechanism of action or whether its signature against food vacuole could not be detected at the drug concentration and treatment times tested for this analogue.

The metabolomics data also suggests that the quick-killing mechanism of action of azithromycin analogues is multi-factorial. GSK-5 and C1, featuring di- and mono-chlorinated phenyl functional group respectively (i.e. not a chloroquine-like 4-aminoqunoline functional

group), shared non-haemoglobin peptide signatures similar to that of chloroquine. In addition, GSK-5 was found to down-regulate haemoglobin derived peptides, similar to the food-vacuole activated control drug DHA. In contrast, C1 was found to upregulated expression of haemoglobin derived peptides, a signature that was unique amongst the compounds tested. Together, these data provide additional evidence that quick-killing of some azithromycin analogues does indeed act against the parasites' food vacuole during trophozoite stages and suggests that GSK-5 and C1 interrupt metabolomic process that do not occur over the conditions tested with chloroquine.

GSK-5 and azithromycin shared a secondary metabolomic signature where metabolites associated with the mitochondrial located tricarboxylic acid (TCA) cycle were reduced (Chapter 4). As blood stage parasites exclusively use glycolysis for ATP production, this life stage is not reliant on the activity of a conventional TCA cycle to survive (528, 529, 621). Azithromycin and GSK-5, however, noticeably reduced the availability of fumarate and malate. These metabolites are catalysed by two essential TCA enzymes, fumarate hydratase and Malate quinone oxidoreductase (MQO), into malate and oxaloacetate, respectively, which are used for purine salvage (528-530, 622). MQO is of considerable interest due to its additional involvement in reducing ubiquinone to ubiquinol, thus inhibiting this enzyme would likely collapse the mitochondrial ETC potential and abrogate pyrimidine syntheses (528, 529, 622, 623), similar to atovaquone's blood stage activity (Chapter 1) (reviewed in (186) and (153)). However, atovaquone's mechanism of action against blood stage parasites is comparatively slower than what I found for quick-killing azithromycin analogues (131). Thus, while the activity of azithromycin and analogues against the mitochondrial TCA cycle would likely contribute to quick-killing, it is unlikely that this activity alone would facilitate the rapid and broad phenotype of this mechanism. Notably, MQO is also absent in mammals, but conserved across all Apicomplexa (624-626), including the related parasite *T. gondii* (626), which is also inhibited by the quick-killing activities of azithromycin and analogues (165, 488). Given that both azithromycin and GSK-5 were shown to affect the metabolic functions of parasite's food vacuole and mitochondrion, this data demonstrates that mechanism of quick-killing is multi-factorial for different azithromycin analogues, even against a singular life stage.

Previous studies have proposed that azithromycin analogues featuring a quinoline or chloroquinoline moiety may act like hybrid molecules that combine both azithromycin's apicoplast targeted delayed death and a chloroquine-like activity targeting the food vacuole (484-486). If this was the case, it is possible that azithromycin acts as a delivery mechanism by

transporting and releasing the quinoline/chloroquinoline moiety into the parasite in such a way that potentiates quick-killing. Despite the prevalence of chloroquine resistance, a number of compounds with chloroquine-like activities are being developed including: AQ-13, (248, 269) (Immtech and Tulane University), a chloroquine derivative that has progressed though Phase II trials (270) (Chapter 1); ferroquine, a ferrocenic analogue of chloroquine currently undergoing Phase IIb trials with the ozonide, artefenomel (OZ439) (Medicines for Malaria Venture) (241, 627); and Methylene Blue (University of Heidelberg), an inhibitor of glutathione reductase and haem polymerisation that completed Phase II trials with primaquine in 2017 (628). There are also several potent chloroquine hybrid molecules currently emerging, including DM1157 (DesignMedix) (289), a reversed chloroquine now undergoing Phase I trials (290) (Chapter 1), and a number of 'aminoquinoline hybrids' undergoing pre-clinical development (629, 630) (reviewed in (631, 632)). Thus, potentiating a chloroquine like activity through hybridisation with azithromycin is similar to strategies currently undergoing clinical development. However, my studies also uncovered several differences between how azithromycin analogues and chloroquine like drugs work which further enhance the attractiveness of developing quick-killing azithromycin analogues.

7.2.2 Azithromycin's quick-killing mechanism, similarities and differences with chloroquine.

Before I discuss the similarities and differences between azithromycin and analogues quick-killing activity and chloroquine's mechanism of action, it is instructive to review chloroquine's known activity against the malaria parasite in more detail. Chloroquine is a diprotic weak base that, at physiological pH (~7.4), exists in an mono-protonated state (CQ^{+}) that freely and rapidly diffuses though the RBC and parasites membranes (633, 634). Upon reaching the acidic food vacuole, chloroquine is trapped by protonation (CQ^{++}) and accumulates to high-nanomolar concentrations (167, 634, 635). Chloroquine is widely accepted to kill parasites via binding to haem and the growing hemozoin crystal, resulting in the accumulation of toxic haem and causing oxidative damage to membranes and/or proteins (166, 167, 636). However, there is evidence to suggest that chloroquine may act multi-factorially by altering the pH of the food vacuole and by inhibiting glycolysis via binding to lactate dehydrogenase, in addition to its haeme 'capping' activity (637-640).

The reduced sensitivity of the chloroquine/mefloquine/pyrimethamine resistant DD2 line to azithromycin analogues featuring a chloroquinoline moiety, coupled with the similarities overserved in the metabolomics profiles between azithromycin and chloroquine treated parasites, suggests that azithromycin and analogues may share a similar mechanism of action to that of chloroquine. However, azithromycin and analogues also display a number of properties that differ to chloroquine and other haeme targeting 4-aminoquinolines including:

i) Quick-killing activity was demonstrated for azithromycin, which does not feature a chloroquine like functional group, and improved quick-killing activities were demonstrated across a range of functional groups including phenyls, naphthalenes, quinolines and chloroquinolines from both compound libraries (GlaxoSmithKline and Shandong University, China (Chapters 4 and 5)). While quinolone and chloroquinolone functional groups exhibited the most potent quick-killing activity, other functional groups that are not so obviously similar to the structure of 4-aminoquinolines also exhibited improved quick-killing activity.

ii) Azithromycin analogues featuring a number of different functional groups showed improved inhibitory activity against invading *Plasmodium* merozoites, in addition to exhibiting improved inhibition of host-cell invasion and intracellular development for the related Apicomplexan parasite *T. gondii* (165, 488) (Chapter 4). Since neither merozoites nor *T. gondii* digest haemoglobin or produce haemazoin, it is unlikely that the quick-killing activity of azithromycin analogues against these parasites/stages is similar to chloroquine's mechanism (i.e. hemozoin capping). Moreover, analogues with higher in cycle potencies also showed the greatest improvement in invasion inhibitory activity, suggesting that these two mechanisms are related and supporting a non-haemoglobin dependent mechanism of action contributing to quick-killing (165) (Chapter 4).

iii) Finally, azithromycin and analogues are equipotent throughout the entire intracellular blood stage lifecycle, including early rings stages. In contrast, early ring stage parasites remained largely insensitive to chloroquine and the 4-aminoquinoline quinine, consistent with the lack of food vacuole and limited haemoglobin digestion which is only fully established ~12-18 hrs post invasion (mid-ring stage) (27, 28, 131, 150, 151). To our knowledge, no chloroquine derivative has been reported to inhibit the growth of early ring stage parasites at a potency approaching that seen for maturing trophozoite stages. Aguiar et al., (2018) did assess the morphology of late ring stage parasites (12 hrs post invasion) with a chloroquine analogue but used a higher concentration of drug (10x IC_{50}) than what was applied here (2x IC_{90}) (641). Moreover, there was limited visual evidence of inhibition and 12 hr treatments failed to result in noticeable signs

of parasite death (641), unlike the clear signs of parasite stress seen with azithromycin analogues in this study. Taken together, there appears to be limited evidence to suggest that modifying chloroquine potentiates its activity against ring stage parasites to an equivalent extent to what was observed for a range of azithromycin analogues with different functional groups. These data suggest that while the malaria parasites food-vacuole may indeed be a site of azithromycin and analogues quick killing activity against trophozoite stages, it is certainly feasible that azithromycin and analogues inhibit parasite growth by acting promiscuously though other mechanisms.

7.2.3 Azithromycin's properties that would support a multi-factorial mechanism of action.

Azithromycin, like chloroquine, is lipophilic and weakly alkaline which likely potentiates the accumulation of this drug within infected RBCs (167, 429, 430, 484, 485), where it is trapped by protonation within an acidic compartment (431, 635, 642). Studies have demonstrated that azithromycin accumulates ~4-fold higher in infected RBCs compared to uninfected RBCs (643) and that the addition of a pH neutralizing agent prevents the accumulation of this drug within human leukocytes (644). The accumulation of azithromycin within an acidic body, such as the food-vacuole (pH 3.7-6.5) (645), is likely to be strongly favoured, as it is for chloroquine. Azithromycin, is also known to have other activities, including alteration of intraerythrocytic Ca^{2+}, interference of kinase signalling pathways (reviewed in (416)), sequestration within acidic compartments (537) and binding to and decreasing the mobility of phospholipid bilayers (538). It is therefore possible that azithromycin and analogues inhibit parasite growth by acting through a combination of these potential mechanisms, and this may aid in the activity against the parasites' food vacuole and mitochondrion.

Malaria parasites themselves contain a number of acidic compartments including the food vacuole (~5 pH) (646, 647), slightly less acidic organelles, including the Golgi complex (648) and the acidocalcisome (649-651). Thus, it is feasible that the lipophilic properties of azithromycin and analogues potentiates the accumulation of these drugs within various organelles throughout the parasite. As azithromycin is also weakly alkaline (431, 652), the accumulation of this drug will likely alter the pH within any compartment in which it concentrates, thereby effecting the proteins and enzymes required for the function of the organelle or compartment (416, 643). As the pH of a mitochondrion is considered to be slightly more alkaline (7.0-7.8 pH) than the physiological conditions of the cytoplasm (~7.15 pH) (647,

653), azithromycin may be less likely to concentrate within this compartment and so how it could affect this organelle is less clear.

So why do azithromycin and analogues rapidly kill merozoites and early ring stage parasites while chloroquine does not? One possibility is that early-ring stage development is inhibited by a combination of azithromycin's binding to phospholipids and disruption of pH mediated vacuole-vesicle fusion during the early stages of food vacuole genesis and/or haemoglobin digestion (27, 558, 559). These properties may also see azithromycin and analogues inhibit the function of other membrane-bound acidic compartments. Indeed, a number of candidate organelles are also present within the merozoite stage of parasite development, whereas the parasite food-vacuole is absent. Specifically, the parasites rhoptry organelles (a specialised invasion organelle conserved across Apicomplexa) of *T. gondii* (654, 655) (reviewed in (656)), are acidic, and thus similar to acidic lysosomes in eukaryotes, wherein azithromycin is known to accumulate at high concentrations (429, 430). Moreover, *Plasmodium* merozoites also store Ca^{2+} in high levels (657) that not only acidifies the parasite, but is also required to trigger secretion of the merozoite invasion organelles (658, 659) and other signalling events essential for merozoite invasion into new RBCs (660). It is therefore possible that azithromycin and analogues rapidly accumulate within the membrane-bound acidic compartments of the merozoite (i.e. the rhoptries, acidocalcisome, Golgi complex) (648, 650, 655) and inhibit RBC invasion by interfering with rhoptry section and/or Ca^{2+} signalling (reviewed in (19), Appendix I).

7.2.4 Limitations of mechanistic insights and future directions.

These studies have brought us closer to understanding azithromycin and analogues quick-killing mechanism of action. However, more needs to be done to define the key structural contributors to quick-killing activity and whether a similar mechanism of action works across diverse stages of the parasite lifecycle.

The results of this study showed that a great deal of structural diversity can improve azithromycin's quick-killing activity, with a diverse range of functional groups as well as variations in their site of attachment seen to potentiate quick-killing activity of azithromycin analogues. The diversity in the libraries obtained, the broad quick-killing potency of the analogues and the limited number of specific structural variants (i.e. only small numbers of analogues featuring minor iterative changes that would have allowed us to probe specific SAR changes in detail) means that no unique compound was identified that could be prioritised to

probe the mechanism of quick-killing specifically. While these results have provided a diversity of starting points for future development, there is no clear indication as to which structure may afford the best quick-killing activity against a specific mechanism of action. However, if the quick-killing mechanism of action is indeed multi-factorial, it may be that a range functional groups act against different targets, leading to variations in activity and complicating future SAR optimisation.

One of the major limitations of these metabolomic studies employed in this work was the fact that this analysis only provided a 'snap-shot' of the parasite's metabolomic profile at late-trophozoite stage. As the parasites food-vacuole would provide an enlarged acidic compartment to concentrate these drugs, it stands to reason that the similar signature of azithromycin and analogues to chloroquine was obtained as a consequence of addressing the profiles of trophozoite stage parasites (27, 28). Paradoxically, azithromycin and analogues are also active throughout the entire blood stage lifecycle, including against merozoite invasion of the RBC and very early ring stages (<6 hrs) where the food-vacuole has yet to fully form and chloroquine's activity is minimal (27, 28, 131, 150, 151). Therefore, addressing the metabolomic signatures of early-ring stage parasites treated with azithromycin and analogues could shed more light on the potential for multifactorial mechanism of quick-killing activity. However, metabolomics on ring stages will likely be technically challenging as these parasites make up only a fraction of the metabolic potential of the much larger infected RBC and are difficult to purify away from uninfected RBCs, which may increase the background of human RBC metabolites. A more viable alternative in terms of concentrating parasite material may be to examine the metabolomic signature late in schizogony, when the merozoites are fully formed and the function of the food vacuole diminished. However, as growth during this stage of parasite development has largely stalled, measurable changes in parasite metabolites may be minimal. Determining whether a metabolomic analysis of azithromycin and analogue treatment is feasible at early ring or late schizont stage will be a matter for future experiments that lie outside the scope of this current study.

Changes within the parasites food vacuole upon drug treatment could be addressed by loading parasites with a pH sensitive dye, such as fluorescein-dextran (661), or with a pH sensitive transfectant parasite, such as GFP-PM2, which expresses a pH sensitive fusion protein within the food vacuole (637, 646). Physiological shifts in pH, due to the accumulation of weakly alkaline drug, could be investigated by measuring parasite fluorescence, wherein inhibitory activity or food vacuole alkalinisation would be expected to decrease fluorescence (662).

Similarly, the activity of azithromycin and analogues against the mitochondria could be addressed using a fluorescence probe, such as Mitotracker (663, 664). In this instance, such an assay could provide some insight into the functional state of the mitochondria upon drug treatment using fluorescence microscopy. Alternatively, morphological changes in parasite organelles upon treatment of azithromycin and analogues could be investigated by analysing parasite ultrastructures via scanning election microscopy (387). Such a technique would provide a 3D arrangement of the parasite compartments, including the food vacuole, and provide insight into the aberrant morphologies of specific organelles and shed more light on the target. Potential difficulties with these strategies include the multi-factorial nature of quick-killing and the potency of analogues, which may result in off target effects and kill the parasite prior to accurately detecting the output. Thus, determining the optimal life-stage and drug concentrations for these assays would be critical for these future experiments.

In this study, I compared growth of the chloroquine, pyrimethamine and mefloquine resistant parasite line DD2 to growth of the sensitive line D10-PfPHG in the presence of azithromycin and analogues. While a similar approach has been used to broadly characterise the effect of multi-drug resistance on azithromycin analogue activity (418, 483, 485), in this instance it would have been more informative to compare isogenic lines wherein one line was sensitive to chloroquine and the other resistant (139, 140, 665). Such a strategy was achieved when assessing activity against artemisinin resistant parasites in this study (226). Using isogenic chloroquine resistant and sensitive lines may have enabled the small reduction in potency observed for chloroquinoline azithromycin analogues within the multidrug resistant DD2 line to be assessed accurately in the absence of genetic differences and additional drug resistance mechanisms. Another option to probe similarities with chloroquine specifically is through the addition of a chloroquine reversal agent, such as verapamil, that could be used to address if interference of *Pf*CRT efflux enhances the activity of chloroquinoline analogues (287, 288).

Further investigation using these approaches will likely shed additional light on the activity of azithromycin and analogues and their shared similarities with chloroquine, but this approach may not address whether these drugs act against a non-food vacuole target. If the main avenue for azithromycin and analogue activity is through concentrating in membrane bound compartments, it may be possible to explore this in more detail in merozoites. The malaria merozoite houses several membrane-bound compartments including the rhoptry, which takes up approximately ~1/3 of the merozoite volume (666) and contains significant reserves of Ca^{2+} (657). One avenue is to use live filming in the presence of a Ca^{2+} sensitive fluorescent probe to

track changes in Ca^{2+} homeostasis upon treatment with azithromycin and analogues (667). If a disruption in Ca^{2+} homeostasis is observed in the rhoptry and other acidic compartments upon treatment with azithromycin and analogues, this could be a mechanism by which these drugs inhibit merozoite invasion. Future studies may have the opportunity to investigate these possibilities and shed further light on azithromycin and analogues multi-factorial quick-killing mechanism of action.

7.3 Potential to develop azithromycin as an antimalarial with dual mechanisms of action.

In addition to developing azithromycin's quick-killing secondary mechanism of action, I also investigated whether analogues featuring improved quick-killing activity maintained delayed death, to address if azithromycin could be developed into a drug with dual mechanisms of action. However, the wide-spread and long-term use of azithromycin as an antimalarial whilst maintaining the drugs antibacterial activity does raise concerns for selection of macrolide-resistant pathogens (542, 668) and dysbiosis of the host's commensal bacteria (545, 669). Indeed, altered composition and diversity of faecal microbiota is observed in children following azithromycin treatment (14 days) (545, 670, 671), although a full recovery of the faecal microbiome was observed long-term after cessation of treatment (12-36 months) (545). If further development requires azithromycin analogues to feature no antibiotic activity, analogues wherein the antibiotic activity has been removed or the drug has been engineered so that only the malaria parasites apicoplast ribosome is targeted could be investigated in future studies. In the following, I discuss these potential development avenues to build on the improvements of azithromycin's quick-killing activity and summarise evidence on whether these pathways could be feasible, which is based on the results of my own and other studies.

7.3.1 Pathway 1. Quick-killing antimalarial with bacterial targeting delayed death activity.

Quick-killing azithromycin analogues featuring an additional functional group to the macrolactone ring were found to maintain delayed death activity via targeting the bacterial-derived ribosomes. Engineering azithromycin to have improved quick-killing activity while maintaining delayed death activity has several advantages for malaria treatment including enabling rapid clearance of blood stage parasites and preventing parasite recrudesce through azithromycin's prophylactic activity (130, 160). Indeed, combining delayed death activity with

more potent quick-killing antimalarial activity would likely improve treatment outcomes through both rapid and prophylactic killing of infecting parasites. Moreover, if a dual-acting azithromycin analogue is used in combination with another antimalarial, it is feasible that all 3 independent mechanisms of action would have to be overcome in order for the parasite to become resistant, offering the potential as a 'resistance proofed', single-dose cure. Following the model of resistance selection for ACTs presented by Hastings et al., (2011) (205), in clinical infection, the probability of a mutation conferring *P. falciparum* drug resistance per replication cycle for a single mechanism of action is estimated at ~10^{-9} (672, 673). For two unlinked mechanisms of action the probability of resistance arising to both mechanisms spontaneously is ~10^{-18}. Finally, the probability of resistance arising across three unlinked mechanisms of action, even accounting for reportedly higher rates of mutation in the apicoplast DNA (~10^{-4}) (674) that could confer resistance to azithromycin, is ~10^{-22} (205). Given that infection of *P. falciparum* within a human is proposed to result in upwards of 10^{11} blood stage parasites (205), the probability that parasites would become resistant to a drug combination featuring an azithromycin analogue with dual modalities in addition to a third, highly effective, drug such as an artemisinin is in the order of 1 in 100 billion infections.

Another potential advantage of developing a quick-killing azithromycin analogue that maintains antibiotic activity is the fact this combination would also allow easier treatment of malaria and pathogenic bacterial co-infections. As an antibacterial, azithromycin is routinely used in successful mass drug administration (MDA) campaigns for Trachoma (675, 676), and is associated with both improved pregnancy outcomes (477, 480) and reductions in childhood mortality (463, 464). Following MDA of azithromycin for Trachoma, secondary benefits recorded include reductions in skin, gastrointestinal and respiratory infections caused by bacterial pathogens (468, 677-679) as well as reductions in the clinical malaria burden (463, 464). When azithromycin is used in combination with sulfadoxine/pyrimethamine for Intermittent Presumptive Treatment in Pregnancy (IPTp), improved outcomes were also associated with control of bacterial pathogens as well as malaria (480, 680). Finally, while an increase of macrolide resistant pathogenic bacteria have been observed following MDA (681, 682), this resistance appears transient and declines shortly after cessation of antibiotics (683-686). In this context, maintaining azithromycin's antibiotic activity may be advantageous in some regions or clinical settings in co-treating both bacterial and parasite infections.

7.3.2 Pathway 2. Quick-killing antimalarial with no delayed death activity.

As highlighted in studies that followed MDA of azithromycin for Trachoma treatment, macrolide resistant bacteria do arise in areas of heavy azithromycin usage (681, 682). Therefore, a clear case can be made that the widespread use of an antimalarial that has antibiotic activity may not be desirable. Under this scenario, the antibacterial activity of azithromycin could be removed though modification to azithromycin's desosamine sugar. In this study, appending a functional group to the desosamine sugar was shown to slightly improve quick-killing potency in comparison to similar groups added to the macrolactone ring (Chapter 4). However, the addition of a functional group to the desosamine sugar also abrogated binding to bacterial-like ribosomes and antibiotic activity, thus reducing concerns for selection of bacterial resistance (542, 545, 668, 669). As quick-killing is active across the blood stage lifecycle, clinical application of this mechanism would likely provide rapid parasite clearance and relief of symptoms. However, as these analogues would have lost the prophylactic activity of delayed death, there is a risk that quick-killing alone will be insufficient to provide a single dose cure and combination therapies featuring only azithromycin's quick-killing mechanism of action will lack the resistance proofing and ability to mop up recrudescent parasites that an analogue with dual mechanisms of action could provide.

7.3.3 Pathway 3. Quick-killing antimalarial with non-bacterial targeting delayed death activity.

A third pathway for development of quick-killing azithromycin analogues would see the drug modified such that delayed death activity against the parasites apicoplast is maintained, but the antibacterial activity is engineered out of the compound. This would be the ideal compound to develop as it would quickly clear parasites, provide long-term protection against recrudescence and reinfection, reduce the risk of resistance developing and have no off-target effects on bacterial communities. However, to the best of our knowledge, a macrolide that preferentially targets the parasites apicoplast ribosome over bacterial ribosomes has yet to be reported. In bacterial systems, species-specific differences between bacterial ribosomes has been shown to impact on macrolide binding and activity (reviewed in (411)). Therefore, future studies could investigate engineering azithromycin in such a way that activity against bacterial ribosomes is abolished but the drug maintains activity against the apicoplast ribosome. In order to facilitate this drug engineering, it would be helpful to determine the structure of the apicoplast ribosome, however, this has not been achieved to date. In the meantime, further screens of macrolide libraries investigating delayed death activity against the malaria parasite in absence of

antibacterial activity would offer the best pathway forward to develop malaria specific inhibitors.

7.4 Considerations and future directions for repurposing azithromycin as an antimalarial with dual modalities.

In this study, I characterised a series of azithromycin analogues that featured dual-modalities against malaria parasites; nanomolar quick-killing of blood stages and the slow, prophylactic activity of delayed death. While metabolomic data suggests that azithromycin's quick-killing activity includes a similar profile to that of chloroquine, azithromycin and some analogues demonstrate several advantageous properties over chloroquine and other 4-aminoquinilone hybrid molecules that may be worth pursuing from a drug development perspective. Azithromycin analogues were potent inhibitors across the blood stage lifecycle and maintained activity against early-ring stage parasites, which could be of particular benefit for rapid clearance of parasites during clinical infection (154, 155). Additionally, the majority of analogues tested exhibited limited cross-resistance against drug resistance mechanisms seen in malaria endemic areas, including artemisinin and chloroquine resistance and we presented evidence to suggest that quick-killing is likely multi-factorial and, at the very least, targeting the activity of the food vacuole and mitochondria. These data suggest that existing resistance in the field is likely limited and that parasites may find it difficult to overcome such a promiscuous inhibitor that features at least 2 distinct mechanisms of action (205).

We showed here that azithromycin's quick-killing and delayed death activities are independent and confirmed two different avenues for azithromycin's antimalarial re-development: as a drug that has both delayed death & quick-killing activity or as a drug that features only quick-killing activity. As previous studies precluded addressing the quick-killing activity these of analogues *in vivo* (484-486), ideally, this activity should be explored using the appropriate mouse models.

Previously, azithromycin analogues from the GSK series were shown to feature favourable toxicity and bioavailability profiles as well as being effective in *in vivo* rodent malaria models (418, 482-486). However, these *in vivo* rodent studies drug treated for 3 days, the period of time for delayed death to be active in the *P. berghei* model of malaria (418, 484, 486). Thus, it was not clear whether quick-killing activity was impacting on growth, or whether the predominant activity was through apicoplast targeting delayed death. To specifically address whether quick-killing activity of analogues is effective *in vivo* in the absence of delayed death activity

screening of lead analogues in: i) a *P. berghei* model resistant to azithromycin's delayed death activity; or ii) short-term drug treatments (in cycle equivalent for *P. berghei* is 24 hrs) could be used. Ideally, dual-acting analogues displaying favourable safety profiles, long half-lives and the potential to be developed as single-dose cures could be prioritised for assessment in a humanised rodent model that can be infected with *P. falciparum* (687) (reviewed in (688)).

One potential issue for the clinical application of next-generation azithromycin analogues for treatment of malaria is the link between macrolides and potentially fatal cardiac arrhythmias (*torsade de pointes*) (432, 433, 689). This complication is very rare, occurs after high drug dosage and is usually observed in patients with a pre-existing cardiac disease and/or other medical conditions (436, 437) (reviewed in (434)). As drug-induced cardiotoxicity risks arises from off-target effects on the hERG-encoded K+ channel, the interactions between hERG and lead analogues could addressed early in the development process using standard *in vitro* assays (e.g. IonWorks HT) (690). However, given widespread use of azithromycin, the rarity of these complications and the potential to develop quick-killing analogues with nanomolar potencies, it is possible that analogues with improved antimalarial properties will not exhibit these issues, especially if a lower dose is required for successful treatment.

7.5 Activity of novel tri-peptide proteasome-like inhibitors.

Plasmodium proteasomes have been recently validated as antimalarial targets, with these protein complexes shown to have an essential role throughout the parasite's entire lifecycle (325, 366). As proteasome inhibitors synergise with artemisinins and as artemisinin resistant parasites rely on the proteasome to help survive artemisinin treatment (218, 370, 579), the development of malaria specific proteasome inhibitors is an attractive avenue. One of the major concerns in developing proteasome inhibitors as antimalarials, however, is the potential of host toxicity due to cross-reactivity with the human proteasome (reviewed in (577) and (366)).

I investigated the antimalarial activity of a small panel of peptide vinyl sulfone and aldehyde tri-peptide inhibitors that were synthesised based on published proteasome-targeting compounds (372, 373, 691). These analogues demonstrated low nanomolar activity against blood stages of both *P. falciparum* and *P. knowlesi* that were comparable to the published antiparasitic potencies of leading vinyl sulfone and superior to published aldehyde scaffolds (368, 369, 373). Importantly, these inhibitors also demonstrated selective activity against

parasites over human cells, suggesting these drugs provide a viable starting point to develop new antimalarials.

Despite being developed from known proteasome inhibitors, these compounds killed parasites though a mechanism of action unrelated to inhibition of the parasite proteasome. Neither vinyl sulfones nor aldehydes caused a build-up of ubiquitinated proteins in basic proteasome inhibition assays that would be indicative of proteasome inhibition (218, 367, 370). Moreover, the most inhibitory vinyl sulfone (PI-1) failed to synergise with DHA, in contrast to the strong synergism observed for published peptide vinyl sulfones and other true proteasome inhibitors (368, 370, 371, 572). It is important to note that similarly structured peptide vinyl sulfones and aldehydes have been shown to also target cysteine proteases, such as falcipains, in *P. falciparum* (330, 334, 337, 338). As DHA requires free haem for the excision of the endoperoxide bridge for its antimalarial activity, inhibition of an essential cysteine protease involved in haemoglobin catabolism could contribute to the lack of synergism between DHA and PI-1 (126, 559). Therefore, this opens the possibility that these inhibitors are targeting other proteases, with the lack of synergism between DHA and PI-1 supporting that falcipains are a possible alternative target (reviewed in (328) and (321)).

Currently, several studies have explored *Plasmodium* proteases as drug targets, including: i) subtilisin proteases (SUB1 and SUB2) that are essential for merozoite egress and invasion (692, 693), ii) aspartic proteases (plasmepsins) involved in protein export (PlmV), invasion (Plm IX and X) and haemoglobin digestion (PlmI, II, PlmIII/HAP and PlmIV) (348, 350), iii) both eukaryotic and prokaryotic *Plasmodium* proteasome proteases (325) and iv) aminopeptidases (304) (reviewed in (19) and (327)) (Chapter 1, Appendix I). Falcipains 2 and 3 have also been extensivity investigated due to their essential role in haemoglobin digestion, with a number of promising peptide mimetics, including vinyl sulfones (337, 338) and aldehydes (330, 334) designed to target these proteins demonstrating nanomolar *in vitro* potencies. Indeed, both FP-2 and FP-3 are essential in *P. falciparum*, and both share a 66% homology in their catalytic domains, which suggests that a single drug could target both proteases (331, 694, 695).

However, development of tri-peptide falcipain inhibitors is not without its own challenges. *In vivo* rodent models showed similar vinyl sulfones had a limited ability to clear blood stage parasitaemia (40-80% cure), with this limited *in vivo* activity attributed to the peptide's poor oral availability and pharmacokinetic profiles (333, 696). Off-target effects of vinyl sulfones is also important to consider since similar drugs have been shown to target human caspases

(599, 697, 698). However, given the low nanomolar activity and high selectivity of the compounds against parasites identified in this study, the novel chemotypes tested are, at the very least, worth further investigation as potential antimalarials.

7.5.1 Feasibility of hypoxia inducible pro-drugs.

Finally, in an effort to further reduce off-target activity against host proteases and potentially other antimalarials, we investigated whether a hypoxia activated prodrug strategy could work against malaria parasites. We initially hypothesised that the malaria parasites exclusive use of glycolysis (185), dissociation of haem-bound oxygen during haemoglobin digestion (149, 166) and hypoxic culture conditions (1% O_2) (492) may provide an environment with enough reducing potential to activate the pro-drug *in vitro* (586). Despite these conditions, however, the HAPs were not activated in standard culture conditions relative to oxygen rich culture conditions, suggesting that oxygen levels were too high to cleave the bio-reductive sidechain. Since initial chemistry trials identified that reduction of the sidechain occurred best in the presence of the strong reducing agent ($Zinc^{2+}$), it may be that the bio-reductive linker requires significantly more reducing potential than what is available with our *in vitro* culture conditions. Further studies could investigate whether these drugs could be artificially activated by culturing parasites with the drug in a hypoxia chamber capable of creating an environment with <1% O_2 (492, 512, 586) or by supplementing the culture media with a reducing agent, such as DDT, to initiate oxidative stress (605).

As these avenues many provide evidence for the activation of HAPs against malaria *in vitro*, it remains questionable as to whether this strategy will be feasible under *in vivo* conditions unless the bio-reductive linker is significantly improved to allow targeted reduction in infected RBCs. Recent studies in the field of cancer drug development suggest further optimisation is required for the HAP strategy, as limited efficacy was seen in Phase II/III trials for HAP targeting hypoxic tumours, with three leading anti-cancer HAPs failing clinical trials (reviewed in (587)). Clinical failure was, in part, attributed to the variability of tumour environments that resulted in poor tissue penetration of the drug, inadequate oxygen inhibition as well as oxygen independent activation, resulting in tolerability and toxicity issues (587). Therefore, if this avenue is to be pursued for malaria, it is clear that further optimisation will be required. Use of a pro-drug strategy to treat malaria would prevent cross-reactivity with unwanted targets within host cells by activating the drug action at a specific site. In malaria, this strategy would be especially relevant if the human host shares equivalent homologues of the drug target. Moreover, as a plethora of prodrug classes exist (reviewed in (616) and (617)),

this opens the possibility that other pro-drug strategies may be better suited for targeting malaria parasites going forward.

The application of pro-drugs for treatment of malaria is implemented with clinically used antimalarials, including artemisinins (583-585) (reviewed in (240)) and proguanil (129, 176), with both requiring activation after administration in order to initiate their activity against the malaria parasite. A pro-drug strategy that could be implemented against infected RBCs, instead of a HAP strategy, is the inclusion of a haeme activated endoperoxide bridge, as observed in artemisinins and ozindes (214, 240, 699), that could be linked to a masking sidechain to block binding to a protease until the drug is activated at the site of haemoglobin digestion. However, simpler strategies to reduce off-target activity against host proteases, such as engineering compound structures to prevent cross-reactivity with host enzymes, is also feasible and is currently being undertaken for *Plasmodium*-specific proteasome inhibitors across a number of studies (368, 369, 572). In this study, while I was unable to show HAP activity, I did characterise 4 drug chemotypes with improved antimalarial activity over the proteasome inhibitor MG-132 (330, 373). While the activity of these chemotypes appears to be unrelated to the proteasome, their potency against blood stage malaria parasites and low mammalian cell toxicity makes these drugs interesting starting points for further development in collaboration with the laboratory of Prof. Andrew Abell (The University of Adelaide).

7.6 Final conclusions.

My work has advanced our current understanding of azithromycin's mechanism of action against malaria parasites and shed new light on whether the drugs quick-killing activity could be developed as an additional mechanism of action against malaria parasites. I demonstrated that quick-killing was completely independent of apicoplast-targeted delayed death activity and that azithromycin and its analogues are active throughout the entire blood stage lifecycle, making them desirable as clinical treatments. While the mechanism of quick-killing was not completely characterised, evidence suggests that azithromycin and analogues likely act though multi-factorial modes of action with the parasite's food vacuole and mitochondria being likely targets. While further validation is required, a single drug featuring multi-factorial activity would be especially useful in clinical settings as the parasite would require multiple resistance mutations to overcome this activity.

I identified a number of azithromycin analogues with varying functional groups that exhibit low-nanomolar IC_{50} values and improved activity against the invading merozoite, thereby providing a good starting point for redevelopment of this antibiotic as an antimalarial. As azithromycin's two mechanisms are independent, we confirmed that two different avenues could be pursued for azithromycin's antimalarial re-development: i) delayed death and quick-killing, or ii) quick-killing only. Finally, we provided a starting point in developing novel, proteasome inhibitor-based compounds as inhibitors of malaria parasite growth, though the mechanism of action needs to be fully characterised and additional medicinal chemistry modifications explored to improve potency and further reduce mammalian cell toxicity. It is hoped that the knowledge and compounds identified here will form the basis for future drug development activity leading to new antimalarials for use in combination therapies.

Chapter 8. References.

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Appendix I. Targeting malaria parasite invasion of red blood cells as an antimalarial strategy.

Appendix I. Statement of authorship.

Statement of Authorship

Title of paper	Targeting malaira parasite invasion of red blood cells as an antimalarial strategy
Publication Status	☒ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Amy L Burns, Madeline G Dans, Juan M Balbin, Tania deKoning-Ward, Paul R Gilson, James G Beeson, Michelle J Boyle, Danny W Wilson FEMS Microbiology Reviews. 2019. 43(3). 223-238

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Overall percentage (%)	55%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Appendix I.

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Contribution to the Paper	Conceived the manuscript, designed and co-wrote the manuscript. Contribution 10%
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Appendix I. Targeting malaria parasite invasion of red blood cells as an antimalarial strategy.

FEMS Microbiology Reviews, fuz005, 43, 2019, 223–238

doi: 10.1093/femsre/fuz005
Advance Access Publication Date: 11 February 2019
Review article

REVIEW ARTICLE

Targeting malaria parasite invasion of red blood cells as an antimalarial strategy

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One sentence summary: Malaria invasion of red blood cells is an essential step in parasite replication and this review discusses targets and drug chemotypes being developed to stop invasion and growth.

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Editor: Christiaan van Ooij

ABSTRACT

Plasmodium spp. parasites that cause malaria disease remain a significant global-health burden. With the spread of parasites resistant to artemisinin combination therapies in Southeast Asia, there is a growing need to develop new antimalarials with novel targets. Invasion of the red blood cell by *Plasmodium* merozoites is essential for parasite survival and proliferation, thus representing an attractive target for therapeutic development. Red blood cell invasion requires a co-ordinated series of protein/protein interactions, protease cleavage events, intracellular signals, organelle release and engagement of an actin-myosin motor, which provide many potential targets for drug development. As these steps occur in the bloodstream, they are directly susceptible and exposed to drugs. A number of invasion inhibitors against a diverse range of parasite proteins involved in these different processes of invasion have been identified, with several showing potential to be optimised for improved drug-like properties. In this review, we discuss red blood cell invasion as a drug target and highlight a number of approaches for developing antimalarials with invasion inhibitory activity to use in future combination therapies.

Keywords: malaria; merozoites; invasion; antimalarial(s); *P. falciparum*; *P. vivax*

INTRODUCTION

Malaria is a mosquito borne disease caused by parasites of the genus *Plasmodium*. The majority of the ~445 000 malaria related deaths in 2016 were caused by *P. falciparum* and occurred in sub-Saharan Africa (Murray *et al.* 2014; WHO 2017). In addition, *P. vivax, P. malariae, P. ovale* (comprised of two different subspecies; *P. ovale curtisi* and *P. ovale wallikeri* (Sutherland

Received: 8 December 2018; **Accepted:** 11 February 2019

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et al. 2010)) and two zoonotic species, *P. knowlesi* and *P. simium* (Singh *et al.* 2004; Brasil *et al.* 2017), are recognised as significant contributors to global malaria disease burden. While intervention against *Anopheles* mosquito vectors and the success of artemisinin-based combination therapies have contributed to marked decreases in disease burden since the year 2000, there is growing concern regarding the spread of *P. falciparum* strains throughout Southeast Asia which are resistant to artemisinin-based drugs and their partner drugs utilized in combination therapies (Dondorp *et al.* 2009; Ariey *et al.* 2014; Ashley *et al.* 2014; Tun *et al.* 2015; Das *et al.* 2018). Resistance to other clinically used antimalarials, such as chloroquine and sulfadoxine-pyrimethamine, is also widespread globally (Plowe *et al.* 1997; Trape *et al.* 1998; Mehlotra *et al.* 2001). Thus, there is an urgent need to bring to market new antimalarials with novel mechanisms of action which are active against all drug-resistant strains, effective against all human pathogenic *Plasmodium* spp. and can clear parasitemia rapidly for improved clinical outcomes (Burrows *et al.* 2017). Targeting multiple lifecycle stages would improve the effectiveness of combination therapies across endemic areas and help slow the development of drug resistance.

Human infections begin with the bite of a mosquito vector and release of malaria sporozoites, with the sporozoite then traveling to the liver and invading hepatocytes (reviewed in Aly, Vaughan and Kappe 2009). After rapid multiplication of liver stage parasites, the mature hepatic schizont ruptures and releases red blood cell (RBC) invading merozoites into the blood stream. In the case of *P. falciparum*, after merozoite invasion of a RBC a 48 hour cycle of growth, multiplication, RBC rupture and release of 16–32 new merozoites ensues (reviewed in White *et al.* 2014). The number of merozoites produced per cycle of growth and the length of the blood stage lifecycle varies between human malaria species. A small portion of blood stage parasites (<1%) differentiate into sexual stage gametocytes, which are transmitted to *Anopheles* vectors during blood meal feeding (reviewed in Liu, Miao and Cui 2011).

As all malaria pathology is caused by blood stage parasites, and this is when infection is diagnosed and clinical symptoms occur, antimalarials used for treatment of clinical disease or clearance of parasitemia predominantly target this stage of the lifecycle. One emerging strategy to kill blood stage parasites is to target merozoite invasion of the RBC with antimalarials. RBC invasion is an extracellular step in the blood stage lifecycle which is essential for parasite proliferation. A model for the sequential process of invasion, from late stage merozoite development, priming of invasion ligands, merozoite release from the schizont (Fig. 1), through RBC contact and invasion (Fig. 2), is described: briefly (i) merozoites attach to the RBC, (ii) the apical tip of the egg-shaped merozoite contacts the RBC, (iii) invasion ligands from organelles situated at the apical tip (the rhoptry and micronemes) are secreted upon calcium signals and an irreversible interaction known as the tight junction is formed, (iv) the actin-myosin invasion motor engages, protease cleavage events are triggered as the RBC membrane is pulled around the parasite to form the parasitophorous vacuole and (v) the invasion pore is fused behind the invaded parasite (Dvorak *et al.* 1975; Gilson and Crabb 2009; Weiss *et al.* 2015). An important consideration in terms of drug-development is that RBC invasion requires a series of co-ordinated and often irreversible events to occur in sequence, with even small perturbations of this complex process likely to limit parasite survival *in vivo*.

RBC invasion is the essential first step in the disease causing blood stage of the lifecycle and the extracellular merozoite is exposed directly to the bloodstream. Vaccine development targeting merozoite invasion is well advanced with vaccines against merozoite surface protein 1 (MSP1, Phase 2b; Genton *et al.* 2003; Ogutu *et al.* 2009), MSP2 (Phase 2b; Genton *et al.* 2003), apical membrane antigen 1 (AMA1, Phase 2b; Thera *et al.* 2011), reticulocyte binding homologue 5 (Rh5, Phase 1a; Payne *et al.* 2017), erythrocyte binding antigen (EBA-175, Phase 1a; Koram *et al.* 2016) and others reaching clinical trials, but with limited efficacy demonstrated to date (reviewed in Draper *et al.* 2015; Beeson *et al.* 2016). However, many essential proteins and protein/protein interactions required for invasion are unique to malaria parasites and are highly conserved between isolates, making them strong targets for antimalarial development. Given this, there are several broad approaches that could be used therapeutically to block merozoite invasion. The importance of key parasite-parasite and parasite-RBC protein interactions on the merozoite surface highlights the possibility of blocking protein-protein interactions directly; potentially by targeting RBC receptor(s). In addition, protease cleavage events, calcium signalling, the action of the invasion motor and structural changes are also key processes in RBC invasion which could be targeted by drugs. Importantly, a drug that blocks a merozoite's ability to invade immediately and permanently ends the parasite's lifecycle, with this cidal activity potentially having benefits in terms of reducing the risk of tolerance leading to resistance and removing the parasites ability to transition to mosquito transmissible gametocytes. However, it has only been in recent years that protocols have been developed to test invasion-inhibitory compounds against *P. falciparum* merozoites directly *in vitro*), with the availability of these techniques now allowing improved screening and characterisation of new invasion-inhibitory chemotypes.

This review will give an overview on compounds that inhibit invasion, either through inhibition of an upstream invasion priming event in the developing schizont (Fig. 1) or during the merozoite invasion process (Fig. 2), that have shown potential to be developed into antimalarial drugs. Targeting RBC invasion has been established as a proof-of-concept through the demonstration of potent inhibitory activity of numerous compounds *in vitro* (summarised in Supplementary Table S1) and through several *in vivo* studies using animal models (Xiao *et al.* 1996; Zenonos *et al.* 2015; Nasamu *et al.* 2017; Pino *et al.* 2017). Numerous chemical starting points and targets have been identified that could be the basis for the development of potent inhibitors for therapeutic use, as described below. We compare this concept to the clinical use of inhibitors which block HIV entry into host cells (Barre-Sinoussi *et al.* 1983; Gallo *et al.* 1983) to demonstrate that RBC invasion is a viable therapeutic target. In addition, we consider studies from the related apicomplexan parasite *Toxoplasma gondii*, for which a number of optimisable inhibitors have been developed against targets analogous to proteins in malaria parasites. As resistance to frontline artemisinin-based combination therapies continues to spread (Dondorp *et al.* 2009; Ariey *et al.* 2014; Ashley *et al.* 2014), this review is a timely reminder that malaria invasion of RBCs provides an 'Achilles heel' within the parasite's lifecycle that is of increasing interest for antimalarial development.

VIRAL ENTRY INHIBITORS AS A MODEL FOR DEVELOPMENT OF RBC INVASION-INHIBITORY ANTIMALARIALS

Viruses are highly successful and widespread lifeforms that require establishment of an infection inside a host cell in order

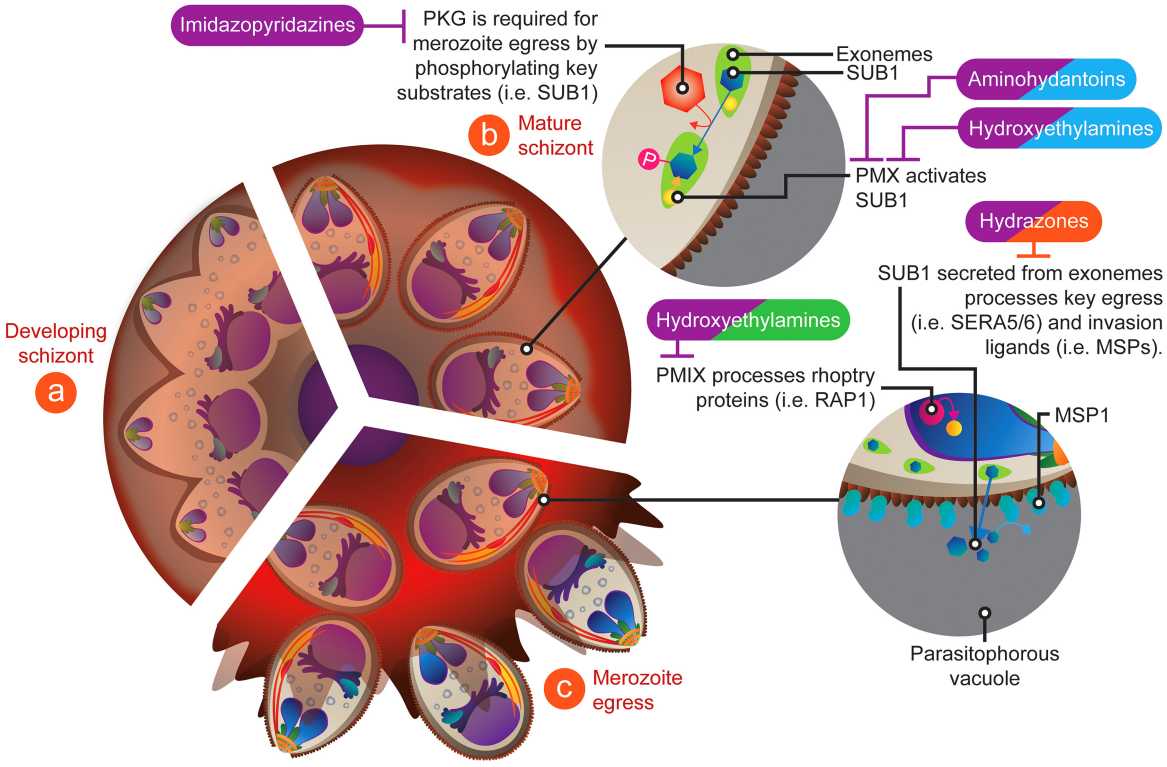

Figure 1. Druggable targets during merozoite development where inhibitors block downstream invasion of the RBC. **(a)** Late stage merozoite development showing partial formation of merozoite membranes and invasion organelles. **(b)** Merozoite formation is completed in mature schizonts. The PVM becomes permeable and PfPKG is activated, leading to activation and discharge of subtilisin-like protease 1 (PfSUB1) from the exonemes. The protease Plasmepsin X (PMX) also resides in the exonemes and is required to process PfSUB1 into an active form. **(c)** Cleavage of PfSUB1 by exoneme resident Plasmepsin X leads to activation of the egress regulating papain-like proteases SERA5 and SERA6, with loss of SERA 5/6 activity preventing merozoite egress from schizonts. Release and activation of PfSUB1 also leads to the cleavage of a number of merozoite invasion ligands including MSP1, MSP6, MSP7, AMA1, with the rhoptry antigen RAP1 processed by Plasmepsin IX (PMIX). Whilst these ligands are largely not required until merozoite contact with the RBC and invasion commences (see Fig. 2), inhibition of these cleavage events around schizont egress is associated with loss of invasion. Inhibitors have been labelled using a dual-colour system that allows their activity against merozoite development/egress (this figure) and their latter effects against invasion (Fig. 2) to be highlighted for inhibitors of: PMIX (purple/green), PMX (purple/blue), PfSUB1 (purple/orange).

to replicate. As a consequence, the essential step of viral entry into the host cell has been targeted by peptide/protein mimics and small molecule entry inhibitors for a diverse range of viruses including HIV (Kilby *et al.* 1998; Dorr *et al.* 2005), orthomyxoviruses (Malakhov *et al.* 2006), flaviviruses (Wang *et al.* 2009), paramyxoviruses (Lambert *et al.* 1996; Rapaport *et al.* 1995), filoviruses (Watanabe *et al.* 2000) and coronaviruses (Bosch *et al.* 2003). The three-step process of viral entry, consisting of attachment, co-receptor binding, and fusion, precedes release of the viral genome into the host's cytoplasm where new virions are assembled before budding and release of the virions from the cell.

HIV entry into memory CD4 + T-lymphocytes (Klatzmann *et al.* 1984; Ho *et al.* 1995) is reliant on a very small number of viral-protein/host-protein interactions and protease cleavage events. Similar to malaria RBC invasion, HIV cell-entry is orchestrated by a multi-step process and is completed within a fraction of the total viral generation time (defined as time between virion release, infection of a new host cell and generation of daughter viral particles (excluding viral latency), estimated between 48 and 63 hours (2.0–2.6 days) (Dixit *et al.* 2004; Murray, Kelleher and Cooper 2011; Puller, Neher and Albert 2017)). Host cell entry begins with virion attachment, binding to the host's co-receptor and entry; with inhibitors, both clinically approved and in development, identified to target each step (reviewed in Kuritzkes 2009; Henrich and Kuritzkes 2013). Maraviroc, the first licenced chemokine receptor antagonist and first host targeting antiretroviral drug, specifically inhibits entry of HIV isolates by binding to the host cell receptor, C-C chemokine receptor type 5 (CCR5), one of two chemokine receptors that HIV viruses use for entry into the cell (Dorr *et al.* 2005; Wood and Armour 2005). Maraviroc specifically inhibits the entry of CCR5-tropic (R5) viruses and is routinely used in second-line antiretroviral combination therapies against R5 HIV viruses (reviewed in Perry 2010).

After binding of the host cell receptors CCR5 or CXCR4 (CXC chemokine receptor type 4), a conformational change occurs within the virion membrane which exposes the heptad repeat domains on viral envelope glycoprotein gp41 (heptad repeat 1 (HR1) and HR2) (reviewed in Klasse 2012). Enfuvirtide, the first antiretroviral fusion-inhibitor approved for HIV treatment, is a 36-amino acid synthetic peptide that mimics the HR2 region of gp41 that binds to HR1, preventing the formation of the six-helix gp41 bundle that is critical for viral fusion and entry (Kilby *et al.* 1998). Enfuvirtide is typically active against HIV-1 isolates that are resistant to other classes of antiretroviral drugs and is reserved for second-line combination therapies of advanced stage HIV infections (Kitchen *et al.* 2008).

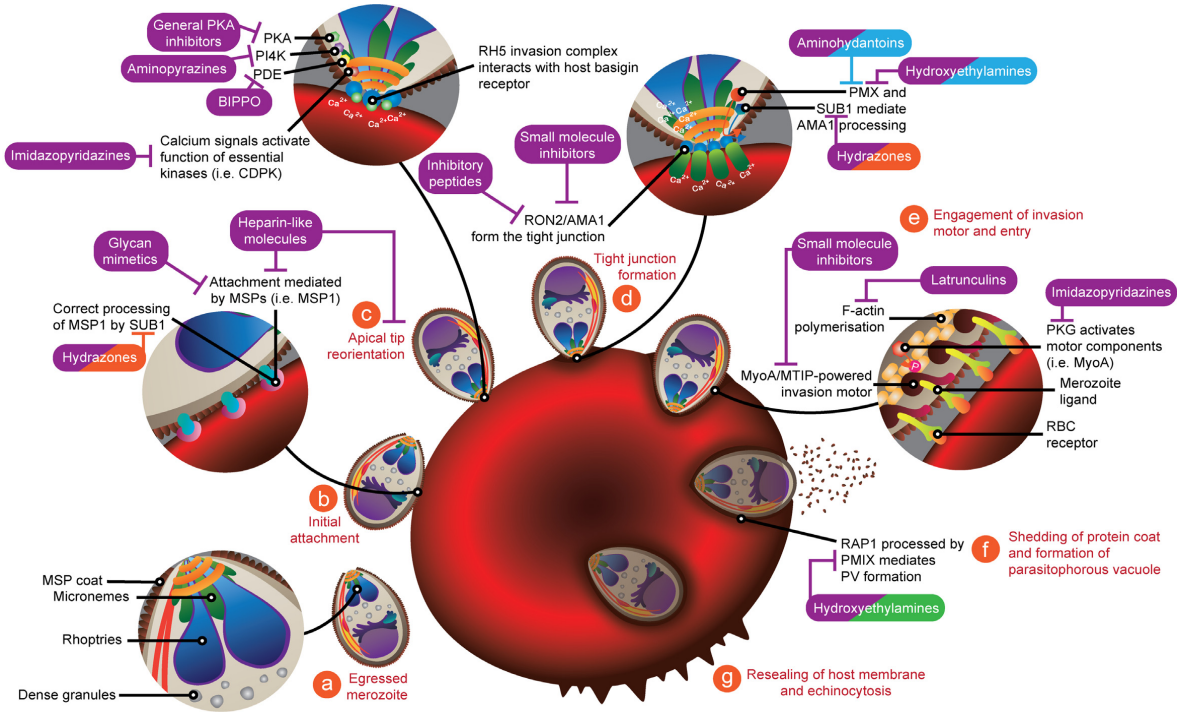

Figure 2. Malaria merozoite invasion of the RBC and invasion inhibitors. **(a)** Merozoites are released into the blood stream after rupture of schizonts (mature blood stage parasites), ready to invade new RBCs. **(b)** Initial attachment requires low-affinity interactions between the surface coat of MSPs, and host receptors on the surface of the RBC. **(c)** Merozoites reorientate such that the apical tip binds to the surface of the RBC and invasion ligands are secreted from the apical tip organelles; the rhoptries and micronemes. The rhoptry antigen PfRH5 binds to its RBC receptor basigin in a key early interaction required for merozoite invasion. **(d)** An irreversible tight-junction is formed when the microneme-secreted protein AMA1 binds to the rhoptry neck protein complex that is embedded on the RBC membrane. **(e)** Entry of the parasite is powered by an actin-myosin motor that pulls the RBC around the invading merozoite while **(f)** the surface coat of MSPs is simultaneously shed. Calcium signalling and phosphorylation by kinases are thought to play a key role in controlling the sequence of events required for invasion during this period. The vacuole membrane fuses behind the invading parasite forming a parasitophorous vacuole. **(g)** Shortly after internalization, a large proportion of RBCs temporarily distort in a process known as echinocytosis. It has been postulated that echinocytosis is caused by rhoptry secretion and rapid entry of Ca^{2+} discharged from the rhoptries into the RBC during invasion, but a more recent explanation suggests that it is incorporation of parasite rhoptry contents into the RBC membrane which leads to RBC membrane ruffling (Dvorak *et al.* 1975, Gilson and Crabb 2009). Examples of drug inhibitors which act at certain stages of the invasion process are labelled in purple. Labels with two colours indicate the inhibitor also has activity around merozoite egress (see Fig. 1).

Although the cellular targets and kinetics of HIV entry into CD4 + T-lymphocytes differ to the requirements of *Plasmodium* invasion into RBCs, these examples of clinically used HIV entry inhibitors for treatment of disease provide an informative comparison for considering the development of antimalarial drugs that target RBC invasion.

PLASMODIUM INVASION INHIBITORS AND PROSPECTS FOR DEVELOPMENT

The majority of current antimalarials target the blood stage of the lifecycle and work through various targets such as; (i) the parasites intracellular food vacuole (chloroquine, artemisinin)(Fidock *et al.* 2000; Klonis *et al.* 2011), (ii) DNA replication (pyrimethamine)(Cowman *et al.* 1988), (iii) mitochondrion function (atovaquone (Fry and Pudney 1992) and proguanil (Dickerman *et al.* 2016)) or, (iv) the apicoplast, the parasite's remnant plastid organelle (doxycycline, azithromycin, clindamycin) (Dahl and Rosenthal 2007; Goodman, Su and McFadden 2007). Currently, no clinically used antimalarial has activity against RBC invasion (Wilson *et al.* 2013), except azithromycin when used at higher concentrations (Wilson *et al.* 2015).

In vitro live-cell filming of *P. falciparum* has shown that RBC invasion, from formation of the tight junction to completion of RBC entry, generally takes less than 1 min (Gilson and Crabb 2009). However, the time taken for a merozoite to commence invasion after egress from a schizont is variable, with one study finding that it took 10 min for 80% of invasion events to be completed *in vitro*. Depending on the drug target, key processes required for RBC invasion could be susceptible to an antimalarial during merozoite development and schizont egress (Fig. 1a–c) or during the process of invasion itself (Fig. 2a–g). To be clinically useful, invasion-inhibitory drugs would need to have a long half-life so that the drug can be maintained in the blood at a high enough level to inhibit invasion as it occurs. A drug with a very short half-life (i.e. artesunate has a half-life of $<$ 30 minutes; Dondorp *et al.* 2009) would not be suitable. Of interest, the half-life of the HIV entry inhibitor maraviroc is ~16 hours (Perry 2010). The myriad of essential and unique targets required to work in a coordinated fashion to enable the rapid process of invasion, combined with the sensitivity of the invasion process to perturbation, provides a promising avenue for new antimalarial development. In this review, we highlight several essential processes targeted in invasion-inhibitory drug development (Figs 1 and 2) and outline some of the compounds that have been tested to date (Supplementary Table S1).

Inhibition of MSPs and RBC receptors using glycan derivatives

Heparin like molecules as invasion inhibitors

Heparin, a member of the glycosaminoglycan family, is a known inhibitor of RBC invasion (Butcher, Parish and Cowden 1988; Boyle *et al.* 2017). A diversity of other sulfated carbohydrates and heparin-like-molecules (HLMs) have also been identified to inhibit invasion of *P. falciparum* merozoites, including curdlan sulfate (Havlik, Rovelli and Kaneko 1994; Evans *et al.* 1998), polyvinyl-sulfonate sodium salt (Kisilevsky *et al.* 2002), suramin (Fleck *et al.* 2003), carrageenans (Adams *et al.* 2005), sulfated cyclodextrins (Crandall *et al.* 2007), fucosylated chondroitin sulfate (Bastos *et al.* 2014), K5 polysaccharides (Boyle *et al.* 2010), inulin sulfate, xylan sulfate, tragacanth sulfate and scleroglucan sulfate (Boyle *et al.* 2017). Furthermore, HLM invasion-inhibitory activity has also been reported for the zoonotic malaria parasite *P. knowlesi* (Lyth *et al.* 2018) and *P. berghei* (Xiao *et al.* 1996), indicating that pan-species invasion inhibition of human malaria parasites is achievable with these molecules. Although precise mechanisms of action for sulfated carbohydrates in inhibiting invasion are not fully understood, HLMs have been reported to inhibit the earliest step in invasion, initial RBC attachment, and to bind MSP1 (Boyle *et al.* 2010), as well as to rhoptry and microneme proteins involved in reorientation and signalling steps of invasion (Fig. 2a-c) (Baum *et al.* 2009; Kobayashi *et al.* 2010; Kobayashi *et al.* 2013). Therefore, it is likely that these compounds target multiple essential ligands in the invasion process, thus reducing the potential for developing drug resistance. Indeed, attempts to generate heparin resistant parasite strains *in vitro* have been unsuccessful (Boyle *et al.* 2010). Although limited studies have been performed to evaluate the activity of HLMs *in vivo*, there is data from animal models (Xiao *et al.* 1996) and human clinical trials supporting their potential development (Havlik *et al.* 2005; Leitgeb *et al.* 2017).

Heparin has been historically used as an adjunct treatment for disseminated intravascular coagulation that can occur in severe malaria (Smitskamp and Wolthuis 1971; Munir *et al.* 1980; Rampengan 1991), but its use was stopped because its anticoagulative properties led to an increased risk of bleeding. Recently, heparins with periodate oxidation of non-sulfated uronic acid residues that greatly reduced anticoagulation activity of heparin (Pisano *et al.* 2005) were shown to be highly inhibitory to RBC invasion (Boyle *et al.* 2017). Similar HLMs have been tested for inhibition of lung cancer growth in mice with no anticoagulation activity reported across a range of tissue (Yu *et al.* 2010). Of further promise, curdlan sulfate (Boyle *et al.* 2010) has a 10-fold reduced anticoagulative activity and testing in a small human trial suggested that treatment reduced malaria disease severity (Havlik *et al.* 2005). A recent phase I clinical trial of the non-anticoagulant HLM sevuparin, a negatively charged polysaccharide manufactured from heparin, limited parasite replication by blocking invasion (Leitgeb *et al.* 2017). HLMs such as sevuparin also disrupt pathogenic mediators such as rosetting and sequestration of infected RBCs, (Udomsangpetch *et al.* 1989; Carlson *et al.* 1992; Rowe *et al.* 1994; Barragan *et al.* 1999; Vogt *et al.* 2006; Kyriacou *et al.* 2007; Skidmore *et al.* 2008; Bastos *et al.* 2014; Saiwaew *et al.* 2017), potentially enabling HLMs to provide dual protective mechanisms of action against severe malaria. Current HLMs that have been identified with antimalarial activity have relatively low potency (Boyle *et al.* 2017) and they have also been reported to have relatively short half-lives when used clinically (i.e. heparin $<$ 1 hour (Perry, Herron and King 1974), sevuparin ~1 hour (Leitgeb *et al.* 2017), curdlan sulfate ~2–3 hours (Gordon *et al.* 1994)). However, the clinically used HLM fondaparinux has a longer half-life (17–21 hours)(Donat *et al.* 2002). Work on improving oral availability of heparin derivatives (reviewed in Neves *et al.* 2016), as well as prolonging HLM drug activity and potency (Hoffart *et al.* 2006; Boyle *et al.* 2017) are ongoing and offer an avenue for HLMs to be developed as antimalarials with invasion-inhibitory activity.

Targeting MSP 1 using glycan mimetics

Initial interactions between the merozoite and the RBC membrane are low affinity, reversible and can occur irrespective of the parasite's orientation. These interactions are mediated by glycosylphosphatidyl inositol (GPI) anchored proteins present on the merozoite's surface (Holder *et al.* 1992; Gilson *et al.* 2006). MSP1 is the most abundant GPI anchored protein on the merozoite surface (Gilson *et al.* 2006) and the proteolytic cleavage of MSP1 to

Appendix I.

83 kDa, 30 kDa, 38 kDa and 42 kDa fragments is essential for RBC invasion (Blackman and Holder 1992). The N-terminal MSP1–83 fragment binds to the RBC receptor glycophorin A and the C-terminal MSP1–42 fragment has a role in binding to band-3 on the RBC surface. (Baldwin *et al.* 2015).

The 19 kDa C-terminal cysteine rich epidermal growth factor (EGF)-like domain of MSP-1 is formed after secondary cleavage of MSP1–42 and this essential proteolytic event has been investigated as a potential vaccine and drug target (Goel *et al.* 2003). Testing of EGF domain inhibitors with anticancer properties against MSP1–19 identified a small-molecule glycan mimetic, 2-butyl-5-chloro-3-(4-nitro-benzyl)-3H-imidazole-4-carbaldehyde (NIC), as a specific inhibitor of MSP1–19 function and parasite invasion (Fig. 2b, c) (Chandramohanadas *et al.* 2014). The invasion-inhibitory activity of NIC was confirmed using live filming of invasion and through the use of purified merozoites. NIC not only inhibited the growth of *P. falciparum* isolates, it also inhibited *P. falciparum* expressing *P. chabaudi* rodent malaria MSP1–19 and *P. vivax* field isolates, with IC_{50}s ~20 μM; indicating the pan-species potential of these molecules against malaria parasite invasion. The authors highlight the possibility of targeting the EGF domain of MSP1–19 using small molecule glycans that are being developed as anti-cancer agents (Fig. 2b) (Sugahara *et al.* 2012), but to date no examples of this have been published and further development of this strategy would be required before clinical applications could be assessed. A potential advantage of developing inhibitors that target MSPs, such as HLMs and glycan mimetics, is that they can target merozoites throughout their extracellular phase; post release from schizonts through to resealing of the parasitophorous vacuole membrane.

Small molecule inhibitors of the tight junction that forms between AMA 1 and the RON protein complex

After binding to the RBC and apical re-orientation, the invading merozoite releases proteins residing within specialised apical secretory organelles, the micronemes and rhoptries, to establish an irreversible zone of attachment called the tight junction (Aikawa *et al.* 1978; Bannister and Mitchell 1989). This tight junction is formed as a result of AMA1 (secreted from the micronemes) binding to the RBC bound rhoptry neck (RON) 2/4/5 (secreted from the rhoptries) protein complex (Alexander *et al.* 2006; Collins *et al.* 2009; Richard *et al.* 2010; Tonkin *et al.* 2011), with a known high affinity interaction demonstrated between AMA1 and RON2 (Srinivasan *et al.* 2011; Tonkin *et al.* 2011). The essential interaction between AMA1 and RON2 has been targeted by vaccine induced antibodies (Hodder, Crewther and Anders 2001; Kennedy *et al.* 2002), inhibitory peptides (Harris *et al.* 2005) and drug development (reviewed in Devine *et al.* 2017) (Fig. 2d). A phase 2b vaccine trial of children in Mali demonstrated high anti-AMA1 antibody titres and protection against clinical malaria caused by parasites harbouring vaccine-like alleles after 6 months, but there was minimal efficacy against clinical malaria overall, highlighting the difficulties with targeting a polymorphic antigen such as AMA1 (Thera *et al.* 2011). However, recent studies report substantial conservation of AMA1 function between species, providing evidence that generating cross-species inhibitory activity may be possible (Drew *et al.* 2018).

Several invasion-inhibitory peptides that target AMA1/RON2 binding have been developed. The 20-amino acid R1 peptide, identified from a random phage display library (Harris *et al.* 2005), exhibits high binding affinity for the 3D7 parasite line AMA1/RON2 complex (KD_{50} ~0.2 μM) (Harris *et al.* 2005). RON2L mimics a conserved peptide region of RON2 and competes with native RON2 for the hydrophobic binding pocket of AMA1, blocking formation of the tight junction and inhibiting RBC invasion (Srinivasan *et al.* 2011). Making use of the high binding affinity of peptides that block AMA1/RON2 interactions, a RON2L(peptide)/AMA1 binding inhibition assay was used to screen 21 733 small-molecule inhibitors for activity against AMA1/RON2 (Srinivasan *et al.* 2013), with three hits suggested to directly inhibit RBC invasion. Modification of the lead compound, NCHC00015280 (a pyrrolopyrimidine; IC_{50} 30 μM), yielded two analogues that showed a three (9.8 μM) and five (6 μM) fold improvement in invasion inhibition (Srinivasan *et al.* 2013). However, subsequent studies failed to show binding of these compounds to AMA1 with an affinity commensurate with their reported growth inhibitory activity (Devine *et al.* 2014; Pihan *et al.* 2015), leading Devine *et al.* (2014) to conclude that these compounds inhibited invasion through an AMA1/RON2 independent manner. Nevertheless, the essential role of the AMA1/RON2 complex for invasion, the availability of complete protein structures for *in silico* screening and optimisation makes inhibitors of AMA1/RON2 complex function an attractive target for further development.

The actin-myosin invasion motor as an invasion-inhibitory target

After formation of the tight junction, the actin-myosin motor is engaged and the RBC membrane is pulled around the merozoite via treadmilling of short actin filaments (F-actin) which are pulled unidirectionally. Invasion is powered by a myosin motor complex embedded in the merozoite's pellicle (inner membrane complex; Fig. 2e) (Soldati, Foth and Cowman 2004) (reviewed in Tardieux and Baum 2016). Given the importance and complexity of the actin-myosin motor, a number of targets have been investigated for antimalarial development.

Inhibitors of actin dynamics as invasion-inhibitory drugs

A number of natural agents, such as cytochalasins (a fungal alkaloid) and latrunculins (from marine sponges) have been reported to disrupt actin polymerisation dynamics and ultimately arrest RBC invasion (Fig. 2e) (Miller *et al.* 1979; Cooper 1987; Johnson *et al.* 2016). Latrunculins bind to actin's monomeric form (G-actin) near the Adenosine Triphosphate (ATP) binding site and prevent polymerisation to filamentous actin (F-actin). A recent study identified key amino acid differences between human and *Plasmodium* spp. actin within the ATP binding pocket and sought to synthesise truncated latrunculin B analogues with improved activity against *P. falciparum* malaria and reduced toxicity against mammalian cells (Johnson *et al.* 2016). Truncated latrunculin analogues achieved a 6-fold improved potency against *in vitro* parasite growth (to 7 μM) and 17-fold higher selectivity over mammalian cell cytotoxicity (Johnson *et al.* 2016). To address whether these analogues had activity directly against parasite invasion, the authors used *T. gondii* invasion inhibition assays since this related apicomplexan parasite shares an identical amino acid sequence around the actin ATP binding pocket as *P. falciparum* (Johnson *et al.* 2016). Lead latrunculin analogues showed a >5-fold improvement in invasion-inhibitory activity against *T. gondii* (to 16 μM), indicating that latrunculin analogues target parasite actin during invasion and that activity against apicomplexan parasites is likely to be conserved (Johnson *et al.*

2016). But the high IC_{50}s of these compounds against both parasites highlights that further development is needed.

Inhibitors of the myosin A/MTIP complex

Myosin A (MyoA) is the F-actin bound motor that powers apicomplexan gliding motility during invasion (Meissner, Schluter and Soldati 2002)(Fig. 2e). The ATP-powered protomotive movement of MyoA is dependent on a conserved complex between MyoA's C-terminal domain and the conserved N-terminal domain of MyoA tail interacting protein (MTIP, called myosin light chain 1 (MLC1) in *T. gondii*) (Bosch *et al.* 2006, 2007). Not only is the interaction between MyoA and MTIP essential for parasite invasion of the RBC, but structural characterisation has also identified distinct differences between *Plasmodium* spp. and human homologs, thus presenting a viable target for drug development (Bosch *et al.* 2006).

Modelling of the interaction between MTIP and a growth inhibitory 15-amino acid C-terminal MyoA peptide (Bosch *et al.* 2006) was used to identify small molecule MTIP/MyoA binding inhibitors in a library of 300 000 compounds (Kortagere 2010). A pyrazole-urea based compound (C416) demonstrated the best growth inhibitory activity (IC_{50} of 0.145 μM) (Kortagere 2010) and further structure-based screening identified several analogues with improved activity over the original peptide (C2–1 IC_{50} 0.047 μM; C3–21 IC_{50} 0.385 μM). C3–21 was investigated further and was found to inhibit gliding motility of mosquito stage sporozoites, a marker assay for actin-myosin based motor function that is shared between sporozoites and RBC invading merozoites (Kortagere 2010). Comparative analysis identified that some compounds inhibited growth of both *P. falciparum* and *T. gondii*, thus suggesting a conserved target between the two divergent parasites. However, many analogues also showed variation in efficacy between *P. falciparum* and *T. gondii*, suggesting structural differences between the MyoA/MTIP interaction can be sufficient to reduce efficacy against different apicomplexan parasites (Kortagere 2010; Kortagere *et al.* 2011).

Another high-throughput screen of 12,160 non-cytotoxic compounds against *T. gondii* tachyzoite host cell invasion identified 21 compounds that inhibited parasite motility (Carey *et al.* 2004). One of these hits, tachyplegin A, was found to covalently bind to TgMLC1 (MTIP in *Plasmodium* spp.) and the resulting post-translational modifications caused loss of MyoA function and inability to drive the invasion motor (Carey *et al.* 2004; Heaslip 2010; Leung *et al.* 2014). These studies have identified a diverse range of chemical scaffolds that inhibit function of the MTIP/MyoA driven invasion motor, a conserved complex that is essential for RBC invasion of apicomplexan parasites.

Inhibitors of protease cleavage events required for RBC invasion

Invasion requires a co-ordinated series of proteolytic cleavage events to enable the correct function of essential proteins. The essential role of serine proteases in schizont rupture and RBC invasion have seen them become a significant target of invasion inhibitor drug development (reviewed in O'Donnell and Blackman 2005). The *P. falciparum* subtilisin proteases PfSUB1 (Blackman *et al.* 1998; Yeoh *et al.* 2007) and PfSUB2 are bacterial-like enzymes that have received significant interest because of the key role they play in the essential processing of proteins required for RBC invasion (Figs. 1 and 2) (Supplementary Table S1). PfSUB1 has been shown to cleave MSPs (MSP1, MSP6 and MSP7), invasion ligands released from the micronemes (AMA1) and rhoptry (RAP1) (Yeoh *et al.* 2007; Koussis *et al.* 2009; Silmon de Monerri *et al.* 2011) and is involved in priming the proteolytic cascade that leads to schizont rupture and merozoite egress (Fig. 1c)(Yeoh *et al.* 2007). Comparison of the stage-specific efficacy of the PfSUB1 inhibitor MRT12113 indicates that the IC_{50} against *P. falciparum in vitro* invasion inhibition (~25 μM) was lower than that for schizont rupture inhibition (~180 μM) (Yeoh *et al.* 2007), highlighting the potential sensitivity of the invasion process to chemical inhibition compared to other stages of blood stage development. Interestingly, a follow-up study identified that even partial inhibition of MSP1 processing at invasion inhibitory concentrations of MRT12113 was associated with invasion inhibition, indicating the sensitivity of the invasion process to chemical inhibition (Koussis *et al.* 2009). More recent studies have begun to optimise inhibitors of PfSUB1 from a range of chemical scaffolds (Gemma *et al.* 2012; Bouillon *et al.* 2013; Giovani *et al.* 2014; Kher *et al.* 2014). *Plasmodium* spp. SUB1 are highly conserved and trials using recombinant proteins suggest that 'pan-species' inhibitors can be developed that target the SUB1 of multiple species (Withers-Martinez *et al.* 2012). Indeed, an *in silico* screen using a 3D homology model of PvSUB1 led to the discovery of Cpd2, a compound that inhibits the activity of both recombinant PvSUB1 and PfSUB1 (Bouillon *et al.* 2013). Furthermore, Cpd2 had an *in vitro* IC_{50} of 0.37 μM against *P. falciparum* parasites and inhibited growth of *P. berghei* rodent malaria parasites in a dose-dependent manner, highlighting the pan-species potential of SUB1 inhibitors (Bouillon *et al.* 2013).

Recently its been demonstrated that the aspartic proteases Plasmepsin IX and X (Nasamu *et al.* 2017; Pino *et al.* 2017) have key roles in RBC invasion (Fig. 2d, f) and invasion/egress (Fig. 1b, c), respectively. Plasmepsin IX is located in the rhoptry organelle in merozoites and loss of this protease causes aberrant rhoptry formation and prevents cleavage of key invasion ligands (Nasamu *et al.* 2017; Pino *et al.* 2017). Plasmepsin X is located in merozoite exonemes (secreted from the merozoite prior to rupture) and is involved in activating SUB1 (essential for invasion and schizont rupture), as well as directly processing ligands excreted from the microneme (Nasamu *et al.* 2017; Pino *et al.* 2017). The activity of Plasmepsin IX and X was effectively inhibited by the hydroxylethylamine aspartic protease inhibitor 49c (Ciana *et al.* 2013) at low nanomolar concentrations, providing evidence that both essential proteases can be targeted by one drug (Pino *et al.* 2017). Furthermore, 49c was effective in a *P. berghei* rodent model of malaria against multiple life-cycle stages, including liver stage parasites and gametocytes (Pino *et al.* 2017). Recombinant Plasmepsin X was found to be inhibited by the orally bioavailable aminohydantoins (Meyers *et al.* 2014; Nasamu *et al.* 2017). Further investigation revealed the aminohydantoins inhibited *P. falciparum* growth *in vitro* at submicromolar concentrations and growth of *P. chabaudi* rodent malaria parasites *in vivo*, providing a second starting point for drug development against Plasmepsin X (Meyers *et al.* 2014; Nasamu *et al.* 2017). Since 49c is a potent inhibitor of Plasmepsin IX (rhoptry biogenesis and invasion ligand processing) and both 49c/aminohydantoins inhibit Plasmepsin X function (activation of the egress/invasion priming PfSUB1, invasion ligand processing), both chemical starting points offer activity against merozoite egress and invasion (Nasamu *et al.* 2017; Pino *et al.* 2017).

Inhibitors of malaria merozoite intracellular signalling

The targeting of cellular signal transduction pathways has successfully been used to treat non-infectious diseases such as cancer and autoimmune diseases (Aggarwal *et al.* 2007; Croce

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et al. 2016; Marciano and Holland 2017) and is now of increasing interest for antimalarial development. One leading drug target involved in signalling during RBC invasion is calcium dependent protein kinase 1 (CDPK1), a parasite kinase not present in the human host (Harper and Harmon 2005) that has key roles in microneme secretion, activation of the actin-myosin motor and other processes required for RBC invasion (Fig. 2b-e) (Green et al. 2008; Bansal et al. 2013; Bansal et al. 2018). PfCDPK1 has been targeted in several high throughput screens (HTS) of compound libraries (Green et al. 2008; Kato et al. 2008; Lemercier et al. 2009; Chapman et al. 2013; Ansell et al. 2014; Chapman et al. 2014). 2,6,9 trisubstituted purines such as purfalcamine inhibited *P. falciparum* parasite growth (IC_{50} of 230 nM) as well as host cell invasion of related *T. gondii* tachyzoites, consistent with a CDPK in *T. gondii* having a key role in invasion (Kato et al. 2008; Lourido et al. 2010; Kumar et al. 2017). However, purfalcamine was unsuccessful in clearing *P. yoelii* rodent malaria parasites *in vivo*, possibly due to poor pharmacokinetics and reduced efficacy against PyCDPK1 (Kato et al. 2008). A second screen identified 3,6-disubstituted imidazopyridazines as compounds of interest, with modification of early leads reducing the growth inhibitory IC_{50} to < 100 nM and providing superior pharmacokinetics to the initial hit compounds (Chapman et al. 2013; Chapman et al. 2014). However, *in vivo* efficacy against *P. berghei* rodent malaria parasites was again limited (Chapman et al. 2013; Chapman et al. 2014). Further investigation revealed that a number of optimised imidazopyridazine PfCDPK1 inhibitors were more likely to be targeting *P. falciparum* cGMP dependent protein kinase G (PfPKG; Green et al. 2015). Based on conflicting evidence for whether PfCDPK1 is essential for blood stage parasite growth, the limited efficacy *in vivo* and variable specificity of inhibitors, it has been suggested that PfCDPK1 may not be suitable for blood stage drug development (Green et al. 2015; Bansal et al. 2018)(reviewed in Cabrera et al. 2018).

PfPKG has also been a focus for antimalarial development since it is expressed in multiple stages of the lifecycle and has different activation properties to mammalian kinases (McRobert et al. 2008; Alam et al. 2015; Govindasamy et al. 2016). Inhibitors of PfPKG are potent inhibitors of merozoite egress from the developed schizont and are being developed as antimalarials (Taylor et al. 2010). Studies have also identified that inhibition of PfPKG blocks invasion of mechanically released merozoites, with speculation that this invasion-inhibitory activity is due to preventing discharge of the invasion priming protease PfSUB1 (Fig. 1b, c) and interfering with phosphorylation of proteins thought to have a role in invasion including PfCDPK1 and invasion motor components (Fig. 2b-e) (Collins et al. 2013; Alam et al. 2015; Das et al. 2015). Recently, a highly potent series of compounds were developed based on an imidazopyridine inhibitor of PKG used for treatment of the apicomplexan parasite *Eimeria tenalla* in chickens. The most active of these, ML10, had an IC_{50} of 2 nM against *P. falciparum* parasite growth *in vitro*. ML10 was also highly efficacious in a *P. chabaudi* rodent model of malaria, with twice daily doses of 100 mg/kg for 4 days reducing parasitemia to undetectable levels in a *P. falciparum* humanized mouse model of malaria (a promising outcome for development of a PKG inhibitor for inclusion in combination therapies.

Development of inhibitors against cAMP-dependent protein kinase A (PKA), which has key roles in microneme secretion (Dawn et al. 2014), phosphorylation of the functional domain of AMA1 (Leykauf, 2010) and activation of the actin-myosin motor (Lasonder et al. 2012), has been less successful. General inhibitors of PKA, such as H89 and KT5720, and its messenger molecule cAMP have been used as biological tools to block PfPKA and to study its function, but these compounds have low potency (IC_{50} typically > 1 μM) (Syin et al. 2001; Beraldo et al. 2005; Leykauf et al. 2010; Salazar et al. 2012) and we are currently unaware of any compounds that have been optimized for activity against PfPKA and cAMP (Buskes et al. 2016; Cabrera et al. 2018).

An alternative strategy to inhibit invasion is to target 3',5'-cyclic nucleotide phosphodiesterases (PDEs) which regulate degradation of cAMP and cGMP into AMP and GMP, respectively. Increased cAMP and cGMP leads to activation of PKA and PKG, respectively, making PDEs significant regulators of signalling during egress and invasion (Fig. 2c) (Collins et al. 2013; Baker et al. 2017). Screening of human PDE inhibitors identified zaprinast (growth inhibitory IC_{50} of 35 μM) (Yuasa et al. 2005) and a pyrazolopyrimidinone, termed BIPPO, (growth IC_{50} of 0.4 μM) (Howard et al. 2015) as having activity against PfPDEα, an isoform that specifically inhibits cGMP. Since PfPDEα has been demonstrated to be dispensable to blood stage parasite growth (Wentzinger et al. 2008) and treatment with BIPPO causes activation of cAMP (PfPDEβ) and cGMP (PfPDEα) dependent pathways, it has been suggested that BIPPO may inhibit multiple PfPDE isoforms due to conservation in active sites (Wentzinger et al. 2008; Howard et al. 2015). Modelling suggests that there are key similarities between human, *Plasmodium* and *Toxoplasma* PDE orthologues that would support this cross-reactivity (Howard et al. 2015). Indeed, BIPPO retains activity against isoforms of human PDEs, including PDE9 (IC_{50} = 30 nM), and selectivity for *Plasmodium* PDEs would need to be greatly improved before this PDE inhibitor could be developed as an antimalarial (Howard et al. 2015).

Although a number of kinases with key roles in RBC invasion have been identified, the development of effective inhibitors against these signalling molecules is still a work in progress with improvements in specificity and potency required for many early leads. However, the feasibility of targeting signalling effector molecules can be demonstrated by recent efforts to develop inhibitors against phosphatidylinositol 4-kinase (PI4K), a key enzyme in protein trafficking required across multiple lifecycle stages, including merozoite development (McNamara et al. 2013). This has led to the 2-aminopyrazine compound UCT943 being taken forward into pre-clinical development (Brunschwig et al. 2018).

Invasion inhibitory starting points originating from diverse or focussed drug libraries

HTS of small molecule or compound libraries have been used extensively to identify growth inhibitory compounds of the asexual blood stages of *P. falciparum* malaria. However, relatively few screens have been directly designed to identify inhibitors of RBC invasion. Medicines for Malaria Venture (MMV) released a 400-compound library in 2011, termed the Malaria Box, which contains a diverse set of compounds that display antimalarial properties (Spangenberg et al. 2013). Subramanian et al. (2018) recently screened the Malaria Box for activity against *P. falciparum* blood stage egress and merozoite invasion inhibitors (Subramanian et al. 2018) and identified 11 out of 26 hits that inhibited the schizont to ring stage transition at an IC_{50} of <500 nM. Upon microscopic examination of blood smears, MMV665878 and MMV006429 treated schizonts ruptured normally, but free merozoites were found attached to RBCs and few successful invasion events were evident, a phenotype typical of invasion inhibitors (Weiss et al. 2015). Further testing

revealed that MMV665878 and MMV006429 were potent invasion inhibitors with up to 50% of invasion events inhibited at concentrations down to 300 nM in assays using purified merozoites (Subramanian *et al.* 2018). Of the 26 compounds identified in this screen, 10 of them have been characterised as inhibitors of PfATP4, a sodium efflux pump on the parasite plasma membrane, indicating either PfATP4 is involved in egress and invasion, or that the compounds have targets additional to PfATP4 (Lehane *et al.* 2014; Subramanian *et al.* 2018).

Screens of the related Apicomplexan parasite *T. gondii* have opened up new starting points for invasion-inhibitory drug development against apicomplexan parasites. *T. gondii in vitro* motility and invasion assays were used as secondary screens against a library of 527 putative kinase inhibitors (Kamau *et al.* 2012). Of the 14 lead compounds with growth inhibitory or enhancing affects, compounds C5 (IC_{50} 1.82 μM) and C1 (IC_{50} 1.36 μM) were found to irreversibly inhibit motility or motility and invasion, respectively. A second study using a fluorescence microscopy based assay screened 1222 covalent inhibitors directly for inhibition of *T. gondii* tachyzoite attachment and invasion, identifying 5 invasion-inhibitory compounds. The leading compound, WRR-086, demonstrated low micromolar (IC_{50} of 5.7 μM) invasion-inhibitory activity. Biochemical and genetic analysis identified a homologue of human DJ-1 (TgDJ-1) as the target of WRR-086, with inhibition of TgDJ-1 linked to loss of microneme secretion and failure to invade (Hall *et al.* 2011). Screening compounds for their invasion-inhibitory activity is providing starting points for the development of drugs with novel mechanisms of action and uncovering new insights into invasion biology of apicomplexan parasites.

The clinically used antibiotic azithromycin as an inhibitor of RBC invasion

The majority of compounds identified that have invasion-inhibitory activity against malaria parasites have no record of clinical use. Recent identification of the invasion-inhibitory activity of the antibiotic azithromycin (Wilson *et al.* 2015) marks one of the few clinically used compounds that have been shown to inhibit *Plasmodium* spp. invasion of RBCs. Macrolide antibiotics are known to target the malaria parasite's remnant plastid (the apicoplast) 70S bacteria-like ribosomal complex (Sidhu *et al.* 2007; Goodman *et al.* 2013). Inhibition of the apicoplast ribosome prevents replication of this essential organelle, resulting in the loss of isoprenoid pyrophosphate (IPP) precursor synthesis and parasite death a full two cycles of growth post treatment (termed delayed death) (Dahl and Rosenthal 2007; Goodman, Su and McFadden 2007). Despite the limitations of a slow killing antimalarial for treatment of disease, azithromycin's safe clinical profile and long half-life (>50 hours) has led to the antibiotic being trialled as a partner drug in artemisinin combination therapies (Cook *et al.* 2006; Sykes *et al.* 2009).

Recently it was found that azithromycin could rapidly inhibit RBC invasion *in vitro* (IC_{50}, 10 μM, in ethanol), which is independent of apicoplast-targeted delayed death (IC_{50}, 0.04 μM, in ethanol) activity (Wilson *et al.* 2015). Although the speed of azithromycin's invasion-inhibitory activity for an otherwise slow acting drug provides a new avenue to develop the drug as an antimalarial, the requirement for a 250-fold higher concentration of azithromycin needed to inhibit invasion currently prevents clinical use of this drug as an invasion inhibitor. However, of note is the identification of several analogues that show >5-fold improvement in invasion-inhibitory activity (Wilson *et al.* 2015, Burns *et al.* Unpublished Data), indicating that improved invasion-inhibitory potency is achievable. Importantly, the most invasion-inhibitory azithromycin analogues also exhibit improved activity against short-term blood stage parasite development and retain activity against the apicoplast, suggesting that azithromycin can be developed to have both fast acting (RBC invasion inhibition and short-term parasite growth inhibition) and apicoplast-targeting delayed death properties (Wilson *et al.* 2015, Burns *et al.* Unpublished Data). Given azithromycin's history of safety, proven activity, long half-life (>50 hours), availability of modified analogues and ease of modification, the identification of azithromycin's invasion-inhibitory activity opens up an attractive starting point to develop an invasion-inhibitory antimalarial with dual-mechanisms of action.

INVASION INHIBITORS IN COMBINATION THERAPIES

A focus of antimalarial development for treatment of clinical disease is on single-dose drug combinations that act broadly across blood-stage development to quickly kill parasites (Burrows *et al.* 2017). As a standalone drug, it is unrealistic to expect an antimalarial which only targets invasion will eliminate all parasites within a matter of hours. However, such a drug could be of benefit in a combination therapy and, as demonstrated in this review, many invasion inhibitors have activity against other lifecycle stages. Combination therapies that have two (or more) safe and efficacious drugs with different mechanisms of action have significant potential advantages, including reducing the risk of developing drug resistance (Hastings 2011). Importantly, the reported mechanisms of action for invasion inhibitors developed to date are not involved in the mechanisms of action of existing antimalarials, limiting the likelihood of cross-resistance.

Evidence from rodent models of malaria suggest that antimalarial monotherapy using drugs that target intracellular parasite growth may not prevent all parasites from progressing through to the next cycle (Khoury *et al.* 2017). Failure to rapidly inhibit blood stage replication may increase the risk of selecting for drug resistance and lead to higher numbers of mosquito transmissible gametocytes posttreatment, thereby contributing to transmission. Targeting invasion directly, the first step in blood stage parasite growth, in a combination therapy would immediately stop progression of parasites into the next cycle of growth, thus limiting opportunities for drug resistance to develop and reducing the number of new gametocytes.

Combining a drug that targets intracellular parasite development (timing of action of current antimalarials) with one that inhibits RBC invasion (extracellular) has intrinsic appeal as targeting these two developmental stages could facilitate rapid clearance of disease causing blood stage parasites and increase drug efficacy. Evidence from monotherapy drug efficacy studies of severe malaria patients suggests that rapid parasite clearance after treatment results in reduced mortality (Dondorp *et al.* 2005). Complicating the speed of parasite clearance, studies have highlighted that a number of clinically used antimalarials have reduced efficacy as malaria parasites transition from mature schizonts, through invasion and into newly established ring stage infections (Painter, Morrisey and Vaidya 2010; Wilson *et al.* 2013; Dogovski *et al.* 2015; Khoury *et al.* 2017). Since each surviving *P. falciparum* schizont is capable of releasing 16–32 new RBC

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invading merozoites, providing additional cover through a drug combination that includes a potent invasion inhibitor has the potential to fast-track parasite clearance.

Since *P. falciparum* invasion occurs roughly every 48 hours, this raises the question as to whether clinical treatment with a drug that has a short half-life risks being ineffective across one growth cycle if administered temporally distant from the next period of rupture and invasion. Studies evaluating circulating and sequestered populations of parasites in infected subjects have generally found a wide developmental age range for parasite populations at the time of sampling; predominantly young parasites in peripheral blood and mostly mature stages for parasites sequestered in capillaries (but younger parasites can also be at high levels) in cerebral malaria, non-cerebral malaria and placental malaria cases (MacPherson *et al.* 1985; Oo *et al.* 1987; Silamut *et al.* 1999; Beeson *et al.* 2002; Pongponratn *et al.* 2003). These studies indicate that there is limited parasite synchronicity *in vivo* and it is likely that invasion inhibitors will encounter invading merozoites soon after administration and regularly across the next 48 hours.

In terms of ideal drug properties, invasion inhibitors should have: (i) a half-life that allows the drug to be maintained at effective concentrations with a dosing regimen no more frequent than daily, and (ii) an effective concentration well below that of each dose, allowing inhibitory concentrations of drug to be available over a time period equivalent to many cycles of parasite invasion and growth. As demonstrated by the clinical use of the HIV entry inhibitor maraviroc (half-life ~16 hours; Perry 2010), maintaining drug concentrations to inhibit pathogen host cell entry over several replication cycles is clinically achievable. In terms of clinically used compounds with invasion-inhibitory antimalarial activity, the half-life is known for azithromycin (>50 hours) and several HLMs (Heparin < 1 hour, sevuparin ~1 hour, curdlan sulfate ~2–3 hours and fondaparinux 17–21 hours), and the half-life will need to be a consideration for any invasion inhibitors with higher potency.

Drug resistance models suggest that the increased selective window (when drug levels fall below the minimal inhibitory concentration) of long-lasting drugs can potentially increase drug resistance selection pressure posttreatment (Stepniewska and White 2008; Kay and Hastings 2015). In contrast, drugs with a short half-life, such as the artemisinins, have a much shorter selective window and are considered less likely to select for resistance due to minimal parasite exposure to sub-inhibitory concentrations (Stepniewska and White 2008); but more frequent dosing is required to maintain treatment efficacy. Therefore, there are several important considerations in selecting ideal drug combinations with the potential impacts on clinical efficacy, reducing the risk of drug resistance and reducing transmission all needing to be assessed for combination therapies that include an invasion inhibitor. Despite the potential benefits of having an invasion-inhibitory drug in antimalarial combination therapies, the therapeutic efficacy of a drug combination featuring both an invasion inhibitor and an intracellular blood stage growth inhibitor *in vitro* or *in vivo* has yet to be assessed directly. Thus, future studies will need to assess the potential synergies of using an invasion inhibitor in a combination therapy as well as model the therapeutic and resistance-proofing benefits of doing so.

CONCLUSION

Targeting RBC invasion is a promising antimalarial drug development strategy because: (i) extracellular parasites are exposed directly to drugs in the bloodstream, (ii) most parasite proteins required for invasion lack human equivalents, offering possibilities for selective inhibition and (iii) blocking invasion immediately stops multiplication of disease causing blood stage parasites. Inhibition of host cell entry is a validated strategy for HIV combination therapies (Kilby *et al.* 1998; Dorr *et al.* 2005) and the predicted viral generation time of HIV (≥48 hours; Dixit *et al.* 2004; Murray, Kelleher and Cooper 2011; Puller, Neher and Albert 2017) is similar to the blood stage lifecycle of *P. falciparum*. Therefore, the clinical use of HIV entry inhibitors provides a proof-of-concept that inhibitors of RBC invasion can have a role in antimalarial combination therapies.

The targets of invasion-inhibitory antimalarials under development are mostly essential, conserved and non-redundant (i.e. Yeoh *et al.* 2007; Boyle *et al.* 2010; Kortagere 2010; Wilson *et al.* 2015; Pino *et al.* 2017). The conservation evident in key, drug targetable, invasion machinery between malaria isolates, different *Plasmodium* spp. and different lifecycle stages (i.e. sporozoite invasion) is leading to the development of pan-invasion inhibitors. Therapeutic inhibition of invasion is likely to have profound effects on parasite viability since the merozoite has a short half-life and failure to invade immediately ends parasite growth and multiplication. This would mitigate the risk that parasites develop drug tolerance and persist as is the case for artemisinin resistance. Merozoite invasion may be more sensitive to treatment than other intracellular RBC stages, as demonstrated by a lower IC_{50} for invasion inhibition (~25 μM) than achieved for rupture inhibition (~180 μM) for the PfSUB1 inhibitor MRT121113 (Yeoh *et al.* 2007). Therefore, improving a drug's activity against the process of merozoite invasion could have a significant impact on parasite clearance and clinical effectiveness. To date, a number of diverse chemotypes with different targets have been identified to inhibit RBC invasion (Supplementary Table S1), but there is tremendous scope to develop new inhibitors of this essential step in parasite growth for use in combination therapies. The search for new invasion-inhibitory targets is helped by the availability of published mature schizont stage proteomic and phosphoproteomic resources (www.plasmodb.org) that highlight potential merozoite specific therapeutic targets for assessment (Solyakov *et al.* 2011; Lasonder *et al.* 2015). Encouragingly, the development of specific assays to quantify the invasion-inhibitory activity of compounds (Wilson *et al.* 2013; Wilson *et al.* 2015; Weiss, Crabb and Gilson 2016) has led to the identification of new chemical scaffolds that inhibit invasion.

Although several compounds with invasion-inhibitory activity have achieved promising levels of potency *in vitro* and *in vivo* (Gemma *et al.* 2012; Bouillon *et al.* 2013; Giovani *et al.* 2014; Kher *et al.* 2014; Meyers *et al.* 2014; Nasamu *et al.* 2017; Pino *et al.* 2017), an important way forward for invasion inhibitor development is to optimise additional compounds with activity in the low nanomolar range to fast-track further development options. Another future research priority is the evaluation of invasion-inhibitory compounds in combination with currently used and new emerging therapeutics that target intraerythrocytic parasite development, including artemisinin. Such studies would better define the properties and timing of action of drugs to be used in optimal combinations. While proof-of-concept for invasion inhibitors has been demonstrated in animal models, further *in vivo* studies are needed to better define the therapeutic potential of the different inhibitor classes alone and in combination. Incorporation of mathematical modelling, as is increasingly being used in drug evaluation and clinical trials, to assess

the ideal properties of invasion inhibitors in combination therapies *in vivo* would be particularly valuable for informing development priorities.

Combining an invasion inhibitor with artemisinin or a similar drug that acts broadly across malaria's blood stages would provide complete drug coverage across this disease causing stage of the lifecycle. Despite the potential to identify potent, specific and broad acting antimalarials targeting invasion, the discovery and development of drugs that act against this essential and exposed step in blood stage replication has been limited. The recent identification of numerous promising drug leads and targets, combined with improved merozoite purification methods and screening strategies, has revealed promising new avenues for the development of next-generation therapeutics for malaria.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSRE* online

Authorship contributions

DWW, MJB, TdKW, PRG and JGB conceived the idea of this review; ALB, MGD MJB, JMB and DWW conducted the literature review and wrote the manuscript. JMB created the figures. All authors contributed to the design, content and editing of this manuscript.

ACKNOWLEDGMENT

JGB is a member of the NHMRC Australian Centre for Research Excellence in Malaria Elimination.

FUNDING

DW received funding from the NHMRC (Fellowship APP1035715, Project Grant APP1143974) and University of Adelaide Beacon Fellowship. MJB received funding from NHMRC (Career Development Fellowship APP1141632 and Project Grant APP112656), and JGB (1077636) and TdKW (1136300) were supported by NHMRC Senior Research Fellowships. ALB, MGD and JMB recieved support from ARC postgraduate scholarships.

Conflict of interest. None declared.

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Appendix I.

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Appendix I S. Table 1 Summary of invasion inhibitor targets and chemotype development covered in Burns et al.. (2019).

Probable Stage [a]	**Proposed process** [b]	**Proposed target**	**Inhibitor**	**Growth / invasion IC_{50} (μM)**	**Reference**
Egressed merozoites	Initial attachment and apical tip reorientation	MSP1	Heparin-like-molecules (HLMs)	19	(517, 700)
		MSP1-19	Glycan mimetic (NIC)	~ 20	(701)
Commitment to invasion	Tight junction formation	AMA1/RON2 interaction	NCGC00262654	6*	(702)
		Unknown	Azithromycin	10*	(165)
Internalising merozoite	Actin-myosin motor	F-actin polymerization	Latrunculin B analogue 15	7 19* (*T. gondii*)	(703)
		MyoA/MTIP interaction	pyrazoleamide (C2-1)	0.047	(704)
		MyoA/MTIP interaction	C3-21	~0.5 (*T. gondii*)	(705)
		TgMLC1 (MTIP)	Tachyplegin A	<100* (*T. gondii*)	(706-708)
Merozoite development and egress	Processing of early invasion ligands	Pf-Subtilisin 1	MRT12113	25*	(692)
		Pf-Subtilisin 1	Quinolylhydrazone(s)	20	(709)
		Pf-Subtilisin 1	Peptidic α-ketoamides	0.9	(693)
		Pf-Subtilisin 1	Difluorostatone-based inhibitors	0.6	(710)
		Pf-Subtilisin 1	E683-0109 (Cpd2)	0.37	(711)

Appendix I.

		Plasmepsin IX and X	Hydroxylethylamine (49c)	0.0006	(361)
		Plasmepsin IX and X	aminohydantoins	<0.5	(362, 712)
Merozoite development, egress and invasion	Intracellular signalling	PfCDPK1	Purfalcamine	0.25	(713)
		PfPKG	ML10	0.002	(714)
		PfPKG and/or PfCDPK1	Imidazopyridazines	0.1	(715, 716)
		PfPDE	BIPPO	0.15	(717)
Not identified	Na^+ and pH homeostasis	PfATP4	MMV665878*	~0.3	(310)
		PfATP4	MMV006429*	~0.3	(310)
Inhibition of host cell attachment and invasion	Inhibition of conoid extension, microneme secretion and motility	Unknown	Inhibitors: 5, 6, 7, 8 & 9	<12.5* (*T. gondii*)	(706)
Tachyzoite development and invasion	Unknown	Kinase	C1	1.36* (*T. gondii*)	(718)
Host cell attachment and invasion	Inhibition of microneme secretion and motility	TgDJ-1	WRR-086	5.7* (*T. gondii*)	(719)

[a] Describes the development stage during which the drug is thought to act. [b] Describes the protein/organelle/process that the drug is proposed to inhibit. * IC_{50} (μM) for direct invasion inhibition or schizont treatment.