

https://dspace.org/

The effect of 7-chloroquinoline derivatives on the life cycle of the malaria parasite

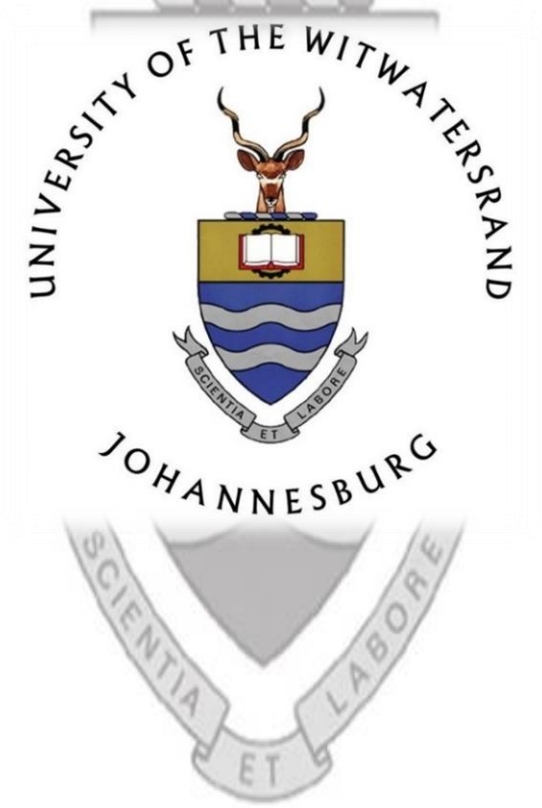
Kathrada, Fatima

2017
https://hdl.handle.net/10539/25350

Downloaded from a DSpace repository. https://dspace.org/

The effect of 7-chloroquinoline derivatives on the life cycle of the malaria parasite

Fatima Kathrada



A dissertation submitted to the Faculty of Health Sciences, University of Witwatersrand, Johannesburg, in the fulfilment of the requirements for the degree of Master of Science in Medicine.

2017

DECLARATION

I, Fatima Kathrada, declare that this dissertation is my own work. It is being submitted for the degree of Masters of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

_____

Fatima Kathrada

Signed at _____ on this _____day of _____ 2017.

DEDICATIONS



I dedicate this work to my parents and family for contributing selflessly to my development and achievements.

To my husband, Mohammed, your unconditional love, support and encouragement has carried me throughout this journey.

To all those who have encouraged me to fly towards my dreams.

To my Creator, without whom none of this would be possible.

'The cure for ignorance is to question'

Prophet Muhammed (PBUH)

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Prof. Robyn L van Zyl, for sharing her vast knowledge, expertise, time, immense guidance and patience with me through this journey. I would also like to thank my co-supervisor, Prof. Maureen Coetzee for her valuable contributions in my vector studies.

I would sincerely like to thank Ms N.J. Van Vuuren, Mr C.T Chen and Mrs N. Shunmoogum-Gounden for their patience, guidance and assistance in the laboratory. A very big thank you to my laboratory partner, Mrs Mustapha, for her support and discussions over tea.

I would like to thank the departmental staff, there are too many to mention, for their discussions and ongoing motivation.

I would like to thank Dr Shune Oliver for her help and assistance with vector work and providing the larvae for my study.

I would like to acknowledge Prof Amir Azam for providing my compounds.

I would like to thank the University of Witwatersrand, the NRF and FRC for financial support.

ABSTRACT

With the widespread resistance to current antimalarials and the great burden of malaria, the aim of this study was to determine the antimalarial activity of novel derivatives against the *Plasmodium falciparum* NF54 strain alone and in combination with quinine using the pLDH assay. The derivatives were designed such that they maintained the core chloroquinoline structure with known antimalarial activity with the addition of further scaffolds with a different mechanism of action in an attempt to produce compounds with good antimalarial activity with a lower probability of developing resistance.

The twenty-seven 7-chloroquinolin-4-yl piperazine-1-yl acetamide (CQPA) derivatives and the nine 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone (CQPPM) derivatives all possessed antimalarial activity with 4 and 8 derivatives from each class respectively showed inhibitory activity below 10 µM. The most active derivative from each set of derivatives, CQPA-**26** and CQPPM-**9** displayed good antimalarial activity with IC_{50} values of 1.29 µM and 1.42 µM, respectively compared to the standard antimalarial, quinine (IC_{50}: 0.18 µM). The two most active derivatives displayed drug-like properties with favourable ionisation properties to confirm its ability to accumulate in the parasites' digestive vacuole. In combination with quinine, these derivatives displayed synergistic and additive interactions, respectively. Morphological studies were carried out over a period of 48 hours to identify which parasite stage was most sensitive to the most active derivative from each class of derivatives. Both derivatives proved to show similar schizonticidal activity as the standard quinine with a lag in progression from the trophozoite stage. The inability to induce haemolysis indicated that the derivatives acted against the parasite and not the host red blood cell (<1% lysis), conferring a good safety profile. The low toxic nature of the derivatives was further demonstrated with the minimal toxicity towards human embryonic kidney epithelial cells and no effect on *Artemia fransiscana* brine shrimp viability (<5% lethality). Although the cyclopropyl and methyl substituted derivatives displayed good activity towards the intra-erythrocytic parasite, all the derivatives lacked larvicidal activity towards *Anopheles arabiensis* (mortality range: 0 - 5%). The derivatives showed no effect on the morphological structure of the *Artemia fransiscana* brine shrimp and the *An. arabiensis* larvae.

This study highlights the good antimalarial activity of these derivatives coupled with a favourable safety profile as antimalarial agents and not larvicides. The introduction of less bulky side chains proved to show a greater antimalarial activity against *Plasmodium falciparum*.

PUBLICATIONS

Publications- full papers

- **Kathrada, F**., Hayat, F., Inam, A., van Zyl, R.L., Coetzee, M., Shin, D., Azam, A., 2017, 'New antiprotozoal agents: Synthesis and biological evaluation of different 4-(7-chloroquinolin-4-yl) piperazin-1-yl)pyrrolidin-2-yl)methanone hybrids', *Bioorganic & Medicinal Chemistry* 27, 460-465

  Contribution made to the publication:

  Conducted all antimalarial (parasite lactate dehydrogenase assay), cytotoxicity (haemolyis and cell viability assay), larvicidal activity, lipid peroxidation experiments, data analysis, interpretation, wrote up methods and contributed to discussion.

- **Kathrada, F**., van Zyl, R.L., Coetzee, M., Azam, A., '7-Chloroquinolin-4-yl piperazine-1-yl acetamide hybrids as novel antiamoebic and antiplasmodial activity', to be submitted to *Bioorganic & Medicinal Chemistry* for publication, 2017

  Contribution made to the publication:

  Conducted all antimalarial (parasite lactate dehydrogenase assay, combination studies) and cytotoxicity (haemolysis, cell viability, brine shrimp lethality assays), larvicidal activity and stage-determination experiments, data analysis, interpretation, wrote up methods and contributed to discussion

Published congress abstracts

- **Kathrada, F**., van Zyl, R.L., Coetzee, M., Oliver, S., Inam, A., Azam, A., 2015, 'The effect of novel 4-(7-chloroquinolin-4-yl) piperazin-1-yl)pyrrolidin-2-yl)methanone derivatives on the life cycle of the malaria parasite', *South African Journal of Infectious Diseases* 30(1), 34

CONFERENCES

Conferences (presented)

- Kathrada, F., van Zyl, R.L., Coetzee, M., Olivier, S., Inam, A., Azam, A. 2015.

  Poster presentation: 'The effect of novel 4-(7-chloroquinolin-4-yl) piperazin-1-yl) pyrrolidin-2-yl) methanone derivatives on the lifecycle of the malaria parasite' WITS School of Therapeutic Sciences Research Day, Parktown, South Africa, 8 September 2015. **Awarded best poster prize in School of Therapeutic Sciences**

- Kathrada, F., van Zyl, R.L., Coetzee, M., Olivier, S., Inam, A., Azam, A. 2015.

  Poster presentation: 'The effect of novel 4-(7-chloroquinolin-4-yl) piperazin-1-yl) pyrrolidin-2-yl) methanone derivatives on the lifecycle of the malaria parasite'. SABCP-TOXSA Congress, Johannesburg, South Africa, 31 August – 2 September 2015. **Awarded second prize for best poster in the SASBCP Young Scientist competition**

- Kathrada, F., van Zyl, R.L., Coetzee, M., Olivier, S., Hayat, F., Inam, A., Azam, A. 2016.

  Poster presentation: 'The effect of 4-(7-chloroquinolin-4-yl) piperazin-1-yl)pyrrolidin-2-yl) methanone derivatives in the management of malaria'. All Africa Congress, 5-7 October 2016, Muldersdrift, South Africa

- Kathrada, F., van Zyl, R.L., Coetzee, M., Olivier, S., Hayat, F., Inam, A., Azam, A. 2016.

  Poster presentation: 'Effect of novel 7-chloroquinolin-4-yl piperazine-1-yl-acetamide derivatives in the management of malaria'. 2nd Malaria Research Conference, 1-3 August 2016, Pretoria, South Africa

TABLE OF CONTENTS

		Page
DECLARATION		i
DEDICATIONS		ii
ACKNOWLEDGEMENTS		iii
ABSTRACT		iv
PUBLICATIONS		v
CONFERENCES		vi
TABLE OF CONTENTS		vii
LIST OF FIGURES		xi
LIST OF TABLES		xiv
LIST OF EQUATIONS		xv
LIST OF ABBREVIATIONS		xvi
Chapter 1: Introduction		
1.1	Prevalence	1
1.2	Parasitology and entomology	2
1.3	Life cycle of mosquito and malaria parasite	3
1.4	Symptoms and diagnosis	4
1.5	Management	5
1.5.1	Prevention by vector Control	5
1.5.2	Indoor Residual Spraying	5
1.5.3	Long-lasting Insecticidal Nets	5
1.6	Management protecting the human Host	5
1.6.1	Treatment	5
1.6.2	Prophylaxis	7
1.7	Development of resistance	8
1.7.1	Resistance by the malaria parasite to current treatment	8
1.7.2	Resistance by the malaria mosquito to vector control methods	8
1.8	Classification of antimalarials	9
1.8.1	4-Aminochloroquinolines	9
1.8.2	Artemisinin	11
1.8.3	Arylamino alcohol derivatives	12
1.8.4	Antifolate drugs	12

1.8.5 Antibiotics 13
1.9 Need for novel antimalarial drugs 14
1.10 Aims & objectives 19
Chapter 2: Methodology
2.1 Compounds and derivatives 20
2.2 Culturing of asexual stages of the parasite 20
2.2.1 Preparation of reagents 20
2.2.2 Collection & preparation of uninfected red blood cells 23
2.2.3 Maintenance of *P. falciparum* NF54 strain culture 23
2.2.4 Giemsa staining 23
2.2.5 Synchronisation of parasites 24
2.3 Antimalarial activity 25
2.3.1 Parasite lactate dehydrogenase assay 25
2.3.2 Experimental setup 25
2.3.3 Data analysis 27
2.3.4 Combination studies 27
2.4 Stage determination 28
2.5 Measurement of drug-likeness 29
2.6 Larvicidal assay 30
2.6.1 Determining concentration for screening compounds 30
2.6.2 Bioassay 31
2.6.3 Data analysis 31
2.7 Toxicity assays 32
2.7.1 Haemolysis assay 32
2.7.1.1 Preparation of test derivatives and classical antimalarials 32
2.7.1.2 Preparation of plates 32
2.7.1.3 Data analysis 33
2.7.2 Cell viability assay 33
2.7.2.1 Preparation of medium & reagents for cell culturing 33
2.7.2.2 Cell Maintenance 33
2.7.2.3 Splitting cells 34
2.7.2.4 MTT cell viability assay 34
2.7.2.5 Data analysis 35

2.7.3 Safety Index 35
2.7.4 Lipid peroxidation assay 35
2.7.4.1 Preparation of reagents 36
2.7.4.2 Lipid peroxidation experimental assay 36
2.7.4.3 Data analysis 36
2.7.5 Brine shrimp lethality assay 37
2.7.5.1 Hatching eggs 37
2.7.5.2 Bioassay 38
2.7.5.3 Data analysis 38
2.8 Statistical analysis 38
Chapter 3: Results
3.1 Antimalarial activity 39
3.2 Combination studies 43
3.3 Stage determination 43
3.4 Solubility & permeability predictions 48
3.5 Predicted ionisation & absorption as a function of pH 48
3.6 Larvicidal activity 49
3.7 Cytotoxicity 51
3.7.1 Haemolysis 51
3.7.2 Cell viability 51
3.7.3 Lipid peroxidation 52
3.7.4 Brine shrimp lethality activity 52
Chapter 4: Discussion
4.1 7-Chloroquinolin-4yl derivatives 54
4.1.1 Overcoming chloroquine resistance 55
4.1.2 7-Chlroquinolin-4yl piperazine-1yl acetamide derivatives 56
4.1.3 7-Chloroquinolin-4yl piperazine-1-yl pyrrolidine-2-yl methanone derivatives 58
4.2 Beta-haematin inhibition 59
4.3 Drug accumulation in parasite digestive vacuole 60
4.4 Protein synthesis inhibition 62
4.5 Inhibition of parasite folate pathways 65
4.6 Combination therapy 67
4.7 Toxicity 67

4.8 Vector effects 69

Chapter 5: Conclusion, limitations & recommendations 72

References 75

Appendices 90

Appendix A: Biosafety ethics clearance: protocol number. 91

Appendix B: Human Ethics clearance to use and purchase human plasma and blood: waiver. 91

Appendix C: Human ethics clearance to obtain drug-free human whole from healthy volunteers. 94

Appendix D: Ethics clearance for the use of mosquitoes and mosquito parasites: reference number. 95

Appendix E: Human ethics clearance certificate for use of human cell lines: reference number. 98

Appendix F: Animal ethics clearance certificate for use of *Artemia* brine shrimp. 99

LIST OF FIGURES

Figure 1.1: The distribution of malaria deaths (a) found in endemic countries (b). 1

Figure 1.2: Number of malaria cases and deaths by year and endemic provinces in South Africa. 2

Figure 1.3: The mosquito life cycle. 3

Figure 1.4: Malaria life cycle illustrating both sexual, asexual intra-erythrocytic and mosquito stages. 4

Figure 1.5: Algorithm for treatment of malaria in South Africa. 6

Figure 1.6: Malaria prophylaxis agents by site of action. 7

Figure 1.7: Structure of quinine and chloroquine. 10

Figure 1.8: Proposed representation of haemozoin formation in *Plasmodium falciparum.* 11

Figure 1.9: Artemisinin (1) dihydroartemisinin (2), doxycycline (3), lumefantrine (4). 12

Figure 1.10: Antifolate drug effects on folate metabolism in *P. falciparum.* 13

Figure 1.11: The delayed death effect of antibiotics on the malaria parasite results from slow growth inhibition. 14

Figure 1.12: Rationale of hybrid drug design. 15

Figure 1.13: Hybrid antimalarial with 4-aminochloroquinoline group (A) linked to an acetamide (C) group via a piperazine linker (B). 16

Figure 1.14: Basic structure of 4-(7-chloroquinolin-4-yl) piperazin-1-yl) pyrrolidin-2-yl)methanone derivatives, bearing a 4-aminoquinoline (A), piperazine (B) and pyrrolidine ring (C) with a sulphonamide group (D). 17

Figure 2.1: The intra-erythrocytic cycle of *P. falciparum* with inset pictures showing the appearance of the parasites at different stages upon Giemsa staining. 24

Figure 2.2: Plate layout in pLDH assay indicating decreasing drug dilutions (from top to bottom) in triplicate (horizontally) with colour changes indicating parasite presence (dark purple) and no parasite (clear to light purple). 26

Figure 2.3: Visualisation of possible pharmacological interactions between two compounds in combination using an isobologram. 26

Figure 2.4: Workflow of methodology in applying the Lipinski rule of five. 30

Figure 2.5: Morphological appearance of HEK cells. 34

Figure 2.6: The design of the brine shrimp hatchery setup. 37

Figure 3.1: Log sigmoid dose-response curve indicating the antimalarial activity of

the most active compound from the 7-chloroquinolin-4-yl piperazine-1-yl acetamide (CQPA-26) and 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone (CQPPM-9) derivatives in comparison to the standard antimalarial agent, quinine. 39

Figure 3.2: Isobolograms showing the drug interactions between the most active CQPA-**26** (A) and CQPPM-**9** (B) derivatives with quinine. 43

Figure 3.3: Changes in total parasitaemia between the most active CQPA-**26** from the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives in comparison to the standard quinine and untreated (drug-free) control (A) with an indication of the difference between total parasitaemia at the IC_{50} and IC_{90} values of CQPA-**26** (B). 44

Figure 3.4: Changes in total parasitaemia between the most active derivative from 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone derivatives (CQPPM-**9**) in comparison to the standard quinine and untreated (drug-free) control (A) with an indication of the difference between total parasitaemia at the IC_{50} and IC_{90} values of CQPPM-**9** (B). 45

Figure 3.5: The effects of CQPA-**26** and CQPPM-**9** and quinine (IC_{50} oncentration) on parasite morphology and development over time at the IC_{50}. 46

Figure 3.6: Effects of each derivative (IC_{50}) and standard quinine (IC_{50}) on each stage (rings (A), trophozoites (B), schizonts (C)) in the lifecycle of the parasite over time (hr). 47

Figure 3.7: LC_{50} value (0.05 ± 0.004 µM) determination of DDT against *An. arabiensis* larvae. 49

Figure 3.8: The cell viability (IC_{50}) of the 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone derivatives against the HEK-293 cells in comparison to quinine and the positive control camptothecin at 50 µM. 52

Figure 4.1: Rationale in structural design of the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives (B) and 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives (C) compared to the standard chloroquinoline (A). 55

Figure 4.2: Antimalarial activity and cytotoxicity of the key 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives. 57

Figure 4.3: Antimalarial activity and cytotoxicity of the key 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives. 58

Figure 4.4: Proposed mechanism of action of quinoline-based drugs showing degradation of haemoglobin and detoxification mechanisms of the parasite. 59
Figure 4.5: Theoretical binding sites of derivatives aminochloroquinoline core with the haemozoin crystal. 60
Figure 4.6: Proposed structure-activity relationships according to studies by Egan *et al.,* (2000). 61
Figure 4.7: Diffusion and accumulation of chloroquine into the parasite digestive food vacuole due to the pH gradient. 62
Figure 4.8: Protein synthesis pathways in the *Plasmodium* parasite. 63
Figure 4.9: Proposed mechanism of binding of chalcone to histidine site of falcipain enzyme. 64
Figure 4.10: Structural similarities between 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives (a), Proguanil (b), and 2-(4-(substituted benzoyl)-1,4-diazepan-1-yl)-N-phenylacetamide derivatives (c). 65
Figure 4.11: Structural similarities of sulphonamide moiety in the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives (a) compared to the 7-chloro-4-aminoquinolyl derived sulphonamides (b). 66
Figure 4.12: Binding sites of novel chloroqunoline sulphonamide hybrid in cancer cells with structural similarities (chloroquinoline & sulphonamide scaffolds) as the 7-chloroquinolin-4-yl piperazine derivatives in this study 69
Figure 4.13: Structural differences and similarities between novel chalcone (a) and acridine isomers (b) with larvicidal properties in comparison to the 7-chloroquinolin-4-yl piperazine-1-yl acetamide (c) and 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone (d) derivatives showing no larvicidal properties. 70
Figure 5.1: Proposed structure-related mechanisms of action of 7-chloroquinolin-4-yl piperazine-1-yl acetamide (A) and 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone (B) derivatives in this study. 73

LIST OF TABLES

Table 1.1: Mean percentage mortalities of samples of *An. arabiensis* following exposure to listed insecticides by class. 9

Table 2.1: The core structure and side chains of the 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone derivatives. 21

Table 2.2: The core structure and side chains of the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives 22

Table 2.3: Concentration ratios at which derivatives were combined with quinine (B-H) and positive controls containing single compounds alone (A & I). 27

Table 3.1: Results showing antimalarial activity and cytotoxicity profile of the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives. 40

Table 3.2: Results showing antimalarial activity and cytotoxicity profile of the 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone derivatives. 42

Table 3.3: Predicted solubility and permeability of the most active derivatives using Lipinski's rule of five. 48

Table 3.4: Predicted ionisation of the most active derivatives. 49

Table 3.5: The lack of larvicidal activity by the derivatives (0.5 µM) on *Anopheles* larvae compared to untreated and DDT treated (0.5 µM) larvae. 50

Table 3.6: Table showing no effect of derivatives on *Artemia franciscana* nauplii after 24 hours compared to the potassium dichromate (positive control) and the negative (blank) control. 53

Table 4.1: Chemical structures of larvicides approved by the (WHO). 71

List of Equations

Equation 2.1: Calculation to determine percentage parasitaemia of a thin blood smear. 23

Equation 2.2: Calculation to determine FIC using the IC_{50} values of the compound alone and in combination. 27

Equation 2.3: Henderson-Hasselbalch equation to determine the percentage ionisation for weak acids and bases. 29

Equation 2.4: The equation used to calculate the percent mortality of larvae caused by the compound tested. 31

Equation 2.5: Determination of the percent haemolysis caused by the parasite on the red blood cells. 32

Equation 2.6: Determination of percent cell viability (growth) taking the reference and test wavelengths into account. 35

Equation 2.7: The safety index of the derivatives in targeting *P. falciparum* in preference to human cell lines. 35

Equation 2.8: Determination of the percent lipid peroxidation inhibited by the test compound, where std: standard control. 36

Equation 2.9: Determination of the percent mortality of nauplii caused by the derivatives and control after a 24 hours period. 38

List of abbreviations

˚C	Degrees Celsius	M	Molar
µl	Microlitre	ml	Millilitre
µM	Micromolar	min	Minute
µm	Micrometre	MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
ACT	Artemisinin combination therapy	NAD	Nicotinamide adenine dinucleotide
APAD	Acetylpyridine adenine dinucleotide	$NaHCO_3$	Sodium bicarbonate
DDT	Dichloro diphenyl trichloroethane	NaOH	Sodium hydroxide
DHFR	Dihydrofolate reductase	NBT	Nitro blue tetrazolium
DHPS	Dihydropteroate synthase	nM	Nanomolar
DMEM	Dulbecco's modified eagles medium	PBS	Phosphate buffered solution
DMSO	Dimethyl sulfoxide	PES	Phenazine ethosulphate
SA-DOH	South Africa Department of Health	pH	Potential of hydrogen
FIC	Fractional inhibitory concentration	pKa	Acid dissociation constant
FPIX	Ferriprotoporphyrin IX	pLDH	Parasite lactate dehydrogenase
g	Gravitational constant	RBC	Red blood cell
g/L	Grams per litre	Ro5	Rule of five
H	Hydrogen	rpm	Revolutions per minute
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid	RPMI	Roswell Park Memorial Institute medium
hr	hour	Σ	Sum
IC_{50}	Concentration required to inhibit 50% parasite/cell growth	s.d.	Standard deviation
IC_{90}	Concentration required to inhibit 90% parasite/cell growth	SI	Safety index
IRS	Indoor residual spraying	TRIS	Trisaminomethane
IU	International units	UV	Ultra-violet
LC_{50}	Concentration required to kill 50% larvae	v/v	Volume per volume
LDH	Lactate dehydrogenase	Vis	Visible
LLIN	Long lasting insecticidal nets	w/v	Weight per volume
Log P	Partition coefficient	WHO	World health organisation

Chapter 1: Introduction

1.1 Prevalence

Malaria remains one of the main global health problems, causing 429 000 deaths annually, with 92% of deaths and 90% of cases occurring in Africa (WHO, 2016). According to the latest estimates by the World Health Organization (WHO), there were 212 million malaria cases in 2016, with 13 countries accounting for 75% of malaria deaths (WHO, 2016) (Figure 1.1). Malaria transmission is generally stable in Western and Central Africa, unstable in Eastern Africa and unstable to absent in Southern Africa (Kibret *et al*., 2015).

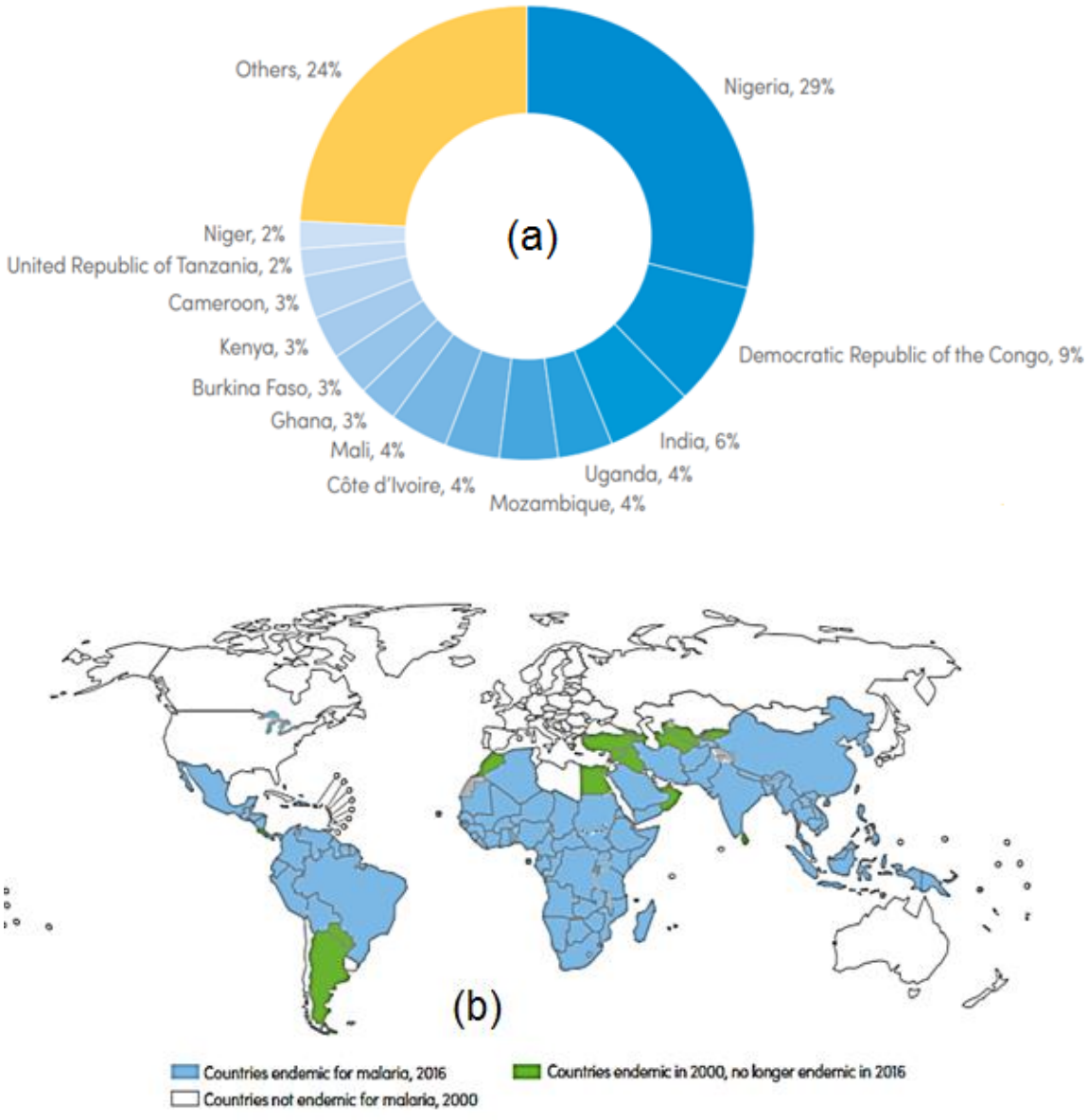


Figure 1.1: The distribution of malaria deaths (a) found in endemic countries (b) (WHO, 2016).

In South Africa, malaria is currently endemic and restricted to the low altitude regions of three provinces, namely Limpopo, Mpumalanga, and KwaZulu-Natal (Maharaj *et al.,* 2012). Neighbouring countries such as Zimbabwe and Mozambique also have malaria-endemic areas and contribute predominantly to imported cases of malaria in South Africa (Maharaj *et al.,* 2013). Studies in 2010 indicate the highest death rate being reported in Limpopo (Figure 1.2).

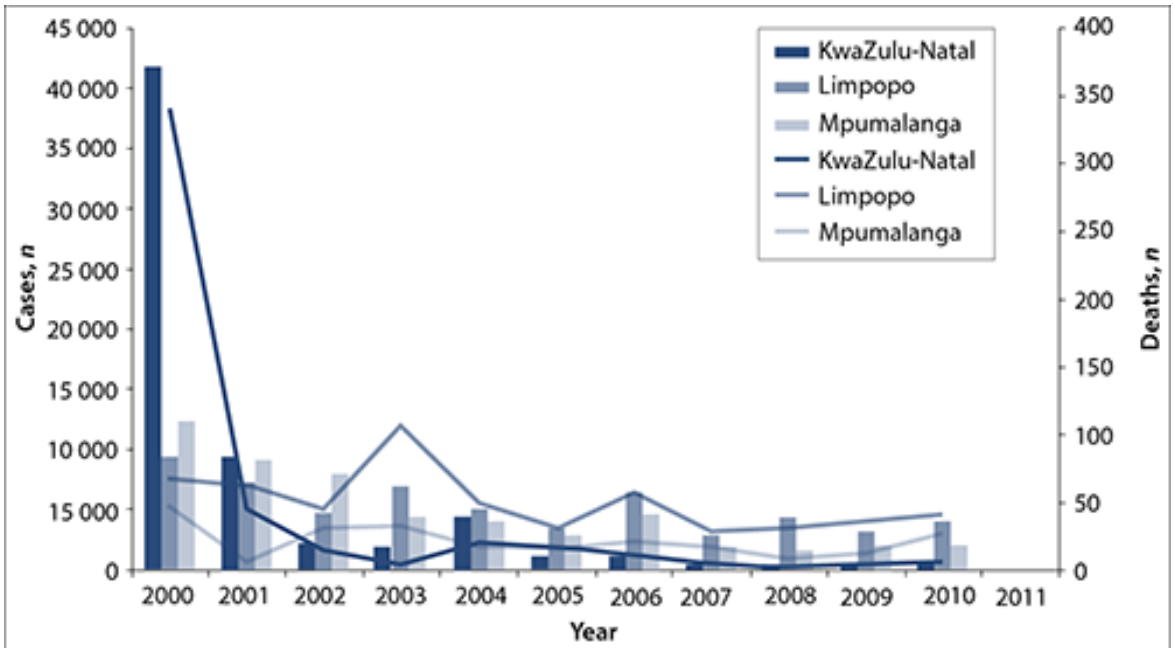


Figure 1.2: Number of malaria cases (bar graph) and deaths (line graph) by year and endemic provinces in South Africa (Maharaj *et al.,* 2013).

1.2 Parasitology and entomology

Malaria is caused by infection of red blood cells with the protozoan *Plasmodium* parasite, and transmitted by the bite of the female *Anopheles* mosquitoes, which depends on a blood meal to mature her eggs (WHO, 2013). There are five species of malaria parasites that infect humans, namely; *P. falciparum, P. vivax, P. malariae, P. knowlesi* and *P. ovale* (WHO, 2013). The predominant causative malaria parasite in South Africa is *P. falciparum,* transmitted primarily by *Anopheles arabiensis* mosquitoes. Malaria transmission occurs mostly between September and May, peaking in March (Maharaj *et al.,* 2012).

1.3 Life cycle of mosquito and malaria parasite

The adult mosquito lays eggs that develop through several aquatic stages to adulthood (Figure 1.3). The eggs are laid on water surfaces and hatch into larvae within 2-3 days.

The larvae go through four growth stages and after 2-3 weeks eventually form pupae. The adult mosquito develops inside the pupae and emerges within 2-3 days. Larvae feed on bacteria and algae in the water, while the pupa does not feed during the development of the adult. The adult female transmits the malaria parasite to the human host by injecting sporozoites via her saliva during biting (Service, 1980).

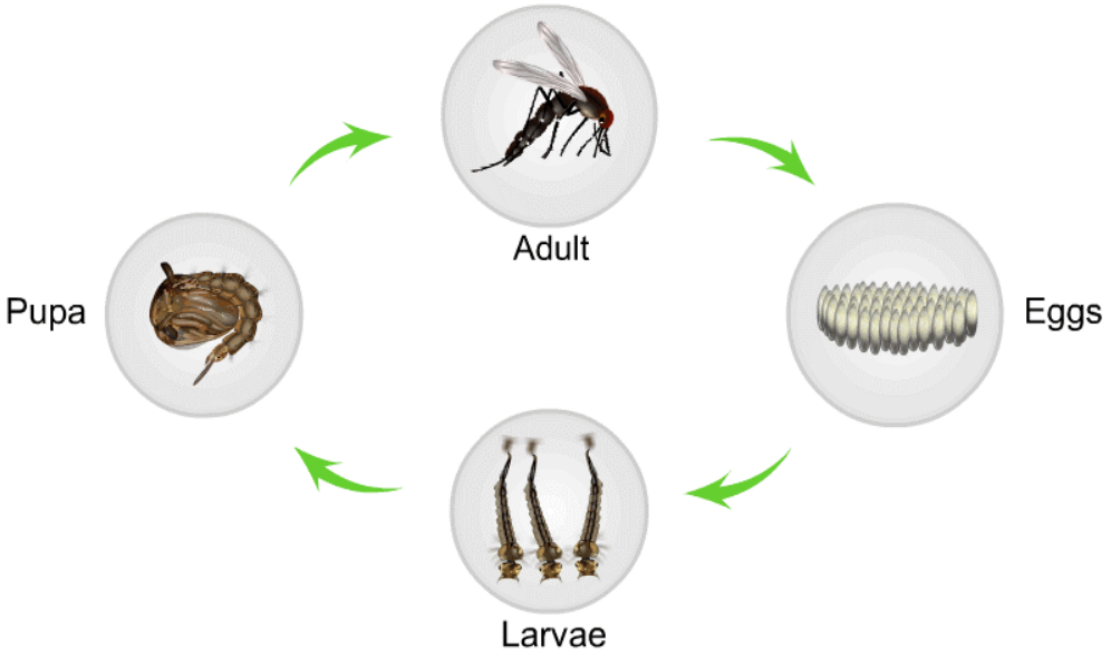


Figure 1.3: The mosquito life cycle (Virtual Biology, 2014).

Sporozoites found in a mosquito's salivary gland are injected into the human host skin during a blood meal that the mosquito requires to develop her eggs (only female mosquitoes suck blood) (Figure 1.4). The sporozoites that reach a blood vessel travel to the liver and traverse several cells before developing in a hepatocyte in which the parasite numbers grow and develop into merozoites. Merozoites are released from the hepatocytes in vesicles and travel through the heart to the lungs, where the vesicles eventually disintegrate and release merozoites that can invade red blood cells to initiate the cyclic asexual stage of the malaria parasite. This cycle is repeated, causing fever each time the parasite erupts from the red blood cell. Symptoms can take 10-14 days to develop after the host has been bitten by an infective mosquito. Some of the infected red blood cells leave the asexual stage and instead of replicating, the merozoites develop into sexual forms called gametocytes which circulate in the blood stream. When this stage of the parasite is ingested by the mosquito during blood feeding, the male and female gametocytes mate in the mosquito's midgut, penetrate the midgut wall to form oocysts in which the sporozoites develop. The oocyst then bursts and the sporozoites migrate to the salivary glands. At this stage, which takes about 10-14 days, the mosquito

becomes infective and will transfer the parasites to the next human host she feeds on. (Service, 1980; Klein, 2013).

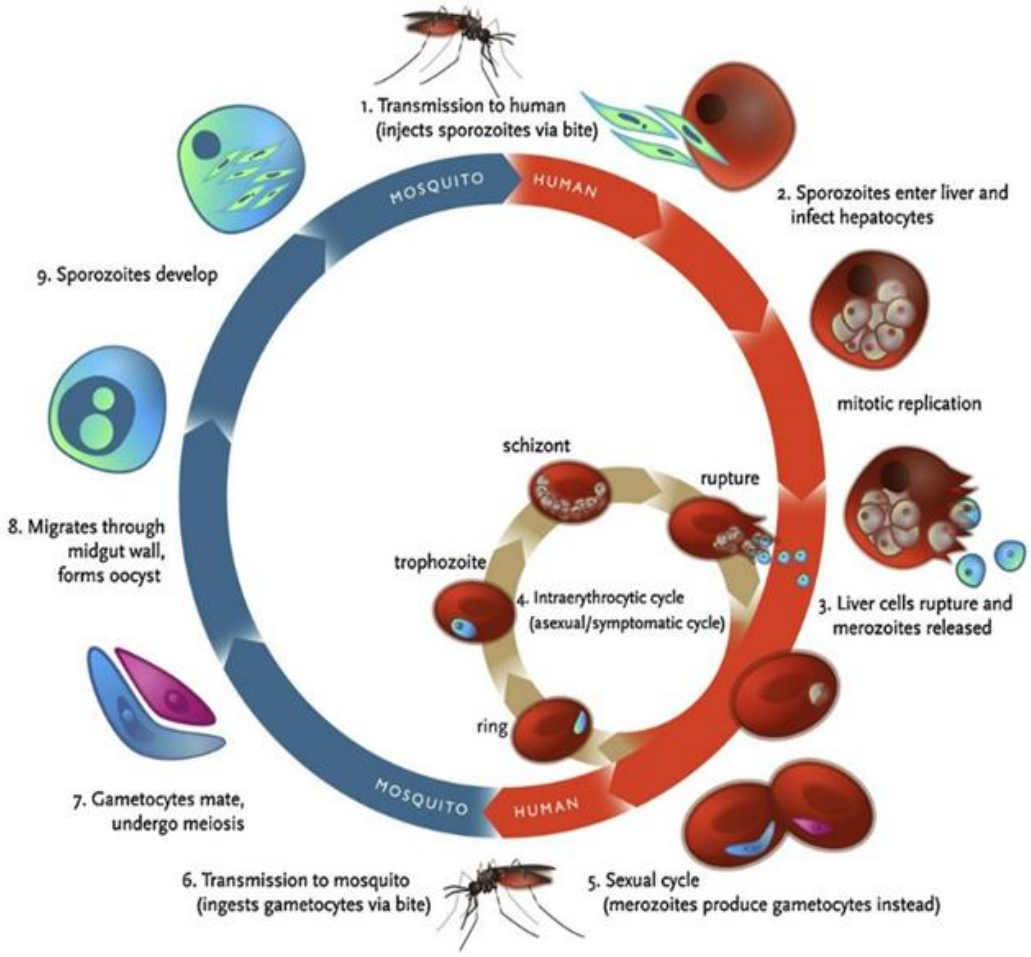


Figure 1.4: Malaria life cycle illustrating both sexual and asexual intra-erythrocytic and mosquito stages (Klein, 2013).

1.4 Symptoms and diagnosis

Symptoms may appear in cycles and occur at different intensities. The cyclic pattern of the malaria symptoms is due to the life cycle of malaria parasites as they develop and are essential indicators of a potential infection (WHO, 2013). Early detection and diagnosis of malaria is imperative to reduce malaria transmission. Delayed diagnosis and inappropriate treatment are associated with increased morbidity and mortality. The first symptoms of malaria may be mild and presents with fever, rigors, headaches and body pains (SA-DOH, 2016). A definitive diagnosis should be made by examining the parasite under a microscope via a Giemsa-stained blood smear (Blumberg, 2015).

1.5 Management

Malaria cases can be prevented by vector control measures (preventing the mosquito from biting the human hosts), or by chemoprevention with the use of drugs that kill infections (WHO, 2016).

1.5.1 Prevention by vector control

Vector control plays a major role in decreasing malaria transmission in endemic areas. IRS and long-lasting insecticidal nets (LLIN's) are the key methods in vector control (Brooke *et al*., 2013).

1.5.2 Indoor residual spraying

IRS involves spraying the interiors of homes using insecticides, such as dichloro-diphenyl-trichloroethane (DDT), carbamates, or pyrethroids on average twice a year depending on the insecticide used and the duration of the transmission season. IRS reduces transmission by killing mosquitoes that enter the home. Thus, the outdoor resting components of the vector species populations are largely unaffected by IRS and are responsible largely for the low-level seasonal malaria transmission in South Africa (Brooke *et al.,* 2013).

1.5.3 Long-lasting insecticidal nets

LLIN's last longer than the conventional insecticidal nets. Together with insecticidal protection, they also serve as a physical barrier between the human and mosquito. Between 2000 and 2012, the numbers of households owning at least one bed net increased from 3% to 53% (WHO, 2014). However, between 2013 to 2014, the distribution of bed nets to sub Saharan Africa dropped (WHO, 2014). This opened a gap for resurgences in malaria transmission.

1.6 Management protecting the human host:

1.6.1 Treatment

Prompt treatment with the appropriate drugs is vital in reducing malaria morbidity and mortality. The choice of antimalarial agents is dependent on the severity of the illness and the pattern of drug resistance of the parasite in the geographical area where malaria was acquired. In the late 1940s, quinine was used for both treatment and prophylaxis, while during the 1970s treatment changed to chloroquine. With the emergence of chloroquine resistance, treatment moved to sulphadoxine-pyrimethamine in 1988 and then to artemether-lumefantrine in 2001 (Blumberg *et al.,* 2014). Artemisinin

is combined with other classes of drugs to form artemisinin combination therapy (ACT). The current first-line treatment for uncomplicated *P. falciparum* malaria is the artemisinin combination of artemether with lumefantrine (Figure 1.5). Second-line treatment of uncomplicated malaria is oral quinine, plus doxycycline or clindamycin. Parenteral artesunate is the preferred treatment of severe malaria with quinine (intravevenous) used when artesunate is unavailable, followed by oral treatment once the patient can tolerate it. Mixed infections of *Plasmodium* are treated with ACT followed by primaquine (Figure 1.5) (Blumberg, 2015; SA-DOH, 2016).

In high risk provinces, the recommended first-line treatment for uncomplicated malaria is accessible at any level of public healthcare. Parenteral forms of antimalarials are only accessible at hospital levels. Uncomplicated malaria in South Africa is treated at an outpatient level, except for high-risk population groups which are treated on admission to hospital (Ukpe *et al.,* 2013).

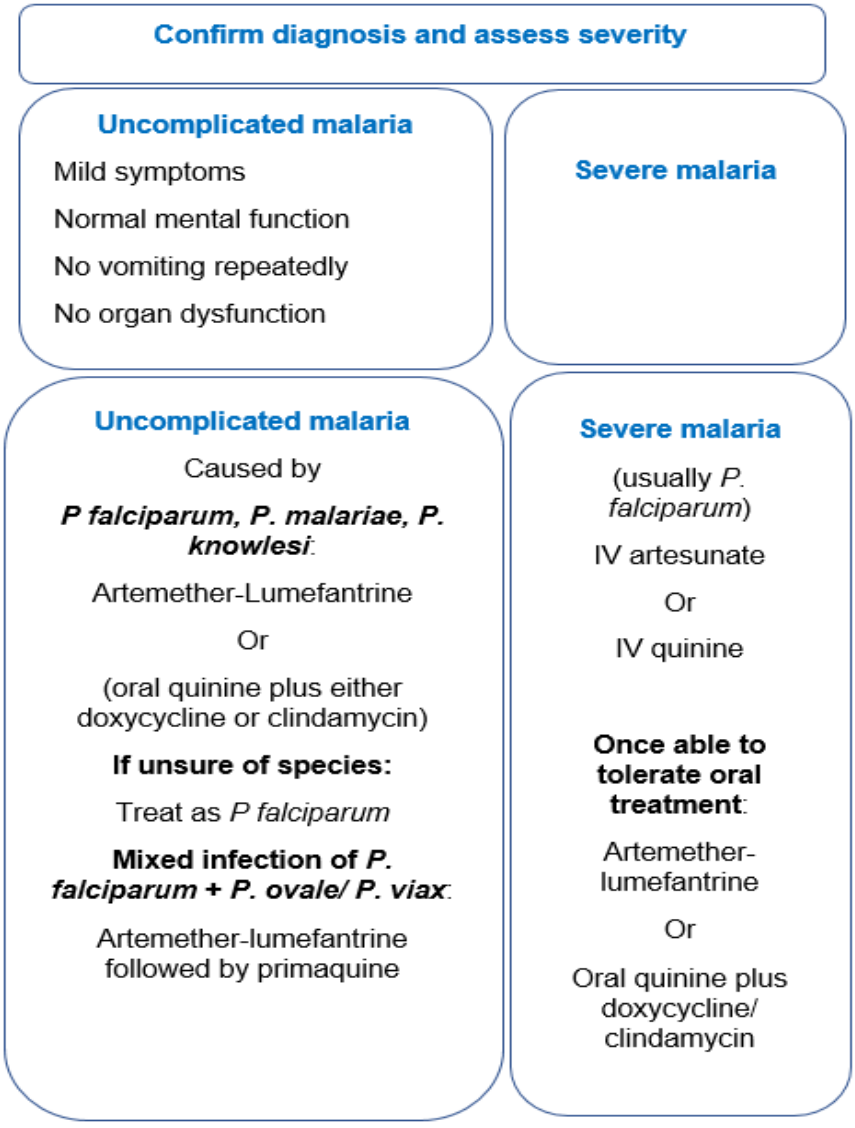


Figure 1.5: Algorithm for treatment of malaria in South Africa (SA-DOH, 2016).

1.6.2 Prophylaxis

The determination of necessitating chemoprophylaxis depends on the area travelling to, the risk the traveller has of being exposed to the mosquito and contracting malaria. The greater the risk, the greater the need for chemoprophylaxis. There are currently three regimens used for chemoprophylaxis in South Africa as recommended by the South African Department of Health (SA-DOH):

- Mefloquine that is taken weekly starting one week before entering the area, weekly while in the area, and four weeks after returning.
- Doxycycline that is taken daily starting one day before entering the area, daily while in the area and four weeks after returning.
- Atovaquone-proguanil that is taken daily one or two days before entering the area, daily while in the area and for 7 days on returning (SA-DOH, 2016).

The reason that mefloquine and doxycycline are to be continued for a month after returning from malaria risk area is that they only act on parasites within red blood cells, whereas atovaquone-proguanil acts on parasites within hepatocytes as well as their erythrocytic stage (Figure 1.6) (Schwartz, 2012).

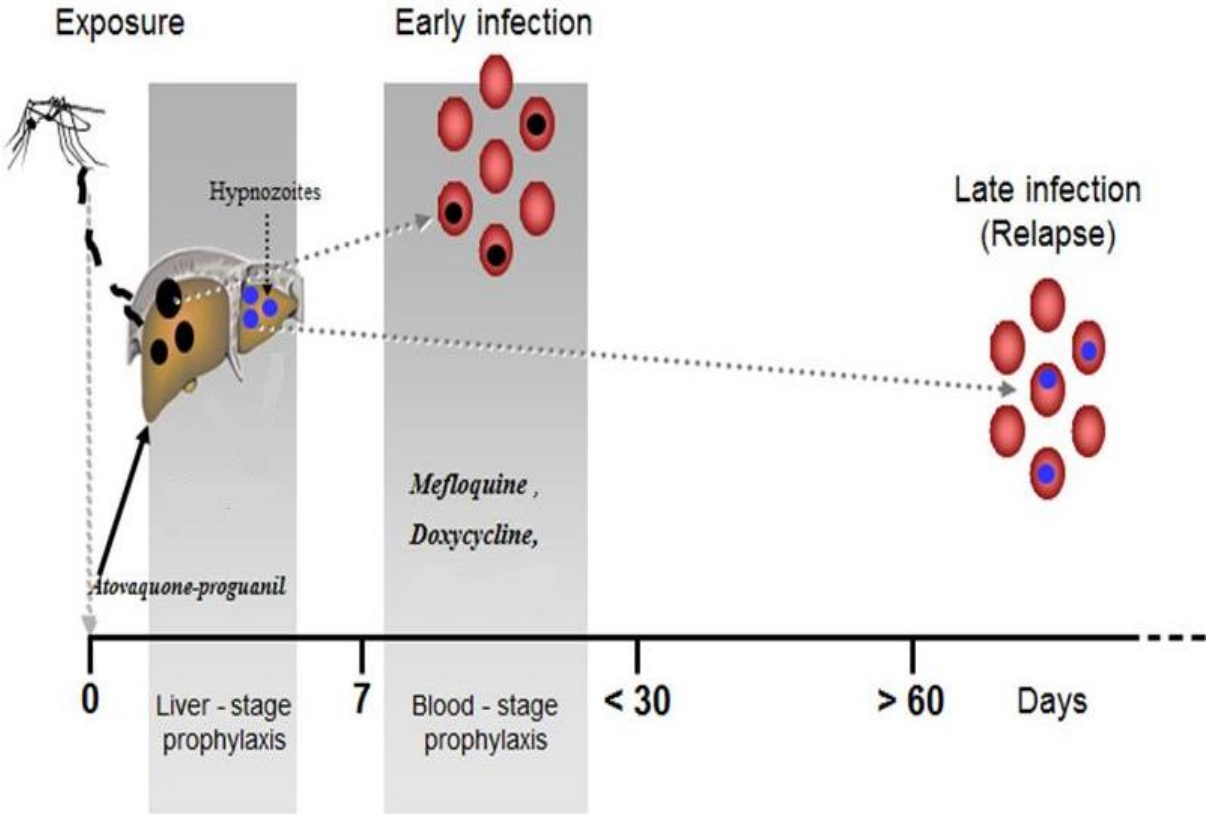


Figure 1.6: Malaria prophylaxis agents by site of action (Schwartz, 2012).

1.7 Development of resistance

The biggest threat to treatment and vector control is the emergence of resistance, by the parasites to current drug treatments, and mosquitoes to the insecticides used in vector control programmes.

1.7.1 Resistance by the malaria parasite to current treatment

According to the WHO, parasite resistance exists to antimalarial drugs in three of the malaria species, namely: *P. falciparum, P. vivax* and *P. malariae.* This results in incomplete clearance of the parasite following treatment with an antimalarial (WHO, 2014). Drug resistance leads to treatment failures and increases the burden of the infection. Resistance is usually initiated through a spontaneous mutation that gives rise to a reduced level of sensitivity of the parasite to the antimalarial. Resistance can become firmly established within a parasite species and can last for long periods of time (WHO, 2015). In all endemic provinces in South Africa, markers associated with sulphadoxine-pyrimethamine and chloroquine resistance were prevalent by 2003 (Hargreaves *et al.,* 2003, Maharaj *et al.,* 2012).

Western Cambodia was the first to report artemisinin resistance, with failure rates of artemisinin combination therapies rising (Ashley *et al.,* 2014). Reports of resistance to artemisinin has spread to Southeast Asia. Artemisinin resistance is characterised by slow clearance of the parasite. On the Thailand Myanmar border, the mean half-life for parasite clearance increased from 2.6 hours in 2001 to 3.7 hours in 2010 (Ashley *et al.,* 2014).

The artemisinin resistance is linked to marked point mutations in a region called the K-13 propeller region which alter the protein synthesis pathways in the parasites development (Straimer *et al*., 2015). The emergence of artemisinin resistance, has raised great concern for the future efficacy of antimalarial treatment and an increased risk for the spread to the African continent (Ashley *et al.,* 2014).

1.7.2 Resistance by the malaria mosquito to vector control methods

The increased use of insecticides in malaria vector control programmes has led to resistant genes in many vector populations and continues to be of concern as the number of resistance reports continue to increase. Recent reports of insecticide resistance in South Africa indicated that *Anopheles. arabiensis* was resistant to DDT, but sensitive to pyrethroids. However, *An. funestus* was found to be resistant to pyrethroids, but sensitive to DDT and carbamates (Hargreaves *et al.,* 2003; Maharaj *et al.,* 2012). According to the WHO, 60 of the 78 reporting countries, reported resistance to at least one insecticide and 49 countries reported resistance to insecticides in two or

more insecticide classes (WHO, 2015). In 2014, pyrethroid resistance was the most commonly reported insecticide class (WHO, 2015). The 2015 data of a study conducted on *An. arabiensis* affirmed the presence of pyrethroid and DDT resistance in KwaZulu-Natal (Brooke *et al.,* 2015) (Table 1.1).

Table 1.1: Mean percentage mortalities of samples of *An. arabiensis* following exposure to listed insecticides by class (Brooke *et al.,* 2015).

Insecticide (concentration)	Insecticide class	Sample size	Mean % mortality	Resistance (R) or susceptibility (S)
Deltamethrin (0.05%)	Pyrethroid	191	87.21	R
DDT (4%)	Organochloride	140	83.85	R
Bendiocarb (0.1%)	Carbamate	145	94.1	R
Pirimiphos-methyl (0.25%)	Organophosphate	45	100	S
Fenitrothion (1%)	Organophosphate	44	100	S
Control (untreated)	-	234	2.74	-

Vector control strategies using IRS and LLIN's, play a vital role in reducing malaria transmission and case number in South Africa. The increase in distribution and intensity of resistance to insecticides compromises the effectiveness and gains of vector control methods. The insecticidal resistance could lead to increases in malaria incidence and mortality (Brooke *et al.,* 2015).

1.8 Classification of antimalarials

Early detection and treatment of uncomplicated malaria is essential to prevent the development of severe malaria to reduce the time frame in which a person carries the parasite in the blood reducing the risk of further transmission and subsequently death (WHO, 2016).

1.8.1 4-Aminochloroquinolines

Quinine, first extracted from the cinchona bark in the 1600's, is one of the oldest antimalarials. Once its therapeutic potential was realised, research into synthetic analogues based on the quinine template (Figure 1.7) began, resulting in compounds such as the 4-aminochloroquinoline, chloroquine (Figure 1.7) (O'Neill *et al.,* 2012).

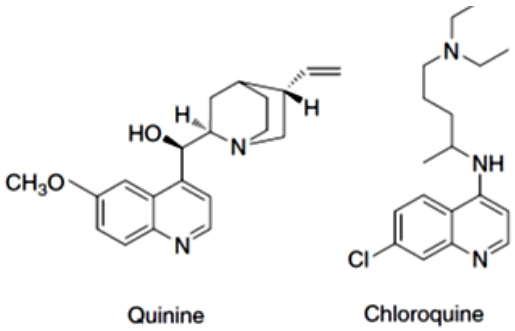


Figure 1.7: Structure of quinine and chloroquine (O'Neill *et al.,* 2012).

Chloroquine was first synthesised in 1934 and became the most widely used antimalarial by the 1940's (O'Neill *et al.,* 2012). Chloroquine is effective against the erythrocytic form of chloroquine sensitive strains of the *Plasmodium* parasite in which chloroquine is able to accumulate at high concentrations within the parasite vacuole. The success of this class as antimalarials has been based on its antimalarial efficacy, low toxicity and cost-effectiveness (O'Neill *et al.,* 2012).

Chloroquine, lumefantrine, mefloquine and quinine antimalarial inhibitory effects are due to their ability to cleave ferric haem which eventually leads to death of the parasite. Parasites grow by degrading haemoglobin after invading red blood cells in the acidic part of the parasite's vacuole (Egan, 2008). The resultant free ferric haem or ferriprotoporphyrin IX (FPIX) is toxic to the parasite, which polymerises it to non-toxic haemozoin for survival (Figure 1.8). The 4-aminochloroquinolines like chloroquine exhibit their antimalarial action by inhibiting the formation of the haemozoin, leading to the accumulation of free toxic FPIX. Free toxic FPIX leads to a cascade of events that result in death of the parasite (Egan, 2008). The aminoquinoline drugs prevent the formation of the haemozoin crystal by binding to the FPIX or forming a drug-FPIX complex (Pisciotta & Sullivan, 2008). The toxicities involve morphological changes in the parasite, haemoglobin accumulation, FPIX accumulation, membrane function impairment, destruction of glutathione, inhibition of proteolytic enzymes and the release of calcium from acidic stores (Fitch, 2003).

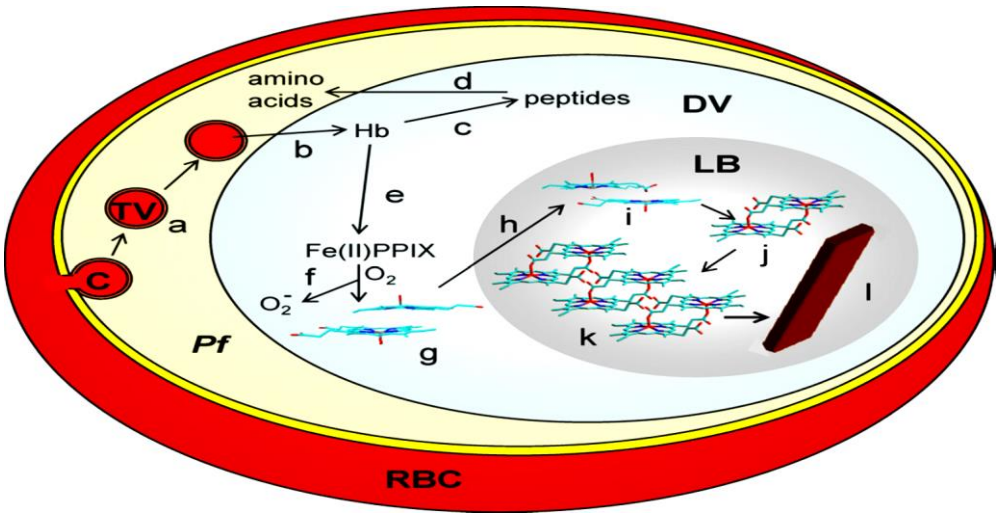


Figure 1.8: Proposed representation of haemozoin formation in *Plasmodium falciparum*. Red blood cell (RBC) cytoplasm ingested into parasite (Pf) via a cytosome (C) and transported to the digestive vacuole (DV) by transport vesicles (TV). Once delivered, haemoglobin (Hb) is digested to short peptides (c) that are exported to the DV and degraded to amino acids (d). The haemoglobin releases ferriprotoporphyrin IX which undergo various processes (e,f,g) and are eventually delivered to the lipid body (LB) where assembly of the haemozoin crystal take place (i,j,k) (Egan, 2008).

1.8.2 Artemisinin

Artemisinin(1) combination therapy is the standardised first-line treatment for *P. falciparum* malaria in endemic areas and has markedly reduced global morbidity and mortality rates due to malaria (WHO, 2016). The artemisinin compounds include artemisinin, artemether, arteether and artesunate. The active metabolite, dihydroartemisinin (2) acts rapidly by inhibiting major metabolic processes in the parasite and killing them. Artemisinin exhibits antimalarial activity against multiple stages of parasite development (Muangphrom, 2016). Artemisinin is a sesquiterpene lactone with an endoperoxide bridge (1) that requires activation to generate the active free radical species (2). The proposed mechanism of action of the artemisinins involves cleavage of the peroxide bridge by free or haem-bound iron to produce reactive free radicals. The free radicals alkylate and oxidise proteins resulting in rapidly killing of the parasite (Krungkrai, 2016). Artemisinins have positively impacted on malarial treatment with their high efficacy and fast action.

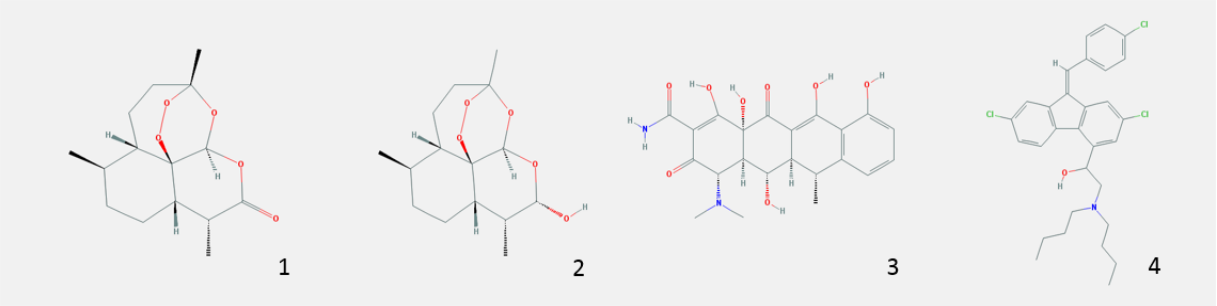


Figure 1.9: Artemisinin (1) dihydroartemisinin (2), doxycycline (3), lumefantrine (4) (Krishna *et al.,* 2004).

1.8.3 Arylamino alcohol derivatives

Lumefantrine, quinine and mefloquine are among the drugs classed as arylamino alcohols. Lumefantrine (4) is used in combination with artemether and interacts in a synergistic manner (Schlitzer, 2007). The mechanism of action of arylamino alcohols involve interference with haem digestion. The arylamino alcohol derivatives accumulate in the parasite food vacuole and inhibit haem digestion (quinine) and form toxic complexes with free haem (mefloquine and lumefantrine) (Schlitzer, 2007).

1.8.4 Antifolate drugs

These classes of antifolate antimalarials interfere with folate metabolism, a process essential to malaria parasite survival (Nzila, 2006) (Figure 1.10). This is a valuable target as it is specific for the parasite and not the host.

Class 1: Dihydrofolate reductase (DHFR) is an enzyme that catalyses NADPH (nicotinamide adenine dinucleotide phosphate)-dependant reduction of dihydrofolate to tetrahydrofolate in the malaria parasite. Compounds that inhibit DHFR include proguanil and pyrimethamine. These drugs act as tissue shizonticides and are slow-acting blood schizonticides. Proguanil is used in with atovaquone for treatment of *P. falciparum* infection (Blumberg, 2015).

Class 2: Sulphonamides such as sulfadoxine act as inhibitors of the enzyme dihydropteroate synthase (DHPS) in the folate pathway in the parasite. They act as analogues of para-aminobenzoic acid and competitively inhibits DHPS in the pathway from forming dihydropteroate (Schlitzer, 2007). The combination of sulfadoxine with pyrimethamine (DHFR inhibitor) has synergistic effects increasing its effectiveness against *P. falciparum* (Schlitzer, 2007). Unfortunately, this synergy and the effectiveness of antifolates have declined due to widespread resistance worldwide. Resistance to the DHFR and DHPS inhibitors is conferred by single mutations of the gene encoding for

the respective enzyme, resulting in substitutions in the amino acid chain (Saifi *et al*., 2013).

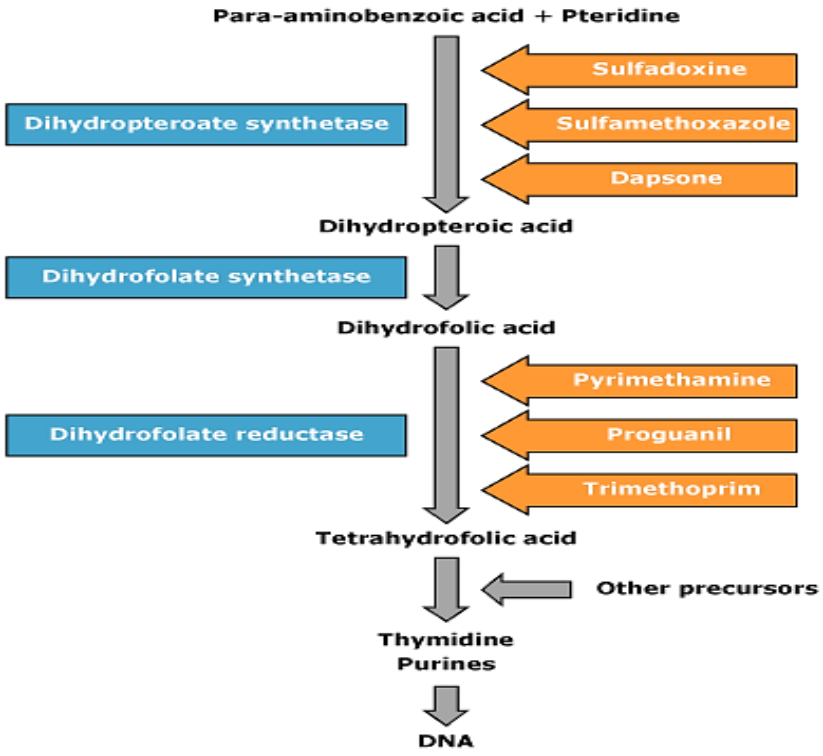


Figure 1.10: Antifolate drug effects on folate metabolism in *P. falciparum* (adapted from Chango *et al*., 2011).

1.8.5 Antibiotics

Antibiotics are used in combination with rapid acting drugs as they are slow acting, with a late onset of antimalarial activity during the second replication cycle of the parasite. The antibiotics have a characteristic delayed death effect by only killing the parasite once it has invaded the new host cell. Antibiotics such as doxycycline and clindamycin exert antimalarial activity through their action on the parasites apicoplast. The antibiotic blocks the expression of the apicoplast genome resulting in a loss of functional apicoplasts in developing merozoites (Dahl, 2006).

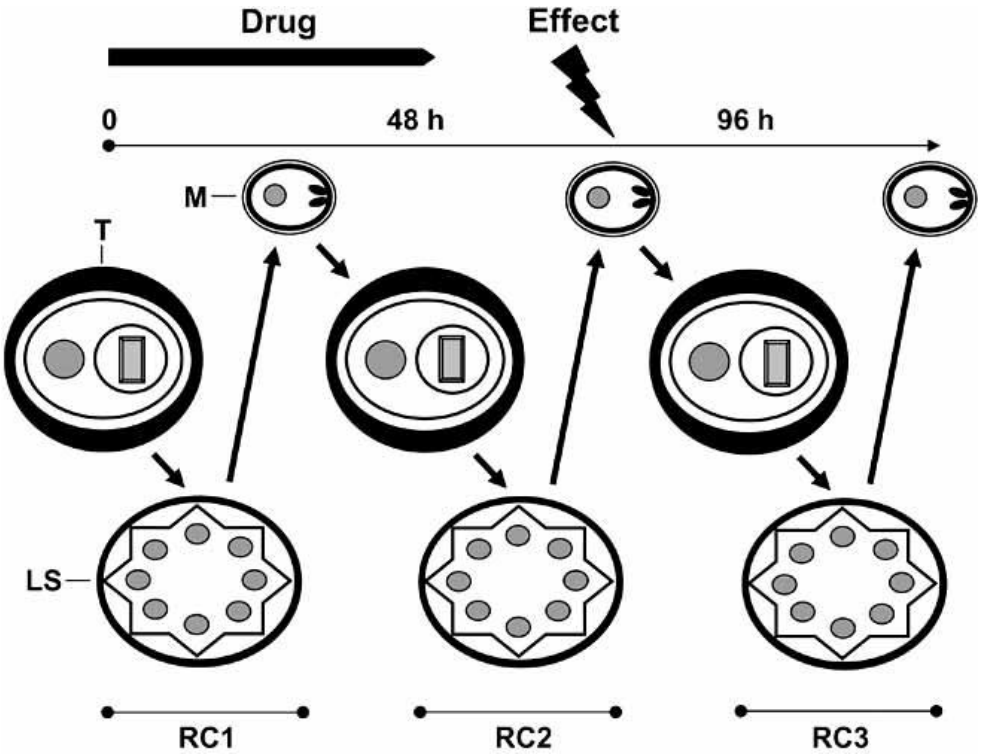


Figure 1.11: The delayed death effect of antibiotics on the malaria parasite results from slow growth inhibition (Pradel *et al*., 2010).

During the first replication cycle (RC1), in which antibiotics are administered, merozoites (M) are formed and infect new erythrocytes. The parasites develop to trophozoites (T). During the second replication cycle (RC2) the parasites are arrested in the schizont (S) stage with no formation of new merozoites. The third replication cycle (RC3) is therefore inhibited (Figure 1.11). Doxycycline (Figure 1.9 (3)) is the most widely used antibiotic to treat malaria in combination with quinine, as well as prophylactically where mefloquine or atovaquone-proguanil is contra-indicated. Clindamycin is considered safe in pregnancy and young children, in contrast to doxycycline which is contraindicated in these patients (Schlitzer, 2007; Blumberg, 2015).

1.9 Need for novel antimalarial agents

With the current line of therapy being faced with lack of efficacy due to resistant strains of the parasites, development of novel derivatives in treating malaria is vital. Current antimalarial drugs mostly target the asexual blood stage of the parasite's life cycle. Moving towards elimination of malaria, parts of the parasites life cycle beyond the blood stages and possibly a dual target (against erythrocytic and vector stages) needs to be investigated.

Current antimalarials mainly act by inhibiting haemozoin formation necessary for survival of the parasite, while others interfere with folate metabolism. Current drugs that target these mechanisms such as chloroquine and proguanil respectively have shown great efficacy and relative safety. However, with the development of resistance to these drugs, alternative ways to inhibit these sites or multiple sites with redesigned derivatives need to be studied.

The current antimalarial trend, involves combination therapy of two or more agents into a single tablet (artemether-lumefantrine) to improve compliance and efficacy (WHO, 2015). However, extensive spread of resistance to classical antimalarials necessitates the search for promising antimalarials with novel chemical structures and mechanism of action (Muregi *et al.,* 2010). The recent widespread interest in hybrid molecules over combination molecules has been backed by encouraging efficacy and toxicity reports (Muregi *et al.,* 2010). Hybrid drugs involve linking two molecules with individual activity into a single agent. This provides a dual functionality effect (Inam et *al*., 2015). Hybrid drugs show advantageous characteristics over combination therapy in terms of cost constraints and have a lower risk of drug-drug adverse interactions. However, the disadvantage is that it poses difficulties in adjusting the ratio of activity at the different targets (Muregi *et al.,* 2010). In recent deliberate rational drug design of antimalarials, hybrid molecules have been designed with multiple targets (Figure 1.12).

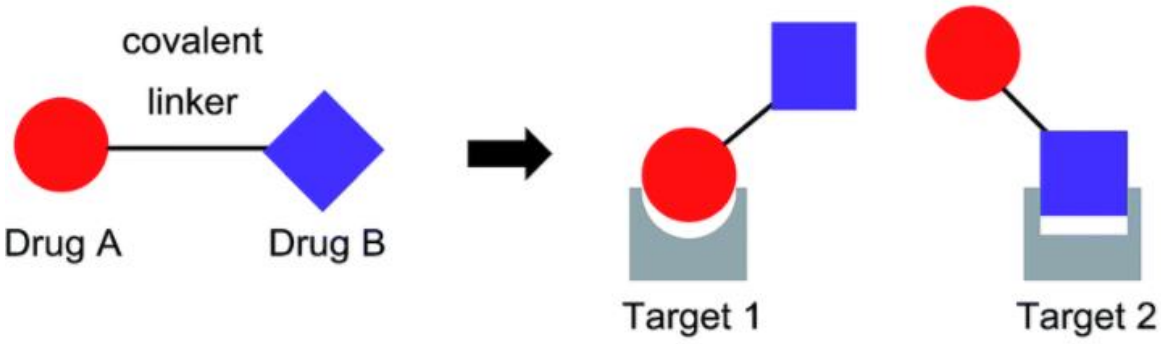


Figure 1.12: Rationale of hybrid drug design (Klhan *et al.,* 2017).

One example involves the development of a trioxaquine hybrid molecule containing a trioxane linked to a quinolone. The trioxaquine hybrid boasts the advantage of dual activity of the quinoline and trioxane fragments; with the quinoline showing antimalarial activity similar to that of potent antimalarials such as chloroquine and the trioxane moiety displaying similar alkylating activity to the artemisinins. The trioxaquine hybrid offered more effective antimalarial activity than the individual components in combination (Muregi *et al.,* 2010). This would imply that the interaction of the

pharmacophores of two molecules in a single hybrid is superior to the individual molecules in combination. This is useful in designing drugs such as the aminoquinolines, where the resistance is due to inability to access the target rather than alteration of the target. This study employs the principle of 'covalent bitherapy' by developing hybrid molecules that restore the activity of other drug classes that have become ineffective due to resistance.

The first set of compounds in the current study, 7-chloroquinolin-4-yl piperazine-1-yl-acetamide derivatives is comprised of a 4-aminochloroquinoline group linked via a piperazine group to a substituted acetamide (Figure 1.13). The rationale behind the design and synthesis of these compounds was that the 4-aminochloroquinoline will promote haem binding, the piperazine will act as a linker and the acetamide group will exhibit DHFR inhibitory effects (Rastelli *et al.,* 2003; Madapa *et al.,* 2009).

The second set of hybrid compounds evaluated in this study is the 4-(7-chloroquinolin-4-yl) piperazin-1-yl)pyrrolidin-2-yl)methanone derivatives (Figure 1.14); where it was proposed that the sulphonamide group would inhibit DHPS, the piperazine will act as a linker and the 4-aminoquinoline group would inhibit haem binding.

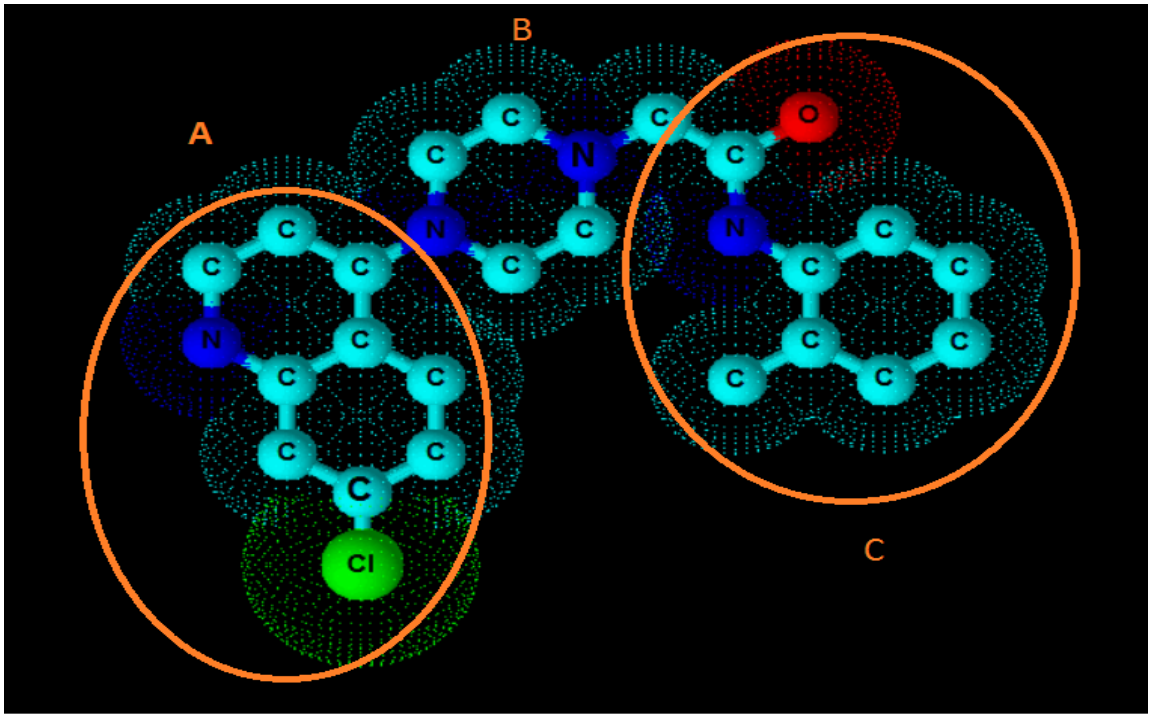


Figure 1.13: Hybrid antimalarial with 4-aminochloroquinoline group (A) linked to an acetamide (C) group via a piperazine linker (B) (personal communication, Azam, 2014).

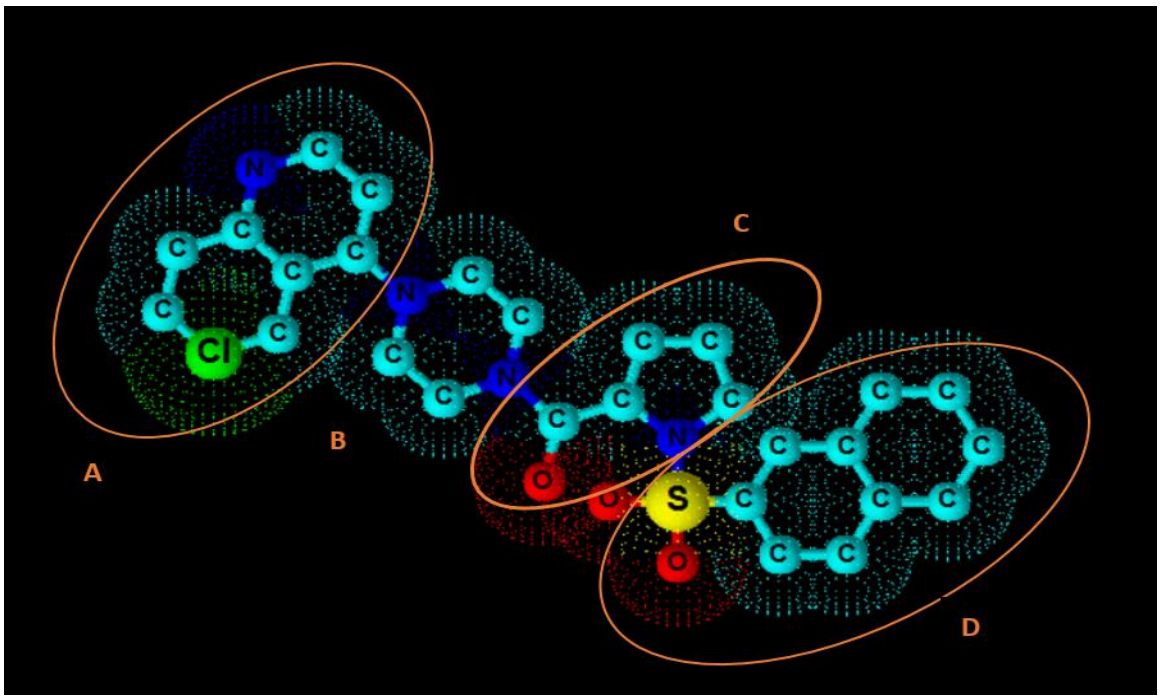


Figure 1.14: Basic structure of 4-(7-chloroquinolin-4-yl) piperazin-1-yl) pyrrolidin-2-yl)methanone derivatives, bearing a 4-aminoquinoline (A), piperazine (B) and pyrrolidine ring (C) with a sulphonamide group (D) (personal communication, Azam, 2014).

The components of these hybrids compounds were selected based on specific features of the individual compounds:

- Both sets of derivatives contain an aromatic nucleus similar to chloroquine which is known to have high binding affinity for haem in the malaria parasite (Leed *et al.,* 2002). The specificity of chloroquine in accumulating and binding to a unique target within the malaria parasite illustrates its' importance in including similar structural features in to novel hybrid compounds.
- Clindamycin, an antibiotic that contains a pyrrolidine ring has shown positive antimalarial results against *P. falciparum* and is used in combination with either quinine or chloroquine (Lell *et al.,* 2002).
- The sulphonamide group on the second group of derivatives is proposed to show similar activity to sulphonamides in inhibiting the enzyme DHPS in the folate sythesis pathway. A sulphonamide, sulphadoxine is used in combination with pyrimethamine in treating malaria in pregnancy and young children (Rogerson *et al.,* 2000).
- The acetamide group on the first set of derivatives in this study, is characteristic of proguanil, an antimalarial that inhibits the enzyme DHFR in the folate synthesis pathway (Hogh *et al.,* 2000).

In recent studies, piperazine and pyrrolidine derivatives showed antimalarial activity against *P. falciparum* (Mendoza *et al.,* 2011). Studies by Ekoue-Kovi *et al.,* (2009), showed that introducing a sulphonamide side chain to chloroquine analogues showed high antimalarial activity and low resistance index. These studies provide a platform for the rational design of dual functionality hybrid molecules with two or more pharmacophores in the development of promising novel antimalarials. This strategy, was undertaken in this study to create novel antimalarial hybrids which are able to restore the efficacy of traditional pharmacophores and surmount the rapid development of resistance.

Despite the availability of current antimalarials, and the use of vector prevention methods, mortality rates in endemic areas are still high (WHO, 2016). The emergence of resistance to current antimalarials, as well as to insecticides places the importance on developing new antimalarials to overcome the current resistance. Despite the resistance to the aminoquinoline core structure, this pharmacophore displays unique antimalarial ability and low host cell toxicity, which warrants its' inclusion into new hybrid drugs. The design and synthesis of the new drugs in this study are centred around the basic pharmacophore of the aminoquinoline, with the addition of side chains to decrease resistance as well as increase the overall antimalarial activity of the hybrid derivatives.

1.10 Aims & Objectives

The aim of this study was to investigate the *in vitro* antimalarial activity against *P. falciparum* and larvicidal activity against *An. arabiensis* of 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives and 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone derivatives.

In order to achieve this, the main objectives of this study were as follows:

- To determine the antimalarial activity of a total of 36 novel derivatives using the parasite lactate dehydrogenase (pLDH) assay.
- To evaluate the effect of lead derivatives in combination with standard antimalarials to assess drug interactions.
- To determine the larvicidal effects of the derivatives on *Anopheles arabiensis* mosquitoes to assess a possible dual action of the derivatives.
- To assess the toxicity of the derivatives against the host red blood cell using the red blood cell haemolysis assay.
- To determine the selectivity index of the derivatives for *P. falciparum* parasites compared to human epithelial cells.
- To determine the stage-specific morphological effects of the most active compounds to elucidate antimalarial mechanisms of action.
- To evaluate the drug-like properties of the most active derivatives using Lipinski's predictions.
- To evaluate toxicity of the derivatives toward *Artemia fransiscana* (brine shrimp) using a lethality assay.

Chapter 2: Methodology

2.1 Compounds and derivatives

All compounds were purchased from reputable commercial suppliers at the best quality. The derivatives were synthesised, characterized and the structures confirmed by spectroscopic techniques by Prof A. Azam from the Department of Chemistry at Jamia Islamia Millia, New Delhi (India). The purity of the compounds were established on the basis of CHNS elemental analysis in which values lie in the range ±0.3. Two sets of compounds were synthesised, twenty seven 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives (Table 2.2) and nine 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone derivatives (Table 2.1). The derivatives were prepared in dimethyl sulfoxide (DMSO) to a 10 mM stock and stored in 1 ml aliquots at -70˚C until needed for experiments.

2.2 Culturing of asexual stages of parasite

Culturing and maintenance of the chloroquine-sensitive strain of *P. falciparum* (NF54) malaria parasites was carried out in accordance with the methods described by Trager and Jansen (1977), Freese *et al*. (1988) and departmental protocols (Appendix A). The *P. falciparum* (NF54) strain was used as it is the most pathogenic form of malaria found in humans exclusively (Klein, 2013). Furthermore, the intention of further gametocyte studies with the *P. falciparum* strain provided the basis in using the NF54 strain. Aseptic techniques were used in a laminar flow unit with 70% ethanol to wipe down containers and surfaces.

2.2.1 Preparation of reagents

The **phosphate buffered saline (PBS**) was prepared by dissolving 4 mM potassium chloride (Sigma Aldrich®), 2 mM potassium phosphate monobasic (Fluka®), 4 mM sodium chloride, 0.004 M sodium phosphate dibasic dehydrate (Sigma Aldrich®) in autoclaved MilliQ® water and then the pH adjusted to 7.4 with 10 mM NaOH (Sigma Aldrich®). The PBS solution was autoclaved and stored at 4˚C.

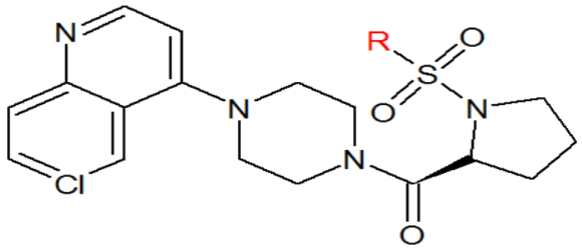
The **incomplete culture medium** was prepared in autoclaved MilliQ® water with 10.4 g/L Roswell Park Memorial Institute (RPMI) medium 1640 (Gibco®), 30 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer (Boehringer Mannheim®), 0.02 M anhydrous glucose (Merck®), 0.3 mM hypoxanthine (Sigma Aldrich®) and 0.1 mM gentamicin (Sigma Aldrich®). The preparation was stirred for one hour and filter sterilised through a 0.22 µm filter (Sterivex™). The **incomplete experimental media** was prepared as the incomplete culture medium with the omission of gentamicin and stored at 4˚C.

Lipid-rich bovine serum albumin (AlbuMAX®) (Gibco®) was prepared (Appendix B) in incomplete experimental medium as a 5% (w/v) solution and stirred vigorously for at least 2

hours at 37˚C. The solution was filter sterilised using a 0.22 µm filter (Sterivex™) and stored at -20˚C.

Complete culture medium was prepared in incomplete culture medium with 10% (v/v) AlbuMAX® (Section 2.2.1) and 4.2 ml 5% (w/v) sodium bicarbonate (Sigma Aldrich®) under sterilised conditions.

Table 2.1: The core structure and side chains of the 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone derivatives.



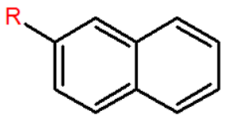
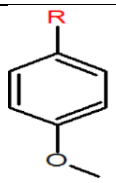
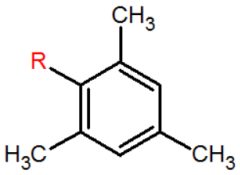
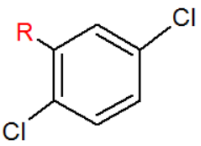
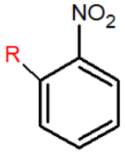
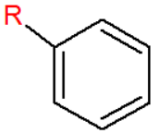
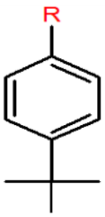
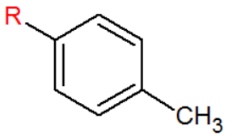
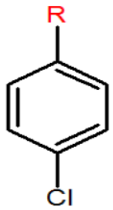
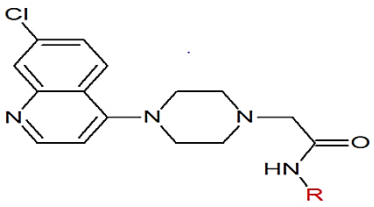
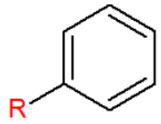
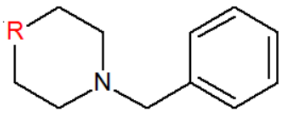
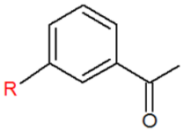
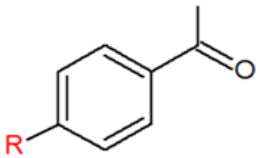
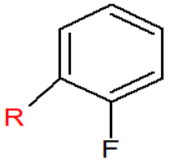
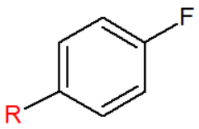
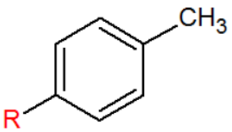
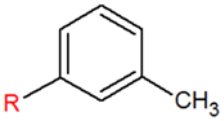
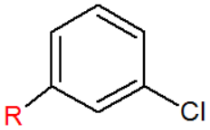
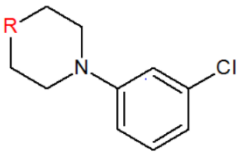
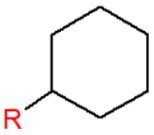
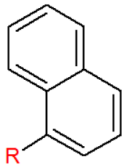
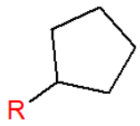
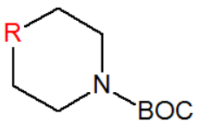
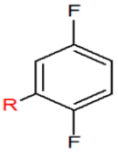
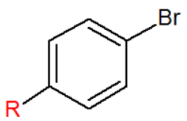
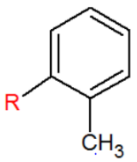
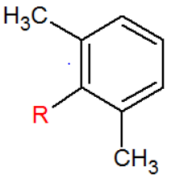
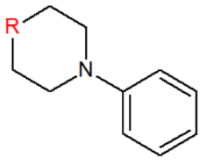
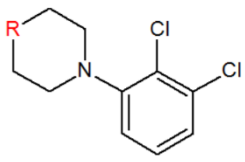
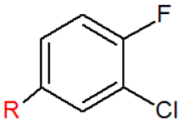
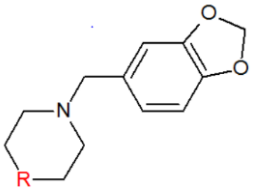
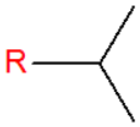
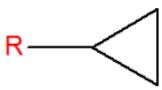
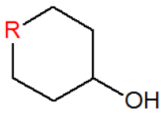
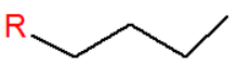
Compound designation	R-group	Compound designation	R-group
CQPPM-**1**		CQPPM-**6**	
CQPPM-**2**		CQPPM-**7**	
CQPPM-**3**		CQPPM-**8**	
CQPPM-**4**		CQPPM-**9**	
CQPPM-**5**			

Table 2.2: The core structure and side chains of the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives.

Compound designation with R Group substituent



 CQPA-**1**	 CQPA-**2**	 CQPA-**3**	 CQPA-**4**
 CQPA-**5**	 CQPA-**6**	 CQPA-**7**	 CQPA-**8**
 CQPA-**9**	 CQPA-**10**	 CQPA-**11**	 CQPA-**12**
 CQPA-**13**	 CQPA-**14**	 CQPA-**15**	 CQPA-**16**
 CQPA-**17**	 CQPA-**18**	 CQPA-**19**	 CQPA-**20**
 CQPA-**21**	 CQPA-**22**	 CQPA-**23**	 CQPA-**24**
 CQPA-**25**	 CQPA-**26**	 CQPA-**27**	

2.2.2 Collection and preparation of uninfected red blood cells

Human ethics was obtained from the University of the Witwatersrand Human Research Ethics committee (clearance certificate number: M140669, Appendix C) to draw blood from healthy volunteers. Whole, fresh blood was collected in Vacuette® blood vial tubes containing an anticoagulant (acid citrate dextrose) from healthy volunteers who were not on antimicrobials, anticoagulants or antimalarials. The blood was washed by centrifugation at 671 g for 5 min to remove the white blood cells, plasma and platelets. The supernatant was discarded and RBCs washed three times with PBS (pH 7.3) after which incomplete experimental media (Section 2.2.1) was added to maintain a 50% haematocrit and stored at 4˚C for a maximum of 10 days before discarding.

2.2.3 Maintenance of *P. falciparum* NF54 strain culture

Bioethics clearance was obtained from the University of Witwatersrand Human Research Ethics committee to culture malaria *P. falciparum* parasites (clearance certificate number:07-11-2017, Appendix D). *P. falciparum* (NF54 strain) was cultured in a tissue culture flask (Sigma Aldrich®) at a 5% haematocrit and 5% parasitaemia. On a daily basis, slides of the cultures were prepared (Section 2.2.4) and examined microscopically. Based on the microscopic findings (Figure 2.1), blood was added when the parasitaemia was >5% trophozoites/schizonts and synchronised when in the ring stage (Section 2.2.5). The spent media from the culture was replaced with complete culture medium (Section 2.2.1) before the culture was gassed for 30 seconds with 3% oxygen, 4% carbon dioxide and 93% nitrogen before being incubated at 37˚C in a static position.

2.2.4 Giemsa staining

Giemsa-stained thin blood smears were prepared from settled cultures on a daily basis to assess parasitaemia, morphology and stage (Figure 2.1). The Rapi-diff stain set (Clinical Sciences Diagnostics) was used to stain the parasite in the red blood cell. The slide was immersed into solution 1 (thiazine dye) once to fix the slide, and immersed into solution 2 (eosin) five times to stain the RBCs, then rinsed in water and dried and immersed in solution 3 (methylene blue) ten times to stain the parasites. The slides were microscopically (Olympus®) examined under oil immersion at 1000X magnification. The stage, morphology and percent parasitaemia (Equation 2.1) was determined after viewing and counting ten fields of the stained thin blood smear.

$$\% \text{ Parasitaemia} = \frac{Total\ number\ of\ parasitized\ RBCs}{Total\ number\ of\ uninfected\ RBCs + parasitised\ RBCs} \times 100$$

Equation 2.1: Calculation to determine percentage parasitaemia of a culture from a thin blood smear.

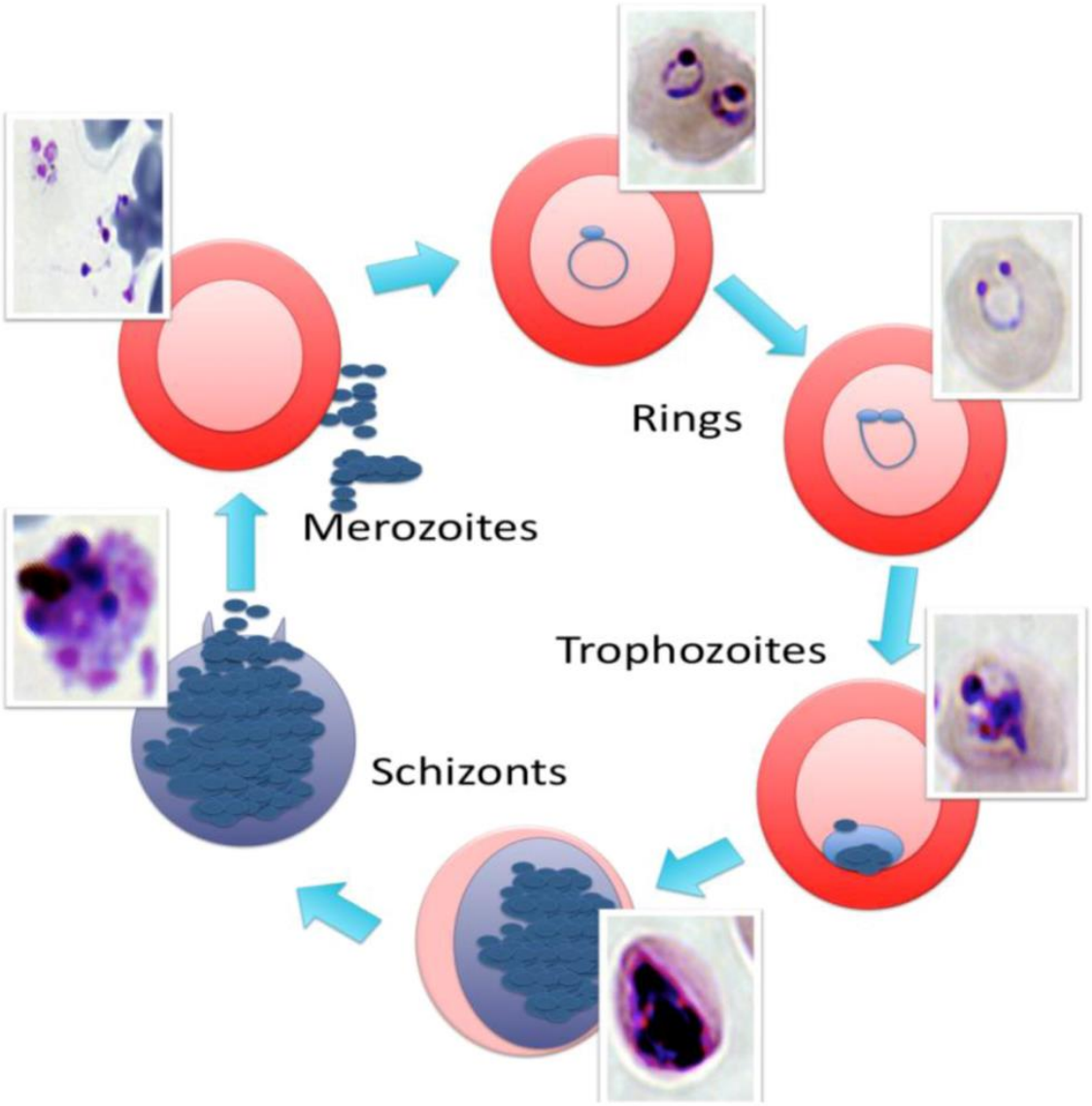


Figure 2.1: The intra-erythrocytic cycle of *P. falciparum* with inset photographs at 1000x magnification showing the appearance of the parasites at different stages upon Giemsa staining.

2.2.5 Synchronisation of parasites

The cultures were synchronised when predominantly in the ring stage (Figure 2.1) with a 5% (w/v) D-sorbitol (Lambros *et al.,* 1979) to allow the D-sorbitol to lyse the trophozoites and schizonts and keep the culture in synchrony. This is based on the differential permeability of the red blood cell membranes where uninfected red blood cells and early ring staged infected red blood cells are less permeable than later staged *Plasmodium*-infected red blood cells (Lambros *et al.,* 1979). This property is used to selectively kill the mature stages of the

parasite by osmotic shock while the uninfected red blood cells and ring stages remain unaffected. The pellet was treated with 20 ml of 5% (w/v) D-sorbitol (Sigma Aldrich®) following a 20 minute incubation at 37˚C. Thereafter the suspension was centrifuged at 400 g for 5 minutes and supernatant discarded. The culture was washed thrice in PBS before adding the complete culture media and being gassed (Section 2.2.3) and returned to the flask to be re-incubated.

2.3 Antimalarial activity

2.3.1 Parasite lactate dehydrogenase assay

The *in vitro* anti-*Plasmodium* activity in this study was determined using a modified method of the parasite lactate dehydrogenase (pLDH) assay as described by Makler *et al.* (1993). Lactate dehydrogenase (LDH) is an essential metabolic enzyme, which catalyses the dehydrogenation of lactate and produces pyruvate with nicotinamide adenine dinucleotide (NAD) as a co-enzyme. The assay distinguishes parasite LDH from host LDH by using 3-acetylpyridine adenine dinucleotide (APAD) as an analogue of NAD (Makler *et al.,* 1993). The pLDH converts APAD to APADH, which in turn reduces yellow nitro blue tetrazolium (NBT) to purple formazan salts. The percentage of parasite survival was determined measuring the conversion of NBT. Malstat™ was used to disrupt the red blood cell membranes, exposing the parasite to NBT for the enzymatic reaction to occur.

The 1.96 mM NBT (Sigma-Aldrich®) and 0.24 mM phenazine ethosulphate (PES) (Sigma-Aldrich®) solutions were freshly prepared for each experiment in MilliQ® water, hand filtered (0.22 µm) and stored at 4˚C in the dark. The Malstat™ reagent was prepared by combining Triton™ X-100 (1 ml/L), 0.5 mM APAD (Sigma-Aldrich®), calcium-L-lactate (0.18 mM) (Sigma-Aldrich®) and trisaminomethane (TRIS) buffer (27.5 mM Tris base (Prolabo®) in MilliQ® water and stirred at 37˚C until dissolved. The freshly prepared solution was filtered (0.22 µm) and stored in the dark.

2.3.2 Experimental set-up

Parasite cultures were synchronised in the ring stage with 5% (w/v) D-sorbitol (Section 2.2.5). Parasitised red blood cells were prepared at a 2% parasitaemia and 2% haematocrit in complete experimental culture media (Section 2.2.1). One hundred microliters of the parasitised red blood cells was plated out in all wells of a 96 well microtitre plate except the blank wells (Figure 2.2). A 2% haematocrit of non-parasitised red blood cells was prepared and plated out in 3 wells (100 µl) of the last row to serve as an uninfected red blood cell control, with the remainder of the last row serving as the untreated parasitised red blood cell control (Figure 2.2). The plates were incubated in sterile candle jars containing MilliQ® water

to ensure a humidified environment was maintained at 37˚C for 24 hours. The approximate micro-anaerobic conditions (3% CO_2, 17% O_2, 80% N_2) were achieved by burning candles in the candle jar (Trager and Jensen, 1977).

After 24 hours, compounds and controls prepared in incomplete culture medium, were plated out (12.5 µl) and 12.5 µl of incomplete culture medium added to the untreated uninfected and parasitised red blood cell wells. The plates were returned to the candle jar and re-incubated for 48 hours at 37˚C after re-lighting the candles in the candle jar at 24 hour intervals.

After incubation, plates were sealed using parafilm and frozen at -70˚C for 1 hour, after which they were thawed for 2 hours in the incubator at 37˚C in the dark. The contents of the plates were re-suspended on a Thermo™ plate shaker for 2 minutes at 1200 rpm. Maintaining the same plate layout, 25 µl of the re-suspended red blood cell lysate was transferred to a separate sterile 96 well plate. One hundred microliters of Malstat™ and 20 µl of NBT/PES (1:1) solution were added to each well, plates sealed and shaken on a Thermo™ plate shaker for 30 seconds before being incubated for 40 minutes at 37˚C in a sealed plastic container to develop. A 5% (v/v) acetic acid (50 µl) was added to each well to stop the reaction and air bubbles were removed with a hairdryer to prevent inaccurate absorbance readings. The absorbance of the formazan products was read at 620 nm.

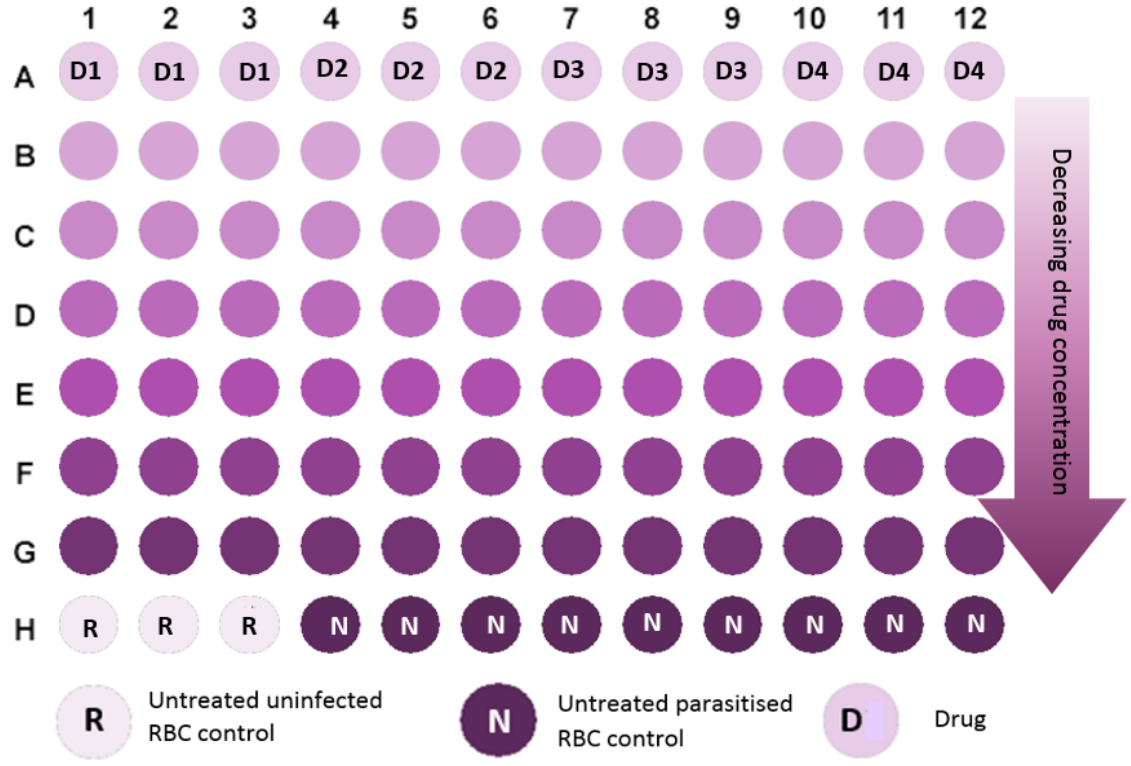


Figure 2.2: Plate layout in the pLDH assay indicating decreasing drug dilutions (from top to bottom) in triplicate (horizontally) with colour changes indicating parasite presence (dark purple) and no parasite (clear to light purple).

2.3.3 Data analysis

For compounds showing >50% inhibition of parasite growth, serial dilutions were prepared to determine the concentration that inhibited 50% of parasite growth (IC_{50} value) and log sigmoidal dose response curves generated using GraphPad® Prism Version 5.02. For compounds inhibiting less than 50% parasite growth, the percent parasite growth inhibition was reported. Each experiment was repeated thrice to produce mean ± standard deviation.

2.3.4 Combination studies

With multi-drug resistance being reported in most parts of the world, new antimalarial regimens advocating combination therapy are in place (WHO, 2015). The concept of combination therapy is based on the potential synergistic effects of using two or more combined drugs to increase efficacy and delay the development of resistance (Berenbaum, 1989). The pLDH assay was used (Section 2.3.1) with an additional two fold dilution factor taken into account to account for the addition of two compounds. Two of the three most active derivatives were combined with quinine. Based on the IC_{50} value of the individual derivatives, the test compound and quinine were combined in different ratios which were prepared such that as the concentration of the one increased, the concentration of the other decreased (Table 2.3). Serial dilutions were prepared from each set of ratios, from which log sigmoid dose response curves were constructed to determine IC_{50} values for the drug alone and the combinations. To assess the interaction between the test compound and quinine, an isobologram was constructed (GraphPad® Prism Version 5.02) and generated using the Fractional Inhibitory concentration (FIC) equation (Equation 2.2).

$$FIC = \frac{\text{IC}_{50}\text{ of compound in combination}}{\text{IC}_{50}\text{ of compound alone}}$$

Equation 2.2: Calculation to determine the FIC using the IC_{50} values of the compound alone and in combination.

Table 2.3: Concentration ratios at which derivatives were combined with quinine (B-H) and positive controls containing single compounds alone (A & I).

Concentration (µM)	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
Quinine	50	50	35	20	7.5	0.2	0.02	0.002	0
Derivative	0	0.05	0.5	0.75	1.5	25	35	50	75

The values on the isobologram above the line were interpreted as being antagonistic, below as synergistic, and those near the line as additive (Figure 2.3). To verify the interaction interpreted from the isobologram, the sum of the FIC values (∑FIC) for the derivative with quinine at each combination ratio was calculated and interpreted as follows: <0.5 as synergistic, 0.5-2.0 as additive, while that > 2 as antagonistic (Berenbaum, 1989; van Zyl *et al.,* 2010).

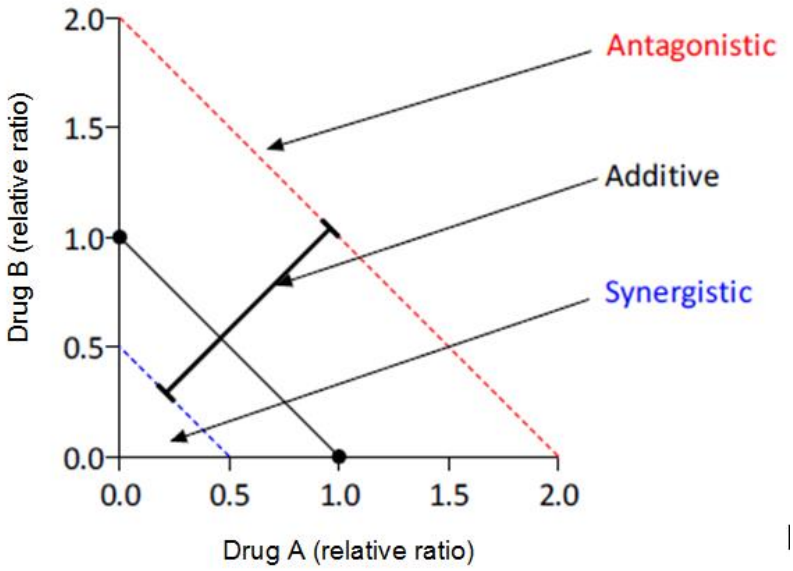


Figure 2.3: Visualisation of possible pharmacological interactions between two compounds in combination using an isobologram (adapted from Berenbaum *et al.,* 1989).

2.4 Stage determination

This study was carried out to determine the specific effects of the two most active derivatives on the asexual stage in the life cycle of the malaria parasite (Moseley, 2012). The IC_{50} and IC_{90} values of the most active derivative from each class of compounds was determined from the log sigmoid dose-response curves (Section 2.3.3). Working solutions of the derivatives at the IC_{50} and IC_{90} values were prepared in incomplete experimental culture media to determine concentration effects on the parasite. The study was carried out in 25 cm^3 tissue culture flasks (Sigma Aldrich®) to enable easy removal of blood samples. Parasites were synchronised in the ring stage (Section 2.2.5) and prepared at a 2% parasitaemia and 2% haematocrit in complete culture media. The synchronised parasites (5.4 ml) were placed in each flask to which the derivatives (600 µl) were added. A negative control was prepared with a 2% haematocrit and 2% parasitaemia in the absence of any drug. The standard antimalarial agent, quinine was also prepared at its IC_{50} and IC_{90} values for comparison. The

cultures were gassed in an atmosphere of 3% oxygen, 4% carbon dioxide and 93% nitrogen for 15 seconds, sealed and placed in the incubator at 37˚C in a static position. Every 8 hours the flasks were removed from the incubator and agitated gently to re-suspend the contents to remove samples (20 µl) to make thin blood smears (Section 2.2.4) from the centrifuged sample (671 g for 5 minutes). After each time interval of preparing a blood smear, the cultures were re-gassed and returned to the incubator. The blood smears were analysed microscopically at 1000x magnification (Olympus®) to determine total parasitaemia, percent parasitaemia at each stage and to note morphological changes in the parasite.

2.5 Measurement of drug-likeness

Keeping in line with the Medicines for Malaria Venture requirements that the oral bioavailability of new drugs must be determined, as such the solubility and permeability properties of the derivatives were predicted using the Lipinski's rule of five (Ro5) predictions to determine the bioavailability of the derivatives. The drug-likeness of the most active derivatives to be able to permeate into the acidic digestive vacuole of the *P. falciparum* parasite (Figure 1.8), was measured using the physicochemical properties of the derivatives in comparison to the reference drug quinine (Figure 2.4). Drug-likeness refers to the drug-like permeability properties for the purpose of this investigation (Bhal *et al.,* 2007). The Lipinski's Ro5, also known as the Pfizer's rule of five is a rule to evaluate drug-likeness or determine if a chemical compound with a certain pharmacological or biological activity has properties that would make it a likely drug that would be orally bioactive in humans (Lipinski *et al*., 1996). In this study, the partition coefficient was used as a predictor of the lipid solubility of the derivatives. The Lipinski's 'rule of five' concept was applied as outlined by Lipinski *et al*. (1996), such that drugs with a molecular weight less than 500, a partition coefficient (Log P) less than 5, possessing less than 5 H-bonds and less than 10-H bond acceptors were considered to have drug-like properties. Two dimensional structures of the derivatives were constructed using ACD/ChemSketch software (freeware version). Thereafter, solubility and permeability predictions were performed using ACD/iLab version 2. Derivatives that violated more than one of the rule of five parameters were considered as non-drug-like, having poor pharmacokinetic properties.

The ionisation constants (pKa values) as predicted by the ACD/iLab version 2 software, were used to determine the degree of ionisation of the derivatives at physiological pH 7.4 (red blood cell cytosol) and pH 5 (parasite digestive food vacuole), was derived using the Henderson-Hasselbalch equation (equation 2.3) (Po *et al.,* 2001).

$$\%\, Ionisation = \frac{100}{1 + \text{antilog (pKa - pH)}}$$

Equation 2.3: Henderson-Hasselbalch equation to determine the percentage ionisation for weak acids and weak bases.

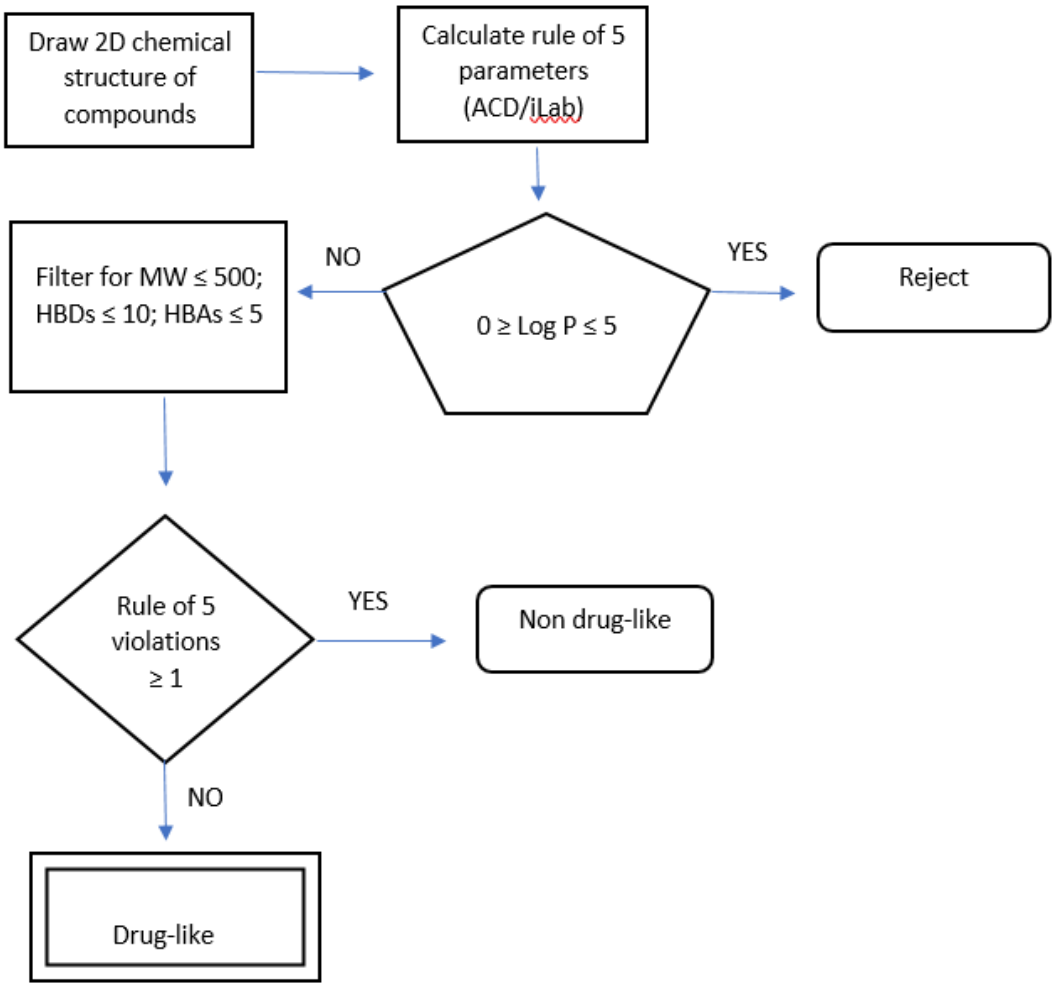


Figure 2.4: Workflow of methodology in applying the Lipinski rule of five (adapted from Bhal *et al.,* 2007).

2.6 Larvicidal assay

All the derivatives were screened against *Anopheles arabiensis* mosquito larvae to determine its activity, according to the WHO protocols with slight modifications (WHO, 2005).

2.6.1 Determining concentration for screening compounds

In order to determine the most appropriate concentration at which to screen compounds against the larvae, the lethal concentration at which 50% of the larvae are killed (LC_{50}) with dichlorodiphenyltrichloroethane (DDT) (Supelco®) (positive control) was obtained and the concentration determined from that value. A stock solution of DDT in ethanol was prepared at a concentration of 1 mg/ml (2817.7 µM) with 1:10 dilutions in ethanol prepared. The experiment was carried out five times as explained below (Section 2.4.2), and the LC_{50} value

determined using Probit analysis with IBM SPSS® Statistics version 22 software. The concentration of screening the derivatives was set at 10 times higher than the LC_{50} of DDT. For purposes of generating a graph, GraphPad® Prism version 5.02 was used and verified to produce the same LC_{50} as probit analysis (IBM SPSS® Statistics version 22).

2.6.2 Bioassay

Animal ethics was obtained from the University of Witwatersrand Animal Research Ethics Committee (clearance certificate: 07-11-2017-O, Appendix D). Mosquito larvae were obtained from a DDT-susceptible strain of *Anopheles arabiensis* (KGB) housed in the Botha de Meillon Insectary at the National Institute for Communicable Diseases, Johannesburg and fed standard larval food twice a day. Batches of 25 third to fourth instar larvae (male or female) of *An. arabiensis* (KGB) were gently introduced into disposable plastic cups (173.2 cm^2) containing a total of 25 ml of distilled water. To each test cup, 250 µl (0.5 µM) test derivative or positive control DDT (0.5 µM) or untreated background control with water or untreated solvent controls of DMSO or ethanol was added and incubated for 24 hours at 25-28˚C. The larvae in each cup were fed standard larval food by tapping enough powder to cover the water surface. After 24 hour incubation, dead larvae were gently prodded and non-responsive larvae recorded as dead. Percentage mortality was calculated according to Equation 2.4.

$$\%\ \text{Mortality} = \frac{(\%\ \text{survival in untreated control}) - (\%\ \text{survival in treated control})}{\%\ \text{survival in untreated control}} \times 100$$

Equation 2.4: The equation used to calculate the percent mortality of larvae caused by the compound tested.

2.6.3 Data analysis

Each derivative was tested once in each experiment and the experiment carried out 5 times to produce a mean ± standard deviation. Compounds showing significant larivicidal effects were further tested to produce the concentration of derivative required to kill 50% of the *An. arabiensis* larvae (LC_{50}) and generate a log dose-probit regression line using the IBM SPSS® Statistics version 22 program. For compounds showing mortality <10%, the percentage mortality was reported.

2.7 Toxicity assays

Toxicity assays are paramount in the screening of novel compounds. The essence is not merely determining the safety of a compound, but also to characterise the possible toxic effects it can produce.

2.7.1 Haemolysis assay

The haemolysis assay was carried out to determine the toxicity profile of the compounds, by determining whether the test derivatives could induce lysis of the red blood cell membrane by evaluating haemoglobin release. The haemolytic assay was carried out using aseptic technique on healthy freshly obtained red blood cells (Section 2.2.2) as described by Hayat *et al.* (2011).

2.7.1.1 Preparation of test derivatives and classical antimalarials

Stock solutions (10 mM) of test derivatives and positive controls chloroquine (Sigma-Aldrich®) and quinine (Sigma-Aldrich®) were diluted to a screening concentration of 50 µM in incomplete experimental medium (Section 2.2.1) with the appropriate dilution factor taken into account.

2.7.1.2 Preparation of plates

In a sterile 96 well-plate, 25 µl of each of the 50 µM derivatives were plated out in triplicate with 200 µl of a 2% haematocrit red blood cell suspension (Section 2.2.2) and 25 µl of incomplete experimental media. The last row of the plate was used for the controls, with 50 µl of 2.9% (v/v) Triton™ X-100 solution (Sigma-Aldrich®) in PBS (pH 7.4) plated out in 3 wells as the positive, 100% lysis positive control and 50 µl incomplete culture media as a 0% lysis negative control in the remaining wells.

The plates were incubated in a humidified candle jar at 37˚C for 48 hours. Thereafter, the plates were gently agitated and centrifuged at 377 g for 5 min. The supernatant (50 µl) from each well was transferred to a second non-sterile 96 well-plate, with 150 µl water added to each well. The absorbance was read at 412 nm and the percentage haemolysis calculated according to equation 2.5.

$$\%\ Haemolysis = \frac{\text{Absorbance (test compound)} - \text{Absorbance (0\% haemolysis control)}}{\text{Absorbance (100\% haemolysis control)} - \text{Absorbance(0\% haemolysis control)}} X\ 100$$

Equation 2.5: Determination of the percent haemolysis caused by the parasite on the red blood cells.

2.7.1.3 Data analysis

All experiments were carried out in triplicate to produce a mean ± standard deviation. Compounds that produced <40% haemolysis were reported as a percentage haemolysis at 50 µM. For compounds displaying >40% haemolysis, an IC_{50} was determined.

2.7.2 Cell viability assay

The cell viability assay measures the ability of cells to reduce the tetrazolium dye, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), to its insoluble formazan product, resulting in a purple colour. Since this requires a functioning mitochondria, the assay effectively measures the metabolic activity of live cells (Mosmann, 1983).

2.7.2.1 Preparation of medium and reagents for cell culturing

Incomplete medium consisted of 13.53 g/l Dulbecco's Modified Eagles Medium (DMEM) (Sigma Aldrich®) and 44 mM $NaHCO_3$ (Sigma Aldrich®) double distilled MilliQ® water and stirred until dissolved. The solution was filter sterilised through a 0.22 µm filter and stored at 4˚C.

To prepare **complete culture medium** to maintain the cells, the incomplete medium was supplemented with 10% foetal bovine serum and stored at 4˚C.

The **MTT** salt solution was prepared as a 12 mM solution in PBS (pH 7.3) and filter sterilised through a 0.22 µm filter and stored at 4˚C.

2.7.2.2 Cell maintenance

Human embryonic kidney epithelial (HEK-293) cells were maintained at 37˚C in a humidified 5% CO_2 environment (clearance certificate: W-CJ-161129-2, Appendix E). Cells were viewed microscopically at 40x magnification (Olympus® CKX41) to determine if the cells appeared healthy and confluent (Figure 2.4). Spent media was discarded and cells rinsed once with 10 ml sterile PBS (pH 7.3). Fresh complete culture medium (20 ml) was then added to the cells together with 200 µl penicillin 100 IU/ml^{-1} and streptomycin 100 IU/ml^{-1} solution. The culture was then returned to the incubator or trypsinised to split the confluent cells (Section 2.5.2.3) or to be used in experiments (Section 2.5.2.4).

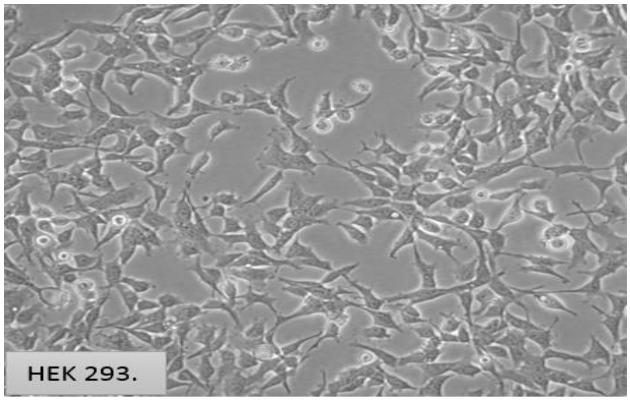


Figure 2.5: Morphological appearance of HEK-293 cells.

2.7.2.3 Splitting cells

The cells were split when they were confluent, as viewed microscopically (Figure 2.5). The media was discarded and cells rinsed once with PBS (10 ml) as above. Thereafter, 3 ml 10% trypsin was added. The flask was incubated at 37˚C for 1 minute and agitated to lift cells. Complete culture media (3 ml) was added to inactivate the trypsin and the cell suspension transferred to a 50 ml sterile tube, and re-suspended in complete culture medium up to 10 ml. This was used in the cell viability assay (Section 2.5.2.4). One millilitre of the suspension was used to re-establish the culture in fresh complete culture media to which 200 µl penicillin/streptomycin was added and incubated for a further 48 hours before this process needed to be performed again.

2.7.2.4 MTT cell viability assay

The cell suspension as prepared above (Section 2.5.2.2), was adjusted to 10 000 cells per well for all viability experiments. The cell density of this latter cell suspension was determined by staining the cells with 0.2% (w/v) tryphan blue (Sigma Aldrich®) and microscopically counting the cell number on a haemocytometer at 100x magnification to determine the average cell count per ml. The cell suspension was adjusted in complete experimental culture medium such that $1x10^4$ cells/180 µl were plated per well, except to the background control wells which contained no cells only 180 µl complete culture medium. The remainder of the row served as the untreated controls with 100% cell growth. The plate was incubated for 24 hours at 37˚C in a humidified environment with 5% CO_2. After incubation, the derivatives or controls (20 µl) were plated out in triplicate with camptothecin (Molekula®) as the positive control and 20 µl DMEM added to background and untreated control wells. The plates were once again incubated under the same conditions for 48 hours. Thereafter, wells were treated with 40 µl MTT (12 mM) for 2 hours and the plates were centrifuged at 671 g for 5 minutes before the supernatant (180 µl) was discarded and replaced with 180 µl DMSO to solubilise the formazan crystals. The plates were agitated slightly on a Thermo plate shaker for 2 minutes at 1000 rpm and left to stand for a further 10

minutes in the dark, to ensure complete dissolution of the crystals. The test absorbance was read at 540 nm (purple formazan colour), with a reference wavelength of 690 nm (unreacted yellow MTT) using a UV-Vis plate spectrophotometer (Labsystems Multiskan LC). The percent cell growth was calculated using Equation 2.6.

$$\%Cell\ viability = \frac{\text{Absorbance drug (540 nm)} - \text{Absorbance drug (690 nm)}}{\text{mean absorbance untreated control(540 nm)} - \text{mean absorbance untreated control(690 nm)}} \text{x100}$$

Equation 2.6: Determination of percentage cell viability (growth) taking the reference and test wavelengths into account.

2.7.2.5 Data analysis

Serial dilutions were prepared for compounds that inhibited >60% cell growth, to determine the IC_{50} values and generate log sigmoidal dose response curves using GraphPad® Prism Version 5.02. For compounds that inhibited <60% cell growth, the percentage inhibition at 50 µM was reported. All experiments were repeated at least in triplicate to produce a mean ± standard deviation.

2.7.3 Safety Index

The safety index was calculated as the selective activity of the compounds against the *P. falciparum* parasites in comparison to their cytotoxicity on the nomalised cell line. The safety index was calculated as a cytotoxicity to antiplasmodial activity ratio as in Equation 2.7 (Manohar *et al.,* 2010).

$$Safety\ Index\ (SI) = \frac{cytotoxicity\ (IC_{50})}{Antimalarial\ activity\ (IC_{50})}$$

Equation 2.7: The safety index of the derivatives in targeting *P. falciparum* in preference to human cell lines.

2.7.4 Lipid peroxidation assay

Reactive oxygen species are thought to be involved in the pathogenesis of malaria and in the progression of the disease (D'Souza *et al.,* 2006). The lipid peroxidation assay as outlined by Gulcin (2007) was used to assess if the compounds induced death to the parasites via this pathway as a potential mechanism of action.

2.7.4.1 Preparation of reagents

Sodium phosphate buffer (0.2 M): A 100 ml solution of $NaH_2PO_4.H_2O$ (2.76 g) (Sigma-Aldrich®) and 200 ml solution of $NaH_2PO_4.2H_2O$ (7.12 g) (Sigma-Aldrich®) were prepared individually in MilliQ® water and 78 ml and 122 ml of each solution respectively was combined. The pH was adjusted to 7 and the solution stored in a glass container at 4˚C.

Ammonium thiocyanate (3.94 M): Ammonium thiocyanate (30 g) (Merck®) was dissolved in 100 ml MilliQ® water in a fume hood and stored at 4˚C away from light.

Ferrous chloride (20 mM): Ferrous chloride (0.127 g) (Fluka®) was freshly prepared on the day of experiment where it was dissolved in 50 ml 3.5% (v/v) HCl and further diluted to a 5 mM working solution.

Linoleic acid emulsion: The emulsion was freshly prepared on the day of the experiment**.** Linoleic acid (51.2 µl) (Fluka®) and Tween-20 (51.2 µl) (Sigma-Aldrich®) were mixed and the volume made up to 10 ml with sodium phosphate buffer (pH 7) (Section 2.8.1).

2.7.4.2 Lipid peroxidation experimental assay

Twenty five microliters of each derivative (50 µM) was plated out to triplicate wells except for the last row with DMSO (25 µl) as the untreated control. The derivatives and control were reacted with the linoleic acid emulsion (125 µl) and sodium phosphate buffer (100 µl) in a non-sterile 96 well plate. The plates were shaken for 1 minute at 1000 rpm and incubated at 37˚C for 24 hours. Thereafter, 25 µl of the reaction was reacted with 70% ethanol (150 µl), ammonium thiocyanate (40 µl) and ferrous chloride (40 µl) and compared to the positive control, Trolox™ (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). The absorbance was read at 492 nm and the inhibition of lipid peroxidation was calculated as per Equation 2.8.

$$\%\ Lipid\ peroxidation\ inhibition = \frac{Absorbance\ (untreated\ control)\ -\ Absorbance\ (test\ compound/std)}{Absorbance\ (untreated\ control)} \text{x}100$$

Equation 2.8: Determination of the percent lipid peroxidation inhibited by the test compound, where std: standard control.

2.7.4.3 Data analysis

All experiments were carried out in triplicate to produce a mean ± standard deviation. Compounds which caused <40% lipid peroxidation inhibition were reported as a percent peroxidation inhibition.

2.7.5 Brine shrimp lethality assay

The brine shrimp lethality assay was carried out to determine the toxicity of the derivatives on a whole organism in comparison to human cell lines. The method was adapted from the lethality assay as reported by Ruebhart *et al.* (2009).

2.7.5.1 Hatching eggs

To hatch the *Artemia franciscana* eggs, 0.5 g of the cysts were incubated at 25˚C for 24 hours in 500ml salt water (32 g/l) (Aquarium Systems®), under a continuous source of artificial light (60 Watts), while being aerated vigorously by a rotary pump (Figure 2.6). After 24 hours, the newly hatched shrimp (nauplii) were placed in a sorting tray with 200 ml salt water and held for 15 minutes under the artificial light source to concentrate the nauplii to a suitable density as they are attracted to light. Thereafter the nauplii were plated out for use in the bioassay.

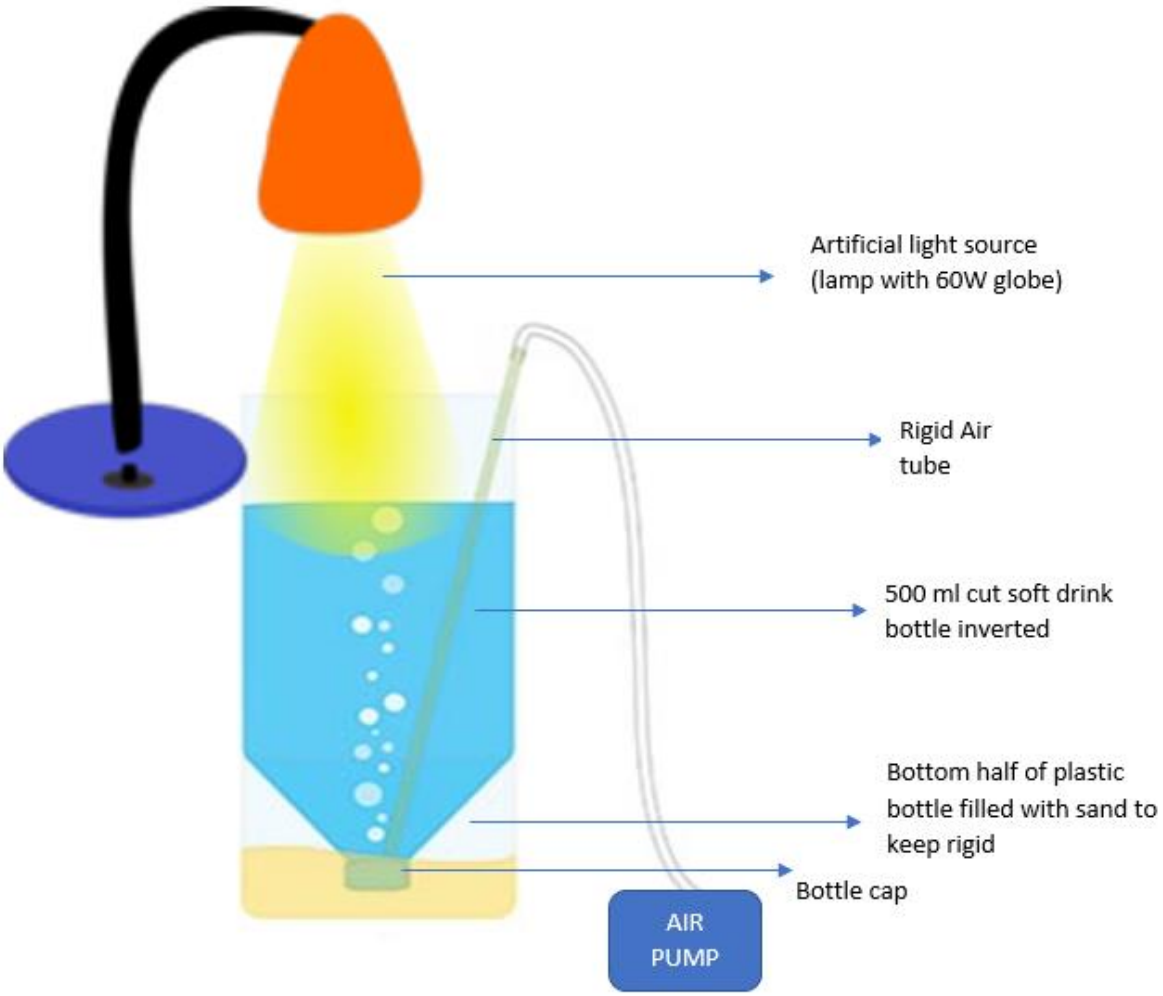


Figure 2.6: The design of the brine shrimp hatchery setup.

2.7.5.2 Bioassay

Animal ethics was obtained by the University of Witwatersrand Animal Research Ethics Committee (clearance certificate: 07-11-2017-O, Appendix F) for the use of Artemia brine shrimp. An average of 20-40 nauplii were plated out (50 µl) in each well of a sterile 48 well plate. Salt water (32 g/L) (197.5 µl) was added to each well, followed by 2.5 µl of derivative (0.5 µM) in triplicate to be screened. All drugs were prepared in DMSO ensuring it equates to <1%. Potassium dichromate (0.5 µM) (125 µl) was plated out as the positive control and a drug-free control containing salt water was used as a negative control. Once plated out, the nauplii were viewed under a microscope (8x magnification; Zeiss©) to record any dead nauplii before incubation (dead at T_0). The plates were incubated for 24 hours at 25˚C. After 24 hours the number of dead nauplii in each well was microscopically counted under 8x magnification (dead at T_{24}). Thereafter, in order to determine the total number of nauplii in each well, 100 µl of acetic acid (5%, v/v) was added to all wells to kill the nauplii. The total number of nauplii was then counted (total nauplii) and used to calculate the percentage mortality using equation 2.9.

$$\% \text{ Mortality} = \frac{\text{Dead nauplii @ T24} - \text{Dead nauplii @ T0}}{\text{Total nauplii}} \times 100$$

Equation 2.9: Determination of the percent mortality of nauplii caused by the derivatives and controls after a 24 hour period.

2.7.5.3 Data analysis

Each experiment was repeated in triplicate to produce a mean ± standard deviation. Compounds showing significant mortality of brine shrimp were analysed as in Section 2.7.1 using the IBM Statistics®22 probit analysis. For compounds not showing any significant mortality, the nauplii treated with each compound were further investigated microscopically (Olympus® CKX41) for any significant morphological changes along with those showing significant mortality.

2.8 Statistical analysis

All experiments were carried out in at least triplicate to produce a mean ± standard deviation (s.d.) and each experiment independently repeated three times. To determine if there was a statistically significant difference between a test compound and control, an unpaired students T-test was performed using GraphPad® Prism Version 5.02 software, with a p-value of less than 0.05 considered to be significant.

CHAPTER 3: Results

3.1 Antimalarial Activity

All twenty-seven 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives and the nine 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone derivatives inhibited more than 40% malaria growth on initial screening at 50 µM (range: 52.04 - 97.23% inhibition). The derivatives were further assessed and displayed antimalarial activity with an IC_{50} range of between 1.29 – 53.98 µM (Table 3 .1) and 1.42 – 19.62 µM (Table 3.2). Derivative CQPA-**26** showed the most promising antimalarial activity from the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives against the *P. falciparum* parasite (IC_{50} :1.29 ± 3.35 µM) (Figure 3.1) followed by CQPA-**25** with an IC_{50} value of 1.72 µM compared to quinine (IC_{50}: 0.18 ± 0.05 µM). Derivative CQPPM-**9** showed the most promising activity from the 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone derivatives against the *P. falciparum* parasite with an IC_{50} value of 1.42 µM compared to quinine ($p < 0.05$). Derivative CQPA-**6** (53.98 ± 3.01 µM) and CQPPM-**2** (19.62 ± 3.21 µM) were the least active in inhibiting malaria growth.

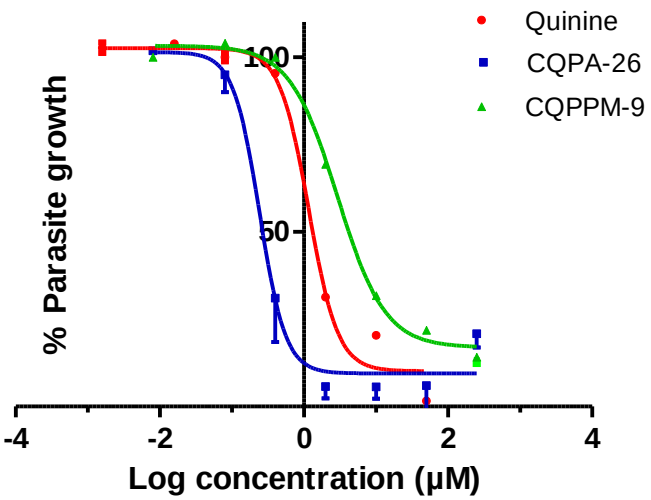
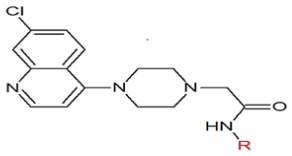
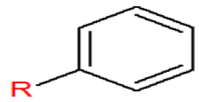
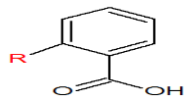
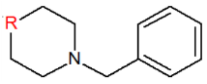
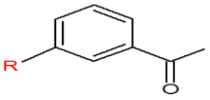
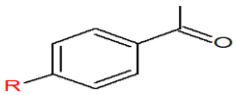
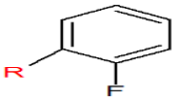
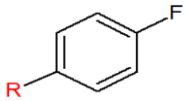
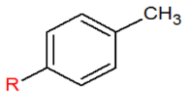
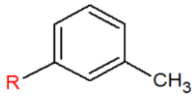
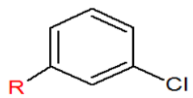
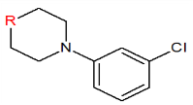
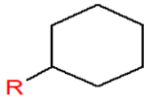
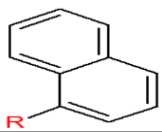
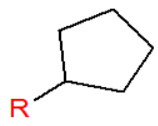
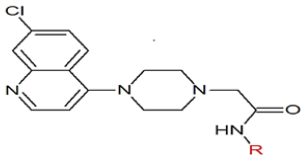


Figure 3.1: Log sigmoid dose-response curve indicating the antimalarial activity of the most active compound from the 7-chloroquinolin-4-yl piperazine-1-yl acetamide (CQPA-**26**) and 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone (CQPPM-**9**) derivatives in comparison to the standard antimalarial agent, quinine.

Table 3.1: Results showing antimalarial activity and cytotoxicity profile of the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives.



Derivative	Chemical Structure R-Group	Antimalarial activity	Cytotoxicity Profile	
		IC_{50} (μM) ± sd	% cell inhibition at 50 μM	% rbc lysis at 50 μM
CQPA-**1**		34.19 ± 1.07	34.91 ± 0.21	0.95 ± 0.37
CQPA-**2**		42.16 ± 3.42	44.08 ± 4.89	1.32 ± 0.59
CQPA-**3**		21.85 ± 3.66	32.65 ± 5.06	1.43 ± 0.39
CQPA-**4**		35.35 ± 7.33	40.5 ± 8.44	1.42 ± 0.41
CQPA-**5**		30.13 ± 7.40	51.68 ± 8.48	0.1 ± 0.01
CQPA-**6**		53.98 ± 3.01	43.05 ± 3.72	0.1 ± 0.01
CQPA-**7**		18.75 ± 3.32	46.49 ± 3.61	0.1 ± 0.01
CQPA-**8**		29.88 ± 5.73	43.47 ± 3.73	0.08 ± 0.04
CQPA-**9**		23.76 ± 3.00	33.62 ± 3.52	0.2 ± 0.11
CQPA-**10**		18.99 ± 3.04	53.85 ± 0.88	0.25 ± 0.18
CQPA-**11**		24.48 ± 5.78	43.23 ± 5.78	1.18 ± 0.55
CQPA-**12**		31.57 ± 2.91	43.86 ± 1.08	0.3 ± 0.17
CQPA-**13**		19.07 ± 1.42	43.43 ± 2.44	0.37 ± 0.29
CQPA-**14**		17.08 ± 2.31	51.38 ± 4.62	0.68 ± 0.41



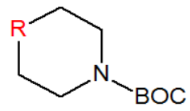
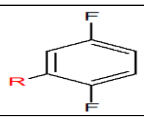
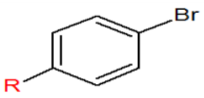
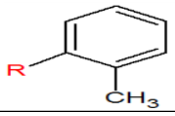
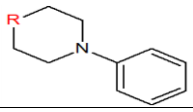
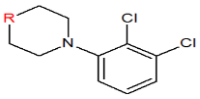
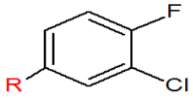
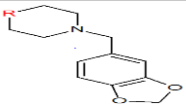
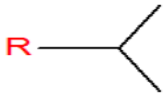
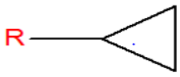
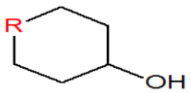
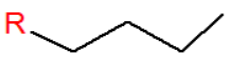
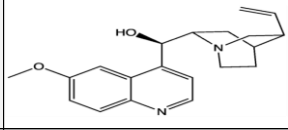
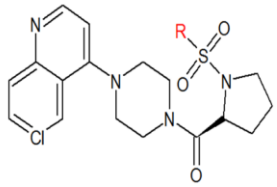
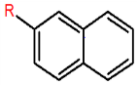
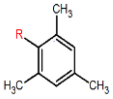
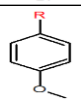
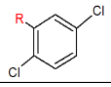
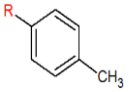
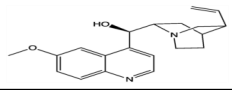
Derivative	Chemical Structure R-Group	Antimalarial activity	Cytotoxicity Profile	
		IC_{50} (µM) ± sd	% cell inhibition at 50 µM	% rbc lysis at 50 µM
CQPA-**15**		11.06 ± 1.97	59.94 ± 6.01	0.42 ± 0.28
CQPA-**16**		35.92 ± 3.11	19.45 ± 9.55	0.67 ± 0.31
CQPA-**17**		24.16 ± 5.73	55.25 ± 2.94	0.79 ± 0.38
CQPA-**18**		13.10 ± 2.89	38.25 ± 4.84	0.51 ± 0.24
CQPA-**19**		1.85 ± 0.52	50.2 ± 5.25	0.28 ± 0.16
CQPA-**20**		18.95 ± 7.53	22.47 ± 3.79	0.09 ± 0.02
CQPA-**21**		9.70 ± 1.51	51.93 ± 4.89	0.15 ± 0.09
CQPA-**22**		51.41 ± 4.96	48.93 ± 10.30	0.21 ± 0.1
CQPA-**23**		10.81 ± 1.60	44.32 ± 2.89	0.14 ± 0.07
CQPA-**24**		13.11 ± 2.59	39.88 ± 0.72	0.49 ± 0.16
CQPA-**25**		**1.72 ± 0.17**	34.93 ± 2.81	0.59 ± 0.46
CQPA-**26**		**1.29 ± 3.35**	54.88 ± 0.48	0.4 ± 0.28
CQPA-**27**		21.79 ± 2.06	42.05 ± 5.44	0.09 ± 0.02
Quinine		0.18 ± 0.05	23.46 ± 3.78	0.1 ± 0.01
Camptothecin		n/a	78.11 ± 6.6	n/a

Table 3.2: Results showing antimalarial activity and cytotoxicity profile of the 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone derivatives.



Derivative	Chemical Structure R-group	Antimalarial activity	Cytotoxicity Profile		Selectivity index
		IC_{50} (μM) ± sd	MTT IC_{50} (μM) ± sd	% rbc lysis at 50 μM ± sd	
CQPPM-**1**		3.45 ± 0.83	24.35 ± 5.75	1.74 ± 0.64	7.06
CQPPM-**2**		19.62 ± 3.21	14.67 ± 2.09	0.85 ± 0.50	0.75
CQPPM-**3**		4.08 ± 0.99	57.66 ± 11.86	0.42 ± 0.25	14.15
CQPPM-**4**		3.21 ± 0.72	53.10 ± 6.32	0.81 ± 0.63	16.54
CQPPM-**5**		6.33 ± 0.89	46.02 ± 5.77	0.09 ± 0.02	7.27
CQPPM-**6**		4.02 ± 0.63	57.67 ± 8.38	0.10 ± 0.01	14.33
CQPPM-**7**		4.21 ± 0.75	37.96 ± 4.12	0.13 ± 0.05	9.02
CQPPM-**8**		5.33 ± 1.20	64.06 ± 3.10	0.18 ± 0.13	12.03
CQPPM-**9**		**1.42 ± 0.32**	81.24 ± 8.73	0.09 ± 0.02	57.14
Quinine		0.18 ± 0.05	275.6 ± 16.46	0.10 ± 0.01	1494.58
Camptothecin		n.t	0.11 ± 0.01	n.t	n.t

n.t: not tested

3.2 Combination studies

The most active derivative from the 7-chloroquinolin-4-yl piperazine-1-yl acetamide group demonstrated a synergistic interaction when combined with quinine and the most active derivative from the 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone group demonstrated an overall additive interaction in combination with quinine. The ∑FIC values for CQPA-**26** and CQPPM-**9** were 0.29 ± 0.0014 and 1.31 ± 0.03, respectively (Figure 3.2).

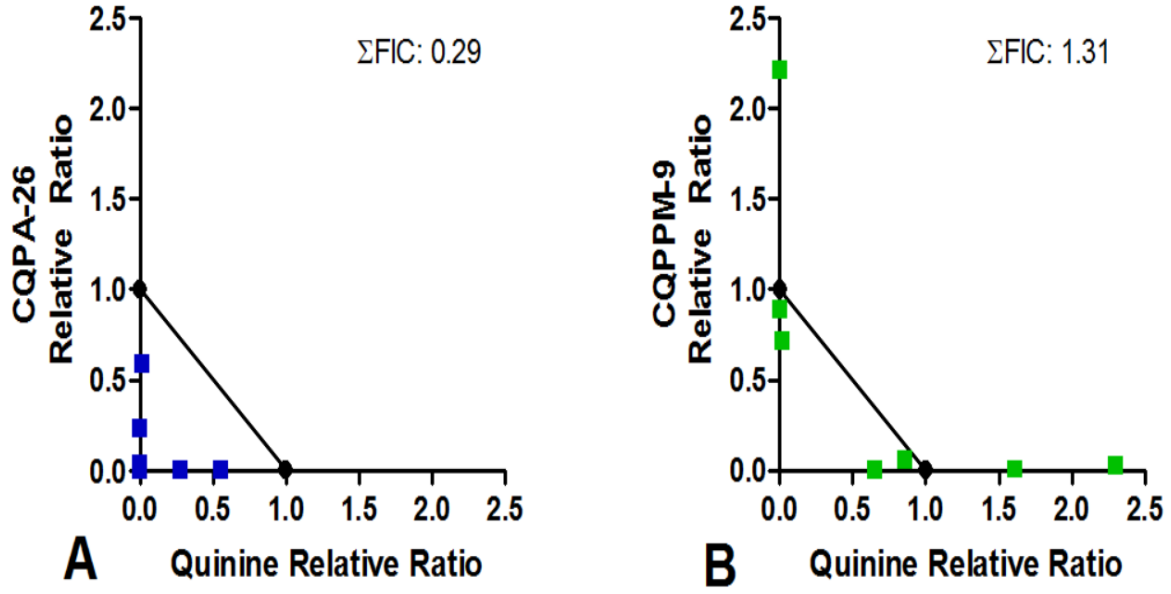


Figure 3.2: Isobolograms showing the drug interactions between the most active CQPA-**26** (A) and CQPPM-**9** (B) derivatives with quinine.

3.3 Stage determination

The total parasitaemia of the most active derivative from the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives, CQPA-**26** and the most active derivative from the 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone derivatives, CQPPM-**9** both showed a decline in the total parasitaemia present over time (Figure 3.3A and 3.4A). The derivatives showed similar stage specific effects at the IC_{50} and IC_{90} concentrations, with differences observed in the parasite numbers being lower at the IC_{90} (Figure 3.3B and 3.4B). The only morphological differences observed between the parasites treated at the IC_{50} and IC_{90} concentrations for both derivatives was the smaller (pyknotic) appearance of the parasites when compared to the untreated control.

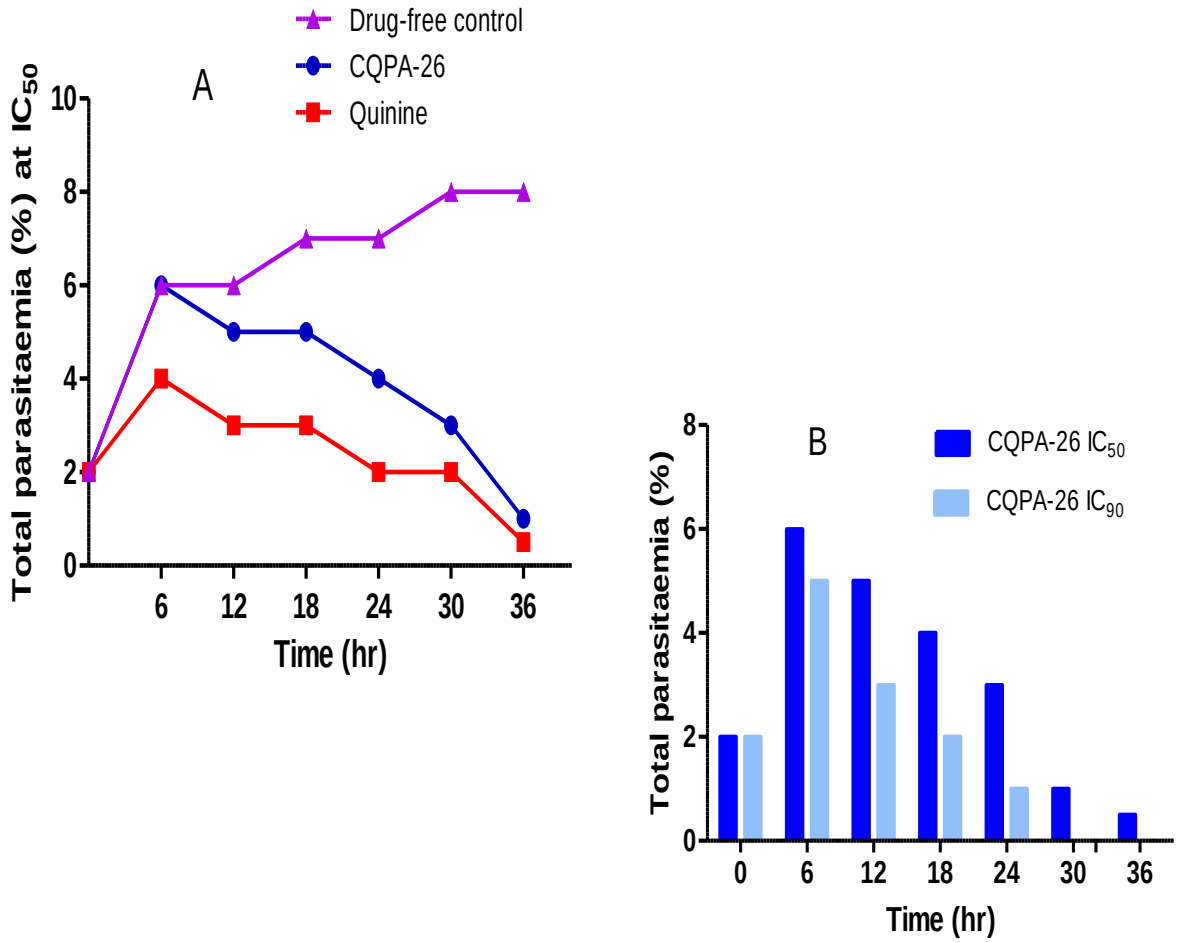


Figure 3.3: Changes in total parasitaemia between the most active CQPA-**26** from the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives in comparison to the standard quinine and untreated (drug-free) control (A) with an indication of the difference between total parasitaemia at the IC_{50} and IC_{90} values of CQPA-**26** (B).

Both derivatives progressed through the ring stage of the lifecycle of the parasite with no notable stage-specific morphological changes. Trophozoites developed within the same time-frame as the untreated control at 24 hrs into the lifecycle. However, derivative CQPPM-**9** showed a lag in progression from trophozoites to schizonts which was similar to the effects of quinine and CQPA-**26** showed no schizont development. At the time when the untreated control showed complete progression into schizonts (100%), 97% of parasites treated with CQPA-**26** and 85% treated with CQPPM-**9** remained in the trophozoite stage, with only 15% of parasites treated with CQPPM-**9** progressing into schizonts. The parasites treated with quinine also showed a lag in progression into schizonts, with 79% remaining in the trophozoite stage (Figure 3.5).

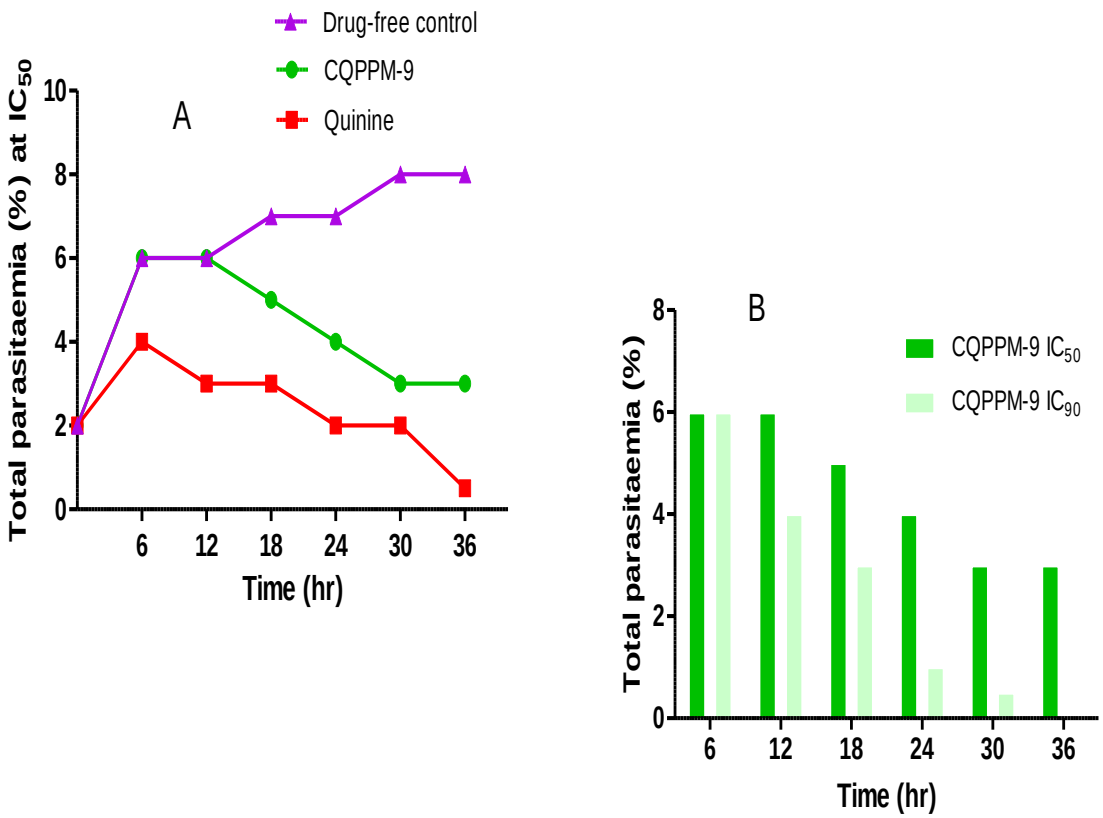


Figure 3.4: Changes in total parasitaemia between the most active derivative from 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone derivatives (CQPPM-**9**) in comparison to the standard quinine and untreated (drug-free) control (A) with the difference IN total parasitaemia at the IC_{50} and IC_{90} values of CQPPM-**9** (B).

Despite the lag in progression from trophozoite to schizont stage, derivative CQPPM-**9** and quinine showed completion of the cycle and progression into the new cycle as evident in the the development of rings (10% and 12%, respectively) compared to the untreated control (73%). However, the average total parasitaemia was very low in comparison to the untreated control. Derivative CQPA-**26**, showed no completion of the cycle, with no progression into new rings (0%) (Figure 3.5 and 3.6).

The parasites treated with the derivative CQPPM-**9** were notably smaller in size (pyknotic) in comparison to the untreated control. Haemozoin formation was visible in the parasites treated with CQPA-**26** and CQPPM-**9**, however the crystals were not as distinct and as compact as in the control. CQPA-**26** parasites showed very light staining whereas CQPPM-**9** produced a very dark intense staining. Parasites treated with derivative CQPA-**26** showed signs of vacuolisation with swelling of the digestive vacuole (Figure 3.5).

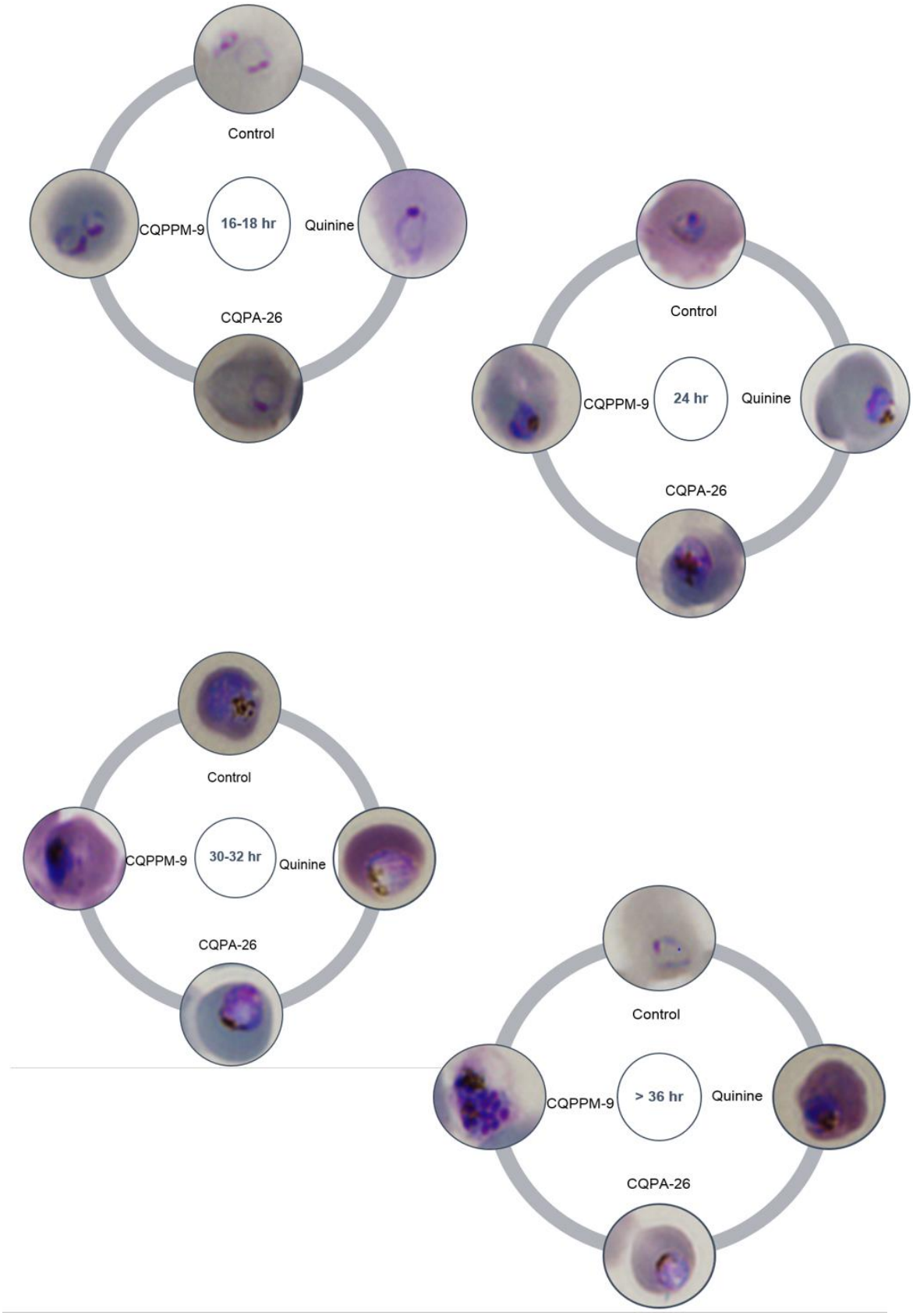


Figure 3.5: The effects of CQPA-**26**, CQPPM-**9** and quinine (IC_{50} concentration) on parasite morphology and development over time.

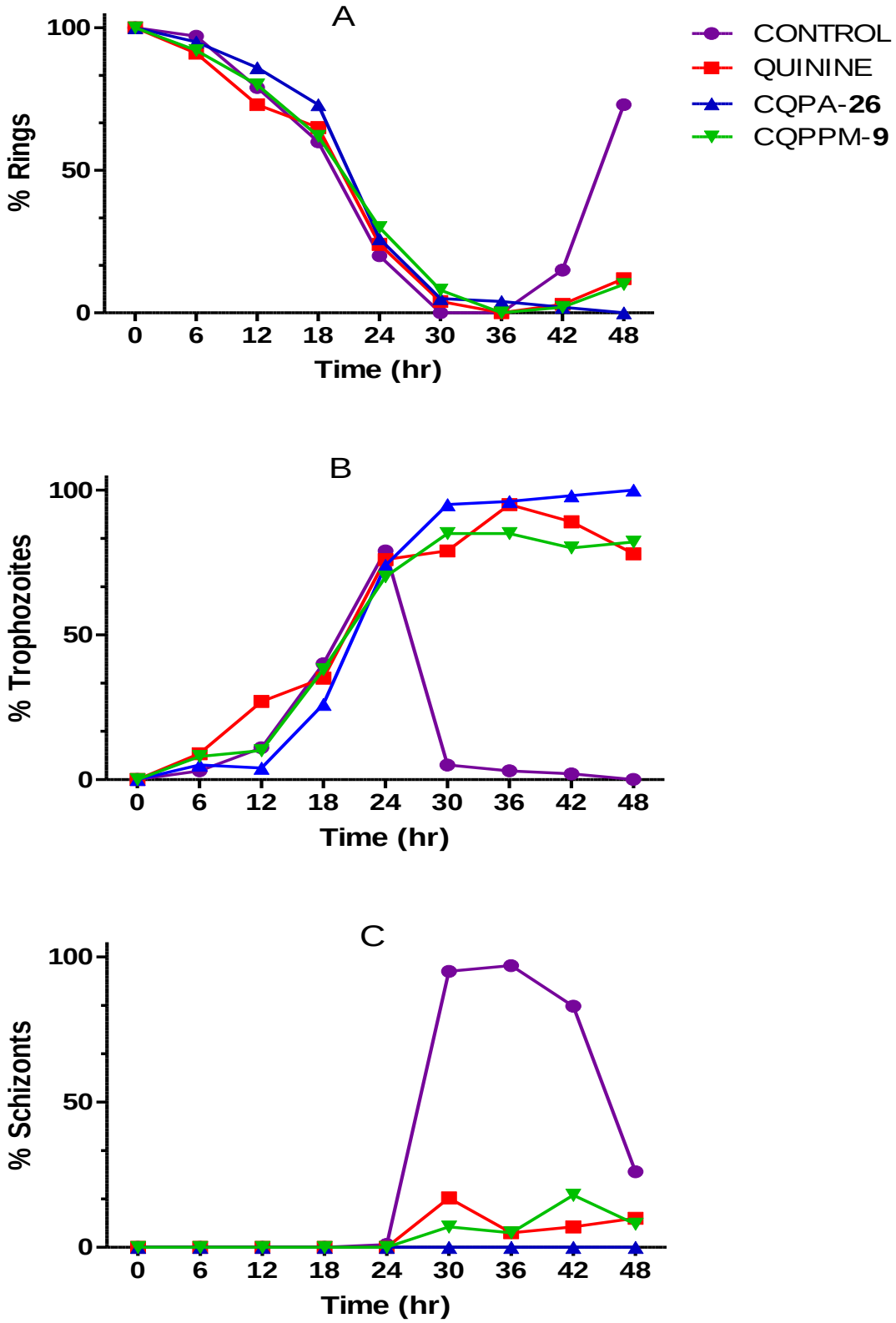


Figure 3.6: Effects of each derivative (IC_{50}) and standard quinine (IC_{50}) on each stage (rings (A), trophozoites (B), schizonts (C) in the life cycle of the parasite over time (hr).

3.4 Solubility and permeability predictions

The most active derivatives both complied with Lipinski's rule of five having no violations. Derivative CQPA-**26** and CQPPM-**9** had molecular weights less than 500 g/mol, hydrogen bond donors less than five and hydrogen bond acceptors less than ten. The log P values of both derivatives tested adhered to Lipinski's rule in the range between 0 to 5 (Table 3.3). These predictions prove these derivatives to be drug-like compared to quinine with good solubility.

Table 3.3: Predicted solubility and permeability of the most active derivatives using Lipinski's rule of five.

Compound	MW (g/mol) <500	Hydrogen bonds donors <10	Hydrogen bond acceptors <5	Log P <5	Rule of five violations
CQPA-**26**	388.89	1	6	1.77	0
CQPPM-**9**	487.01	0	7	2.68	0
Quinine	324.42	1	4	3.44	0

3.5 Predicted ionisation and absorption as a function of pH

The ionisation of the most active compounds were predicted at a pH similar to that of the red blood cell cytosol (pH 7.4) and at a pH similar to that of the digestive vacuole of the parasite (pH 5) (Table 3.4). Quinine, being a diprotic weak base and in its unprotonated form, diffuses through the membranes of the parasitised erythrocyte and accumulates in the acidic digestive vacuole. Once inside the vacuole, it becomes protonated and as a consequence becomes trapped in the acidic compartment of the food vacuole (Figure 1.8). CQPA-**26** displays similar properties to quinine in being diprotic and since it is not ionised in the red blood, 100% diffuses into the food vacuole where it accumulates. In contrast, only 3% of CQPPM-**9**, traverses into the food vacuole, as the majority of it is ionised and trapped in the red blood cell (96.93%). However, of the small percentage of CQPPM-**9** found in the food vacuole, almost all of it (99.99%) is ionised and trapped to confer its effect (Table 3.4).

Table 3.4: Predicted ionisation of the most active derivatives.

Compound	pKa	% ionisation @ pH 7.4	% ionisation @ pH 5	% GIT Absorption
CQPA-**26**	15.3, 8.4[1]	0	100	100
CQPPM-**9**	8.9[1]	96.93	99.99	3.07
Quinine	9.7, 5.07[2]	0.5	54	99.5

[1]pKa predicted using ACD/iLab version2

[2]pKa values from Fegas *et al.* (2006)

3.6 Larvicidal activity

The twenty-seven 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives and the nine 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone derivatives displayed no larvicidal activity compared to DDT (100% dead) at 0.5 µM which was 10 times the LC_{50} value for DDT (LC_{50}: 0.05 ± 0.004 µM) (Figure 3.7). The first set of derivatives displayed mortality to the larvae up to 5% (range: 0-5%), while all of the second set of compounds displayed 0% mortality against the larvae.

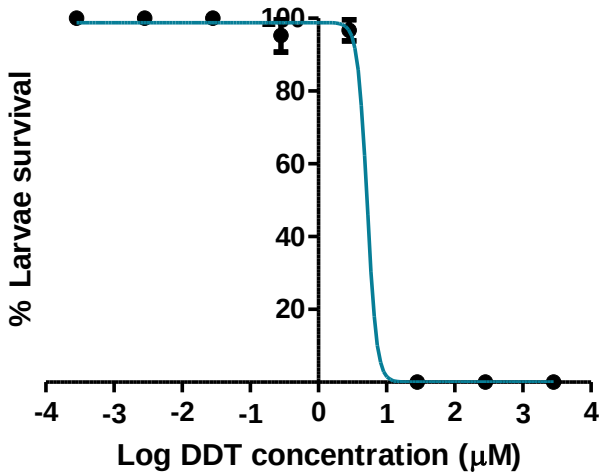
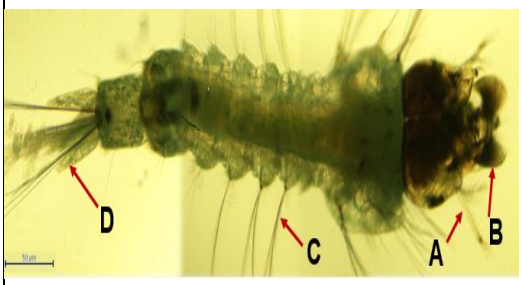
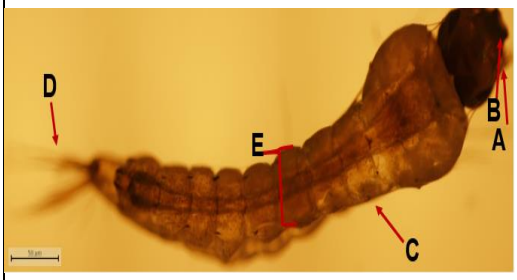


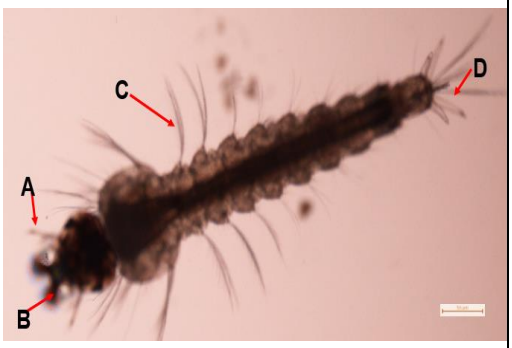
Figure 3.7: LC_{50} value (0.05 ±.004 µM) determination of DDT against *An. arabiensis* larvae.

The progression of larvae from one growth stage to the next in the larval life cycle was not halted in any of the larvae treated with the derivatives (Figure 1.3). All larvae treated with the derivatives were able to progress to the pupae stage. The treated larvae retained normal movement when prodded in the cervical region (Table 3.5). In comparison, the DDT treated larvae showed complete impairment of movement with no progression in growth due to death at 24 hours.

Table 3.5: The lack of larvicidal activity by the derivatives (0.5 µM) on *Anopheles* larvae compared to untreated and DDT treated (0.5 µM) larvae.

Treat-ment	Effect after 24 hrs	Morpho-logical changes	Pupae development	*An. arabiensis* (KGB) larvae
Drug-free control	Alive Mobility: no change	*Normal appearance. *Antennae (A) & mouth brush (B) prominent. *Lateral hair (C) on abdomen & tracheal gills (D) clearly visible.	Pupae developed. Progressed to growth stages.	
DDT positive control	Dead Mobility: retarded	*Shrinkage of larvae. *Antennae (A) & mouth brush (B) shrivelled. *Lateral hair (C) on abdomen not visible. *Narrowing of abdomen (E). *Tracheal gills still visible (D).	No pupae developed. Growth stages halted.	
CQPA-**26**	Alive Mobility: No change	*No size change. *All segments intact. *Antennae (A) & mouth brush (B) prominent. * Abdominal lateral hair (C) & tracheal gills (D) clearly visible.	Pupae developed. Progressed to normal growth stages.	

…Table 3.5 continued

Treat-ment	Effect after 24 hrs	Morpho-logical changes	Pupae development	*An. arabiensis* (KGB) larvae
CQPPM-**9**	Alive Mobility: no change	*Size remained consistent. *All segments intact. *Antennae (A) & mouth brush (B) prominent. *Lateral hair (C) & tracheal gills (D) on abdomen intact.	Pupae developed. Progressed to growth stages normally.	

3.7 Cytotoxicity

3.7.1 Haemolysis

The inhibitory effects appeared to be directed against the intra-erythrocytic parasite rather than affecting the membrane integrity of the red blood cell host, as no haemolysis was observed for the 7-chloroquinolin-4-yl piperazine-1-l acetamide derivatives (range:0.1-1.43% lysis) compared to quinine (0.1%) and the Triton-X100 positive control (100% lysis) when screened at 50 µM (Table 3.3). Similarly, the 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone derivatives caused no significant red blood cell lysis (range: 0.1-1.74% lysis) (Table 3.4).

3.7.2 Cell viability

The 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives showed a good safety profile with low toxicity to human embryonic kidney epithelial cells (% inhibition average: 1.93 - 53.85%) (Table 3.3). However, the 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone derivatives inhibited the human embryonic kidney epithelial cells more than the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives and proved to be more to toxic to the human kidney epithelial cells (IC_{50} range: 14.67- 81.24 µM) than quinine (IC_{50}: 275.6 ±16.46 µM) (Figure 3.8). None of the derivatives were as toxic as the anticancer drug camptothecin (IC_{50}: 0.11 µM) (positive control).

.

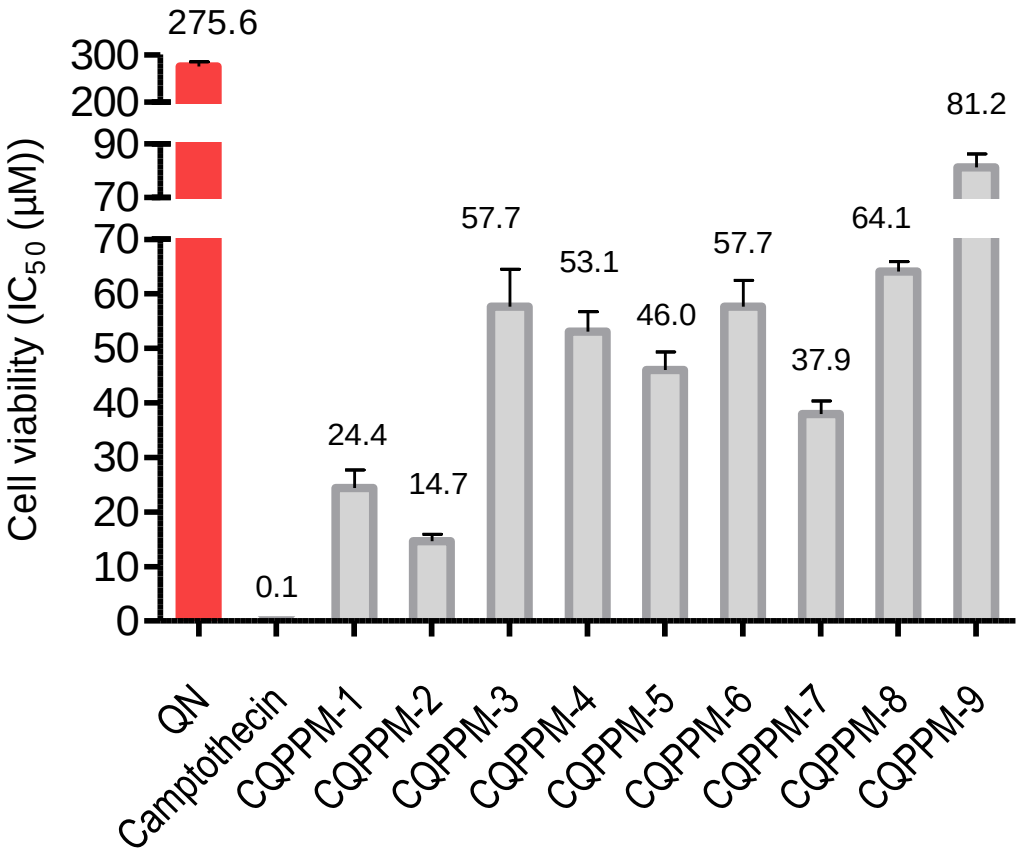


Figure 3.8: The cell viability (IC_{50}) of the 4-((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone derivatives against the HEK-293 cells in comparison to quinine and the positive control camptothecin at 50 µM.

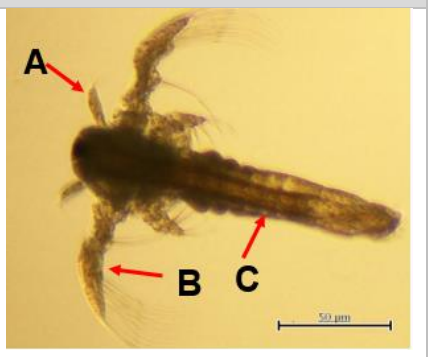
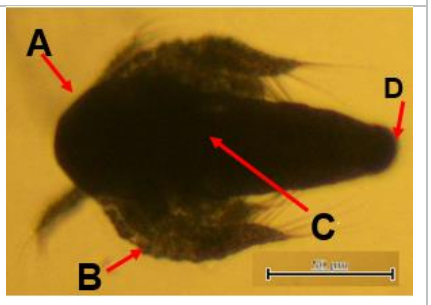
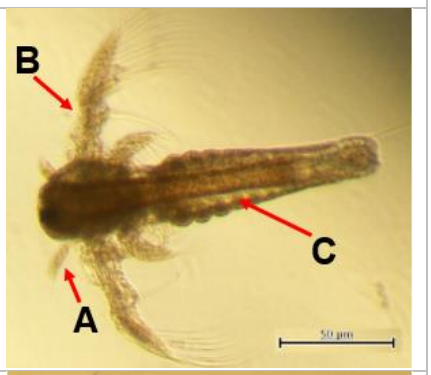
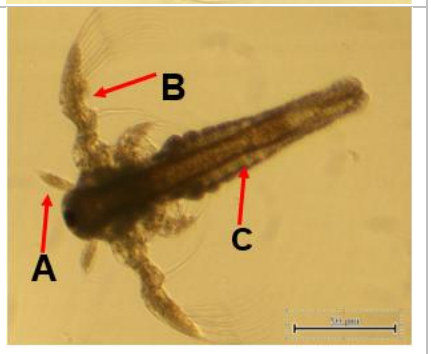
3.7.3 Lipid peroxidation

None of the derivatives (50 µM) inhibited lipid peroxidation (range: 0 - 3.06% inhibition) compared to Trolox (74.01% ± 0.14) in comparison to quinine (0.01% ± 1.04 inhibition).

3.7.4 Brine shrimp lethality activity

All the derivatives showed minimal lethality to brine shrimp (0 - 4.7%) at 0.5 µM compared to potassium dichromate (>60% mortality) at 50 µM. All nauplii treated with the derivatives showed no structural morphological changes when viewed under a microscope (Table 3.6). Their mobility was not decreased or altered in any way when compared to the healthy untreated nauplii.

Table 3.6: Table showing no effect of derivatives on *Artemia franciscana* nauplii after 24 hours compared to the potassium dichromate (positive control) and the negative (blank) control.

Treatment	Effect (Dead/alive)	Morphological changes	*Artemia fransiscana* nauplii
Drug-free control (negative control)	Alive (0% mortality) Mobility: free, fast movement	*No change in size. *Antennae (A) & swimming appendages (B) moved freely. *Colour remained consistent (C).	
Potassium dichromate (positive control)	Dead (100% mortality) Mobility: None	*Nauplii shrivelled (D). *Antennae (A) & swimming appendages (B) shrivelled. *Colour darkened & thickening of cervical region (C).	
CQPA-**26**	Alive (0% mortality) Mobility: Free, fast movement	*Size remained the same. *Swimming appendages (B) & antennae (A) moved normally. *Colour remained the same (C).	
CQPPM-**37**	Alive (0% mortality)	*Nauplii size remained unchanged. *Antennae (A) & swimming appendages (B) showed no changes *Colour remained the same (C).	

CHAPTER 4: Discussion

The growing resistance of *P. falciparum* against the current line of drug therapies has warranted the need for the development of novel compounds. The two sets of derivatives in this study were based on the concept that since the standard antimalarial drug, chloroquine has a novel mechanism of action to target the parasite and has a good safety profile in all age groups including pregnancy, its core chloroquinoline structure was maintained and additional side chains added to produce single agent compounds with a possible dual action. The 7-chloroquinolin-4-yl piperazine-1-yl acetamide and 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives in this study contained an aromatic nucleus similar to chloroquine (Figure 4.1) combined with other scaffolds such as acetamides or sulphonamides. The aim was to achieve the combined effects of each of the pharmacophores via a piperazine linker in a single compound in the move towards novel drug discovery.

4.1 7-Chloroquinolin-4yl derivatives

The 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives were synthesised such that the aminochloroquinoline group remained the same with the addition of an acetamide group linked by a piperazine linker (Figure 4.1). A study by Kumawat *et al.,* (2011) employed the same concept keeping aminochloroquinoline as the core structure with an attached propyl group linked to a thiazine group. These compounds showed moderate results as antimalarials against the chloroquine-sensitive *P. falciparum* RKL-2 strain with a percentage of dead parasites between 5.0-18.5% in comparison to chloroquine (50%). The second group of derivatives synthesised namely the ((7-chloroquinolin-4-yl) piperazine-1-yl) pyrrolidin-2-yl) methanone derivatives bearing an aminochloroquinoline core structure with the addition of piperazine and pyrrolidine rings with a sulphonamide group (Figure 4.1). Studies by Salahuddin *et al*., (2013) illustrated that structural modifications to a chloroquinoline pharmacophore has good antimalarial activity. This study evaluated the antimalarial effect of novel chloroquinoline based sulphonamide hybrids against the chloroquine-resistant (FCR-3) strain with good antimalarial activity (IC_{50} range: 1.16-8.54 µM) in comparison to quinine (IC_{50}: 0.17 µM) (Salahuddin *et al*., 2013). Structure-activity relationship studies illustrated that the 4-aminochloroquinoline nucleus is necessary for the groups antimalarial activity and accumulation in the parasite vacuole (Kumawat *et al.,* 2011). These evidences encouraged the development of the derivatives in this study such that modifications of the side chains gave rise to drugs with desirable bioactivity.

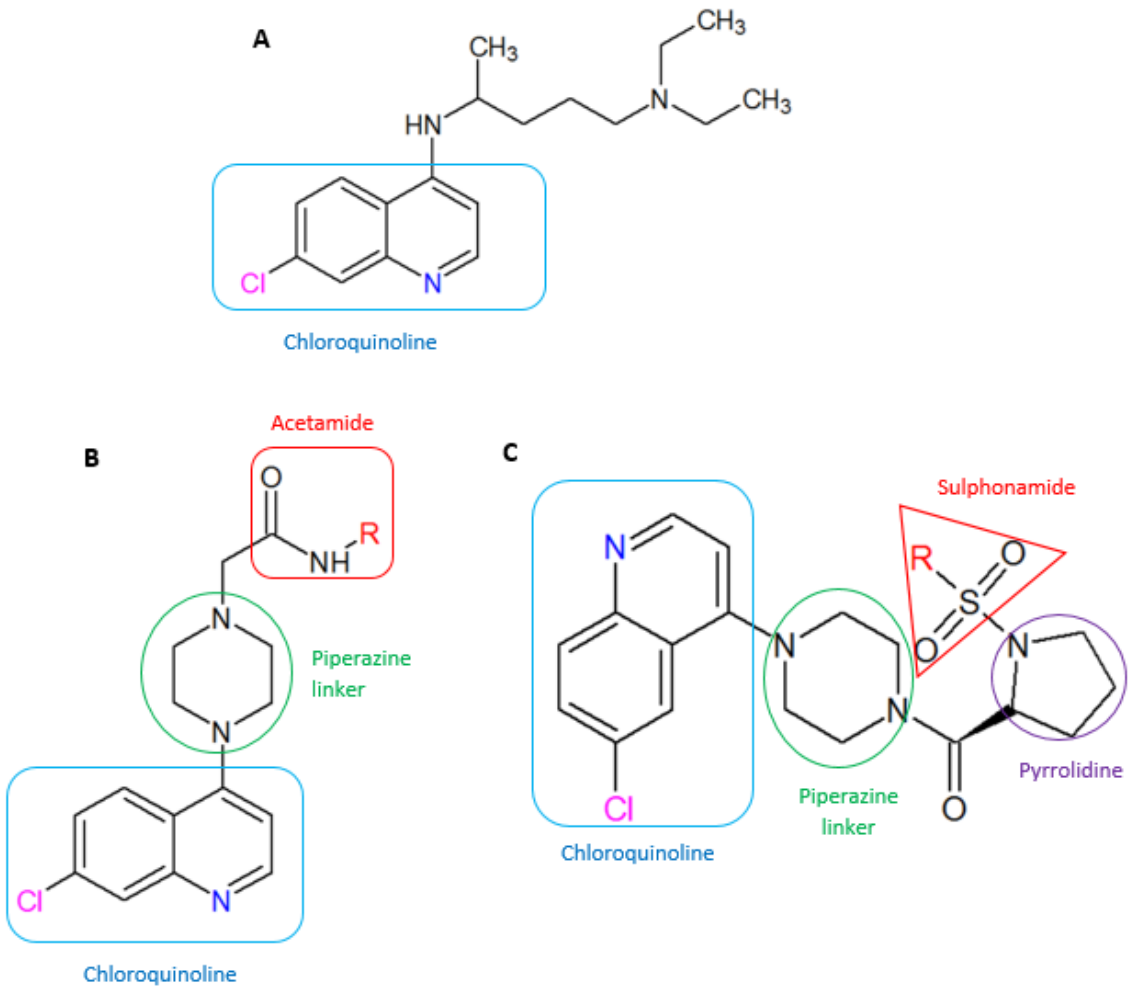


Figure 4.1: Rationale in structural design of the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives (B) and 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives (C) compared to the standard chloroquinoline (A).

4.1.1 Overcoming chloroquine resistance

Resistance to chloroquine is largely through alteration of the drugs accumulation mechanism in the digestive vacuole of the parasite. Mutations in the *P. falciparum* chloroquine resistance transporter (pfCRT), a protein found in the parasite digestive vacuole, aids efflux of the positively charged chloroquine from the digestive vacuole. This leads to decreased accumulation of chloroquine in the parasite to levels which are safe for the parasite (Boudhar *et al*., 2016). With chloroquine not being used routinely, the drug pressure has been removed resulting in the loss or down-regulation of the resistance gene, making chloroquine and its mechanism of action viable again (Boudhar *et al*., 2016). This explains the rationale in keeping the chloroquinoline core structure in the design of the derivatives used in this study with the addition of acetamide or sulphonamide and pyrrolidine side chains to increase

the target sites against the parasite, while maintaining the efficacious core structure with known antimalarial activity.

4.1.2 7-Chloroquinolin-4-yl piperazine-1-yl acetamide derivatives

The chloroquinoline class has been identified as drugs with potent antimalarial activity with minimum inhibitory concentrations in the range of 0.05 to 0.11 µM (Gupta *et al.,* 2008). The 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives in this study inhibited the NF54 strain of the *P. falciparum* at varying degrees with minimum 50% inhibitory concentrations between 1.29 to 53.98 µM (Table 3.3). The 27 derivatives did not alter the host red blood cell membrane integrity indicating that the activity was directed towards the intra-erythrocytic parasite (0.1-1.74% lysis) (Table 3.4). This illustrates a favourable property for the use of these derivatives as antimalarials. Considering the pathology of malaria which includes red blood cell haemolysis during parasite lifecycle (Figure 1.4), the antimalarial drug should not further compromise the patients uninfected red blood cells (Table 3.3). Derivatives CQPA-**26,25,19** showed five times higher antimalarial activity than CQPA-**21** (Table 2.1; Figure 4.2). This could be due to the introduction of the piperazine group in CQPA-**21**. The addition of a fluorine group in the *ortho* position of the aromatic amine decreased the antimalarial activity by three fold to produce the least active derivative CQPA-**6** (IC_{50}: 53.98 µM) (Table 2.1). However, positioning the fluorine group in the *para* position in the aromatic amine increased the antimalarial activity 3 fold (CQPA-**7** IC_{50}: 18.75 µM)). This could be due to the effect of the *ortho* substituents which decrease the basic properties of the compound and thus decreasing its antimalarial effect. Studies by Sparatore *et al.,* (2005*),* showed similar findings where substituents decreasing basicity caused decreased activity against the D-10 and W-2 *P. falciparum.* Contrastingly, moving of the position of a methyl group from the *para* position as in derivative CQPA-**8** to the *meta* position in CQPA-**10**, only contributed to a slight increase in antimalarial activity (Figure 4.2). This is consistent with the findings of chloroquinoline-pyrimidine hybrids with a similar structure to the derivatives in this study (Kumar *et al.,* 2014). The addition of bulky groups did not improve antimalarial activity. A similar trend was observed in a study by Inam *et al., (*2015*)*, where the reduction in both antimalarial (3D7-strain) and antiamoebic activity in the chloroquinoline-acetamide hybrids was attributed to the imposed steric hindrance of the bulky groups. The two most active 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives, CQPA-**26** and CQPA-**25**, with IC_{50} of 1.29 µM and 1.72 µM, respectively against the *Plasmodium falciparum* parasite showed no toxic effects against the host red blood cell and showed favourable results against normal cell lines with a percentage cell inhibition of 54.88% and 34.93%, respectively (Table 3.3). The activity of the cyclopropyl derivative, CQPA-**26** could be due to the closing of the triangular side chain of the isopropyl derivative CQPA-**24** (Figure 4.2). Furthermore, the

increased activity of derivative CQPA-**26** could be correlated to the addition of the strong electron donating group (-OH) which would increase its basic properties and increase its activity against the parasite, particularly its accumulation in the parasite digestive vacuole. In studies by Sparatore *et al*., (2005), a decreased activity was seen when electron withdrawing groups were added to the side chain reducing the basic properties of the drug. In a study by Boudhar *et al.,* (2016), a similar approach as in this study was undertaken where hybrid compounds were developed in which the 4-aminochloroquinoline scaffold was maintained and linked to other scaffolds with a different mechanism of action, in an attempt to resensitise chloroquine-resistant parasites. The hybrid compounds in this latter study all showed IC_{50} values below 500nM against the Hb3 and 3D7 parasite strains (Boudhar *et al.,* 2016). This study highlighted the ability of new hybrids in resensitising chloroquine-resistant parasites.

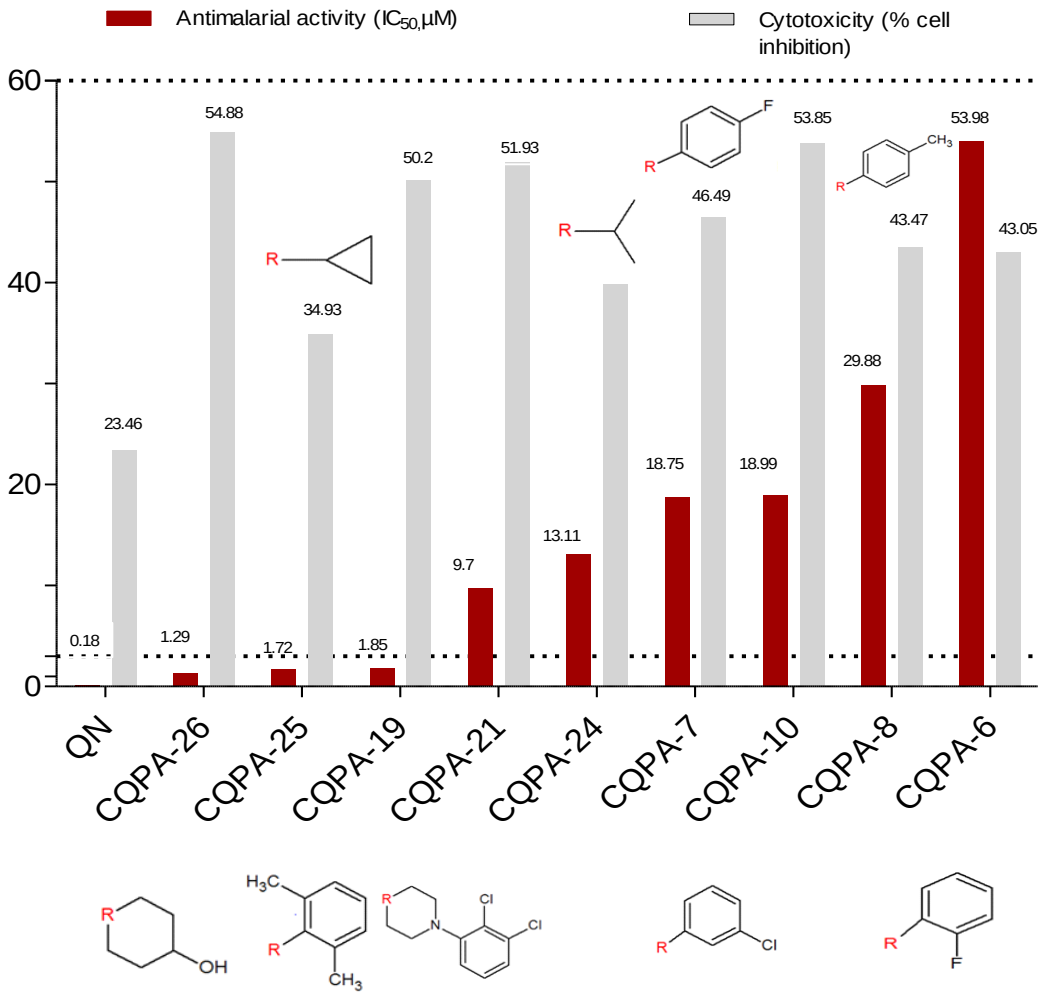


Figure 4.2: Antimalarial activity and cytotoxicity of the key 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives.

4.1.3 7-Chloroquinolin-4yl piperazine-1 yl pyrrolidine-2yl methanone derivatives

The nine 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives showed antimalarial activities with all IC_{50} values below 20 µM (IC_{50} range: 1.42-19.62 µM) (Table 3.4). The inhibitory effects appear to be directed against the intra-erythrocytic parasite rather than the membrane of the red blood cell host, as no haemolysis was observed for all nine compounds (<1% lysis) compared to chloroquine (<0.1%) (Table 3.4). A similar trend was observed in a study by Salahuddin *et al.,* (2013), in which a new series of 4-aminocloroquinoline based sulphonamides showed no significant lysis of the human red blood cells. Derivative CQPPM-**9** displayed the best safety profile (safety index: 57.14) and proved to be the most potent in inhibiting *P. falciparum* (NF54) from the nine derivatives. The methyl substitution of derivative CQPPM-**9** increased the antimalarial activity compared to the *tert*-butyl substitution of derivative CQPPM-**4** (4-fold) (Figure 4.3). The presence of a chlorine atom has been shown to increase the antimalarial potency of a compound, but this trend was not echoed with the 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives (Inam *et al*., 2014).

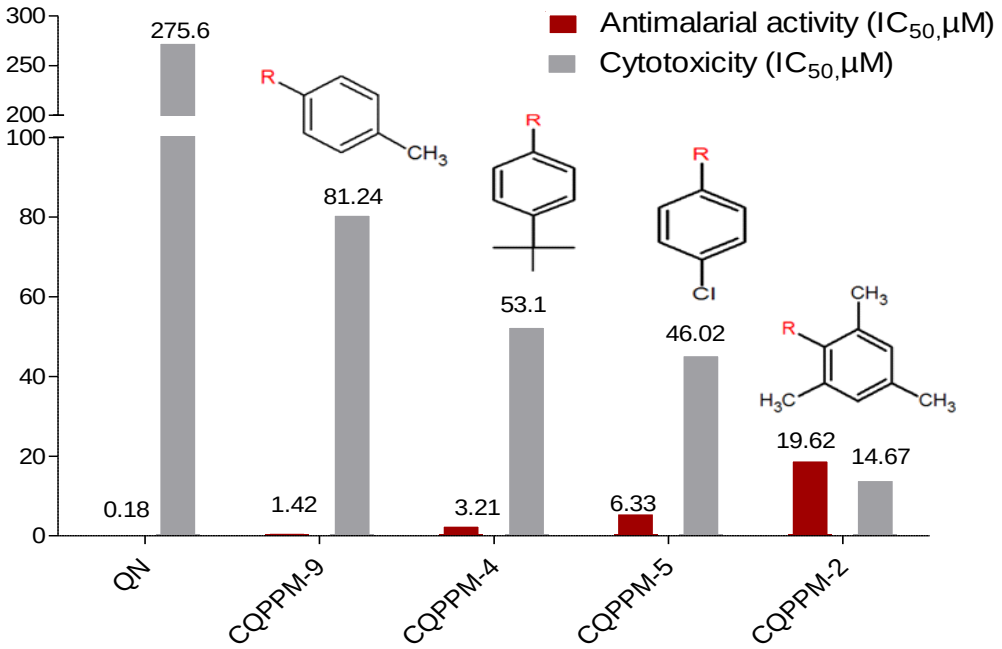


Figure 4.3: Antimalarial activity and cytotoxicity of the key 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives.

4.2 Beta-haematin inhibition

The presence of an aromatic nucleus has shown positive results in malaria chemotherapeutics for its haematin binding affinity (Figure 4.4). The presence of the 7-chloroquinoline moiety in antimalarial compounds has been shown to be a necessary factor in binding to haematin in the parasites' acidic food vacuole, thereby inhibiting haemozoin formation (Gupta *et al.,* 2008). The presence of the 7-chloroquinoline moiety in both the 7-chloroquinolin-4-yl piperazine-1-yl acetamide and 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives could be the possible mechanism of action of these derivatives (Figure 4.1). This is backed by a study by Inam *et al,* (2015) which showed that the aminochloroquinoline moiety of the chloroqunoline-acetamide hybrids inhibited β-haematin formation. The aromatic quinoline nucleus present in these derivatives is necessary to intercalate onto the surface of the FPIX to form a drug-haem complex (Leed *et al.,* 2002). The drug-haem complex is incorporated into the growing dimer chains preventing further sequestration of toxic haem and disrupting membrane function (O'Neill *et al.,* 2012) (Figure 4.4).

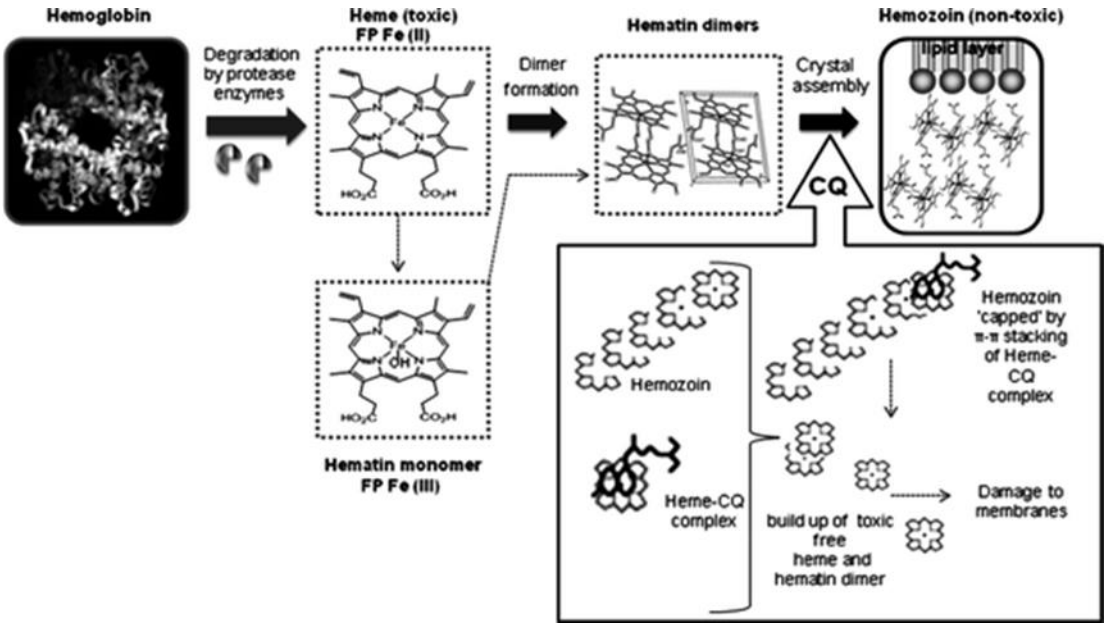


Figure 4.4: Proposed mechanism of action of quinoline-based drugs showing degradation of haemoglobin and detoxification mechanisms of the parasite (O'Neill *et al.,* 2012).

The quinoline ring present in both the 7-chloroquinolin-4-yl piperazine-1-yl acetamide and 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives may intercalate with the porphyrin groups on the surface of the haematin crystal (Figure 4.5 (**A**)). The quinoline

nitrogen group can form a hydrogen bond with the vinyl group of the porphyrin ring (Figure 4.5 (**B**)).

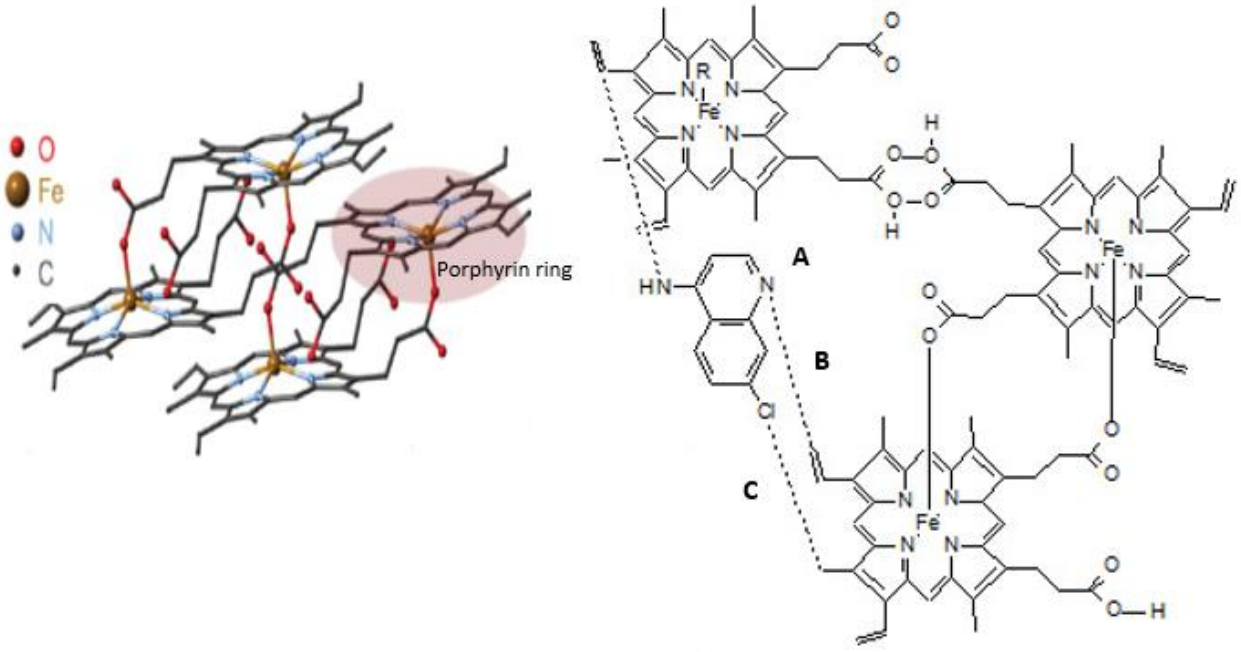


Figure 4.5: Theoretical binding sites of derivatives aminochloroquinoline core with the haemozoin crystal (Buller *et al*., 2002; Weissbuch & Leiserowitz, 2008).

Furthermore, the chlorine substituent in the quinoline ring has been shown to contribute to increased antimalarial activity and inhibition of haemozoin formation (Egan *et al.,* 2000) (Figure 5.4). The 7-chloroquinoline moiety in both the 7-chloroquinolin-4-yl piperazine-1-yl acetamide and 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives possess the quinoline group in its core structure, as well as the chlorine substituent in the quinoline ring which is consistent with the studies by Egan *et al,* (2000) in conferring β-haematin inhibitory properties as a mechanism of action. The 7-chloro group of the derivatives can interact with a methyl group of a neighbouring porphyrin moiety of haematin (Figure 4.5 (**C**)).

4.3 Drug accumulation in parasite digestive vacuole

Quinolines target the food vacuole of the intraerythrocytic malaria parasite. The weak basic properties of these drugs allow them to accumulate in the acidic vacuole of the *P. falciparum* parasite via transmembrane pH gradients (Yayon *et al.,*1985) (Figure 4.7). Studies by Yayon *et al,* (1985) suggest that the antimalarial activity of chloroquine is exerted by alkalanisation of accumulated drug interfering with vital cellular functions of the food vacuole. The 7-chloroquinolin-4-yl piperazine-1-yl acetamide and 7-chloroquinolin-4-yl piperazine-1-yl

pyrrolidin-2-yl methanone derivatives contain a protonatable quinoline nitrogen (Figure 4.6: nitrogen in blue) that would increase the accumulation of the derivatives in the parasite vacuole. The increased antimalarial activity of the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivative CQPA-**26**, could be due to increased diffusion and accumulation (100%) into the parasite food vacuole (Table 3.4). This derivative shows similar properties to chloroquine in conferring ion trapping properties due to its diprotic weak base nature (Figure 4.6). This is further supported by its ionisation capabilities and good solubility from drug-likeness predictions using Lipinski's rule of five (Table: 3.3, 3.4). The weak basic nature of the derivatives could contribute to its mechanism of action in that by increasing the pH of the parasite digestive food vacuole, it would affect the functionality of the acid proteases of the parasite, thereby preventing haemoglobin digestion and resulting in parasite death (Figure 4.7; 4.8) (Homewood *et al.,* 1972).

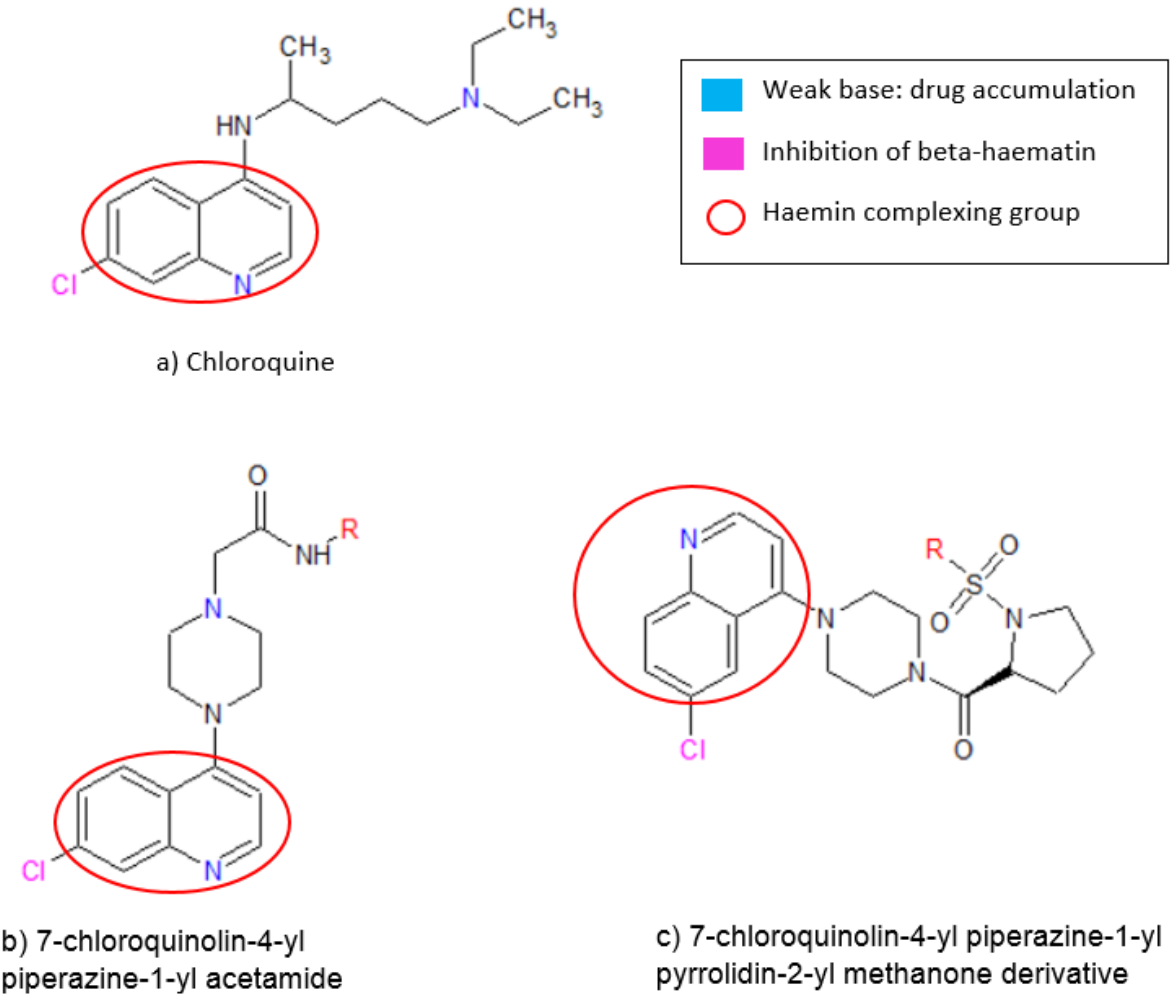


Figure 4.6: Proposed structure-activity relationships according to studies by Egan *et al.,* (2000).

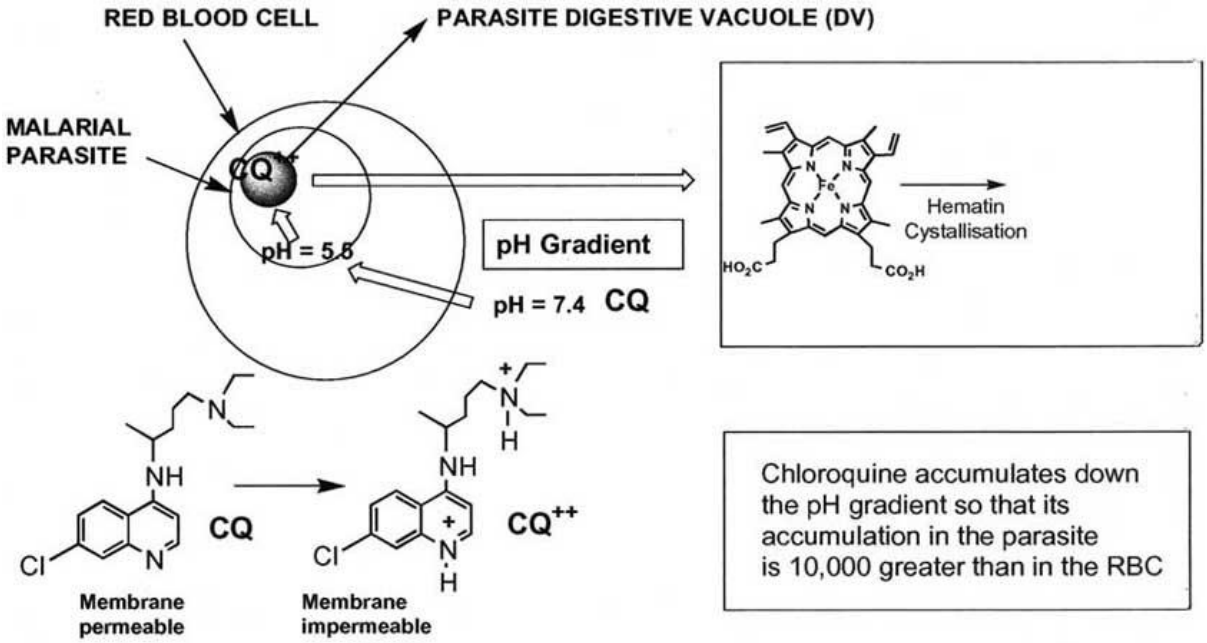


Figure 4.7: Diffusion and accumulation of chloroquine into the parasite digestive food vacuole due to the pH gradient (O'Neill *et al.,* 2012).

4.4 Protein synthesis inhibition

Plasmodium proteases are involved in pathways that are essential for the parasite survival. This makes them a suitable target for drug development due to their enzymatic mechanisms and active site structures (Deu, 2017). Apart from the proposed mechanism of action of quinolines in inhibiting haemozoin formation, it has been proposed that quinolines inhibit parasite protein synthesis (Rosenthal *et al*., 1996). This occurs in the parasitic digestive food vacuole following degradation of haemoglobin into haem and globin (Figure 1.8) and the latter is further degraded to amino acids that are used as building blocks for parasitic protein in a process involving haemoglobinases (Dominguez *et al*., 1997). Given that malarial parasites have limited capacity for *de novo* amino acid biosynthesis, the majority of amino acids required for the parasite's own protein biosynthesis and energy metabolism are supplied by haemoglobin degradation products. Malaria parasites express a number of proteases in the food vacuole for the degradation of haemoglobin imported from the host's red blood cell (Dominguez *et al*., 1997). The haemoglobin degradation process follows a well-ordered pathway as outlined in Figure 4.8.

Aspartic protease inhibitors and the cysteine protease inhibitors are two classes of these enzyme inhibitors with antimalarial effects. It can be proposed that the most active 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivative, CQPA-**26** possessed inhibitory

properties similar to a cysteine protease inhibitor. Inhibition of falcipain, a cysteine protease, was shown to halt parasite development and an abnormal appearance of the food vacuole with accumulated undergraded haemoglobin (Dominguez *et al.,* 1997). Derivative CQPA-**26** caused similar morphological changes as those seen with falcipain inhibitors where the parasitic food vacuole appeared swollen, with parasite development halted in the trophozoite stage and no progression to the schizont stage. Parasites treated with derivative CQPA-**26** did not complete the cycle to form new ring-stage parasites (Figure 3.1, >36hrs). Studies by Dominguez *et al*., (1997), show that phenothiazines inhibited falcipain with morphological abnormalities showing vacuolization of the parasite cytoplasm and limited development beyond the trophozoite stage. This inhibition could be due to the binding of the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivative to the falcipain active site in a manner analogous to that predicted for chalcones (Mishra *at al.,* 2008). Like the chalcones, derivative CQPA-**26** shows structural stability in the acidic vacuole due to its weak base properties (Section 3.5) which allows for binding to the falcipain active site (Mishra *at al.,* 2008). Furthermore, the spacing of the nitrogen atoms in the derivatives is proposed to be necessary and more effective in binding to the enzyme active site (Mishra *at al.,* 2008).

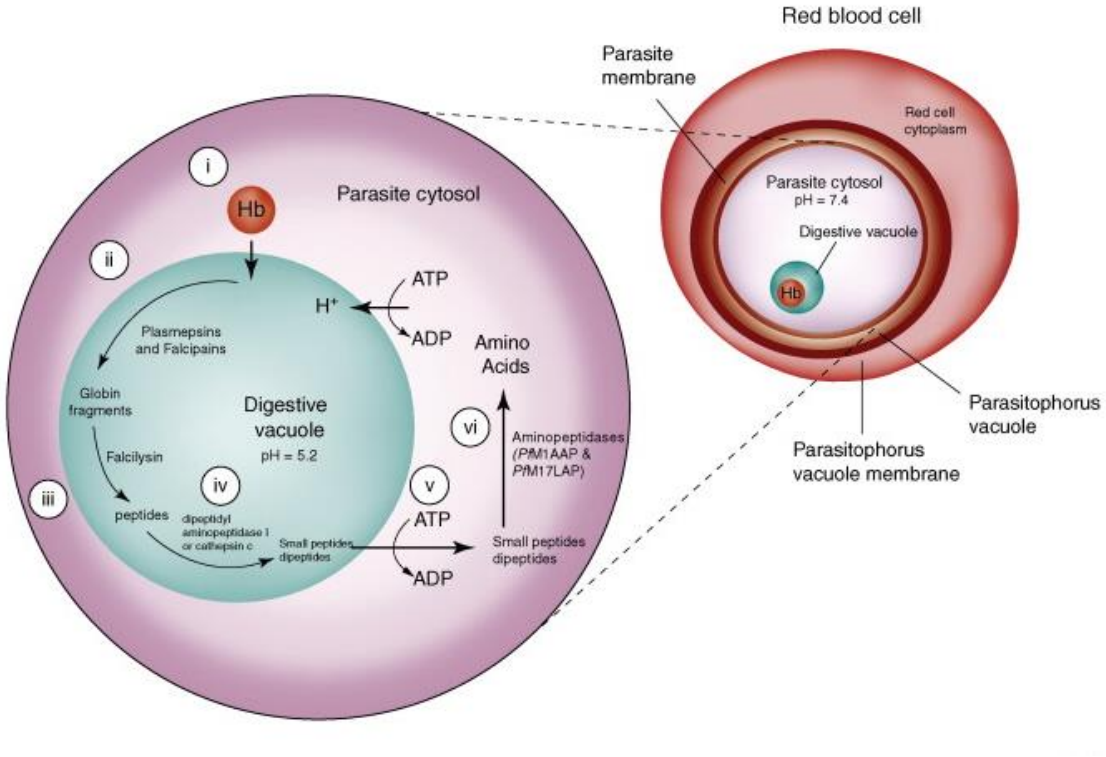


Figure 4.8: Protein synthesis pathways in the *Plasmodium* parasite indicating digestion of host haemoglobin in the parasites digestive vacuole (i). Endopeptidases such as aspartic proteases (plasmepsins) and cysteine proteases (falcipains) (ii) initiate catabolism (iii), reducing peptides to dipeptides (iv). These smaller peptide fragments are transported to the parasite cytosol (v) where they are further broken down to amino acids (vi). (Skinner-Adams *et al.,* 2010).

Mahesh *at al.* (2014) outlines the necessary pharmacophore essential for falcipain inhibitors. The study outlines that an aromatic moiety is necessary with hydrogen bond donors and acceptors of between 0 to 2 and 2 to 6, respectively; as well as a basic nature of the molecule confers falcipain inhibitory activity. Derivative CQPA-**26** contains all of these necessary structural features, with hydrogen bond donors (1) and acceptors (6) within the range (Table 3.3) and the amino moiety conferring the basic nature. Mahesh *et al.* (2014), illustrated in a group of compounds which contained an acetamide moiety like CQPA-**26** showed good antimalarial activity against *Plasmodium* falcipain enzyme (>60%) at low concentrations (10 µM) and attributed its activity to the acetamide moiety. Like the chalcones, the free nitrogen in the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives allow for protonation or hydrogen bonding with the active histidine site of the falcipain enzyme as proposed by Mishra *et al.* (2008) (Figure 4.8). It can be deduced that the activity of derivative CQPA-**26** against could be driven by the covalent interaction with falcipain.

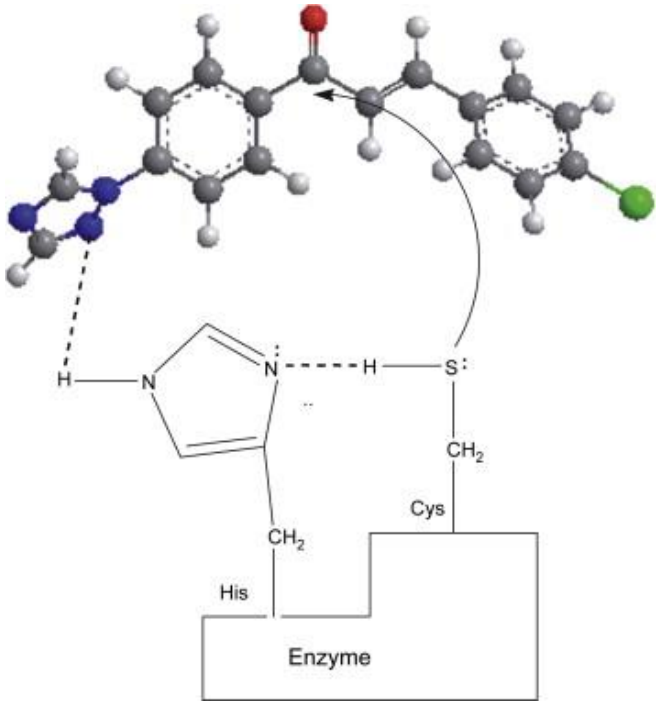


Figure 4.9: Proposed mechanism of binding of chalcone to histidine site of falcipain enzyme Mishra *et al.,* (2008).

Unlike parasites treated with CQPA-**26**, parasites treated with 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone, CQPPM-**9** derivative, did progress eventually to the schizont stage (after 36 hrs). However, progression was much slower than the control

and quinine (30-32 hours) with a reduction in haemozoin production (Figure 3.5). The observation of pyknotic trophozoites detailed in this study with derivative CQPPM-**9**, with no effect on early ring stages and eventual progression to the schizont stage could imply that the derivative exerts its toxic effects in the late stages of the erythrocytic lifecycle where DNA synthesis peaks. This trend is in line with the trends seen with all antifolates and supports the proposed mechanism as a folate inhibitor due to the presence of the sulphonamide moiety (Ekoue-kovi *et al.,* 2009) (Figure 4.11). It prevented the emergence of a new life cycle with no new rings being formed compared to the control, thus arresting development in the schizont stage (Figure 3.5). The CQPA-**26** derivative did not progress to the schizont stage and predominantly remained in the trophozoite stage, with swelling of the vacuole and diminished haemozoin formation which supports the earlier proposed mechanism in inhibiting haemozoin formation (Figure 3.5).

4.5 Inhibition of parasite folate pathways

The presence of the amide in the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives is characteristic to the structure of proguanil and could exhibit similar DHFR inhibitory effects in the parasites folate pathway as an additional mechanism of action (figure 4.8) (Rastelli *et al*., 2003; Madapa *et al.,* 2009). This results in inhibition of parasite DNA synthesis (Figure 1.10). A study by Mahesh *et al.* (2014), in which acetamide derivatives similar to the acetamide side chains in the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives showed good antimalarial activity against *Plasmodium* at low concentrations (10 µM) due to its acetamide moiety (Figure 4.10).

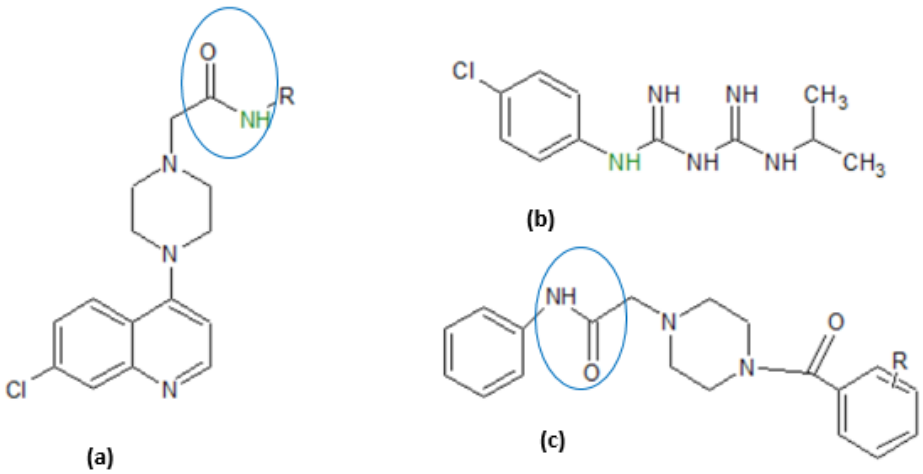


Figure 4.10: Structural similarities between 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives (a), Proguanil (b), and 2-(4-(substituted benzoyl)-1,4-diazepan-1-yl)-N-phenylacetamide derivatives (c) (Mahesh *et al.,* 2014).

It is proposed that the sulphonamide side chain present in the 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives (Figure 4.11a) also play a role in inhibiting the folate pathway in the parasite, but does so by inhibiting the enzyme DHPS (Hogh *et al.,* 2000). Studies show that the introduction of a sulphonamide side chain to chloroquine analogues displayed higher antimalarial activity and a low resistance index (Ekoue-kovi *et al.,*2009). From studies by Jatakiya et al.,(2014), it was deduced that the sulphonamide substitution was necessary for better antimalarial activity against *Plasmodium* in a group of novel *N*-substituted piperazine derivatives against the 3D7 strain of *P. falciparum.* The derivatives in this study displayed a higher antimalarial activity with a sulphonamide substituted group at the *para* position in comparison to a methyl, methoxy or chloro substitution in the same position (Jatakiya *et al*., 2014). The 7-chloro-4-aminoquinolyl derived sulphonamides (Figure 4.11b), showed antimalarial activity in the range nanomolar range upon introduction of the sulphonamide moiety against the HB3 (chloroquine sensitive) (IC_{50} range: 10-272 nM) and the Dd2 (chloroquine resistant) (IC_{50} range: 23-260 nM) strains of *P. falciparum* (Ekoue-kovi *et al.,* 2009). This could be due to the dual mechanism of action of the chloroquinoline in inhibiting haemozoin formation and the sulphonamide moiety in interrupting the folate pathway (Figure 1.10). A novel hybrid set of compounds were studied by Boudhar *et al.,* (2016), in which a similar approach was undertaken as in this study to create a novel hybrid combining a haem-binding aminochloroquinoline with a dihydropteroate synthase-inhibiting sulphonamide group. These hybrids displayed promising antimalarial activity against the chloroquine-sensitive 3D7 strain and the chloroquine-resistant K1 strain with IC_{50} values below 500 nM for both strains (Boudhar *et al.,* 2016).

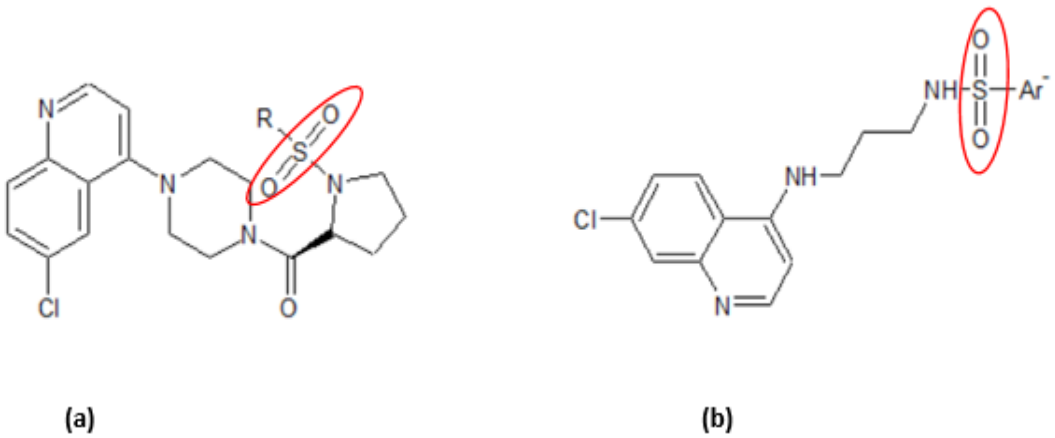


Figure 4.11: Structural similarities of sulphonamide moiety in the 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives (a) compared to the 7-chloro-4-aminoquinolyl derived sulphonamides (b) (Ekoue-kovi *et al.,* 2009).

4.6 Combination therapy

The current most effective antimalarial treatment available is an artemisinin-based combination therapy (ACT). Unfortunately, in places such as Thailand and Cambodia, ACT has shown prolonged clearance rates (Boudhar *et al.,* 2016). The threat of developing resistance to antimalarials has led to the search of novel combination therapies to prevent drug resistance. The structure of the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives owing to different mechanisms of target in the parasite could be the reason for the synergistic combination effect with quinine. In the treatment of microbial infections, combination therapy is preferred to monotherapy to delay the emergence of resistant parasites (Bell, 2005). The most active derivative from the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives showed a synergistic effect in combination with quinine (Figure 3.2A). This proves to be favourable as in addition to reducing the emergence of resistance, combination therapy allows for the reduction in doses needed of the individual agents thereby minimizing adverse effects and costing of the treatment regimen (Bell, 2005). With derivative CQPA-**26** containing the basic core structure as quinolines, the addition of side chains which introduce different target pathways in the parasite, allows for a synergistic effect without competition for binding sites nor inhibition of one drug to the other at the same binding sites within the parasite. Unlike chloroquine and quinine with the same target pathways contributing to an antagonistic combination effect (Bell, 2005).

Derivative CQPPM-**9** showed an overall additive effect when combined with quinine (Figure 3.2B). The additive combination effect could be due to the quinine acting on the late trophozoite stage of the parasite and CQPPM-**9** acting from the early trophozoite stages of the parasite lifecycle. Acting on different stages, allows for an additive effect without enhancing nor inhibiting the effects of the individual drugs. The individual FIC at the highest derivative concentration, showed an antagonistic effect in comparison to the overall additive effect. The high concentration of derivative binding to haem may result in reduced concentrations of free haem for quinine to bind to and therefore bringing about an antagonistic effect similar to that seen with combinations like mefloquine and chloroquine (Bell, 2005).

4.7 Toxicity

The search for novel compounds in the treatment of malaria is complicated as the antimalarials need to target the parasite within the host red blood cell without affecting the integrity of the host cells. All of the derivatives did not affect the red blood cell membrane

stability, causing red blood cell membrane lysis of <2% compared to quinine (0.1%) at a concentration of 50 µM (Section 3.7.1). This was a favourable effect, demonstrating that the antimalarial activity was directed towards the intra-erythrocytic parasite. The favourable safety profile was further displayed by the 7-chloroquinolin-4-yl piperazine-1-yl acetamide derivatives with little effect on cell viability (% inhibition 1.93-53.85%) (Table 3.1), requiring high concentrations, hence a high therapeutic window (Boudhar *et al.,* 2016). The 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives, however proved to be more toxic to human kidney epithelial cells (IC_{50} range: 14.67 - 581.24 µM) than quinine (IC_{50}: 275.6 µM). The most active antimalarials from both groups, CQPA-**26** and CQPPM-**9**, displayed the least effects on cell viability (IC_{50}: 54.88 and 81.24 µM) (Table 3.1 and 3.2), increasing the therapeutic window of these derivatives. The low toxicities associated with the derivatives was further echoed with no lethality towards the brine shrimp (0 - 4.75 mortality) compared to potassium dichromate (>60%) (Table 3.6). The quinoline derivatives have been proven to exhibit diverse biological activities including anticancer properties (Ghorab *et al.,* 2016). A recent study evaluated the anticancer properties of quinoline as well as sulphonamide hybrids, similar in structure to those in this study and showed good cytotoxic activity against lung (IC_{50} range: 44.34 - 76.73 µg/ml), colorectal (IC_{50} range: 28.82 - 104.78 µg/ml), and breast cancer (IC_{50} range: 26.54 - 115.11 µg/ml) lines compared to the reference drug DCF (124.87, 114.12, 113.94 µg/ml), as a PI3K inhibitor, which is a vital signal transductory enzyme (Figure 4.12) (Ghorab *et al.,* 2016). Such studies provide a basis for further evaluation of these derivatives in the search for compounds with multiple activities against malaria, vectors and cancers.

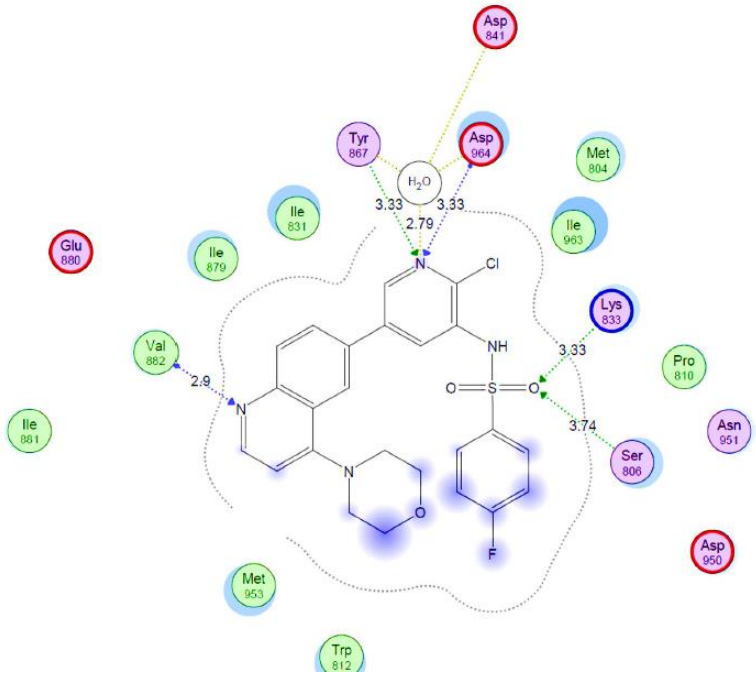


Figure 4.12: Binding sites of novel chloroqunoline sulphonamide hybrid in cancer cells with structural similarities (chloroquinoline and sulphonamide scaffolds) as the 7-chloroquinolin-4-yl piperazine derivatives in this study (Ghorab *et al.,* 2016).

4.8 Vector effects

The pharmacological properties of both the 7-chloroquinolin-4-yl piperazine-1-yl acetamide and 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives were directed towards the intra-erythrocytic stage of the malaria parasite life cycle rather than the vector stages, displaying no larvicidal activity compared to DDT (Section 3.6)

Larvicidal management to reduce adult mosquito population involves effective control of mosquito larvae with the least impact on human health and the environment. Studies show a growing resistance to insecticides such as DDT and the environmental concerns associated with insecticides used in larval control (Mohammed *et al.,* 2015). Most studies focus on larvicides of natural and botanical origin due to their limited adverse effects on the environment and beneficial organisms. A study by Begum *et al.,* (2011) looked at the larvicidal properties of novel chalcones. Looking at the structure-activity relationships, chalcones with an electron donating group on ring A or B showed high toxicity to the larvae of the common house *Culex quinquefasciatus* (Begum *et al.,* 2011) (Figure 4.13 (a)). Based on the findings by Begum *et al.,* (2011), the presence of the electron withdrawing group present in the 7-chloroquinolin-4-yl piperazine-1-yl acetamide and 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives in this study, could be the reason for their ineffective larvicidal properties (Figure 4.13 (b & c)). In another study in which acridone derivatives were screened for larvicidal activity, pyrazolo fused acridine isomers showed promising larvicidal activity against the yellow fever virus vector *Aedes aegypti* (Roopan *et*

al., 2017). These acridine isomers contain the same heterocycle nucleus as the derivatives in this study which is imperative for antimalarial activity, but differ in the presence of additional heterocycles (Figure 4.13 (d)). This provides a basis for adapting the current derivatives by adding an additional heterocycle to improve larvicidal activity, while maintaining its antimalarial core structure.

Looking at the structural differences between the 7-chloroquinolin-4-yl piperazine-1-yl acetamide and 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives in comparison to current insecticides used, a common differential feature is the lack of the chloroquinoline aromatic nucleus in current insecticides which is an important feature in conveying antimalarial properties and would suggest that mimicking these structures would lead to compounds with no antimalarial properties and only larvicidal properties (Table 4.1). Furthermore, adapting the same chemical structure as current insecticides would not be ideal as it would not resolve the rising resistance to these insecticides and the toxicities associated with them. It is possible that in evaluating the structural differences of the derivatives in this study to current insecticides, removal of the fused rings in the 7-chloroquinolin-4-yl piperazine-1-yl acetamide and 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives would produce larvicidal activity. However, whether the antimalarial activities would still be present will need to be re-evaluated.

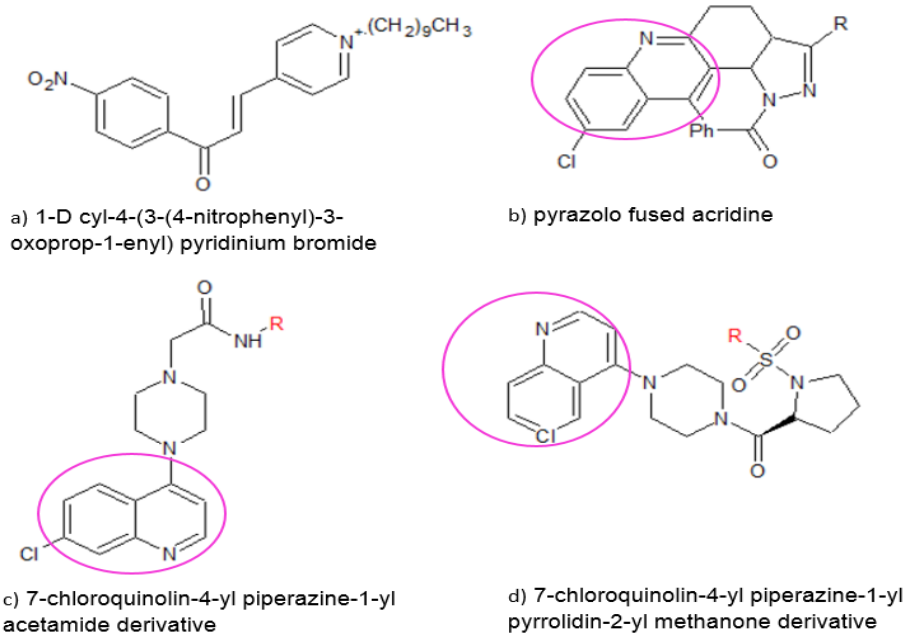
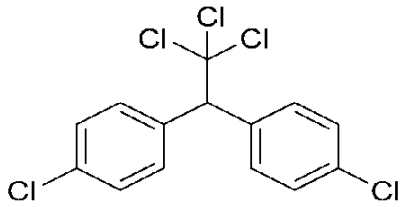
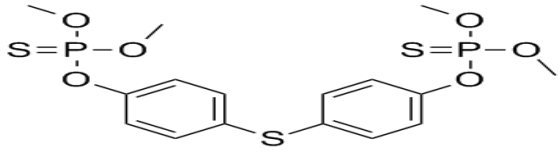
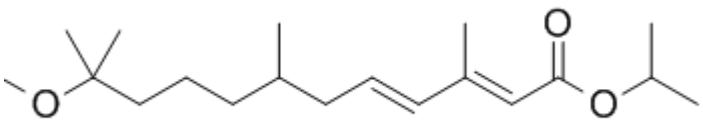
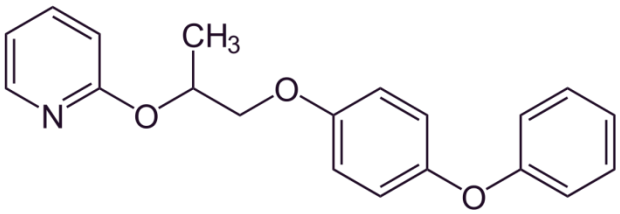


Figure 4.13: Structural differences and similarities between novel chalcone (a) and acridine isomers (b) with larvicidal properties in comparison to the 7-chloroquinolin-4-yl piperazine-1-yl acetamide (c) and 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone (d) derivatives showing no larvicidal properties.

Table 4.1: Chemical structures of larvicides approved by WHO.

Larvicide	Chemical structure
DDT	
Temephos	
Methoprene	
Pyripoxifen	

CHAPTER 5: Conclusion, limitations & recommendations

Both classes of derivatives provided an ideal scaffold with numerous proposed mechanisms of actions that makes it worthwhile to further investigate these derivatives. The chloroquinoline core structure similar to that in the standard antimalarial chloroquine confers good antimalarial activity and beta-haematin inhibition that has been studied extensively (Gupta *et al.,* 2008). Drawing from the existing widespread resistance to the chloroquinoline class of drugs, the novel derivatives in this study built on the basic core structure by adding side chains with different mechanisms of action to increase activity and reduce the possible potentiation in resistance. The one set of derivatives contained an acetamide group linked to a chloroquinoline group by a piperazine linker (Figure 5.1A), whilst the second set contained a sulphonamide moiety linked to a chloroquinoline core by a piperazine and pyrrolidine linker (Figure 5.1B). The acetamide and sulphonamide groups are known to inhibit folate synthesis thereby inhibiting parasite DNA synthesis (Figure 5.1) (Rastelli *et al*., 2003; Hogh *et al.,* 2000). Derivatives CQPA-**26**, CQPA-**25** and CQPPM-**9** in particular are worth further study due to their good antimalarial activity and low toxicity against the host red blood cell and human epithelial (HEK-293) line (Table 3.1 and 3.2). These derivatives were less active than the standard antimalarial, quinine, but further structural modifications may increase the antimalarial activities, while maintaining their safety profiles. The addition of a quinolizidine ring to a chloroquinoline core structure of both sets of derivatives may increase the antimalarial activity, as it did against the W-2 chloroquine-resistant strain of *P. falciparum* with antimalarial activity ten times that of chloroquine (Sparatore *et al*., 2005). Furthermore, studies by Salahuddin *et al*.,(2013), showed that the addition of an acetylaniline side chain to an aminochloroquinoline-based sulphonamide increased antimalarial activity of the structure to yield the most active compound from the group with an IC_{50} value of 2 µM against the chloroquine-resistant FCR-3 strain of *P. falciparum.*

Looking at the structural differences between the 7-chloroquinolin-4-yl piperazine-1-yl acetamide and 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives in comparison to current insecticides, a common differential feature is the lack of the chloroquinoline aromatic nucleus in current insecticides. This is an important feature in conveying antimalarial properties and would suggest that mimicking these structures would lead to compounds with no antimalarial properties and only larvicidal properties (Table 4.1). Furthermore, adapting the same chemical structure as current insecticides would not be ideal as it would not resolve the developing resistance to these insecticides and the toxicities associated with them. Perhaps, in evaluating the structural differences of the derivatives in this study to current insecticides, removal of the fused rings in the 7-chloroquinolin-4-yl

piperazine-1-yl acetamide and 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone derivatives would produce larvicidal activity. However, whether the antimalarial activities would still be present will need to be re-evaluated.

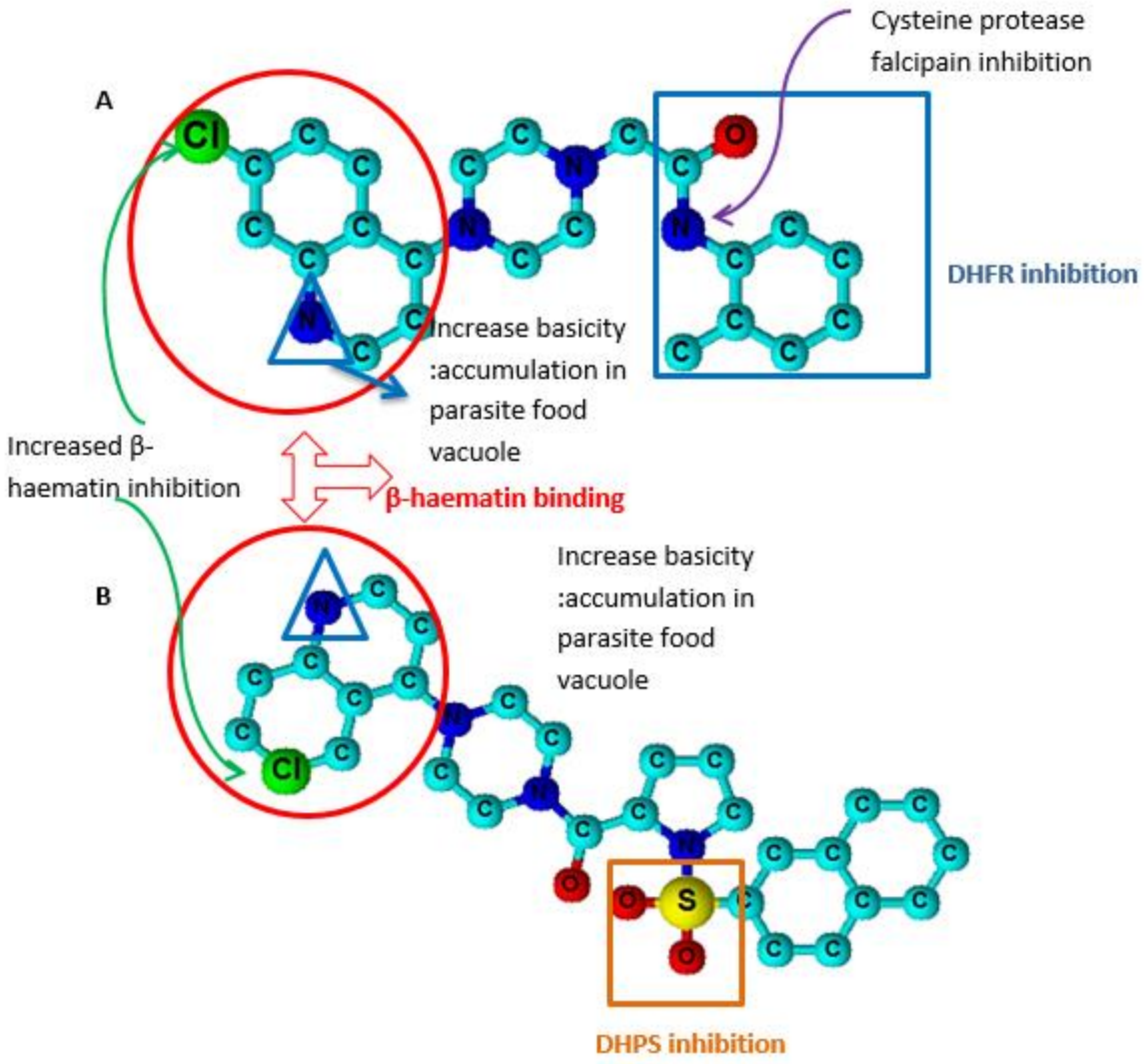


Figure 5.1: Proposed structure-related mechanisms of action of 7-chloroquinolin-4-yl piperazine-1-yl acetamide (A) and 7-chloroquinolin-4-yl piperazine-1-yl pyrrolidin-2-yl methanone (B) derivatives in this study.

The most active derivatives CQPA-**26** and CQPPM-**9** displayed drug-like properties based on the Lipinski's rule of five (Ro5), with the predicted % ionisation at pH 5 indicating that they would accumulate in the parasite digestive vacuole like quinine and chloroquine (Table 3.3, 3.4). This is indicative of the acid-base properties facilitating its mode of action. Derivative CQPA-**26** displayed a synergistic combinatory effect with quinine (ΣFIC: 0.29), whilst

CQPPM-**9** displayed an additive effect in combination with quinine (ΣFIC: 1.31). This showed potential for both of these derivatives to be used in combination with quinine without altering the antimalarial activity negatively.

The negligible effect of the derivatives on the mosquito vector itself is suggestive that these compounds should not be further investigated for any larvicidal activity but rather focus on their intra-erythrocytic antimalarial activity. Furthermore, testing the activity of the derivatives against the sexual stages of the parasite life cycle may be worthwhile to not only treat the human host but to block transmission. However, a favourable non-toxic effect was shown by all derivatives against brine shrimp (0-4.7% lethality), which further highlights the favourable safety profile of the derivatives. With the multiple mechanisms of actions, further experimentation on the mechanisms of action in inhibiting beta-haematin formation, folate synthesis and the direct effect on the *Plasmodium* protease enzymes would be feasible. Based on this study, the limited supply of the derivatives with no access to additional compounding of the derivatives was a major limitation preventing further experimentation on the mechanisms of action especially the β-haematin inhibitory effects. The morphological effects of derivative CQPA-**26** and CQPPM-**9** in causing a lag in progression beyond the trophozoite stage of the parasite with no progression to a new lifecycle (Figure 3.5), indicates that other modes of action needs to be explored. The structure-activity information from this study can be used for further development and synthesis of improved second generation derivatives. In addition to antimalarial properties, further studies with regards to multiple biological activities such as the anticancer and antiprotozoal properties of the derivatives would be promising as studies of compounds with a similar structural scaffold as the derivatives in this study showed positive results (Inam *et al.,* 2015).

Reference List

Abass, M., Mostafa, B.B., 2005, 'Synthesis and evaluation molluscicidal and larvicidal activities of some novel enaminones derived from 4-hydroxyquinolinones: Part IX', *Bioorganic & Medicinal Chemistry* 13, 6133-6144

Aguiar, A.C., Santos, R., Figueirdo, F.J.B., Cortopassi, W.A., Pimentel, A.S., Franca, T.C.C., Meneghetti, R., Krettli, A.U., 2012, 'Antimalarial activity and mechanisms of action of two novel 4-aminoquinolines against chloroquine-resistant parasite', *PLoS One* 7 (5). Accessed on 20/02/2014 at: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0037259

AI, S., Sidek, H.M., Surif, S., 2012, 'Comparison of the MAKLER & HINRICHS (1993) technique versus application of HEPES lysis solvent in determining the activities of *Plasmodium* Lactate Dehydrogenase (pLDH) in *Plasmodium berghei*-infected erythrocytes', *International Conference on Applied Life Sciences.* Accessed on 15/05/2014 at: https://www.intechopen.com/books/howtoreference/international-conference-on-applied-life-sciences/comparison-of-the-makler-hinrichs-1993-technique-versus-application-of-hepes-lysis-solvent-in-determ

Ashley, E.A., Dhorda, M., Fairhurst, R.M., 2014, 'Spread of artemisinin resistance in *Plasmodium falciparum* malaria', *The New England Journal of Medicine* 371 (5), 411-423

Avery, M.A., Alvim-Gaston, M., Vroman, J.A., Wu, B., Ager, A., Peters, W., Robinson, B.L., Charman, W., 2002, 'Structure-activity relationships of the antimalarial artemisinin', *Journal of Medicinal Chemistry* 45, 4321-4335

Basco, L.K., Marquet, F., Makler, M.M., Bras, J.L., 1995, '*Plasmodium falciparum* and *Plasmodium vivax*: Lactate dehydrogenase activity and its application for *in vitro* drug susceptibility assay', *Experimental Parasitology* 80, 260-271

Begum, L.A., Roy, N., Laskar, R.A., Roy, K., 2011, 'Mosquito larvicidal studies of some chalcone analogues and their derived products: structure-activity relationships', *Medicinal Chemistry Research* 20, 184-191

Bell, A., 2005, 'Antimalarial drug synergism and antagonism: mechanistic and clinical significance', *FEMS Microbiology Letters* 253, 171-184

Berenbaum, M.C., 1989, 'What is synergy?', *Pharmacological Reviews* 41 (2), 93-141

Bhal, S.K., Kassam, K., Peirson, I.G. and Pearl, G.M., 2007, 'The Rule of Five revisited: applying log D in place of log P in drug-likeness filters', *Molecular Pharmaceutics* 4 (4), 556-560

Blumberg, L., Frean, J., Moonasar, D., 2014, 'Successfully controlling malaria in South Africa', *South African Medical Journal* 104 (3), 224-227

Blumberg, L.H., 2015, 'Recommendations for the treatment and prevention of malaria: Update for the 2015 season in South Africa', *South African Medical Journal* 105 (3), 175-178

Boudhar, A., Ng, X.W., Loh, C.Y. *et al*., 2016, 'Overcoming chloroquine resistance in malaria: design, synthesis, and structure-activity relationships of novel hybrid compounds', *Antimicrobial agents and Chemotherapy* 60 (5), 3076-3089

Brogdon, W.G., Fiore, A., Kachur, S.P., Slutsker, L., Wirtz, R.A., 2014, 'Insecticide resistance and malaria', *Centre for strategic & International Studies.* Accessed on 20/02/2014 at *https://csis-prod.s3.amazonaws.com/s3fs-public/legacy_files/files/publication/141202 _Brogdon_InsecticideResistance_Web.pdf*

Brooke, B., Koekemoer, L., Kruger, P., Urbach, J., Misiani, E. & Coetzee, M., 2013, 'Malaria vector control', *South African Medical Journal* 103 (10), 784-788

Brooke, B.D., Robertson, L., Kaiser, M.L., Raswiswi, E., Munhenga, G., Venter, N., Wood, O.R., Koekemoer, L.L., 2015. 'Insecticide resistance in the malaria vector *Anopheles arabiensis* in Mamfene, KwaZulu-Natal', *South African Journal of Science* 111 (11-12), 1-3

Brown, G.D., 2006, 'Artemisinin and a new generation of antimalarial drugs', *Education in Chemistry* 43 (4), 97-99

Buller, R., Peterson, M.L., Almarsson, O., Leiserowitz, L., 2002, 'Quinoline binding site on malaria pigment crystal: a rational pathway for antimalarial drug design', *Crystal Growth & Design* 2 (6), 553-562

Buzzi, S., Presser, A., von Freyhold, M., 2007, 'Determining a viable protocol for the derivatisation of artemisinin into dihydroartemisinin', *Report for Medicine for Malaria Ventures.* Accessed on 18/09/2015 at: https://assets.publishing.service.gov.uk/media/57a08bf9ed915d3cfd0010a6/4_Derivatisation_DHA-AS-Dec14_fincor.pdf

Calvalcanti, E.S.B., deMorais, S.M., Lima, M.A.A., Santana, E.W.P., 2004, 'Larvicidal activity of essential oils from Brazilian plants against *Aedes egypti* L.', *Memorias do Instituto Oswaldo Cruz* 99 (5), 541-544

Chango, A., Abdennebi-Najar, L., 2011, 'Folate metabolism pathway and *Plasmodium falciparum* malaria infection in pregnancy', *Nutrition reviews* 69 (1), 34-40

Chauhan, S.S., Sharma, M., Chauhan, P.M.S., 2014, 'Trioxaquines: hybrid molecules for the treatment of malaria', *Drug News & Perspective* 23 (10), 632-646

Cheng, S., Liu, J., Tsai, K., Chen, W., Chang, S.T., 2004, 'Chemical composition and mosquito larvicidal activity of essential oils from leaves of different *Cinnamomum osmophloeum* provenances', *Journal of Agricultural and Food Chemistry* 52, 4395-4400

Dahl, E., Shock, J.L., Shenai, B.R., Gut, J., DeRisis, J.L., Rosenthal, P.J., 2006, 'Tetracyclines specifically target the apicoplast of the malaria parasite *Plasmodium falciparum*', *Antimicrobial Agents Chemotherapy* 50 (9), 3124-3131

D'Alessandro, S., Silvestrini, F., Dechering, K., *et al*., 2013, 'A *Plasmodium falciparum* screening assay for anti-gametocyte drugs based on parasite lactate dehydrogenase detection', *Journal Of Antimicrobial Chemotherapy* 68, 2048-2058

Dechy-Cabaret, O., Benoit-Vical, F., Loup, C., *et al.,* 2004, 'Synthesis and antimalarial activity of trioxaquine derivatives', *European Journal of Medicinal Chemistry* 10 (7), 1625-1636

Dechy-Cabaret, O., Benoit-Vical, F., 2012, 'Effects of antimalarial molecules on gametocyte stage of *Plasmodium falciparum*: The debate', *Journal of Medicinal Chemistry* 55, 10328-10344

Deu, E., 2017, 'Proteases as antimalarial targets: strategies for genetic, chemical, and therapeutic validation', *The FEBS Journal* 284, 2604-2628

Dominguez, J.N., Leon, C., Rodrigues, J., de Dominguez, N.G., Gut, J., Rosenthal, P.J., 2005, 'Synthesis and antimalarial activity of sulphonamide chalcone derivatives', *Il Farmaco* 60, 307-311

Domínguez, J.N., López, S., Charris, J., Iarruso, L., Lobo, G., Semenov, A., Olson, J.E., Rosenthal, P.J., 1997, 'Synthesis and antimalarial effects of phenothiazine inhibitors of a *Plasmodium falciparum* cysteine protease', *Journal of Medicinal Chemistry* 40 (17), 2726-2732

Druihle, P., Moreno, A., Blanc, C., Brasseur, P.H., Jacquier, P., 2001, 'A calorimetric *in vitro* drug sensitivity assay for *Plasmodium falciparum* based on a highly sensitive double-site lactate dehydrogenase antigen-capture enzyme-linked immunosorbent assay', *The American Society of Tropical Medicine and Hygiene* 64, 233-241

D'Souza, V., D'Souza, B., 2006, 'Comparative study on lipid peroxidation and antioxidant vitamins E and C in *falciparum* and *vivax* malaria', *Indian Journal of Clinical Biochemistry* 21 (2), 103-106

Dua, V.K., Ojha, V.P., Roy, R., Joshi, B.C., Valecha, N., Devi, C.U., Bhatnagar, M.C.,

Sharma, V.C., Subbarao, S.K., 2004, 'Anti-malarial activity of some xanthones isolated from the roots of *Andrographis paniculata*', *Journal of Ethnopharmacology* 95, 247-251

Egan, T.J., 2000, 'Structure-activity relationships in aminoquinolines: effect of amino and chloro groups on quinoline-haematin complex formation, inhibition of beta-haematin formation, and antiplasmodial activity', *Journal of Medicinal Chemistry* 43 (2), 283-91

Egan, T.J., 2008, 'Haemozoin formation', *Molecular & Biochemical Parasitology* 157, 127-136

Egan, T.J., 2004, 'Haemozoin formation as a target for the rational design of new antimalarials', *Drug Design Reviews* 1, 93-110

Ekoue-kovi, K., Yearick, K., Iwaniuk, D.P., Natarajan, J.K., Alumasa, J., de Dios, A.C., Roepe, P.D., Wolf, C., 2009, 'Synthesis and antimalarial activity of new 4-amino-7-chloroquinolyl amides, sulphonamides, ureas and thioureas', *Bioorganic & Medicinal Chemistry* 17 (1), 270-283

Fabbri, C., de Cássia Mascarenhas-Netto, R., Lalwani, P., Melo, G.C., Magalhães, B.M., Alexandre, M.A., Lacerda, M.V., Lima, E.S., 2013, 'Lipid peroxidation and antioxidant enzymes activity in *Plasmodium vivax* malaria patients evolving with cholestatic jaundice', *Malaria Journal* 12 (1), 315

Fegas, R., Adoui, A., Hamdi, A., Siouffi, A.M., 2006, 'Reversed-phase HPLC seperation of quinine and its diastereoisomer quinidine in pharmaceutical tablets', *Asian Journal of Chemistry* 18 (3), 1705

Fitch, C.D., 2003, 'Ferriprotoporphyrin IX, phospholipids and the antimalarial actions of quinoline drugs', *Life Sciences* 74 (16), 1957-1972

Fivelman, Q.L., Walden, J.C., Smith, P.J., Folb, P.I., Barnes, K.I., 1999, 'The effect of artesunate combined with standard antimalarials against chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum in vitro*', *Royal Society of Tropical Medicine & Hygiene* 93, 429-432

Francis, S.E., Sullivan Jr, D.J., Goldberg, A.D.E., 1997, 'Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*', *Annual Reviews in Microbiology* 51 (1), 97-123

Freese, J.A., Sharp, B.L., Ridl, F.C., Markus, M.B., 1988, 'In vitro cultivation of Southern African strains of *Plasmodium falciparum* and gametocytogenesis', *South African Medical Journal* 73 (12), 720-722

Ghorab, M.M., Alsaid, M.S., Al-Dosari, M.S., Nissan, Y.M., Al-Mishari, A.A., 2016, 'Novel chlroquinoline derivatives incorporating biologically active benzenesulfonamide moiety: synthesis, cytotoxic activity and molecular docking', *Chemistry Central Journal* 10 (18), 1-13

Govindarajan, M., Jebanesan, A., Pushpanathan, T., 2008, 'Larvicidal and ovicidal activity of *Cassia fistula* Linn. leaf extract against filarial and malarial vector mosquitoes', *Parasitology Research* 102, 289-292

Govindarajan, M., Sivakumar, R., 2011, 'Mosquito adulticidal and repellent activities of botanical extracts against malarial vector, *Anopheles stephensi Liston* (Diptera: Culicidae)', *Asian Pacific Journal of Tropical Medicine* 4 (12), 941-947

Greenwood, B.M., Fidock, D.A., Kyle, D.E., Kappe, S.H., Alonso, P.L., Collins, F.H., Duffy, P.E., 2008, 'Malaria: progress, perils, and prospects for eradication', *The Journal of clinical investigation* 118 (4), 1266-1276

Gregson, A., Plowe, C.V., 2005, 'Mechanisms of resistance of malaria parasites to antifolates', *Pharmacological Reviews* 57, 117-145

Gülçin, İ., Berashvili, D. and Gepdiremen, A., 2005, 'Antiradical and antioxidant activity of total anthocyanins from *Perilla pankinensis decne*', *Journal of Ethnopharmacology* 101 (1), 287-293

Gülçin, I., 2007, 'Comparison of in vitro antioxidant and antiradical activities of L-tyrosine and L-Dopa', *Amino acids* 32 (3), 431-438

Gupta, L., Srivastava, K., Singh, S., Puri, S.K., Chauhan, P.M.S., 2008, 'Synthesis of 2-[3-(7-chloro-quinolin-4-ylamino)-alkyl]-1-(substituted phenyl)-2,3,4,9-tetrahydro-1H-β-carbolines as a new class of antimalarial agents', *Bioorganic & Medicinal Chemistry Letters* 18 (11), 3306-3309

Hargreaves, K., Hunt, R.H., Brooke, B.D., Mthembu, J., Weeto, M.M., Awolola, T.S., Coetzee, M., 2003, '*Anopheles arabiensis* and *An. quadriannulatus* resistance to DDT in South Africa', *Medical and Veterinary Entomology* 17, 417-422

Hayat, F., Moseley, E., Salahuddin, A., Van Zyl, R.L., Azam, A., 2011, 'Antiprotozoal activity of chloroquinoline based chalcones', *European Journal of Medicinal Chemistry* 46 (5), 1897-1905

Hawley, S.R., Bray, P.G., Park, B.K., Ward, S.A., 1996, 'Amodiaquine accumulation in *Plasmodium falciparum* as a possible explanation for its superior antimalarial activity over chloroquine', *Molecular and Biochemical Parasitology* 80 (1), 15-25

Høgh, B., Clarke, P.D., Camus, D., Nothdurft, H.D., Overbosch, D., Günther, M., Joubert, I., Kain, K.C., Shaw, D., Roskell, N.S. and Chulay, J.D., 2000, 'Atovaquone-proguanil versus chloroquine-proguanil for malaria prophylaxis in non-immune travellers: a randomised, double-blind study', *The Lancet* 356 (9245), 1888-1894

Homewood, C.A., Warhurst, D.C., Peters, W., Baggaley, V.C., 1972, 'Lysosomes, pH and the antimalarial action of chloroquine', *Nature* 235 (5332), 50-52

Inam, A., Siddiqui, S.M., Macedo, T.S., Moreira, D.R.M., Leite, A.C.L., Soares, M.B.P., Azam, A., 2014, 'Design, synthesis and biological evaluation of 3-[4-(7-chloro-quinolin-4-yl)-piperazine-1-yl]-propionic acid hydrazones as antiprotozoal', *European Journal of Medicinal Chemistry* 75, 67-76

Inam, A., Van Zyl, R.L., van Vuuren, N.J., Chen, C.T., Avecilla, F., Agarwal, S.M. and Azam, A., 2015, 'Chloroquinoline–acetamide hybrids: a promising series of potential antiprotozoal agents', *RSC Advances* 5 (60), 48368-48381

Imam, H., Alla, A.W.H.A., Yagi, S.M., 2013, 'Evaluation of the larvicidal, antiplasmodial and cytotoxicity properties of *Cassia arereh* del. stem bark', *European Journal of Medicinal Plants* 3 (1), 78-87

Jatakiyya, V.P., Chhipa, N.M.R., Sen, D.J., 2014, '*In-vitro* antimalarial activity by sybr-green-I based fluorescence assay on *N*-substituted piperazine derivatives synthesised by higher greener solvents and ionic liquids *on Plasmodium falciparum*', *World Journal of Pharmacy and Pharmaceutical Sciences* 3 (2), 2653-2692

Jensen, J.B., Trager, W., 1977, '*Plasmodium falciparum* in culture: use of outdated erythrocytes and description of candle jar method', *The Journal of Parasitology* 63 (5), 883-886

Kalani, K., Cheema, H.S., Tripathi, H., Khan, F., Daroker, M.P., Srivastava, S.K., 2015, 'QSAR-guided semi-synthesis and *in vitro* validation of antiplasmodial activity in ursolic acid derivatives', *Royal Society of Chemistry* 5, 32133-32143

Kamatou, G.P.P., Viljoen, A.M., Van Vuuren, S.F., Van Zyl, R.L., 2006, 'In vitro evidence of antimicrobial synergy between *Salvia chamelaeagnea and Leonotis leonurus'*, *South African Journal of Botany* 72 (4), 634-636

Kappe, S.H., Vaughan, A.M., Boddey, J.A., Cowman, A.F., 2010, 'That was then but this is now: malaria research in the time of an eradication agenda', *Science* 328 (5980), 862-866

Kibret, S., Lautze, J., McCartney, M., Wilson, G.G., Nhamo, L., 2015, 'Malaria impact of large dams in sub-Saharan Africa: maps, estimates and predictions', *Malaria Journal* 14 (1), 339-351

Klahn, P., Bronstrup, M., 2017, 'Bifunctional antimicrobial conjugates and hybrid antimicrobials', *Natural Product Reports* 34, 832-885

Klein, E.Y., 2013, 'Antimalarial drug resistance: a review of the biology and strategies to delay emergence and spread', *International Journal of Microbial Agents* 41 (4), 311-317

Krishna, S., Uhlemann, A., Haynes, R.K., 2004, 'Artemisinins: mechanism of action and potential for resistance', *Drug Resistance Updates* 7, 233-244

Krungkrai, J., Krungkrai, S.R., 2016, 'Antimalarial qinghaosu/artemisinin: the therapy worthy of a nobel prize', *Asian Pacific Journal of Tropical Medicine* 6 (5), 371-375

Kuhn, Y., Rohrbach, P. and Lanzer, M., 2007, 'Quantitative pH measurements in *Plasmodium falciparum*-infected erythrocytes using pHluorin', *Cellular Microbiology 9* (4), 1004-1013

Kumar, D., Khan, S.I., Tekwani, B.L., Ponnan, P., Rawat, D.S., 2014, 'Synthesis, antimalarial activity, heme binding and docking studies of 4-aminochlroquinoline-pyrimidine based molecular hyrbids', *The Royal Society of Chemistry* 4, 63655-63699

Kumawat, M.K., Singh, U.P., Singh, B., Prakash, P., Chetia, D., 2011, 'Synthesis and antimalarial activity evaluation of 3-(3-(chloroquinolin-4-ylamino)propyl)-1,3-thiazinin-4-one derivatives', *Arabian Journal Of Chemistry* 9 (1), 643-647

Lambros, C., Vanderberg, J.P., 1979, 'Synchronization of *Plasmodium falciparum* erythrocytic stages in culture', *The Journal of Parasitology* 65 (6), 418-420

Leed, A., DuBay, K., Ursos, L.M., Sears, D., de Dios, A.C., Roepe, P.D., 2002, 'Solution structures of antimalarial drug-heme complexes', *Biochemistry* 41 (32), 10245-10255

Lell, B., Kremsner, P.G., 2002, 'Clindamycin as an antimalarial drug: review of clinical trials', *Antimicrobial agents and Chemotherapy* 46 (8), 2315-2320

Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 1997, 'Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings', *Advanced Drug Delivery Reviews* 23 (1-3), 3-25

Loria, P., Miller, S., Foley, M., Tilley, L., 1999, 'Inhibition of the peroxidative degradation of haem as the basis of action of chloroquine and other quinoline antimalarials', *Biochemistry Journal* 339, 363-370

Madapa, S., Tusi, Z., Sridhar, D., Kumar, A., Siddiqi, M.I., Srivastava, K., Rizvi, A., Tripathi, R., Puri, S.K., Shiva Keshava,G.B., Shukla, P.K., Batra, S., 2009, 'Search for new

pharmacophores for antimalarial activity (Part 1): Synthesis and antimalarial activity of new 2-methyl-6-ureido-4-quinolinamides', *Bioorganic and Medicinal Chemistry* 17, 203-221

Maharaj, R., Morris, N., Seocharan, I., Kruger, P., Moonasar, D., Mabuza, A., Raswiswi, E., Raman, J., 2012, 'The feasibility of malaria elimination in South Africa', *Malaria Journal* 11, 423

Maharaj, R., Raman, J., Morris, N., Moonasar, D., Durrheim, D.N., Seocharan, I., Kruger, P., Shandukani, B., Kleinschmidt, I., 2013, 'Epidemiology of malaria in South Africa: from control to elimination', *South African Medical Journal* 103 (10), 779-783

Mahesh, R., Mundra, S., Devadoss, T., Kotra, L.P., 2014, 'Design, synthesis and evaluation of 2-(4-(substituted benzoyl)-1, 4-diazepan-1-yl)-N-phenylacetamide derivatives as a new class of falcipain-2 inhibitors', *Arabian Journal of Chemistry.* Accessed on 20/06/2016 at:*http://dx.doi.org/10.1016/j.arabjc.2014.11.008*

Makler, M.T., Ries, J.M., Williams, J.A., Bancroft, J.E., Piper, R.C., Gibbins, B.L., Hinrichs, D.J., 1993, 'Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity', *The American Society of Tropical Medicine and Hygiene* 48 (6), 739-741

Manohar, S., Rajesh, U.C., Khan, S.I., Tekwani, B.L., Rawat, D.S., 2010 'Novel 4-aminoquinoline pyrimidine-based hydrids with improved in vitro and in vivo antimalarial activity', *Medicinal Chemistry Letters* 3 (7), 555-559

Mavundza, E.J., Maharaj, R., Chukwujekwu, J.C., Finnie, J.F., Van Staden, J., 2014, 'Screening for adulticidal activity against *Anopheles arabiensis* in ten plants used as mosquito repellent in South Africa', *Malaria journal* 13 (1), 173

Meunier, B., 2008, 'Hybrid molecules with a dual mode of action: dream or reality', *Acc Chem Res* 41 (1), 69-77

Malaria, R.B., 2005, 'World malaria report 2005', *World Health Organization and UNICEF* Accessed on 15/01/2015 at:*http://www.who.int/malaria/publications/atoz/9241593199/en/*

Malaria, R.B., 2015, 'World malaria report 2015', *World Health Organization.* Accessed on 5/06/2016 at:*http://apps.who.int/iris/bitstream/10665/200018/1/9789241565158_eng.pdf*

Mendoza, A., Perez-Silanes, S., Quiliano, M. *et al*., 2011, 'Aryl piperazine and pyrrolidine as antimalarial agents. Synthesis and investigation of structure-activity relationships', *Experimental Parasitology* 128, 97-103

Mishra, R., Mishra, B., Moorthy, H.N., 2006, 'Dihydrofolate Reductase Enzyme: A potent

target for antimalarial research', *Asian Journal of Cell Biology* 1 (1), 48-58

Mishra, N., Arora, P., Kumar, B., Mishra, L.C., Bhattacharya, A., Awasthi, S.K., Bhasin, V.K., 2008, 'Synthesis of novel substituted 1, 3-diaryl propenone derivatives and their antimalarial activity in vitro', *European Journal of Medicinal Chemistry* 43 (7), 1530-1535

Mohammed, B.R., Abdulsalam, Y.M., Deeni, Y.Y., 2015, 'Insecticide resistance to *Anopheles spp*. mosquitoes (Diptera: Culicidae) in Nigeria: a review', *International Journal of Mosquito Research* 2 (3), 56-63

Morphy, R., Rankovic, Z., 2005, 'Designed multiple ligands. An emerging drug discovery paradigm', *European Journal of Medicinal Chemistry* 48 (21), 6523-6543

Mosmann, T., 1983, 'Rapid calorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays', *Journal of Immunological Methods* 65, 55-63

Mott, B.T., Eastman, R.T., Guha, R. *et al*., 2015, 'High-throughput matrix screening identifies synergistic and antagonistic antimalarial drug combinations', *Scientific Reports* 5, 13891-13905

Mouatcho, J.C., Golding, J.P.D., 2013, 'Malaria rapid diagnostic tests: challenges and prospects', *Journal of Medical Microbiology* 62, 1491-1505

Mouatcho, J.C., Munhenga, G., Hargreaves, K., Brooke, B.D., Coetzee, M., Koekemoer, L.L., 2009, 'Pyrethroid resistance in a major African malaria vector *Anopheles arabiensis* from Mamfene, northern KwaZulu-Natal, South Africa', *South African Journal of Science* 105 (3-4), 127-131

Muagphrom, P., Seki, H., Fukushima, E.O., Muranaka, T., 2016, 'Artemisinin-based antimalarial research: application of biotechnology to the production of artemisinin, its mode of action, and the mechanism of resistance of *Plasmodium* parasites', *Journal of Natural Medicine* 70, 318-334

Muregi, F.W., Ishih, A., 2010, 'Next-generation antimalarial drugs: hybrid molecules as a new strategy in drug design', *Drug Development Research* 71, 20-32

Najila, M.J.S., Rain, A.N., Kamel, A.G.M., Zahir, S.I.S., Khozirah, S., Hakim, S.L., Zakia, I., Azizol, A.K., 2002, 'The screening of extracts from Goniothalamus scortechinii, Aralidium pinnatifidum and Andrographis paniculate for anti-malarial activity using the lactate dehydrogenase assay', *Journal of Ethnopharmacology* 82, 239-242

Naughton, J.A., Nasizadeh, S., Bell, A., 2010, 'Downstream effects of haemoglobinase

inhibition in *Plasmodium falciparum*-infected erythrocytes', *Molecular and Biochemical Parasitology* 173 (2), 81-87

Noedl, H., Wongsrichanalai, C., Wernsdorfer, W.H., 2003, 'Malaria drug-sensitivity testing: new assays, new perspectives', *Trends in Parasitology* 19 (4), 175-181

Nosten, F., Brasseu, P., 2002, 'Combination therapy for malaria', *Drugs* 62 (9), 1315-1329

Nwabor, O.F., Dibua, U.M., Ngwu, G.I., *et al.,* 2014, 'Evaluation of the antimicrobial and larvicidal potentials of seed extracts of *Picrilima nitida'*, *International Research Journal of Natural Sciences* 2 (2), 23-30

Nzila, A., 2006, 'The past, present and future of antifolates in the treatment of *Plasmodium falciparum* infection', *Journal of Antimicrobial Chemotherapy* 57, 1043-1059

Olliaro, P., 2001, 'Mode of action and mechanisms of resistance of antimalarial drugs', *Pharmacology & Therapeutics* 89 (2), 207-219

O'Neill, P.M., Barton, V.E., Ward, S.A., Chadwick, J., 2012, '4-Aminoquinolines: chloroquine, amiodaquine and next generation analogues', *Treatment and Prevention of Malaria*, 19-44

Opsenica, I., Opsenica, D., Lanteri, C.A., *et al.,* 2008, 'New chimeric antimalarials with 4-aminoquinoline moiety linked to a tetraoxane skeleton', *European Journal of Medicinal Chemistry* 51 (19), 6216-6219

Pabón, A., Escobar, G., Vargas, E., Cruz, V., Notario, R., Blair, S., Echeverri, F., 2013, 'Diosgenone synthesis, anti-malarial activity and QSAR of analogues of this natural product', *Molecules* 18 (3), 3356-3378

Panella, N.A., Dolan, M.C., Karchesy, J.J., Xiong, Y., Peralta-cruz, J., Khasawneh, M., Montenieri, J.A., Maupin, G.O., 2005, 'Use of novel compounds for pest control: Insecticidal and acaricidal activity of essential oil components from heartwood of alaska yellow cedar', *Journal of Medical Entomology* 42 (3), 352-358

Park, I., Lee, S., Shin, S., Park, D., Ahn, Y., 2002, 'Larvicidal activity of isobutylamides identified in *Piper nigrum* fruits against three mosquito species', *Journal of Agricultural and Food Chemistry* 50, 1866-1870

Pattanasin, S., Proux, S., Chompasuk, D., Luwiradaj, K., Jacquier, P., Looareesuwan, S., Nosten, F., 2003, 'Evaluation of a new *Plasmodium* lactate dehydrogenase assay for the detection of malaria', *Royal Society of Tropical Medicine and Hygiene* 97, 672-674

Perumalsamy, H., Kim, N., Ahn, Y., 2009, 'Larvicidal activity of compounds isolated from *Asarum heterotropoides* against *Culex pipiens pallens, Aedes egypti* and *Ochlerotatus togoi* (Diptera: culicidae)', *Journal of Medical Entomology* 46 (6), 1420-1423

Pink, R., Hudson, A., Mouriès, M.A., Bendig, M., 2005, 'Opportunities and challenges in antiparasitic drug discovery', *Nature Reviews Drug Discovery* 4 (9), 727-740

Piper, R., Lebras, J., Wentworth, L., Hunt-Cooke, A., Houze, S., Chiodini, P., Makler, M., 1999, 'Immunocapture diagnostic assays for malaria using *Plasmodium* lactate dehydrogenase' *The American Society of Tropical Medicine and Hygiene* 60 (1), 109-118

Pisciotta, J.M., Sullivan, D., 2008, 'Hemozoin: oil versus water', *Parasitology International* 57 (2), 89-96

Po, H.N., Senozan, N.M., 2001, 'The Henderson-Hasselbalch equation: its history and limitations', *Journal of Chemical Education* 78 (11), 1499

Pradel, G., Schlitzer, M., 2015, 'Antibiotics in malaria therapy and their effect on parasite apicoplast', *Current Molecular Medicine* 10 (3), 335-349

Radloff, P.D., Philips, J., Nkeyi, M., Kremsner, P.G., Hutchinson, D., 1996, 'Atovaquone and proguanil for *Plasmodium falciparum* malaria', *The Lancet* 347 (9014), 1511-1514

Rahman, M.A., 2011, 'Chalcone: a valuable insight into the recent advances and potential pharmacological activities', *Chemical Sciences Journal* 2011

Raj, R., Mehra, V., Gut., J., *et al.,* 2014, 'Discovery of highly selective 7-chloroquinoline thiohydantoins with potent antimalarial activity', *European Journal of Medicinal Chemistry* 84, 425-432

Rastelli, G., Pacchioni, S., Sirawaraporn, W., Sirawaraporn, R., Parenti, M.D., Ferrari, A.M., 2003, 'Docking and database screening reveal new classes of *Plasmodium falciparum* dihydrofolate reductase inhibitors', *Journal of Medicinal Chemistry* 46 (14), 2834-2845

Rodea-Palomares, I., González-Pleiter, M., Martín-Betancor, K., Rosal, R. and Fernández-Piñas, F., 2015, 'Additivity and interactions in ecotoxicity of pollutant mixtures: Some patterns, conclusions, and open questions', *Toxics* 3 (4), 342-369

Rogerson, S.J., Chaluluka, E., Kanjala, M., Mkundika, P., Mhango, C. and Molyneux, M.E., 2000, 'Intermittent sulfadoxine-pyrimethamine in pregnancy: effectiveness against malaria morbidity in Blantyre, Malawi, in 1997–1999', *Transactions of the Royal Society of Tropical Medicine and Hygiene* 94 (5), 549-553

Roncales, M., Vidal-Mas, J., Leroy, D., Herreros, E., 2012, 'Comparison and optimization of different methods for the *in vitro* production of *Plasmodium falciparum* gametocytes', *Journal of Parasitology Research*, 927148

Roopan, S.M., Bharathi, A., Al-Dhabi, N.A., Arasu, M.V., Madhumita, G., 2017, 'Synthesis and insecticidal activity of acridone derivatives to *Aedes aegypti* and *Culex quinque fasciatus* larvae and non-target aquatic species', *Scientific Reports* 7, 39753-39758

Rosenthal, P.J., 1995, '*Plasmodium falciparum*: effects of proteinase inhibitors on globin hydrolysis by cultured malaria parasites', *Experimental Parasitology* 80 (2), 272-281

Rosenthal, P.J., Meshnick, S.R., 1996, 'Hemoglobin catabolism and iron utilization by malaria parasites', *Molecular and Biochemical Parasitology* 83 (2), 131-139

Rosenthal, P.J., 2004, 'Cysteine proteases of malaria parasites', *International Journal for Parasitology* 34 (13), 1489-1499

Ruebhart, D.R., Cock, I.E., Shaw, G.R., 2008, 'Brine shrimp bioassay: importance of correct taxonomic identification of *Artemia (Anostraca)* species', *Environmental Toxicology* 23 (4), 555

Ruebhart, D.R., Wickramasinghe, W., Cock, I.E., 2009, 'Protective efficacy of the antioxidants vitamin E and Trolox against Microcystis aeruginosa and microcystin-LR in *Artemia franciscana* nauplii', *Journal of Toxicology and Environmental Health Part A* 72 (24), 1567-1575

Saifi, M.A., Beg, T., Harrath, A.H., Altayalan, F.S.H., Al Quraishy, S., 2013, 'Antimalarial drugs: mode of action and status of resistance', *African Journal of Pharmacy & Pharmacology* 7 (5), 148-156

Salahuddin, A., Inam, A., Van Zyl, R.L., Heslop, D.C., Chen, C., Avecilla, F., Agarwal, S.M., Azam, A., 2013, 'Synthesis and evaluation of 7-chloro-4-(piperazin-1-yl)quinolone-sulfonamide as hybrid antiprotozoal agents', *Bioorganic & Medicinal Chemistry* 21, 3080-3089

Service, M.W., 1980, 'A guide to medical entomology', London: Macmillan Press, 226

Schlitzer, M., 2007, 'Malaria chemotherapeutics part 1: history of antimalarial drug development, currently used therapeutics and drugs in clinical development', *Medicinal Chemistry Journal* 2, 944-986

Schwartz, E., 2012, 'Prophylaxis of malaria', *Mediterranean Journal of Hemotology and*

Infectious Diseases 4 (1). Accessed on 18/09/2016 at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3395692/

Silvestrini, F., Alano, P., Williams, J.L., 2000, 'Commitment to the production of male and female gametocytes in the human malaria parasite *Plasmodium falciparum*', *Parasitology* 121 (05), 465-471

Sisodia, B.S., Negi, A.S., Darokar, M.P., Dwivedi, U.N., Khanuja, S.P.S., 2012, 'Antiplasmodial activity of steroidal chalcones: evaluation of their effect on haemozoin synthesis and the new permeation pathway of *Plasmodium falciparum*-infected erythrocyte membrane', *Chemical Biology & Drug Design* 79 (4), 610-615

Skinner-Adams, T.S., Stack, C.M., Trenholme, K.R. *et al*., 2010, 'Plasmodium falciparum neutral aminopeptidases: new targets for antimalarials', *Biophysical Journal* 53 (1), 53-61

Slater, A.F., 1993, 'Chloroquine: mechanism of drug action and resistance in *Plasmodium falciparum*', *Pharmacology & Therapeutics* 57 (2-3), 203-235

Solomon, V.R., Haq, W., Smilkstein, M., Srivastava, K., Puri, S.K. & Katti, S.B., 2010, '4-Aminoquinoline derived antimalarials: Synthesis, antiplasmodial activity and heme polymerization inhibition studies', *European Journal of Medicinal Chemistry* 45 (11), 4990-4996

South African National Department of Health (SA-DOH), 2009,'Guidelines for prevention of malaria in South Africa', *National Department of Health.* Accessed on 14/04/2015 at:*http://www.fidssa.co.za/Content/Documents/Malaria%20Prevention_NDOH%202009.pdf*

South African National Department of Health (SA-DOH), 2016, 'Guidelines for the treatment of malaria in South Africa 2016', *National Department of Health.* Accessed on 10/04/2017 at:*http://www.nicd.ac.za/assets/files/Guidelines%20-%20MalariaTreatment%202016%20-Final%20Draft%2005%20December%202016.pdf*

Sparatore, A., Basilico, N., Parapini, S., Romeo, S., Novelli, F., Sparatore, F., Taramelli, D., 2005, '4-Aminoquinoline quinolizidinyl-and quinolizidinylalkyl-derivatives with antimalarial activity', *Bioorganic & Medicinal Chemistry* 13 (18), 5338-5345

Straimer, J., Gnadig, N.F., Witkowski, B. *et al*., 2015, 'K-13 propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates', *Science* 23 (347), 428-431

Takala-Harrison, S., Jacob, C.G., Arze, C., Cummings, M.P., Silva, J.C., Dondorp, A.M., Fukuda, M.M., Hien, T.T., Mayxay, M., Noedl, H., Nosten, F., 2015, 'Independent emergence of artemisinin resistance mutations among *Plasmodium falciparum* in Southeast Asia', *Journal of Infectious Diseases* 211 (5), 670-679

Tekwani, B.L., Walker, L.A., 2005, 'Targeting the hemozoin synthesis pathway for new antimalarial drug discovery: technologies for in vitro β-hematin formation assay', *Combinatorial Chemistry & High Throughput Screening* 8 (1), 63-79

The Africa Malaria Report, 2003, 'The burden of malaria in Africa', Accessed on 5/10/2014 at:http://www.againstmalaria.com/downloads/RBMBurdenMalariaAfrica.pdf

Ukpe, I.S., Moonasar, D., Raman, J., Barnes, K.I., Baker, L., Blumberg, L., 2013, 'Case management of malaria: Treatment and chemoprophylaxis', *South African Medical Journal* 103 (10), 793-798

Virtual Biology, 2014, 'Lifecycle of a mosquito'. Accessed on 20/01/2017 at https://i1.wp.com/amrita.olabs.co.in/userfiles/1/image/MosquitoLifecycle%281%29.gif

Van Zyl, R.L., Seatlholo, S.T., Viljoen, A.M., 2010, 'Pharmacological interactions of essential oil constituents on the in vitro growth of *Plasmodium falciparum*', *South African Journal of Botany* 76 (4), 662-667

Waliwitiya, R., Kennedy, C.J., Lowenberger, C.A., 2008, 'Larvicidal and oviposition-altering activity of monoterpenoids, *trans*-anethole and rosemary oil to the yellow fever mosquito *Aedes egypti* (Dipter:culicidae)', *Pest Management Science* 65, 241-248

Wang, P., Wang, Q., Aspinall, T.V., Sims, P.F.G., Hyde, J.E., 2004, 'Transfection studies to explore essential folate metabolism and antifolate drug synergy in the human malaria parasite *Plasmodium falciparum'*, *Molecular Microbiology* 51 (5), 1425-1438

Weissbuch, I., Leiserowitz, L., 2008, 'Interplay between malaria, crystalline haemozoin formation, and antimalarial drug action and design', *Chemical Reviews* 108 (11), 4899-4914

White, N., 2004, 'Antimalarial drug resistance', *The Journal of Clinical Investigation* 113 (8), 1084-1092

Whitty, C.J., Rowland, M., Sanderson, F., Mutabingwa, T.K., 2002, 'Malaria', *British Medical Journal* 325 (7374), 1221

Wilson, D.W., Langer, C., Goodman, C.D., McFadden, G.I., Beeson, J.G., 2013, 'Defining the timing of action of antimalarial drugs against *Plasmodium falciparum*', *Antimicrobial Agents and Chemotherapy* 57 (3), 1455-1467

Wongtanachai, J., Silamut, K., Day, N.P.J., Dondorp, A., Chaisri, A., 2012, 'Effects of antimalarial drugs on movement of *Plasmodium falciparum*', *Southeast Asian Journal of Tropical Medicine* 43 (1), 1-9

World Health Organization, 2003, 'The Africa malaria report 2003'. Accessed on 10/09/2014 at: http://apps.who.int/iris/bitstream/10665/67869/1/WHO_CDS_MAL_2003.1093.pdf

World Health Organisation, 2005, 'Guidelines for laboratory and field testing of mosquito larvicides'. Accessed on 14/02/2015 at:http://apps.who.int/iris/bitstream/10665/69101/1/WHO_CDS_WHOPES_GCDPP_2005.13.pdf

World Health Organisation, 2006, 'Guidelines for testing mosquito adulticides for indoor residual spraying and treatment of mosquito nets'. Accessed on 16/04/2015 at:http://apps.who.int/iris/bitstream/10665/69296/1/WHO_CDS_NTD_WHOPES_GCDPP_2006.3_eng.pdf

World Health Organisation, 1998, 'Test procedures for insecticide resistance monitoring in malaria vectors, bio efficacy and persistence of insecticides on treated surfaces'. Accessed on 7/08/2015 at: http://www.who.int/whopes/resources/who_cds_cpc_mal_98.12/en/

World Health Organisation, 2013, 'World Malaria Report', *Geneva: WHO*. Accessed on 18/06/2016 at:*http://www.who.int/malaria/publications/world_malaria_report_2013/report/en/*

World Health Organisation, 2014, 'Status report on artemisinin resistance', *Geneva: WHO*. Accessed on 3/3/2016 at:http://www.who.int/malaria/publications/atoz/status-rep-artemisinin-resistance-sep2014.pdf

World Health Organisation, 2015, 'World Malaria Report', *Geneva: WHO.* Accessed on 4/01/2017 at: http://www.who.int/malaria/publications/world-malaria-report-2015/report/en/

World Health Organisation, 2016, 'World Malaria Report', *Geneva: WHO.* Accessed on 4/01/2017 at: *http://www.who.int/malaria/publications/atoz/update-artemisinin-resistance-sep2014/en/*

Wu, R., Zhu, C., Du, X., Xiong, L., Yu, S., Liu, X., Li, Z., Zhao, W., 2012, 'Synthesis, crystal structure and larvicidal activity of novel diamide derivatives against *Culex pipiens*', *Chemistry Central Journal* 6, 99-104

Yayon, A., Cabantchik, Z.I., Ginsburg, H., 1985, 'Susceptibility of human malaria parasites to chloroquine is pH dependent', *Proceedings of the National Academy of Sciences* 82 (9), 2784-2788

Yeo, S.J., Liu, D.X., Kim, H.S. and Park, H., 2017, 'Anti-malarial effect of novel chloroquine derivatives as agents for the treatment of malaria', *Malaria Journal* 16 (1), 80

Appendices

Appendix A: Biosafety ethics clearance: protocol number 20090503

Appendix B: Human Ethics clearance to use and purchase human plasma and blood: waiver certificate number: W-CJ-131030-1

Appendix C: Human ethics clearance to obtain drug-free human whole blood from healthy volunteers: clearance certificate number: M140669

Appendix D: Ethics clearance for the use of mosquitoes and mosquito parasites: reference number: 07-11-2017-O

Appendix E: Human ethics clearance certificate for use of human cell lines: reference number: W-CJ-161129-2

Appendix F: Animal ethics clearance certificate for use of *Artemia* brine shrimp: reference number: 07-11-2017-O

Appendix A: Biosafety ethics clearance: protocol number

Research Office

INSTITUTIONAL BIOSAFETY COMMITTEE
(R 14/16)

CLEARANCE CERTIFICATE - RENEWAL **PROTOCOL NUMBER: 20090503**

BRIEF DESCRIPTION OF APPLICATION:

Chemotherapeutic properties of novel synthetic and natural compounds

APPLICANT: Dr R van Zyl

SCHOOL/DEPARTMENT : Pharmacy/Pharmacology

DATE CONSIDERED: 20090528 and 20140327

DECISION OF COMMITTEE:

Approved unconditionally

1. This clearance certificate expires on 20190402 and may be renewed on application
2. An annual report must be provided on the anniversary date of this certificate, for as long as the project continues
3. Notification of any proposed modifications must be submitted on the attached form

DATE: 20140403 **CHAIRPERSON**: _____
(Professor A Capovilla)

DECLARATION BY APPLICANT:

To be completed in duplicate and **one copy** returned to the Secretary, Room 10004, 10th floor, Senate House, University.

1. I have read, understood and accepted the approval conditions above
2. I agree to submit a yearly progress report to the Committee and to submit an interim report on the form provided, in the event of any significant unforeseen event, *e.g.* suspension of a drug trial, temporary closure or relocation of my laboratory, etc
3. I note that the University Safety Officer, or his/her representative, may at any reasonable time inspect my laboratory or trial site to ensure compliance with current Health and Safety legislation. I undertake to offer my full co-operation in any such inspection.
4. I have read, understood and will comply with the *recommended standard operating procedures for the handling of biohazardous materials* posted at http://web.wits.ac.za/Academic/Research/Biosafety.htm
5. I declare (delete as appropriate) that:
    a. I have all the approvals required by statute or regulation and by the funding agencies supporting this work, or
    b. that I will not begin work until such approvals are obtained

Signed: _____ **Date:** _____

MSWorks2000/Iain0015/IBCClearRenew.wps

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix B: Human Ethics clearance to use and purchase human plasma and blood

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH 10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: Medical School Room 10M07, 10th Floor. Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265

Ref: W-CJ-131030-1 30/10/2013

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: Prof R van Zyl.

Project title: The chemotherapeutic properties of novel synthetics and natural compounds.

Reason: This is a laboratory study in which human blood and plasma for the in vitro maintenance of *Plasmodium falciparum* for experimental purposes such as drug sensitivity and toxicity studies. There are no human participants.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON - JONES
HREC (MEDICAL)
2013 -10- 30
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy - HREC(Medical) Secretariat : Anisa Keshav, Zanele Ndlovu.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Human Research Ethics Committee (Medical)
50 years 1966-2016

Research Office Secretariat: Faculty of Health Sciences, Phillip Tobias Building, 3rd Floor, Office 301, 29 Princess of Wales Terrace, Parktown, 2193 Tel +27 (0)11-717-1252 /1234/2656/2700
Private Bag 3, Wits 2050, email: zanele.ndlovu@wits.ac.za
Office email: hrec-medical.researchoffice@wits.ac.za
Website: www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

November 23, 2017

It has come to my attention that there is some misunderstanding concerning waivers for a few postgraduate students in the Pharmacology Division, Department of Pharmacy and Pharmacology. The waivers below are all that is needed for ethics approval for the listed postgraduate students' research.

Each postgraduate received fresh blood weekly needed to grow malaria parasites from healthy volunteers in an approved project (renewal number M140669; original number M 090532) of Prof R L van Zyl.

Waiver details:
W-CJ-130301-3 Prof R L van Zyl, Mr T I Mahlangu – student no 667999.
W-CJ-150330-2 Prof R L van Zyl, Mrs N Jansen van Vuuren – student no 0748672W.
W-CJ-160129-2 Prof R L van Zyl, Ms P Thabana – student no 609529.
W-CJ-161129-1 Ms F Kathrada – student no 0602240N, Prof R L van Zyl.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MEDICAL)
2017 -11- 23
JOHANNESBURG

Professor Peter Cleaton-Jones
Chair

Copies: HREC (Medical) Secretariat: Zanele Ndlovu, Rhulani Mkanzi, Lebo Moeng.

Appendix C: Human ethics clearance to obtain drug-free human whole blood from healthy volunteers

R14/49 Dr Robyn L van Zyl

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140669

NAME: (Principal Investigator)	Dr Robyn L van Zyl
DEPARTMENT:	Pharmacy & Pharmacology Medical School
PROJECT TITLE:	The Chemotherapeutic Properties of Novel Synthetic and Natural Compounds (Renewal previously M090532)
DATE CONSIDERED:	29/05/2009 (Initial Approval) 26/06/2014 (Renewal)
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	26/06/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature

Date 21-07-2014

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix D: Ethics clearance for the use of mosquitoes and mosquito parasites

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

ANIMAL RESEARCH ETHICS COMMITTEE (AREC)

Registration number: AREC0101210-002

Solomon Mahlangu House
10th Floor, Room 10004
Jorissen Street
Braamfontein, Johannesburg

7 November 2017

To whom it may concern,

Re: Waiver from Animal Ethics Screening Committee of the University of the Witwatersrand

This letter serves to confirm that Associate Professor Robyn van Zyl (Department of Pharmacy and Pharmacology, School of Therapeutic Sciences, University of the Witwatersrand) does not require full animal ethics clearance for her studies which use *Anopheles* mosquito species to evaluate known or novel synthetic and natural compounds as potential insecticides against *Anopheles* species.

Reasons: Mosquito eggs and larva are used in the study. No animals or animal products are used in the study.

Conditions: All approved biosafety and biosecurity SOPs should be adhered to.

Should you require any further information, do not hesitate to contact me.

Yours sincerely,

Kennedy Erlwanger

(Chairman: Animal Research Ethics Committee, University of the Witwatersrand)

Reference: R van Zyl –Anopheles Waiver 07-11-2017-O

Assoc Prof Kennedy H. Erlwanger
School of Physiology
Faculty of Health Sciences, University of the Witwatersrand
7 York Road, Parktown, 2193
SOUTH AFRICA
Tel: +27 (0)11 717 2454
Email: Kennedy.Erlwanger@wits.ac.za

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

ANIMAL RESEARCH ETHICS COMMITTEE (AREC)

Registration number: AREC0101210-002

Solomon Mahlangu House
10th Floor, Room 10004
Jorissen Street
Braamfontein, Johannesburg

24 November 2017

To whom it may concern,

Re: Professor R van Zyl Waiver (for MSc students) from Animal Research Ethics Committee of the University of the Witwatersrand

This letter serves to confirm that Associate Professor Robyn van Zyl (Department of Pharmacy and Pharmacology, School of Therapeutic Sciences, University of the Witwatersrand) does not require full animal ethics clearance for her studies undertaken by the students detailed below. She has previously been granted a waiver for the studies as detailed in the documents referenced *R van Zyl (Artemia) 07-11-2017-O and R van Zyl-Anopheles waiver 07-11-2017-O*. The students and studies covered by the waiver letters:

1. Ms Fatima Kathrada [0602240N]: The effect of 7-chloroquinoline derivatives on the life cycle of the malaria parasite.

2. Mr Sahil Lala [358351]: The effect of quinolone heterocyclic derivatives on the malaria parasite and Anopheles vector.

3. Ms Teboho Malimabe [152675]: The effect of novel quinoxaline derivatives on the life cycle of malaria parasite and vector.

4. Ms Natasha Jansen van Vuuren [071867W]: The effect of novel compounds on copper homeostasis in *Plasmodium falciparum* and neuroblastoma cells.

5. Mr Thulani Mahlangu [667999]: The chemotherapeutic properties of novel quinazolines and bis-hydrazones compounds.

6. Ms Puleng Thabana [609529: The effect of novel oxo-1, 6 dihydropyrimidine and 2-(quinolone-8-yloyxy) acetohydrazone derivatives on the life cycle of the malaria parasite.

7. Ms W Ramashia [572354]: Transcription analysis of an immune factor in a main Africa malaria vector *Anopheles funestus*.

Conditions: All previously requisite biosafety and biosecurity SOPs should be adhered to.

Should you require any further information, do not hesitate to contact me.

Yours sincerely,

Kennedy Erlwanger

(Chairman: Animal Research Ethics Committee, University of the Witwatersrand)

Reference: R van Zyl –Anopheles & Artemia PGrad students Waiver 07-11-2017-O

Assoc Prof Kennedy H. Erlwanger
School of Physiology
Faculty of Health Sciences, University of the Witwatersrand
7 York Road, Parktown, 2193
SOUTH AFRICA
Tel: +27 (0)11 717 2454
Email: Kennedy.Erlwanger@wits.ac.za

Appendix E: Human ethics clearance certificate for use of human cell lines

Human Research Ethics Committee (Medical)

Golden Jubilee: October 1966 – October 2016

Research Office Secretariat: Faculty of Health Sciences, Phillip Tobias Building, 3rd Floor, Office 301,
29 Princess of Wales Terrace, Parktown, 2193 Tel +27 (0)11-717-1252 /1 234/2656/2700
Private Bag 3, Wits 2050, email: zanele.ndlovu@wits.ac.za
Office email: hrec-medical.researchoffice@wits.ac.za
Website: www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

Ref: W-CJ-161129-1 29/11/2016

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **Ms Fatima Kathrada (Student No 0602240N), Prof R van Zyl.**

Project title: **The effect of 7-chloroquinolin-4yl piperazine-1-yl derivatives on the life-cycle of the malarial parasite.**

Reason: This is a laboratory study using commercial cell lines including HEK-293. There are no human participants.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MEDICAL)
2016 -11- 29
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu, Rhulani Mkansi.

Appendix F: Animal ethics clearance certificate for use of *Artemia* brine shrimp

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

ANIMAL RESEARCH ETHICS COMMITTEE (AREC)

Registration number: AREC0101210-002

Solomon Mahlangu House
10th Floor, Room 10004
Jorissen Street
Braamfontein, Johannesburg

7 November 2017

To whom it may concern,

Re: Waiver from Animal Ethics Screening Committee of the University of the Witwatersrand

This letter serves to confirm that Associate Professor Robyn van Zyl (Department of Pharmacy and Pharmacology, School of Therapeutic Sciences, University of the Witwatersrand) does not require full animal ethics clearance for her studies which use *Artemia* (Brine shrimp) as a toxicological model to evaluate known or novel synthetic and natural compounds.

Reasons: *Artemia* eggs and *nauplii* are used in the study. No animals or animal products are used in the study.

Conditions: All approved biosafety and biosecurity SOPs should be adhered to.

Should you require any further information, do not hesitate to contact me.

Yours sincerely,

Kennedy Erlwanger

(Chairman: Animal Research Ethics Committee, University of the Witwatersrand)

Reference: R van Zyl Waiver (Artemia) 07-11-2017-O

Assoc Prof Kennedy H. Erlwanger
School of Physiology
Faculty of Health Sciences, University of the Witwatersrand
7 York Road, Parktown, 2193
SOUTH AFRICA
Tel: +27 (0)11 717 2454
Email: Kennedy.Erlwanger@wits.ac.za