In vitro efficacy assessment of targeted antimalarial drugs synthesized following *in silico* design

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Submitted in partial fulfilment of the degree:

Magister Scientiae

Pharmacology

Faculty of Health Sciences

University of Pretoria

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February 2017

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Acknowledgements

May the favour of the Lord our God rest upon us; establish the work of our hands for us.
~ Psalm 90:17

First and foremost I would like to give thanks to the Almighty God, for His endless mercy and grace throughout this project. For always guiding and giving me strength to persevere. Without Him, I could not have accomplished this work. Not forgetting to thank all those that made this project possible.

To my family, my parents (Hellen and Michael Matlebjane) and my siblings (Nthabiseng, Ramadikela, Dineo and Kgaugelo) I am grateful for the continuous support, encouragement and the faith you had in me. To my dearest friends, Julia, Jessica, Laura, Keagile thank you for your endless support even when I thought it was impossible to achieve this. To the malaria research group thank you for all your support throughout the project. To the Department of Pharmacology, thank you for allowing me to be able to pursue this project.

To my supervisors, Prof Duncan Cromarty, Prof Lyn-Marie Birkholtz and Dr Andre Stander, thank you for your endless support throughout the degree. Thank you for your kindness, advice, motivation and support it has been of great of help. I am grateful for your effortless struggle to help me achieve my full potential.

May the grace of God be with you always and may He continue to bless you.

Summary

Malaria is a major public health problem that affects millions of lives globally. The increased burden of malaria requires new interventions that will address the eradication of the disease. Current interventions include vector control by using insecticide-treated bed nets and indoor residual spraying, and antimalarial drugs to control the parasite. Parasite resistance has been reported for the currently used effective antimalarial drugs. To pre-empt the impact of parasite resistance a continued development of new antimalarial drugs that have novel mechanisms of action should be pursued. Antimalarial drug discovery requires that potential antimalarial drugs should have different drug targets to those already targeted, to lower the chances of resistance. Potential antimalarial drugs should preferably provide a single radical cure to prevent reproduction at all life cycle stages.

This study tested the effects of *in silico* designed compounds targeting plasmodial Ca^{2+}-dependent protein kinases (CDPK) 1 & 4, FIKK kinases and bromodomain proteins on the *Plasmodium* parasite. These enzymes are involved in gene regulation and are important factors during gene transcription. In *P. falciparum* the gatekeeper kinases contain small hydrophobic pockets near the ATP-binding site. These hydrophobic pockets allow for selective inhibition of these proteins at the ATP-binding site. The compounds were tested *in vitro* to determine their antiplasmodial activity. These compounds are shown to be potential inhibitors of the intra-erythrocytic *P. falciparum* parasites as three of the compounds showed selective cytotoxic activity at less than 1 µM against the chloroquine sensitive laboratory strains (3D7 and NF54). Even though the proteins targeted by these compounds have been previously indicated to play a role at specific stages during the parasite's life cycle, the compounds tested here were not able to target the sexual gametocyte stages of the *Plasmodium* parasite. Further optimisation of these compounds should be performed to improve activity against both the asexual and sexual stages of the parasites.

Keywrords: malaria, *Plasmodium* falciparum, *in silico* design, CDPK, FIKK, kinase, bromodomain protein, ATP-binding site, stage specificity, kill kinetics

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ACT	Artemisinin-based combination therapy
ATCC	American Type Culture Collection
atm	Atmosphere
ATP	Adenosine triphosphate
bp	Base pair
BRD	Bromodomain proteins
CAMK	Calcium/calmodulin-dependent protein kinases
CDC	Centers for Disease Control and Prevention
CDPK	Calcium-dependent protein kinase
CK	Creatine kinase
CK1	Casein kinase 1
CLK	Cell division cycle (cdc) like kinase
C-lobe	Carboxy-terminal lobe
cLogP	Calculated partition-coefficient
c-Src	A non-receptor cellular tyrosine Src kinase
DDT	Dichlorodiphenyltrichloroethane
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DHODH	Dihydroorotate dehydrogenase
DHPS	Dihydropteroate synthase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal bovine serum
FIKK	An Apicomplexa specific family of kinase with a conserved Phe-Ile-Lys-Lys motif

FQ	Ferroquine
GSK	Glycogen synthase kinase
h	Hour
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HepG2	Human liver carcinoma cell line
HMT	Histone methyltransferase
HTS	High-throughput screening
IC_{50}	Half-maximal inhibitory concentration
IMDM	Iscove's Modified Dulbecco's Medium
IRS	Indoor residual spraying
ITNs	Insecticide-treated bed nets
Kac	Acetyl-lysine
LDH	Lactate dehydrogenase
LINCS	Linear Constraint Solver
MAP2	Mitogen-activated kinase 2
MAPK	Mitogen-activated kinase
min	Minute/s
MMV	Medicines for Malaria Venture
MR4	Malaria Research and Reference Reagent Resource Centre
MW	Molecular weight
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form of NAD^+)
NAG	N-acetyl glucosamine
N-lobe	Amino-terminal
NMR	Nuclear magnetic resonance
P. falciparum	*Plasmodium falciparum*

*Pf*SPZ	*P. falciparum* sporozoite vaccine
P. knowlesi	*Plasmodium knowlesi*
P. malariae	*Plasmodium malariae*
P. ovale	*Plasmodium ovale*
P. vivax	*Plasmodium vivax*
PBS	Phosphate buffered saline
*Pf*CRT	*Plasmodium falciparum* chloroquine resistance transporter
Pfmdr1	*Plasmodium falciparum* multidrug resistance 1 gene
PI(4)K	Phosphatidylinositol-4-OH kinase
PK	Protein kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PKI	Protein kinase inhibitor
PTM	Post-translational modification
QED	Quantitative estimate of drug-likeness
RGC	Receptor guanylate cyclases
RI	Resistance index
RMSD	Root mean squared deviation
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
s	Second/s
SP	Sulphadoxine/pyrimethamine
SBDD	Structure-based drug discovery
SBVS	Structure-based virtual screening
SEC	Single exposure chemoprotection
SEM	Standard error of the mean
SERC	Single exposure radical cure
SMFA	Standard membrane feeding assay
SMILES	Simplified molecular input line entry system
SOP	Standard operating procedures
STE	Sterile

t½	Half-life
TCP	Target candidate profile
THF	Tetrahydrofolate
TK	Tyrosine kinase
TKL	Tyrosine kinase-like
TPP	Target protein profile
Tris	tris(hydroxymethyl)amino-methane
TS	Thymidylate sythase
WHO	World Health Organisation

Chapter 1

1. Literature review

1.1. Introduction

Malaria is a major public health problem and affects millions of people worldwide, mostly in the tropical and subtropical regions of the world (CDC, 2016). Currently, 3.2 billion people are at risk of being infected with the disease and 95 countries and territories had on going malaria transmission (WHO, 2015). The World Health Organization (WHO) has reported that in 2015, there were 214 million cases of malaria (WHO, 2015; CDC, 2016). Of the reported cases, 88% occurred in Africa, 10% from Southeast Asia and only 2% were from the eastern Mediterranean (WHO, 2015). Unfortunately, malaria still resulted in mortality in 438 000 individuals, of which 90% occurred on the African continent (Figure 1). Children under 5 years of age are still susceptible to the disease and an estimated 306 000 children under 5 years were reported to have died due to malaria in 2015 (WHO, 2015).

The incidence of malaria cases has decreased from 2000 to 2015 by 37% globally (WHO, 2015). This decline in malaria burden is attributed to the increased use of vector control tools such as indoor residual spraying (IRS) and insecticide-treated bed nets (ITNs) to reduce the mosquito bite rate and to kill mosquitoes (Burrows *et al.*, 2013), as well as the increased funding available, public awareness of symptoms and the use of combination drug therapy (Flannery *et al.*, 2013).
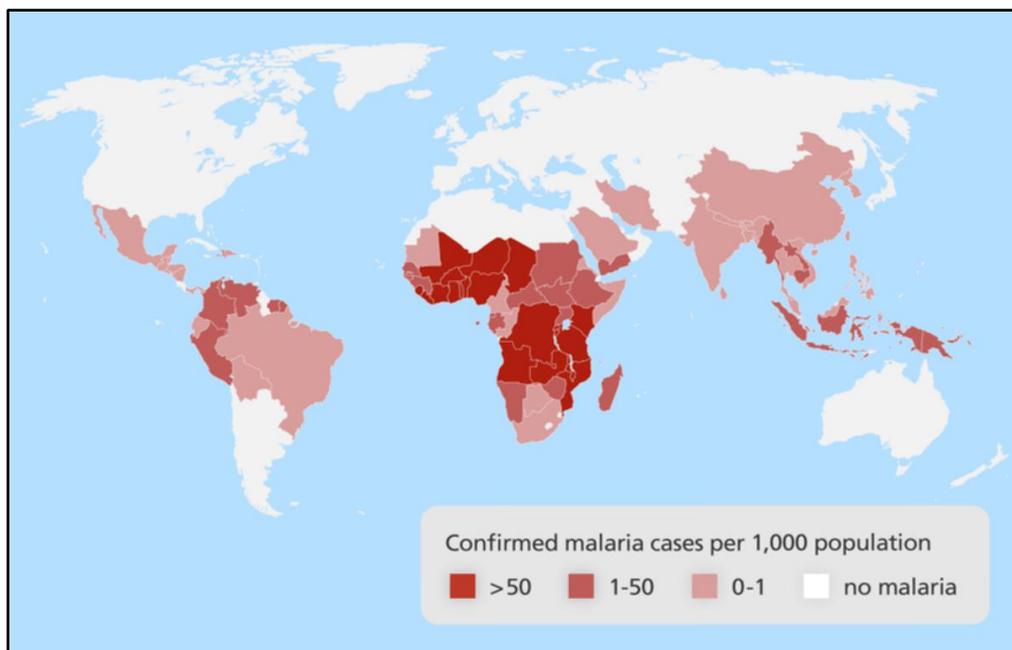
Figure 1: World malaria map showing the distribution of malaria. Malaria transmission occurred in 95 countries and territories. Some countries as indicated on the map have no malaria cases. The image has been reproduced with permission from WHO (WHO, 2014; 2015)

Malaria in humans is caused by 5 species of protozoan parasites that belong to the genus *Plasmodium* (Cowman *et al.*, 2012; Builders, 2013; WHO, 2013; 2014). The species include *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and the zoonotic parasite, *P. knowlesi*. Malaria parasites are transmitted by female *Anopheles* mosquitoes only (Tuteja, 2007; Gamo, 2014). *P. falciparum* and *P. ovale* are the most prevalent forms of the species with *P. falciparum* also being the most lethal. This parasite is predominant on the African continent where most deaths that occur are due to this species (Miller *et al.*, 2013). *P. vivax* has a wider geographic distribution (WHO, 2013; Gamo, 2014) than *P. falciparum* because *P. vivax* is able to develop at lower temperatures and also survive at higher altitudes (WHO, 2013; 2014). Although infection with this species can occur in Africa, the risk of infection is lower because of a general absence of the Duffy gene, which produces a protein that is necessary for *P. vivax* to invade erythrocytes (WHO, 2014), amongst African populations.

Infection with P. vivax and P. ovale can result in a subset of parasites developing into dormant states, known as hypnozoites, that can remain undetected in the liver for months to years (Bousema and Drakeley, 2011; da Cruz et al., 2012; Miller et al., 2013). The patient can be infected but be asymptomatic, however, P. vivax and P. ovale infections can result in a relapse of the disease at any point in time (da Cruz et al., 2012).

1.2.   The life cycle of the malaria parasite

Infection with malaria begins when an infected female *Anopheles* mosquito takes its blood meal from a human host (Tuteja, 2007), injecting infectious *Plasmodium* sporozoites into the human host's bloodstream from within its salivary glands (Figure 2). The exo-erythrocytic cycle begins when the sporozoites migrate to the liver where they invade hepatocytes. In the hepatocytes, the sporozoites mature and divide through schizogony to form a hepatic schizont, which ruptures to release merozoites into the bloodstream. In *P. falciparum* infections, the exo-erythrocytic stage typically takes 8 – 25 days (Tuteja, 2007). The released merozoites enter the bloodstream where they invade erythrocytes (Ramasamy, 1998), initiating the intra-erythrocytic developmental cycle (IDC). The IDC is characterised by an initial development to ring forms, which subsequently develop into metabolically active trophozoites. Multinucleated schizonts are then formed during schizogony, where nuclear material is rapidly replicated; a process characterised by nuclear and cell division without cytokinesis (Tuteja, 2007; Ansell *et al.*, 2014). The schizonts burst to release 20 – 30 merozoites into the bloodstream where they invade new erythrocytes and the IDC repeats (Tuteja, 2007; Biamonte *et al.*, 2013). In *P. falciparum* and *P. ovale* parasites, the IDC takes 48 hours (h), yet takes 72 h in *P. malariae* parasites (Tuteja, 2007).

During the IDC, some trophozoites stochastically differentiate to sexual development [7]. Gametocytogenesis takes place in the bone marrow of the human host and the gametocytes take 10 – 12 days to develop into the (male) micro- and (female) macro-gametocytes (Tuteja, 2007). These mature gametocytes then circulate again in the host's blood stream and can be ingested by a feeding *Anopheles* mosquito and facilitates transmission of the parasites from the human host to the vector (Dixon *et al.*, 2009). In the mosquito, the gametocyte responds to a change in environment (drop in temperature and presence of xanthurenic acid) with male gametocytes exflagellating to form gametes (Ranjan *et al.*, 2009). Fusion of haploid male and female gametes in the gut of the mosquito (Ramasamy, 1998) forms a diploid zygote. Ookinesis then results in an ookinete [10], that forms an oocyst where multiple sporozoites form [11], which then ruptures, releasing large numbers of sporozoites that accumulate in the salivary glands from where the infection cycle is again initiated (Ramasamy, 1998).
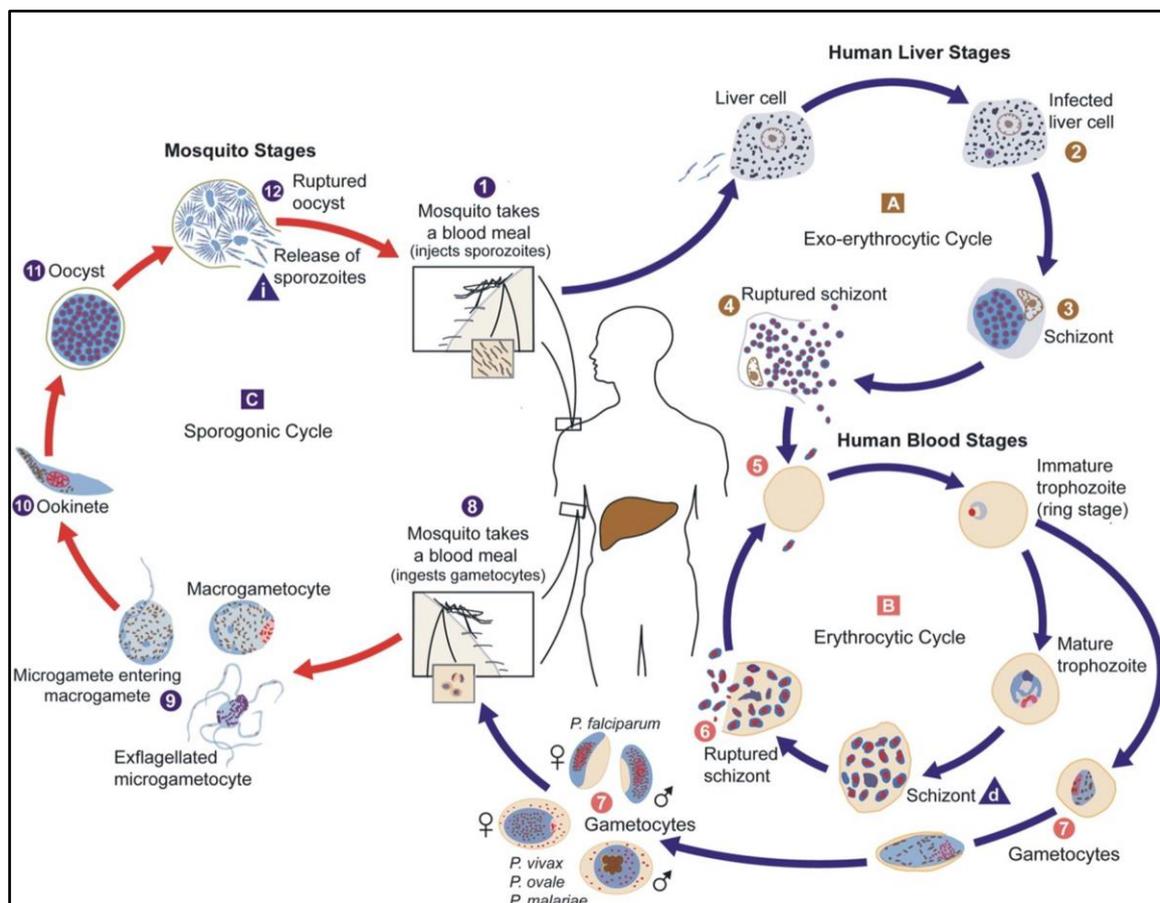
Figure 2: The life cycle of the malaria parasite. The malaria parasite life cycle consists of three stages; A) the exo-erythrocytic cycle, B) the erythrocytic cycle which consists of the intra-erythrocytic developmental stages and the sexual (gametocyte) stages (both the exo-erythrocytic and the erythrocytic cycle take place in the human host and C) the sporogonic cycle which take place in the mosquito. This image has been taken from (Doerig and Grevelding, 2015) reproduced with permission from Elsevier.

1.3. Malaria control

In the hope of eliminating malaria, there are a number of interventions used including strategies that target the mosquito vector, those that safeguard the host from being infected and chemotherapeutics targeting the parasite in symptomatic patients. These individual or combined interventions help to reduce the number of malaria cases and deaths.

1.3.1. Vector control

Vector control strategies aim to prevent transmission of the parasite from the vector, the female *Anopheles* mosquito, to the human host. Vector control includes the use of IRS, ITNs and removing the larva stages of the mosquitoes (whereby mosquitoes are eliminated before they reach maturity) from their habitats (CDC, 2016). IRS works by killing the mosquitoes that come into contact with the insecticide sprayed surface within the houses where the mosquitos tend to settle. This does not prevent mosquitoes from biting humans (CDC, 2014). The first pesticide that was successfully used to control malaria transmission was dichlorodiphenyltrichloroethane (DDT). DDT was used during World War II and was successful in reducing the numbers of soldiers infected by the disease. The use of DDT became widespread with indiscriminate use in agriculture, which resulted in mosquitoes developing resistance to this insecticide. ITNs have been used for malaria control, but only pyrethroid insecticides have been approved for net treatment. There has been an emerging resistance to the insecticides used for ITNs. In order to manage resistance, piperonyl butoxide has been incorporated into some nets, but no evidence has yet been provided to confirm if this has improved the effectiveness of ITNs (CDC, 2014). The limitation with vector control is that mosquitoes have become or are developing resistance to many commonly used insecticides (CDC, 2014; Gamo, 2014). This poses a threat to the use of vector control as a malaria elimination strategy.

1.3.2. Vaccine development

There is currently no effective malaria vaccine on the market (CDC, 2014). Several vaccines have been under development, such as the RTS,S/AS01 (Casares and Richie, 2009; Otto *et al.*, 2010; Arama and Troye-Blomberg, 2014), which has now passed phase III clinical trial and is currently being reviewed (pilot study) (WHO, 2015) and the *P. falciparum* sporozoite vaccine (*Pf*SPZ) (Hoffman *et al.*, 2010). The RTS,S/AS01 vaccine consists of hepatitis B surface antigen virus-like particles and incorporates a portion of the *P. falciparum* derived circumsporozoite protein and a liposome-based adjuvant (Gosling and von Seidlein, 2016). This vaccine targets the pre-erythrocytic stages of the *P. falciparum* life cycle (Alonso *et al.*, 2005). The phase I and IIb results showed that the vaccine was well-tolerated and immunogenic (Alonso *et al.*, 2005). The RTS,S/AS01 vaccine can be used as part of the

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elimination strategies, however, the efficacy of this vaccine will need to be improved (Gosling and von Seidlein, 2016). *Pf*SPZ is a metabolically active sporozoite vaccine that works on the pre-erythrocytic stages of the parasite. Sissoko *et al.* demonstrated in a phase I trial that the *Pf*SPZ vaccine was well tolerated and safe (Sissoko *et al.*, 2017). *Pf*SPZ vaccine will be undergoing clinical trials to increase the efficacy levels.

1.3.3. Malaria chemotherapy

Antimalarial drug treatments aim to reduce transmission and the burden of disease for the patients by targeting the parasite in the intra-erythrocytic stages. Treating malaria with antimalarial drugs has been the most effective way of treating malaria to date. However, increasing parasite resistance poses a challenge for the development of antimalarial drugs. The three main antimalarial drug classes, namely; quinolone-related drugs, folate inhibitors and sesquiterpenes like the artemisinin derivatives have been used as chemotherapeutics for malaria but the parasite is developing resistance to all of these drugs (Gamo, 2014; Hanboonkunupakarn and White, 2016). These drugs will be briefly discussed below.

1.3.3.1. Quinolone related drugs

The quinolone-related drugs (Figure 3) contain a quinolone heteroaromatic nucleus. The first quinolone antimalarial was extracted from the bark of the cinchona tree and was isolated and identified in 1820 as quinine, (White, 1996; Foley and Tilley, 1998). Synthetic derivatives include chloroquine, amodiaquine, mefloquine piperaquine and primaquine (Foley and Tilley, 1998). Chloroquine is a 4-aminoquinoline (Petersen *et al.*, 2011), which is effective against the erythrocytic forms of *P. vivax, P. ovale*, *P. malariae* and chloroquine-sensitive strains of *P. falciparum.* Malaria parasites use the host's haemoglobin to obtain amino acids, and during this process the haem part of the haemoglobin is released, which is toxic to the parasite. To protect itself, the parasite sequesters the free haem and polymerises it to haemozoin as non-toxic crystalline form of haem. Chloroquine is active against the asexual stages of the parasite (Foley and Tilley, 1997) and works by inhibiting the polymerization of haem. Chloroquine accumulates in the acidic food vacuole of the parasite (Foley and Tilley, 1997; Foley and Tilley, 1998) resulting in accumulation of haem that leads to lysis of the erythrocytes and the parasite is killed in the process (Brunton *et al.*, 2008).
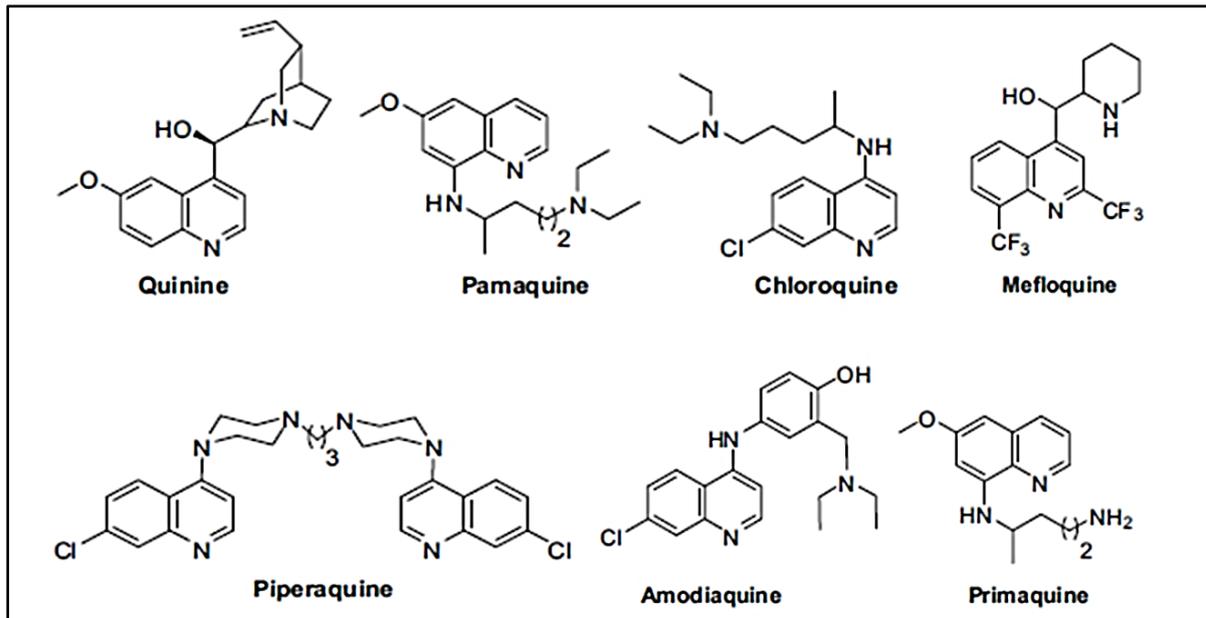
Figure 3: Chemical structures of some of the quinolone antimalarial drugs that are widely used in the treatment of *P. falciparum*. Some examples of the quinolone antimalarials include quinine, pamaquine, chloroquine, mefloquine, piperaquine, amodiaquine, and primaquine.

Chloroquine was used in Africa since the 1950s and remained the drug of choice for years, due to its safety, efficacy and affordability (Petersen *et al.*, 2011; Sinha *et al.*, 2014). The first parasite resistance was observed in Thailand in 1957, resistance reached eastern Africa during the late 1970s (Sinha *et al.*, 2014) and due to this other antimalarial drugs had to be introduced. Chloroquine resistance results from mutations in genes of the parasite transporter proteins that are located on the digestive vacuole membrane. These are mutations in the *P. falciparum* chloroquine resistance transporter (*Pf*CRT – K76T) and mutations in the *Pf* multidrug resistance 1 gene (*Pf*mdr1) which plays a role in chloroquine resistance in those parasites that have altered *Pf*CRT (Miller *et al.*, 2013; Boudhar *et al.*, 2016).

1.3.3.2. Folate antagonists

The malaria parasite does not use folate supplied by exogenous sources but synthesises its own folate *de novo* (Ferone, 1977). Folate inhibitors interfere with the synthesis of folic acid which is required for pyrimidine synthesis and thereby decreases DNA synthesis (Olliaro, 2001). Without an intact folate generating pathway the parasite will not be able to proliferate (Nzila *et al.*, 2005). There are two types of folate inhibitors (some folate inhibitors are depicted in Figure 4); type 1 and type 2. Type 1 inhibitors include the sulphonamides and sulfones and inhibit the enzyme dihydropteroate synthase (DHPS). These drugs mimic *p*-aminobenzoic acid and prevent the formation of dihydropteroate from

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hydroxymethyldihydropterin, a reaction catalysed by DHPS (Olliaro, 2001). DHPS in *Plasmodium* is a bi-functional enzyme that is coupled to 2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridinepyrophosphokinase (PPIK) (Olliaro, 2001).Type 2 inhibitors include pyrimethamine, proguanil, chlorproguanil and clociguanil (Nzila, 2006b). These drugs inhibit dihydrofolate reductase (DHFR). DHFR is coupled to thymidylate synthase (TS) and this prevents the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF), which is essential for the synthesis of purine nucleotides and certain amino acids (Olliaro, 2001). DHFR is, therefore, an enzyme that is essential for both amino acid and nucleic acid synthesis (Anderson, 2005).
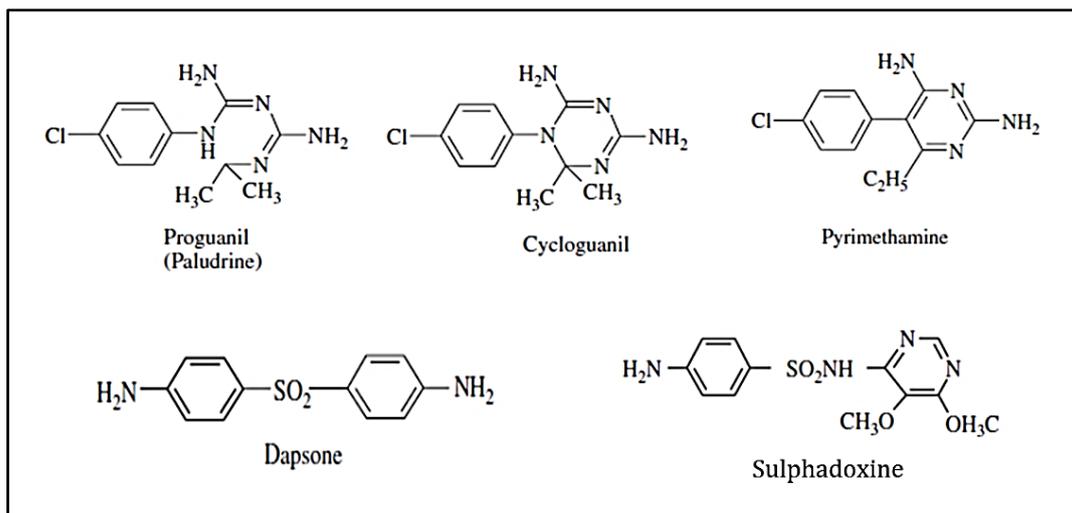
Figure 4: Chemical structures of different antifolates. Some inhibitors of DHFR (proguanil, cycloguanil and pyrimethamine) and DHPS (dapsone and sulfadoxine) are shown.

Folate inhibitors have been used in combination for example as in Fansidar™ (pyrimethamine and sulphadoxine) (Nzila, 2006a), where synergism between the two drugs results from the inhibition of two steps in nucleotide synthesis (Brunton *et al.*, 2008). However, parasite resistance to these drugs and combinations lead to other drugs being recommended (Warhurst, 2002). Buchholz stated that sulphadoxine-pyrimethamine (SP) has had limited use because it can increase the number of gametocytes in malaria patients (Buchholz *et al.*, 2011). Lapdap® (chlorproguanil and dapsone) and Paludrine® (proguanil) (Nzila, 2006a) are examples of other drugs that have been used in the prophylaxis of malaria.

1.3.3.3. Artemisinin derivatives

Artemisinin was first isolated in the 1970s from *Artemisia (A.) annua L.*, a wormwood tree in Asia (Golenser *et al.*, 2006; Miller and Su, 2011; Ho *et al.*, 2014). Artemisinins are a family of sesquiterpene trioxane lactones (Golenser *et al.*, 2006; Ho *et al.*, 2014). The sesquiterpene

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trioxane lactones contain an endoperoxide bridge, which is required for its activity (Golenser *et al.*, 2006; Petersen *et al.*, 2011). Artemisinins are reported to act by forming free radicals when cleaving the endoperoxide bond in its structure (Ho *et al.*, 2014). These compounds are effective against the blood stage of the parasites, as well as the early stages (stage I-III) of gametocyte development (Golenser *et al.*, 2006). Examples of artemisinin-based drugs include dihydroartemisinin (DHA), artesunate and artemether (Figure 5).
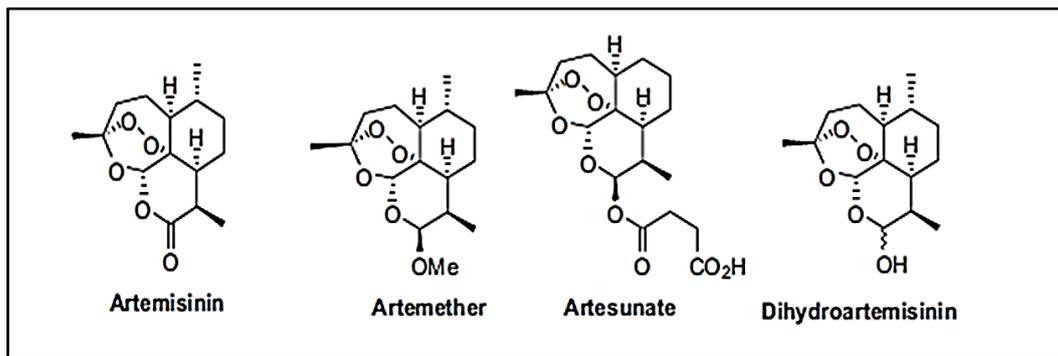
Figure 5: Chemical structures of artemisinin and its derivatives. Artemisinin, artemether, artesunate and the active metabolite of artemisinin, dihydroartemisinin are shown.

Combination therapy is the simultaneous use of two or more drugs that have different mechanisms of action and biochemical targets in the parasite. This is based on the additive potential of two or more drugs to improve the therapeutic efficacy and to also delay the development of resistance to the components of the combination. Combination therapy brings with it a number of advantages, for example, the drug can provide improved efficacy, additive potency and also provide synergistic activity, and delaying the development of resistance (Fidock *et al.*, 2004).

Artemisinin-based combination therapies (ACT) are currently used as the gold standard for malaria treatment and are recommended by the WHO as the first-line antimalarial treatment (Golenser *et al.*, 2006; Klein, 2013). ACTs work on the basis that the short acting artemisinins are combined with longer acting drugs. The reason the artemisinins are used in combination with other drugs is because this results in low relapse rate and high tolerability profile. Examples of ACTs include artesunate – SP, artesunate – mefloquine, artemether – lumefantrine and dihydroartemisinin –Piperaquine.

Artemisinins have shown to be an effective form of treatment against multidrug resistant strains of *P. falciparum*, however, there have been reports of mutations in the multidrug resistance genes of the parasite that were found to alter artemisinin sensitivity (Golenser *et al.*, 2006). Artemisinin resistance has been reported in the greater Mekong sub-region, where resistance is characterised by delayed parasite clearance, and this is reported to be

seen both *ex vivo* and *in vivo* (WHO, 2016). The mutation that is associated with artemisinin resistance and the delayed parasite clearance is located in the Kelch 13 (K-13) propeller domain (WHO, 2016).

With the parasite now demonstrating resistance to all available antimalarial drugs, there is an urgent need for the continued development of new antimalarial drugs with novel mechanisms of action (Olliaro, 2001; Avery *et al.*, 2014). Ideally, these drugs should be able to treat the symptoms that are associated with malaria, clear the parasite from the host and reduce malaria transmission (Miller *et al.*, 2013).

1.4. Antimalarial drug discovery

In the path towards the eradication of malaria and combating parasite resistance to antimalarial drugs, there is continuous search and development of antimalarial drugs. The goal of antimalarial drug development is the development of compounds that will block all stages of the parasite's life cycle (Flannery *et al.*, 2013).

In the search for new antimalarials, Burrows *et al.* (Burrows *et al.*, 2013; Burrows *et al.*, 2017) proposed that new medicines should be able to prevent transmission and relapse of infections with *P. vivax* and *P. ovale*, these medicines should also be able to provide post-treatment prophylaxis and chemoprotection for vulnerable populations (children under 5 years of age and pregnant women). The desired product can then be defined according to target product profiles (TPPs). The TPPs fall into two categories: single exposure radical cure (SERC – TPP1) and single exposure chemoprotection (SEC – TPP2). Treatment with SERC should be a single dose, single exposure and provide a radical cure. Features of SERC include the following: activity against the IDC to treat symptoms associated with the IDC stage infections, activity against the liver stages (to prevent relapse) and be suitable for mass drug administration (MDA) to treat and block malaria transmission (Burrows *et al.*, 2017). Treatment with SEC should be a single exposure that will provide chemoprotection against the 5 species that infect humans and also be used for chemoprevention in endemic areas (Burrows *et al.*, 2013; Burrows *et al.*, 2017; MMV, Annual Report 2015). The MMV has also defined target candidate profiles (TCPs) for the TPPs. Potential molecules should be able to clear the intra-erythrocytic parasites (TCP1), be active against hypnozoites (TCP3) and hepatic schizonts (TCP4), the compound should also be able to block transmission by targeting the gametocyte stages of the parasite (TCP5) and the mosquito vector (TCP6) (Burrows *et al.*, 2017).

The MMV drug discovery pipeline consists of drugs that are in preclinical and clinical development. Drugs in this pipeline have the potential for being part of a single exposure radical cure and some of the drugs have transmission blocking potential. Some of the drug candidates are listed below:

Drugs in preclinical or clinical development (MMV, 2016)

OZ439, which is a trioxolane (second-generation endoperoxide), is currently in Phase IIb combination studies (started in 2015) with Ferroquine (FQ), which inhibits haem detoxification. The combination is known as Artefenomel. Cipargamin (previously known as NITD609) is a spiroindolone, the compound inhibits the P-type sodium (Na^+) transporter ATPase-4 (*Pf*ATP4). Inhibition of *Pf*ATP4 results in an increase in the Na^+ concentration in the parasite. Cipargamin has completed Phase IIa. DSM265 is a triazolopyrimidine and inhibits *Plasmodium* dihydroorotate dehydrogenase (DHODH); the drug is currently in Phase IIa trials in patients with *P. falciparum* or *P. vivax* infections. Another drug in clinical development is the phosphatidylinositol-4-OH kinase (PI(4)K) inhibitor, KAF156 (Gamo, 2014) (previously known as GNF156). KAF156 rapidly kills parasites with a parasite clearance time of less than 48 h and has completed Phase IIa in patients with *P. falciparum* or *P. vivax* infections. MMV048 (3,5-diarylaminopyridine (Younis *et al.*, 2012)) is a PI4K inhibitor, this drug is currently undergoing Phase I clinical trials and human challenge models. The drug was found to have a long half-life (t½) in humans and was set to complete Phase I/Ib studies in 2016.

Drugs currently in pre-clinical development include the *Pf*ATP4 inhibitors, SJ733, PAP2 and GSK030. GSK030 was approved as a pre-clinical candidate in 2014 and has transmission blocking potential based on the results from the standard membrane-feeding assay (SMFA).

1.4.1. *Plasmodium* small gatekeeper kinases

Kinases are enzymes that modify other proteins by phosphorylation, and this usually results in the substrate having a functional change (Tewari *et al.*, 2010). Protein kinases (PK) are important in regulating eukaryotic cellular processes and gene expression (Doerig *et al.*, 2005). The human genome encodes approximately 500 PKs. Most of these PKs share a conserved amino acid sequence and fold (Doerig *et al.*, 2008). The eukaryotic PKs (ePKs) contain a conserved PK catalytic domain for the ATP-binding site. This domain consists of the C-lobe (α-helices), the N-lobe (β-sheets) and a hinge region (Figure 6) (Roskoski Jr, 2004; Wu *et al.*, 2015).
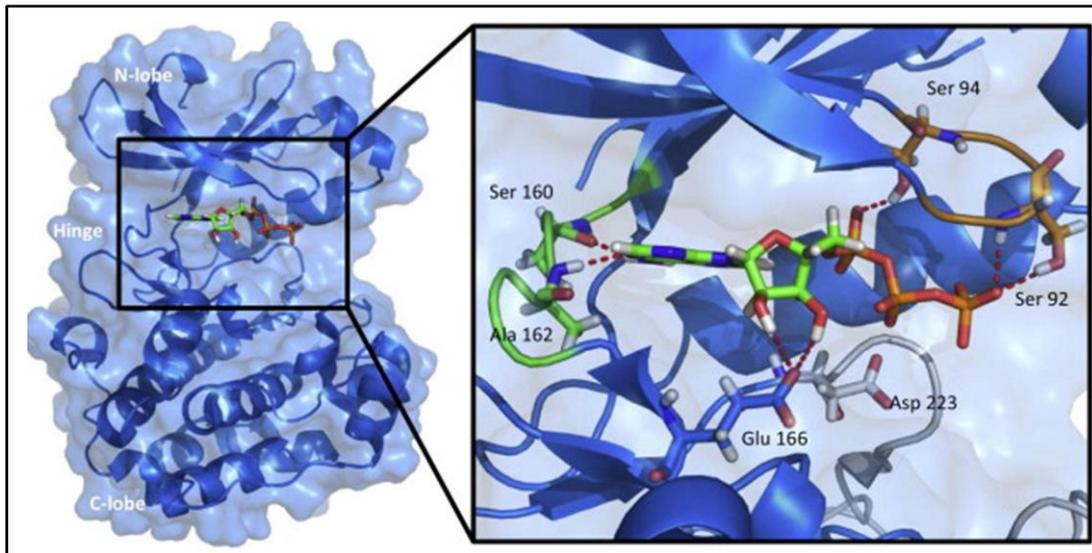
Figure 6: Kinase structure with ATP bound in the active site. The enlarged area shows the structural elements that surround the ATP-binding site (Wu *et al.*, 2015). This image has been reproduced with permission from Elsevier.

PKs have been investigated as drug targets in a number of diseases, including neurodegenerative disorders, inflammation and cancers (Johnson, 2009). There are protein kinase inhibitors (PKI) that have been used successfully in several anticancer therapeutic approaches (Hantschel, 2015). The PK superfamily consists of 8 ePK groups; these include AGC (containing PKA, PKG, PKC family), CAMK (containing CK, MAPK, GSK, CLK family), TK (tyrosine kinases), TKL (tyrosine kinase-like), CK1 (casein kinase 1 family), CMGC, STE and RGC. Small molecule inhibitors of some of the kinase families have been approved for the treatment of cancer (Figure 7) (Wu *et al.*, 2016). Most of these inhibitors target the TK, TKL and CK1 family.
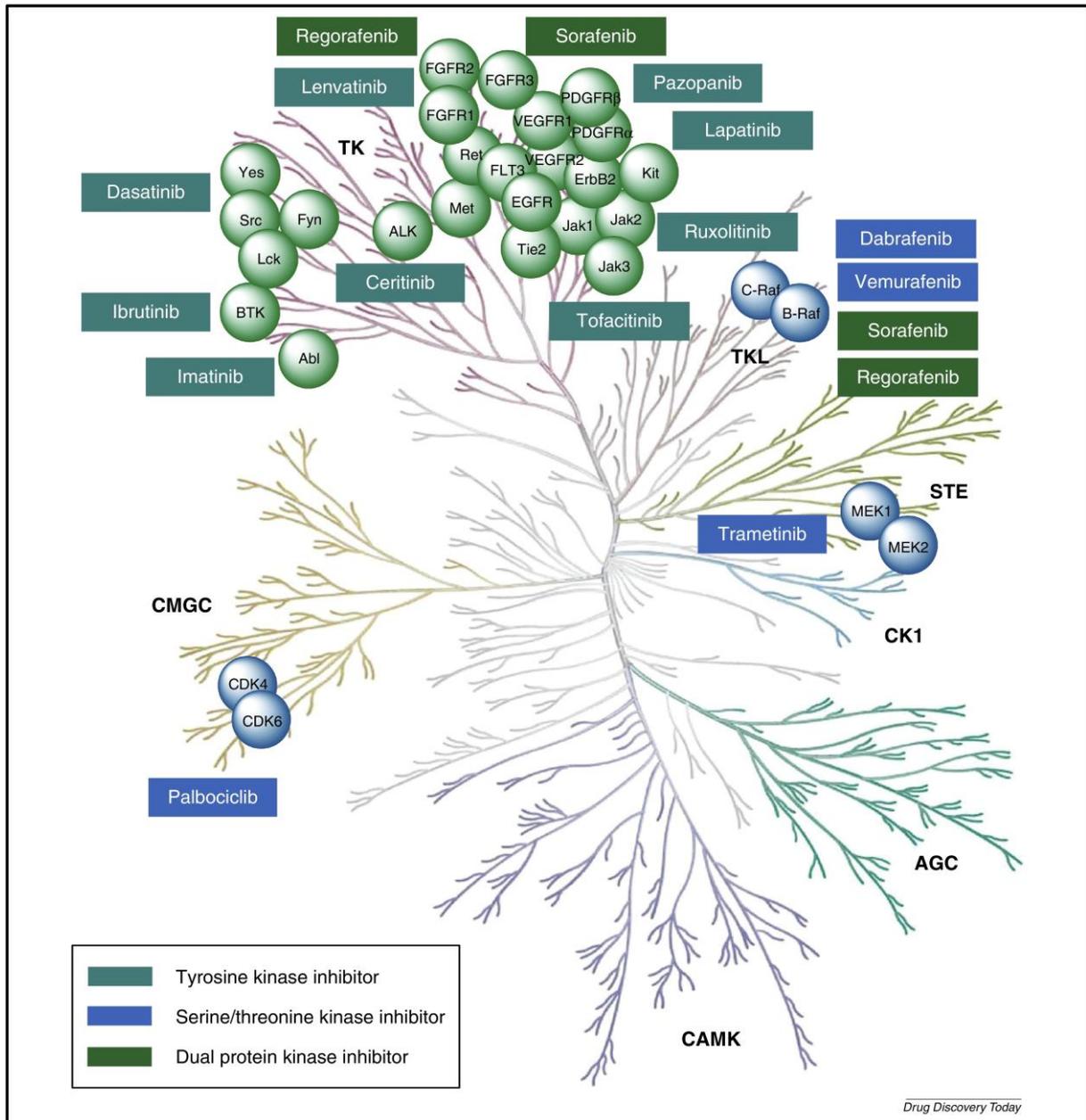
Figure 7: Human kinome tree showing tyrosine and serine/threonine kinases targeted by approved small molecule inhibitors. This image has been reproduced with permission from Elsevier (Wu *et al.*, 2016).

Most parasitic PKs show structural and functional divergence from their host orthologues, with *P. falciparum* kinome encoding approximately 90 PKs and for this reason, selectivity can be achieved (Miranda-Saavedra *et al.*, 2012). For the development of compounds that are selective to the parasite, a family of PKs that are different to the host kinases have to be explored. Figure 8 is an illustration of where specific PKs are present during the *Plasmodium* parasite's life cycle and their function. The PKs are present at all stages of the life cycle and this makes them potential targets. According to Doerig *et al.* one such target is the calcium-dependent protein kinase (CDPKs) family (Doerig *et al.*, 2005). CDPKs are a family of

kinases that are not found in humans, but in plants and alveolates. These kinases contain a kinase domain and calcium-binding regulatory domain (Ward *et al.*, 2004) and are activated by the binding of calcium to their calmodulin-like regulatory domain (Ranjan *et al.*, 2009; Ansell *et al.*, 2014). The CDPKs are also present in the Apicomplexa family such as *Toxoplasma gondii* and *Cryptosporidium parvum* (Ansell *et al.*, 2014).
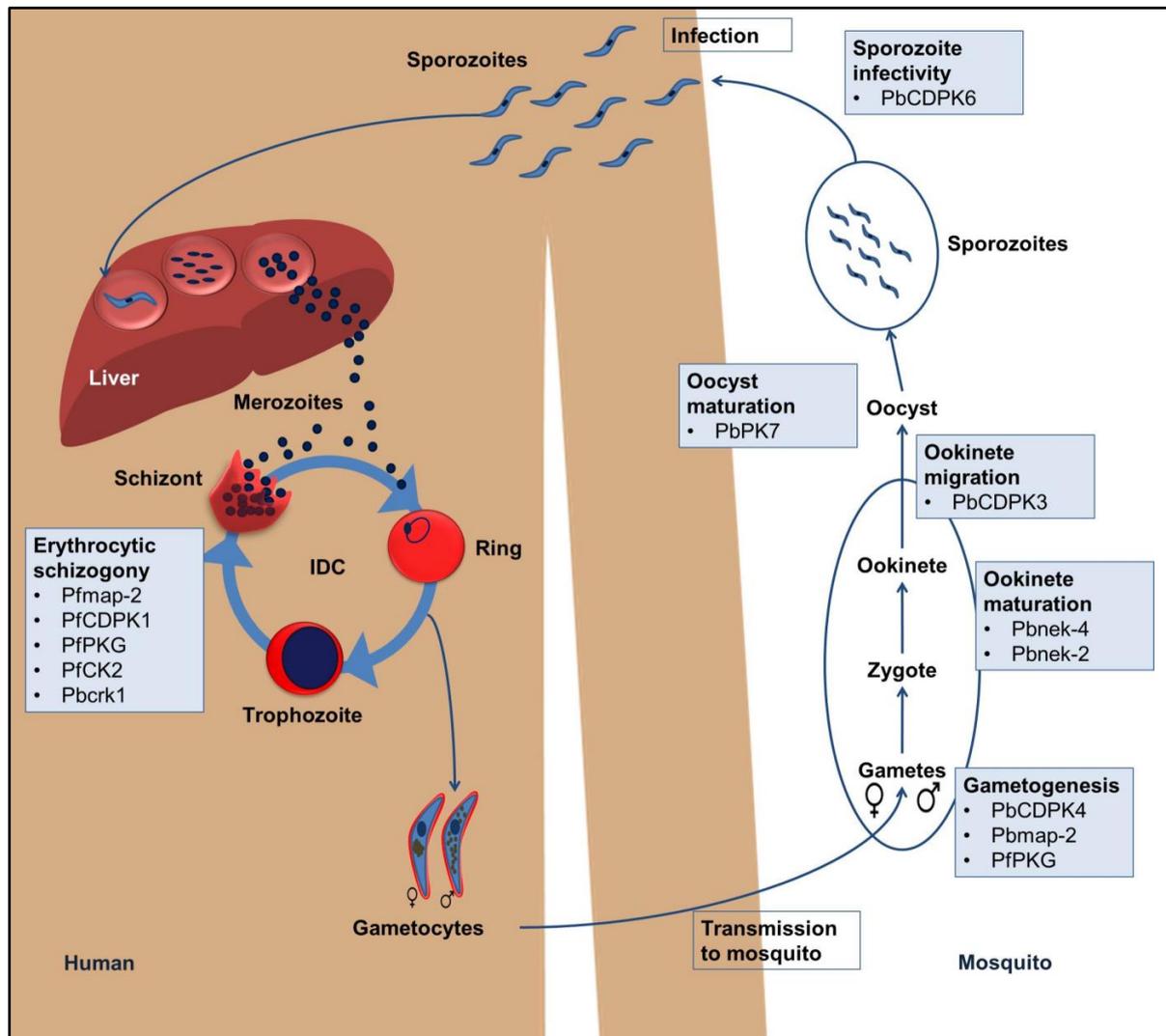
Figure 8: *Plasmodium* protein kinases during the parasite life cycle. Protein kinases are illustrated with their functions. Image generated using GIMP and adopted from Doerig *et al.* (Doerig *et al.*, 2010) with permission from Elsevier.

A major challenge for an antimalarial kinase inhibitor will be to achieve selectivity over human PKs while ideally blocking multiple essential parasite enzymes to prevent the rapid emergence of drug resistance. One way to achieve this would be by focusing efforts on multiple essential targets within a unique subgroup of PKs, such as the CDPKs, which have a domain architecture and mode of regulation otherwise found only in plants.

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Studies show that CDPK1 is essential for parasite proliferation during the IDC and inhibition of the protein's regulatory domain through conditional expression inhibits parasite proliferation at the schizont stage (Ansell *et al.*, 2014). FIKK kinases are a subfamily of these kinases that have been found to be present only in Apicomplexans (Osman *et al.*, 2015). The FIKK family have conserved N-terminal region and C-terminal domain that are characterised by the Phe-Ile-Lys-Lys (these enzymes have been named after this conserved motif) residues (Talevich *et al.*, 2011; Talevich *et al.*, 2012; Osman *et al.*, 2015). In the *P. falciparum* genome, there are 20 sequences that have been identified and in other Apicomplexans such as *P. yoelli*, *P. knowlesi* and *P. vivax* only one sequence has been identified (Ward *et al.*, 2004; Doerig *et al.*, 2005). Some examples of FIKK kinases that have been investigated include FIKK4.1 and FIKK12, which have been implicated in the rigidity of the infected erythrocytes (Nunes *et al.*, 2007). This suggests that these proteins may be essential for the parasite's life cycle (Osman *et al.*, 2015). CDPK4 is expressed in the sexual stages of the parasite and is shown to be important for the development of the parasite in the mosquito (Ward *et al.*, 2004; Ranjan *et al.*, 2009). Ranjan *et al.* demonstrated using Western blot technique that disrupting the CDPK4 gene in *P. berghei* causes defects in sexual reproduction and transmission to the mosquito (Ranjan *et al.*, 2009). In 2010, Tewari identified CDPK1, glycogen synthase kinase 3 (GSK3), casein kinase 1 (CK1) and PKG as essential in asexual erythrocytic stages of *P. berghei* (Tewari *et al.*, 2010). Targeting these kinases could be useful to achieve the goals for TCP1 antimalarials. In the same study CDPK4, mitogen-activated kinase 2 (MAP2), never in mitosis gene A-related kinase 2 and 4 (NEK2 and NEK4) were identified as crucial enzymes in the sexual development of the parasite (Tewari *et al.*, 2010) and targeting these kinases may be relevant to achieve the goals for TCP5 antimalarials.

Within the CDPK family, recent efforts to inhibit CDPK1 (Kato *et al.*, 2008) could be complemented by CDPK5 as an additional target, consistent with its recently confirmed function in *P. falciparum* schizont rupture (Dvorin *et al.*, 2010). CDPK4 is a useful secondary target for a pan-CDPK inhibitor, because of its absolute and early requirement for transmission. Other CDPK family members and CDPK-related CaMKs can be deprioritized because they are either not essential in at least one *Plasmodium* species or, in the case of CDPK2, absent from most *Plasmodium* species infecting humans.

Clinically important inhibitors of human oncogenic tyrosine protein kinases (Src) achieve relative selectivity for their targets by interacting with a hydrophobic pocket at the back of the ATP-binding site, access to which is blocked in most ePKs by a large ''gatekeeper'' residue for example phenylalanine. Where such a gatekeeper is small (serine or threonine), it can be

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exploited as a selectivity filter for rational inhibitor design (Cohen *et al.*, 2005; Zuccotto *et al.*, 2009). The residues are termed gatekeepers as they block or guard access to the hydrophobic pocket that is present close to the ATP-binding site (Doerig *et al.*, 2005). The majority of kinases block this hydrophobic site due to a bulky amino acid in the ATP-binding site (Figure 9A). In order to improve the selectivity of new antimalarial kinase inhibitors, compounds can be designed to specifically target the subgroup of kinases with a small gatekeeper amino acid (Figure 9B). Examples of PKIs that use this principle include Gleevec, which targets Abl kinase and Tarceva, which binds to the ATP-binding site of tyrosine kinase (Doerig *et al.*, 2005).
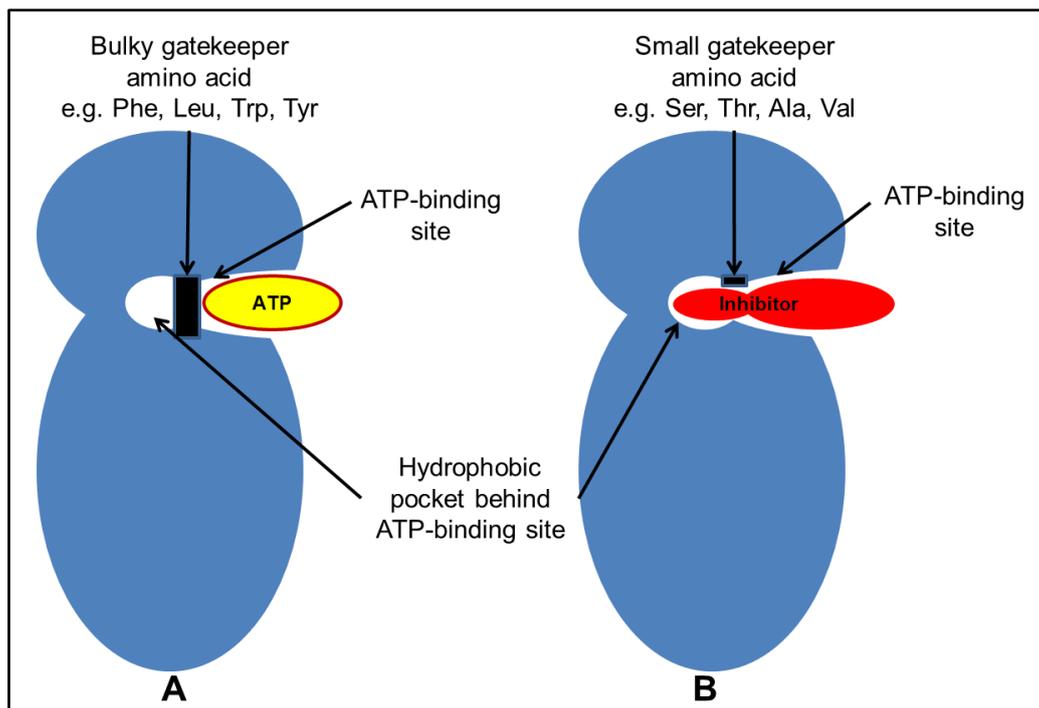
Figure 9: The ATP-binding site of kinases and the hydrophobic pocket behind the binding site. The hydrophobic pocket behind the ATP-binding site of kinases can be blocked by bulky amino acids (A), however, certain kinases have smaller amino acids in this position (B). This can be exploited to design compounds that are very specific towards small gatekeeper kinases.

Selectively targeting small gatekeeper kinases has been demonstrated for a class of anticoccidial inhibitors of *Toxoplasma* PKG and CDPK4 (Donald *et al.*, 2006; McRobert *et al.*, 2008; Tewari *et al.*, 2010; Zhang *et al.*, 2013) and *T. gondii* CDPK1 (Ojo *et al.*, 2010). Selectivity is achieved over the human orthologue by exploiting the small threonine gatekeeper found only in the coccidial enzyme (Donald *et al.*, 2006; McRobert *et al.*, 2008). Alignment of *Plasmodium* kinases has identified PKG, CDPK1, CDPK4 and the FIKK kinases as proteins that have small gatekeeper residues and therefore good targets for the selective inhibition that could result in killing and/or prevention of transmission (Tewari *et al.*, 2010). The CDPK1, CDPK4 and the FIKK kinases are especially attractive targets as they

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are not expressed in animals yet play a crucial role in the life cycle of the parasite (Nunes *et al.*, 2010; Sebastian *et al.*, 2012).

Ciceri *et al.* in 2014 suggested that some kinase inhibitors can act as bromodomain inhibitors, which allows researchers to look for and design compounds with polypharmacological targets; in this case rational design of dual *P. falciparum* kinase-bromodomain inhibitors (Ciceri *et al.*, 2014). Polypharmacology is the ability of a compound to interact with multiple targets (Jalencas and Mestres, 2013; Reddy and Zan, 2013). The goal of combination therapies is the administration of two or more drugs to potentiate an effect, polypharmacology also aims at the same goal, however, using a single drug. Compounds with multiple targets may have more efficacy and be less prone to the development of resistance (Anighoro *et al.*, 2014). Ways in which multitarget drugs can be designed includes docking of ligands into homology models or measured crystal structures of the target proteins; allowing virtual screening of many potential ligands to be performed.

1.4.2. Bromodomain proteins as potential drug targets

Eukaryotic chromatin organisation consists of nucleosomes that have 104 basepairs (bp) of DNA wrapped around histones (Horrocks *et al.*, 2009). Chromatin can be in the form of heterochromatin where DNA is inaccessible to the transcriptional machinery and genes are switched off or euchromatin whereby DNA is accessible to the transcriptional machinery, where genes are switched on (Horrocks *et al.*, 2009; Marek *et al.*, 2015). Epigenetic gene regulation includes writers, erasers and reader proteins. Epigenetic writer proteins such as histone acetyltransferases (HATs), histone methyltransferases (HMTs) and kinases, are involved in laying epigenetic marks on DNA or histones. These marks are read by epigenetic reader proteins such as bromodomains and chromodomains and removed by epigenetic eraser proteins such as histone deacetylases (HDACs), lysine demethylases and phosphatases. Epigenetic gene regulation involves post-translational modification (PTM) of histones and these modifications include methylation, acetylation, sumoylation and phosphorylation (Filippakopoulos and Knapp, 2012). These PTMs play a role in the regulation of transcription of genes for example, histone acetylation and histone methylation (Filippakopoulos and Knapp, 2012). The addition or removal of the epigenetic marks regulates chromatin architecture and therefore influencing gene expression by either making DNA accessible or inaccessible, therefore switching on the transcription of genes or switching off (silencing genes) gene transcription.

Histone acetylation is regulated by HATs and HDACs. HATs catalyse the transfer of an acetyl group to the ε-amino group of lysine side chains on histone proteins (Singh and Misra,

2009) and HDACs operate in an opposing manner, reversing the acetylation of lysine residues. Histone acetylation promotes transcription of DNA as it provides binding sites for proteins that are involved in gene activation for example the bromodomain (BRDs) proteins (Duffy *et al.*, 2012). BRDs are epigenetic reader proteins, which recognise the ε-N-lysine acetylation motifs. The BRDs were first identified in *Drosophila* Brahma (Winston and Allis, 1999; Chung and Witherington, 2011), but are also present in humans, yeast (Chung and Witherington, 2011) and *Toxoplasma*. Human bromodomain containing proteins have been explored for use in oncology, inflammation and infectious diseases (Chung and Witherington, 2011). BRDs are composed of four left-handed α-helices (α$_Z$, α$_A$, α$_B$, α$_C$) (Figure 10), the ZA and BC loops are important for the formation of the hydrophobic acetyl-lysine (Kac) binding pocket (Sanchez *et al.*, 2014; Brand *et al.*, 2015; Ferri *et al.*, 2016). *P. falciparum* parasite has several BRDs including the histone acetyltransferase GCN5 (Loyola and Almouzni, 2004; Duffy *et al.*, 2012). BRDs are required for binding to chromatin and are important in functioning as transcriptional co-activators. The bromodomain proteins contain a hydrophobic acetyl-lysine binding site (Loyola and Almouzni, 2004; Chung and Witherington, 2011), which represents an attractive pocket for the development of small active inhibitor compounds.
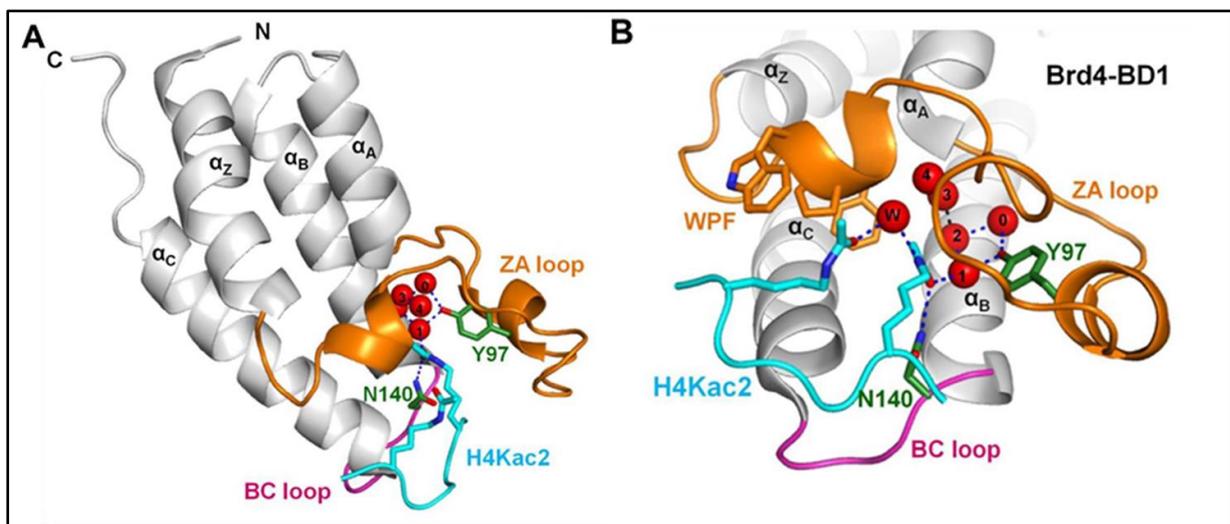
Figure 10: Structure of the human BRD4 in complex with diacetylated histone peptide (H4Kac2) A) shows the overall folding of the four α-helices (Z, A, B and C loops) that make up the BRD in grey. The ZA loop (orange) forms the binding pocket. B) Shows a top view of the BRD and the interactions between hydrogen bonds and Asn140 (N140) and Tyr97 (Y97) residues (Ferri *et al.*, 2016). This image has been reproduced with permission from Elsevier. WPF – residues Tryptophan (W) 81, Proline (P) 82 and Phenylalanine (F) 83

Epigenetic mechanisms have been implicated in a number of diseases such as inflammation, cancer and metabolic disorders (Heerboth *et al.*, 2014; Tocris, 2014). Use of

epigenetic inhibitors in other diseases also provides a basis for the use of epigenetic targets in the treatment of malaria.

1.4.3. Drug design

Techniques that been used for drug design and discovery include high-throughput screening (HTS) through phenotypic screening and target-based screening. Phenotypic screening involves the screening of large compound libraries against whole cells for a phenotypic response (Flannery *et al.*, 2013), for example, the parasite may be screened against a library of known PKIs to identify compounds that are active against the parasite. After screening, the promising compounds' scaffolds can then be identified using cheminformatics and structure-activity relationship (SAR) can be used to determine the effect of chemical modifications on the identified molecules without affecting the potency (Leroy and Doerig, 2008; Flannery *et al.*, 2013).

Target-based screening involves using 3D structures of proteins to identify active compounds by virtual screening. Target-based screening involves structure-based drug design (SBDD), which uses the knowledge of the 3D structure of a target to predict the binding of a molecule to that target (Meng *et al.*, 2011; Lionta *et al.*, 2014). The structure can be obtained from x-ray crystallography, NMR spectroscopy, if a structure is not available for the target, homology modelling may be performed using structures of proteins that are related (Anderson, 2003; Meng *et al.*, 2011). Reguera *et al.* defines *in silico* virtual screening and docking procedures as predictive approaches to design novel drugs against parasite specific targets (Reguera *et al.*, 2014). Using virtual screening helps eliminate compounds that do not bind to the active site (de Beer *et al.*, 2009). As outlined by Lionta *et al.*, structure-based virtual screening (SBVS) provides the advantage of screening thousands of compounds at a much reduced time and cost as compared to HTS. Different approaches of virtual screening can be used such as ligand-based or fragment-based depending on the experimental information that is available. There are however limitations to the use of SBVS, such as protein structures predicted from homology models may result in an increase in false-positive results and compounds that are identified by HTS are most often more bioactive than those identified by virtual screening (Lionta *et al.*, 2014).

1.4.4. Identifying new lead compounds using SBVS against selected kinases and bromodomain proteins of *P. falciparum* parasites

Virtual screening using molecular docking tools is a widely used method for finding potential lead compounds (Ferreira *et al.*, 2015). Molecular docking involves calculating the binding energy and binding pose of a 3D ligand bound to a 3D structure of a protein. The success of

the process depends on the accuracy of the protein template and ligand structure, as well as the algorithm used to find the optimal binding pose and energy. Data from X-ray crystallography experiments help solve the 3D structures of proteins (Berman *et al.*, 2013), however, there are many proteins with known sequences without any corresponding 3D structure. In cases where the 3D structure of the protein is unknown, various computational methods can be used to predict or calculate the structure of a protein without an X-ray crystal structure including template-based (such as homology) modelling and *de novo* modelling (Schmidt *et al.,* 2014). Homology modelling or template-based techniques are considered the most accurate method, as well as most widely used in drug discovery projects for proteins without X-ray crystal structures (Cavasotto and Phatak, 2009). The principle of homology modelling is based on the observation that members of a protein family tend to exhibit similar folds even with sequence similarities of as low as 20% (Chothia and Lesk, 1986; Schmidt *et al.*, 2014). X-ray structures of homologous proteins can be used as templates to generate 3D models of proteins without an X-ray structure. Suitable models of proteins can be generated for molecular docking even when the protein sequence similarity of the homologous protein is as low as 20% (Fan *et al.*, 2009) .

Homology models of the CDPK1, CDPK4 and the FIKK kinases of *P. falciparum* were generated as X-ray structures of these kinases were not available. As discussed, these kinases are small-gatekeeper kinases and attractive targets for the treatment of malaria. In order to further improve the accuracy of the homology models, ligands steering modelling, as well as molecular dynamics simulations were used to generate an ensemble of template proteins to be used in the molecular docking study. Ligand-steered modelling involves modelling a protein around a ligand placed in the binding cavity (Cavasotto *et al.*, 2008). In the case of the present study, it involves known small-gatekeeper kinase inhibitors bound to X-ray structures of the relevant kinase. For example, CDPK1 and CDPK4 proteins from *Cryptosporidium parvum* (3MWU, 2WEI), *Neospora caninum* (4MXA) and *Toxoplasma gondii* (3N51, 3SXF, 3SX9) are complexed with bumped kinase inhibitors and the sequence similarity of these proteins compared to *P. falciparum* CDPK1 and 4 is >70% (Table 1). Building the homology models with the complexed inhibitors ensures that the hydrophobic pocket behind the ATP-binding site (Figure 9) is open and available to dock potential leads into the site.

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Table 1: Structures of bumped kinase inhibitors used for homology modelling

Organism	Protein template	Ligand name	Bumped kinase inhibitor structure
Cryptosporidium parvum	3MWU	BK3	
	2WEI	VGG	
Neospora caninum	4MXA	BK7	
Toxoplasma gondii	3SXF	BK5	
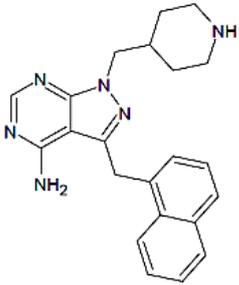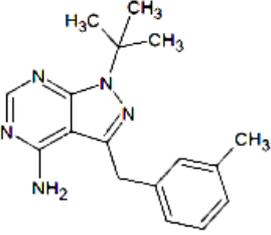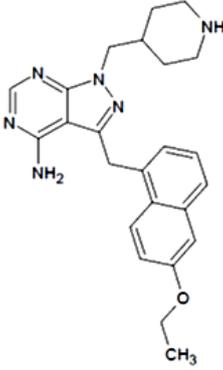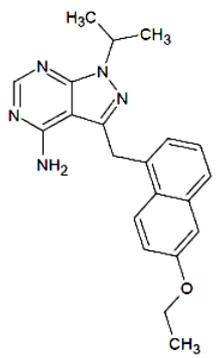
After the completion of homology modelling of the *P. falciparum* CDPK1, CDPK4 and FIKK 9.1 kinase, the bumped kinase ligands (Table 1) were placed back into the homology models

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and a 5ns molecular dynamics simulation was performed in order to relax any potential steric clashes between the ligands and the homology models. In the end, six receptors for FIKK9.1, six receptors for CDPK1, and six receptors for CDPK4 were used for the docking study and they were all respectively based on the *Cryptosporidium parvum* (3MWU, 2WEI), *Neospora caninum* (4MXA) and *Toxoplasma gondii* (3N51, 3SXF, 3SX9) CDPK proteins.

Ciceri *et al.* discovered several kinase inhibitors that are also able to inhibit human bromodomain proteins (Ciceri *et al.*, 2014). This finding, in turn, identified a useful strategy for the rational design of polypharmacological compounds targeting kinases and bromodomain proteins (Ciceri *et al.*, 2014). The CDPKs, FIKK kinases and bromodomain proteins were therefore used as representative targets for the design of small molecules that inhibit these parasite enzymes. Designing compounds that have multiple targets can be advantageous because these compounds will interact with the parasite at multiple sites and thereby have an effect on the parasite. The study of Ciceri *et al.* motivated the search for structures and functions of plasmodial bromodomain proteins in order to design polypharmacological agents capable of targeting small gatekeeper plasmodial kinases and bromodomain proteins. This is the first study that attempts to find dual small-gatekeeper kinase and bromodomain protein inhibitors for the treatment of malaria.

While little is known about the exact role of bromodomain proteins in the various stages of the life cycle of the malaria parasite, several structures of plasmodial bromodomain proteins were found on www.rcsb.org including 3FKM, 4NXJ, 4PY6 and 4QNS. These structures were used in the molecular docking study to find compounds with potential polypharmacological activity against *P. falciparum* small gatekeeper kinases and bromodomain proteins.

For this study, the compounds with antimalarial activity discovered in 2010 by Gamo *et al.* were used to identify potential lead compounds with polypharmacological activity against *P. falciparum* small gatekeeper kinases and bromodomain proteins (Gamo *et al.*, 2010). Gamo *et al.* identified thousands of lead compounds with sub-micromolar *in vitro* activity against the *P. falciparum* 3D7 parasite that also had little effect on human transformed liver HepG2 cells. For most of these lead compounds, the exact mode of action or binding site was unknown. From this list of thousands of compounds, only those with antiplasmodial activity of below 0.5 µM and no activity against HepG2 cells were selected for *in silico* screening against the above-mentioned homology models. The selected compounds were docked into homology models of *P. falciparum* calcium dependent small gatekeeping

kinases CDPK1, *Pf*CDPK4 and *Pf*FIKK9.1. Several groups of compounds were discovered with predicted binding energies <-10 kcal/mol. Compounds that shared similar docking energies to both the selected *P. falciparum* kinases and bromodomain proteins were subsequently optimised for their drug-likeness according to Lipinski and QED parameters while retaining their good docking profiles (Bickerton *et al.*, 2012). This filtering of potential binding and inhibiting compounds resulted in the selection of a group of 5 compounds for evaluation of their antiplasmodial activity *in vitro*.

1.5.   Hypothesis

In silico designed small molecule inhibitors targeting both plasmodial protein kinase and bromodomain protein have antiplasmodial activity

1.6.   Aim

To perform *in vitro* assessment of the antiplasmodial efficacy of *in silico* designed inhibitors of both protein kinase and bromodomain proteins.

1.7.   Objectives

1) To evaluate the antiplasmodial activity of potential protein kinase and bromodomain protein inhibitors against asexual forms of chloroquine sensitive *P. falciparum* 3D7 and NF54 parasites

2) To evaluate the cross-reactivity of the same inhibitors against asexual forms of drug-resistant *P. falciparum* parasite strains (K1 and W2)

3) To determine the stage-specificity and kill kinetics of the most efficacious protein kinase and bromodomain protein inhibitors against asexual parasites

4) To determine antiplasmodial selectivity by comparison to a human hepatocyte cell line

5) To determine if the most efficacious protein kinase and bromodomain protein inhibitors have gametocytocidal activity

1.8.   Outputs

This work was presented at the following conferences and faculty day:

1) Compounds targeting small gatekeeper kinases of the *Plasmodium* proteome show potential antimalarial activity. Matlebjane DMA, Birkholtz L, Stander BA & Cromarty

AD. MOMR 2015 Malaria Research Meeting, 3 – 5 August 2015, Durban, South Africa. Poster presentation

2) *In vitro* efficacy assessment of targeted antimalarial drugs synthesized following *in silico* design. Matlebjane DMA, Birkholtz L, Stander BA & Cromarty AD. Pharmacology and Toxicology congress 2015, Wits Pharmacology Conference 2015, 31 August – 02 September 2015, Johannesburg, South Africa. Poster presentation

3) *In vitro* efficacy assessment of targeted antimalarial drugs synthesized following *in silico* design. Matlebjane DMA, Birkholtz L, Stander BA & Cromarty AD. Health Sciences Faculty Day 2015, 18 – 19 August 2015, University of Pretoria, Pretoria, South Africa. Oral presentation

4) *In silico* designed small-gatekeeper protein kinase and bromodomain selective inhibitors exhibit potential antiplasmodial activity. Matlebjane DMA, Birkholtz L, Stander BA & Cromarty AD. 2nd Annual Malaria Research Conference 2016, 31 July – 02 August 2016, Pretoria, South Africa. Poster presentation

5) *In silico* designed small-gatekeeper protein kinase and bromodomain selective inhibitors exhibit potential antiplasmodial activity. Matlebjane DMA, Birkholtz L, Stander BA & Cromarty AD. Health Sciences Faculty Day 2016, 23 -24 August 2016, Pretoria, South Africa. Oral presentation

6) Compounds designed *in silico* show activity against the malaria parasite. Matlebjane DMA, Birkholtz L, Stander BA & Cromarty AD. All Africa Congress on Pharmacology and Pharmacy, 05 – 07 October 2016, Muldersdrift, South Africa. Oral presentation – received the young scientist award in basic Pharmacology (2nd prize)

Chapter 2

2. Materials and methods

All experiments were conducted in the Malaria Parasite Molecular Laboratory at the Department of Biochemistry, at the University of Pretoria, under the supervision of Prof L Birkholtz. The laboratory and Prof Birkholtz have ethical clearance for the *in vitro* cultivation of malaria parasites in human blood from the Faculty of Natural Sciences Ethics Committee (EC no 120821-077). Additionally, the MSc project received ethical clearance from the Faculty of Health Sciences Research Ethics Committee (459/2015) (see appendix).

The *in silico* designed compounds were synthesized, purified and structurally analysed to confirm structure and purity by WuxiApp Tec in Shanghai, China. Certificates of Analysis were supplied with each compound.

2.1. Materials

RPMI 1640 medium, Gentamycin, HEPES, Glucose, hypoxanthine, D-Sorbitol, Tris, Saponin, Triton X-100, Methanol and N-Acetyl Glucosamine, Iscove's Modified Dulbecco's Medium (IMDM), penicillin G, streptomycin, fungizone and crystal violet were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA). EDTA, NaCl, KCl, KH_2PO_4 and Na_2HPO_4 were purchased from Merck (Darmstadt, Germany). Albumax II was supplied by Invitrogen (Carlsbad, California, USA). Sterile cell culture flasks and plates were procured from Lasec (Johannesburg, SA). The blood bag was supplied by Adcock Ingram (Midrand, SA). Cryotubes, Cryo.S™ were purchased from Greiner Bio-one (Kremsmünster, Austria). Giemsa's azur eosin methylene blue solution and Trypsin-EDTA were purchased from Merck Millipore (Darmstadt, Germany). Boeco C-28A centrifuge from Boeco Germany (Hamburg, Germany) was used. The SYBR Green I fluorescence dye, Dulbecco's Modified Eagle's Medium, and penicillin/streptomycin were purchased from Life Technologies (Carlsbad, California, USA). The Fluoroskan Ascent FL microplate fluorometer and Foetal bovine serum were purchased from ThermoScientific (Waltham, Massachusetts, USA). The luciferase assay reagent, Promega luciferase assay system and Glomax-Multi+ detection system were purchased from Promega (Madison, Wisconsin, USA). The LDH reaction mix kit was purchased from Biovision (Mountainview, California, USA). The triple negative breast cancer cell line, MDA-MB-231, was purchased from Cellonex (Randburg, SA). The gas mixture of 5% CO_2, 5% O_2 and 90% N_2 was supplied by Afrox (Johannesburg, SA).The malaria parasite strains used were obtained from the Malaria Research and Reference Reagent Resource Centre, part of ATCC (MR4) (Virginia, USA). The five compounds (W10, W11,

W12, W13 and W14) that were used in this project were synthesized by WuXi AppTec (Shanghai, China).

2.2. Computer-aided drug design

This component of the work was completed by Dr A Stander (Department of Physiology) and is included here for the sake of clarity and completeness in the context of the rest of the dissertation.

2.2.1. Software

The following software packages were used on an Ubuntu 14.04 LTS system with an Intel Xeon E3-1230 V2 CPU and a GeForce GTX 660Ti GPU: Chimera (Webb and Sali, 2014), Chemsketch (Acdlabs.com, 2015), Open Babel 2.3.2 (OLBoyle *et al.*, 2011), Balloon (Vainio and Johnson, 2007; Puranen *et al.*, 2010), Autodock Vina (Trott and Olson, 2010), GROMACS 4.6.5 (Pronk *et al.*, 2013), the AMBER99sb force field, (Lindorff-Larsen *et al.*, 2010), the Stockholm lipids forcefield (Jämbeck and Lyubartsev, 2012), CUDA 5.0 (Nickolls *et al.*, 2008), ACPYPE (da Silva and Vranken, 2012) using *antechamber* from the AMBER 12 suite (Jämbeck and Lyubartsev, 2014) and g_mmpbsa (Kumari *et al.*, 2014). The Autodock Vina docking package was used for molecular docking. AutoDock Vina is a widely used docking program from the Molecular Graphics Lab at The Scripps Research Institute and is faster and as accurate as Autodock (Trott and Olson, 2010). It is a grid-based docking solution and is based on the X-Score scoring function (Wang *et al.*, 2002; Trott and Olson, 2010). The scoring function is derived using the PDBbind data set and the software implements an efficient Iterated Local Search global optimizer method to find the optimal docking pose. Autodock Vina is faster than Autodock and is also able to reproduce the crystal poses (root mean squared deviation (RMSD) < 2.0) in 78% of the tested protein-ligand complexes, 29% better than Autodock 4 (Trott and Olson, 2010).

2.2.2. Homology modelling of *Plasmodium* CDPK1, CDPK4 and FIKK 9.1

The sequences of the small gatekeeper kinases *Plasmodium falciparum* CDPK1 (NCBI reference sequence: XP_001349680.1), CDPK4 (Swiss-Prot: Q8IBS5.3) and FIKK9.1 kinase (NCBI reference sequence: XP_002808909.1), were obtained from www.pubmed.com. Huang *et al.* identified 9-bit (9 amino acids in the ATP-binding site) selectivity features of the ATP-binding of kinases (Huang *et al.*, 2010). The 9-bit fingerprints from small gatekeeper kinases were used to find suitable homology model templates from www.rcsb.org. *Cryptosporidium parvum* (3MWU, 3IGO, and 2WEI), *Neospora caninum* (4MXA) and *Toxoplasma gondii* (3N51, 3SXF, 3SX9) CDPK1 protein structures were used as templates

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to generate homology models of *Plasmodium falciparum* CDPK1, CDPK4 and FIKK9.1 proteins. In total, 20 individual homology models were generated using Modeller (Webb and Sali, 2014). Chimera was used for inspecting the various homology models and the most stable one was chosen for further molecular dynamics simulations.

2.2.3. Molecular dynamics

Molecular dynamics simulations were performed using GROMACS 4.6.5 (Pronk *et al.*, 2013), and the AMBER99sb force field for proteins (Lindorff-Larsen *et al.*, 2010). The non-bonded force calculations were accelerated through GPU acceleration using CUDA 5.0 (Nickolls *et al.*, 2008). Ligands from the various template proteins were preserved in the homology models. The ligand topologies were prepared with ACPYPE (da Silva and Vranken, 2012) using *antechamber* from the AMBER 12 suite (Jämbeck and Lyubartsev, 2014).

The system was prepared by heating it to 310 K (tau_t=0.2) during a 500 ps constant volume simulation with 2 fs time step using the modified Berendsen thermostat (V-rescale) using velocity rescaling (Berendsen *et al.*, 1984; Bussi *et al.*, 2007). The pressure was equilibrated to 1 atm during a 1000 ps constant pressure simulation with a 2 fs time step using the Parrinello-Rahman parameters for pressure coupling (Nosé and Klein, 1983). In both simulations, all heavy atoms were position restrained with the force constant of 1000 kJ/(mol.nm^2).

For the molecular dynamics run the temperature and pressure were maintained at 310 K and 1 atm using the Berendsen thermostat (V-rescale) and Parrinello–Rahman pressure coupling method. The short-range non-bonded interactions were computed for the atom pairs within the cut-off of 1 nm and the long-range electrostatic interactions were calculated using particle-mesh-Ewald summation method with fourth-order cubic interpolation and 0.12 nm grid spacing (Darden *et al.*, 1993). The parallel Linear Constraint Solver (LINCS) method was used to constrain bonds (Hess, 2008).

2.2.4. Docking Methodology

An ensemble docking study using Autodock Vina on the generated homology models, as well as *P. falciparum* bromodomain proteins from www.rcsb.org. The bromodomain proteins included 4QNS, 4NXJ and 3FKM.

Gamo *et al.* (Gamo *et al.*, 2010) identified thousands of compounds with *in vitro* antiplasmodial activity. However, the mode of action of many of these compounds was

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unknown. More than 1100 compounds with an antiplasmodial IC_{50} of less than 0.75 µM and no activity against the human liver cell line, HepG2, at 10 µM were chosen for the ensemble docking study. The simplified molecular input line entry system (SMILES) annotations of these compounds were converted to 3D structures using Open Babel 2.3.2 (OLBoyle *et al.*, 2011) and Balloon (Vainio and Johnson, 2007; Puranen *et al.*, 2010). Autodock Vina (Trott and Olson, 2010) was used to dock the ligands into receptors. Autodock Vina exhaustiveness was set to 15 with the other parameters set on default (Trott and Olson, 2010).

2.3. Laboratory procedures

2.3.1. Measurements

Methods used in this study were according to the Methods in Malaria Research (6th Ed.) (Moll *et al.*, 2008). The malaria parasite strains used were obtained from the Malaria Research and Reference Reagent Resource Centre (MR4) as part of the American Type Culture Collection (ATCC).

2.3.2. Collection of blood

Blood collection of any blood type from anonymous healthy volunteers was performed by qualified phlebotomists in a controlled environment at the University of Pretoria student health, Hatfield campus. This was carried out according to the National Standards for blood collection using approved standard operating procedures (SOP) for blood donation. The qualified phlebotomists drew approximately 500 ml of blood from the volunteer into a blood bag (containing citrate phosphate adenine anticoagulant) provided by the Department of Biochemistry, University of Pretoria. The blood was transported back to the Department of Biochemistry, the blood was allowed to settle overnight at 4°C, the serum and buffy coat were removed the next day and treated with cupric sulphate and discarded. The erythrocytes were washed in a sterile environment. The erythrocytes were used to culture malaria parasites (for 3 to 4 weeks) in a sterile environment for research purposes. Ethical committee clearance for the collection of blood was obtained from the Faculty of Health Sciences Research Ethics Committee (approval number 459/2015).

2.3.3. Washing of blood

After removing the buffy coat and serum, the erythrocytes were washed with phosphate buffered saline (PBS – 136.89 mM NaCl, 2.68 mM KCl, 1.76 mM KH_2PO_4, 10.14 mM Na_2HPO_4). The erythrocytes were resuspended and centrifuged at 1852x*g* for 10 minutes (min). After centrifugation, the supernatant was removed and the wash step was repeated

two more times. Lastly, the cell pellet was resuspended in an equivalent volume complete culture medium to obtain a 50% haematocrit.

2.3.4. *In vitro* cultivation of asexual malaria parasites (Trager and Jensen, 1976)

To assess the *in vitro* antiplasmodial efficacy of the compounds, several laboratory adapted strains of *P. falciparum* including 3D7 and NF54 (chloroquine sensitive), W2 (chloroquine resistant) and K1 (multidrug resistant) were used. Complete culture medium was composed of RPMI 1640 medium supplemented with 20 mM D-glucose, 200 µM hypoxanthine, 0.2% sodium bicarbonate, 25 mM HEPES, 24 µg/ml gentamycin and 0.5% Albumax II. The parasites were maintained at 37ºC in human erythrocytes in complete culture medium under hypoxic conditions (gas mixture of 5% CO_2, 5% O_2 and 90% N_2).

2.3.5. Thawing of parasite cultures

P. falciparum parasites were thawed from liquid nitrogen cryopreservation (-180 ºC) at 37ºC. Sodium chloride (NaCl) solution was used to thaw parasites (parasites were preserved when at the ring stage – during this stage the erythrocyte membrane is more rigid than when the parasite is in the mature stages (Lambros and Vanderberg, 1979)), starting a high to low concentration (Moll *et al.*, 2008). This prevents water from rapidly entering the cells and also prevents lysis of the erythrocytes. Sterile 12% (w/v) NaCl (0.2 ml) was added drop wise to the parasite culture and transferred to a 50 ml tube. The solution was incubated for 5 min at room temperature after incubation 10 ml of 1.6% (w/v) NaCl was added to the culture thereafter 10 ml 0.9% of NaCl was added followed by centrifugation at 490x*g* for 5 min at room temperature. The supernatant was removed and the pellet resuspended in 10 ml of complete medium and centrifuged at 490x*g* for 5 min. After centrifugation, the supernatant was removed and 6 ml of complete medium was added and the culture placed in a 25 ml flask and incubated at 37ºC for 24 h. The next day the spent medium was removed and 1 ml of 50% washed blood and 9 ml of complete medium was added to reach a culture of 2.5% haematocrit, hypoxic conditions were induced by gassing the flask with the gas mixture of 5% CO_2, 5% O_2 and 90% N_2 for 30 seconds (s) and the culture subsequently maintained in a shaking incubator at 37ºC.

2.3.6. Microscopic evaluation of parasite viability and proliferation

A thin smear of parasite culture was made, fixed onto the microscope slide with methanol for 1 min and stained with Giemsa's azur eosin methylene blue solution (1:19 dilution of Giemsa: distilled water) for 4 min. After staining, the cells were viewed using phase contrast

light microscopy at 100x magnification with oil immersion. Parasitaemia was determined by counting 10 fields containing at least 100 erythrocytes; parasitaemia was calculated using the equation below:

$$\text{Parasitaemia} = \frac{\text{Total number of infected erythrocytes}}{\text{Total number of erythrocytes}} \times 100 \qquad \text{Eq. 1}$$

2.3.7. Sorbitol synchronisation (Lambros and Vanderberg, 1979)

D-Sorbitol was used to synchronise the cultures to obtain parasites compartmentalised to particular life cycle compartments. Ring stage intra-erythrocytic *P. falciparum* parasite cultures (10-12 h post invasion) were collected by centrifugation at 945x*g* to which 4 ml of 5% (v/v) sorbitol was added to the pellet and incubated for 15 min at 37°C. The culture was again centrifuged at 945x*g* and washed 3 times with complete culture medium and finally diluted to 5% haematocrit before continuing culture incubation.

2.3.8. *In vitro* cultivation of sexual gametocyte parasites (Fivelman *et al.*, 2007; Adjalley *et al.*, 2011; Reader *et al.*, 2015)

The *P. falciparum* NF54 strains (NF54-Pfs16-GFP-Luc and NF54-Mal8p1.16-GFP-Luc), a kind donation of D Fidock, Columbia University, USA, (Adjalley *et al.*, 2011) were used to induce gametocytogenesis as per Reader *et al.* (Reader *et al.*, 2015). Culturing, thawing and maintenance of the asexual forms of these parasites were as described above in Section 2.3.4. Asexual parasite cultures with a parasitaemia of 5 – 10% were synchronised twice on Day -7 and Day -5 (as described in Section 2.3.7) before induction of gametocytogenesis.

Gametocytogenesis was first initiated from a culture of intra-erythrocytic *P. falciparum* parasites on Day -3 with a haematocrit of 6% and a parasitaemia of 0.5%. The parasite cultures were transferred to glucose-free medium (medium prepared as described in Section 2.3.4, however without glucose) and maintained under hypoxic conditions (5% CO_2, 5% O_2 and 90% N_2) at 37°C, without shaking. Gametocytogenesis was induced by a stressed combination of nutrient starvation and a drop in haematocrit. On Day -2 the parasites were stressed by having no medium change, this ensured that gametocyte production increased. On Day 0, gametocytes were induced by dropping the haematocrit to 3%.

The *P. falciparum* NF54 parasite cultures for gametocyte production required daily maintenance. During daily medium changes a slide warmer that was set to 37 °C was used in the laminar flow hood to prevent excessive cooling of the *P. falciparum* gametocyte parasite cultures. *P. falciparum* gametocyte parasite cultures were centrifuged at 2419x*g* for 1 min at room temperature and returned to the slide warmer and the supernatant was

aspirated. *P. falciparum* gametocyte parasite cultures were resuspended in preheated (37°C) glucose-free medium. The cultures were maintained in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 37°C, without shaking. From Day 2 onwards the *P. falciparum* gametocyte parasite culture media contained 50 mM N-acetyl glucosamine (NAG) to remove the intra-erythrocytic stage parasites and gametocytes were monitored daily. Gametocytes developed over a period of 10 to 12 days, with the early (I/II/III) and late (IV/V) stage gametocytes being present from Day 1 to Day 6 and Day 7 to Day 12 post gametocyte induction, respectively.

2.3.9. *In vitro* cultivation of HepG2 cells

Human hepatocellular carcinoma cells (HepG2) were used to test the toxicity of the compounds to human cells and confirm selectivity towards malaria parasites. Stored HepG2 cells were removed from liquid nitrogen cryopreservation. The cells were thawed by swirling the vials in a 37ºC water bath. Preheated (at 37ºC), 5 ml of filter sterilised DMEM complete medium (10% heat-inactivated foetal bovine serum (FBS), 1% penicillin/ streptomycin (propagation medium) and Dulbecco's Modified Eagle's Medium (DMEM)) was poured into a 20 cm^2 flask. The cells were slowly added to the medium in the flask and incubated in a 5% CO_2 incubator, at 37ºC. The medium was changed the next day after the cells had attached to the flask to remove any dead cells. The cells were incubated until a confluence of 80% was reached.

HepG2 cells were maintained in DMEM complete medium. The medium was changed every 2 – 3 days. To passage the cells, the medium was aspirated and the cell monolayer rinsed three times with 1xPBS. Pre-warmed (at 37ºC) 0.05% (1.5 ml) trypsin-EDTA was evenly spread on the monolayer and incubated at 37ºC for 5 – 7 min. Released cells were harvested by centrifugation at 500x*g* and the supernatant removed. Trypsin was deactivated by resuspending the cells in 3 ml of DMEM complete medium, and the cells were again collected by centrifugation at 500x*g* for 1 min, the supernatant discarded and 5 ml of DMEM complete medium was added for subsequent maintenance. To count cells, the cells were stained with 0.2% (w/v) Trypan Blue (0.2 g in 100 ml 1xPBS). Cells (20 µl) were mixed with 20 µl of the dye and 15 µl introduced into the haemocytometer cell counter. The cells were viewed using microscopy at 40x magnification and 25 blocks were counted on the haemocytometer. The number of cells was calculated as follows:

$$\text{Total cells/ml = total cells counted} \times \frac{\text{Dilution factor}}{\text{Number of squares}} \times (1 \times 10^4) \text{ cells/ml} \qquad \text{Eq. 2}$$

Cells were seeded at a density of 1×10^4 cells/well and contained complete DMEM medium. The plates were incubated at 37°C for 24 h. In the plate, a background control of medium only was included. The test compounds were added to the seeded cells and serially diluted. The treated cells were incubated with the compound for 48 h at 37°C in a CO_2 incubator.

2.3.10. LDH-Cytotoxicity Colorimetric assay (Weyermann *et al.*, 2005; Nogueira and Rosário, 2010; Smith *et al.*, 2011)

The Biovision LDH cytotoxicity colorimetric assay kit was used. At the conclusion of the 48 h incubation period, 10 µl of cell lysis solution was added to three wells for the high control. The cells were incubated for 15 min at room temperature. After incubation, the plate was shaken for 15 min; this step ensures that the LDH is evenly distributed in the culture medium. The cells were then centrifuged for 10 min at 600x*g*. The clear medium solution (10 µl) was transferred to a clear 96-well plate. One hundred microliters of LDH reaction mix (200 µl of WST Substrate mixed with 10 ml of LDH Assay Buffer) was added to the clear medium solution, mixed and incubated for 20 minutes at room temperature. The absorbance was read at a wavelength of 450 nm using a microtiter plate reader with a reference at 650 nm. Readings were taken until the absorbance values started decreasing. The percentage cytotoxicity was calculated using Excel.

2.3.11. *In vitro* cultivation of MDA-MB-231 cells

The effect of the test compounds was also investigated on tumorigenic, metastatic MDA-MB-231 breast cancer cells. MDA-MB-231 breast cancer cells were cultured in IMDM supplemented with 10% heat-inactivated FBS (56°C, 30 min), 100 µg/ml penicillin G and streptomycin and 250 µg/l of fungizone. The final concentration of DMSO did not exceed 0.05% in cell culture. Experiments were conducted in sterile, flat-bottomed 96-well plates. Exponentially growing cells were seeded at 5 000 cells per well. The cells were incubated for 24 h at 37°C to allow for cell adherence. Following the 24 h incubation period, the cells were exposed to the compounds and incubated for a further 48 h at 37°C.

Crystal violet as a DNA stain was used to quantify fixed monolayer cells via spectrophotometry (Gillies *et al.*, 1986; Kueng *et al.*, 1989). Dose-response curves were determined in order to determine the concentration required to cause 50% inhibition of cell proliferation (IC_{50}) of the compounds on MDA-MB-231 cells. The IC_{50} was determined using GraphPad Prism v5.0 using the non-linear regression curve fit with a variable slope.

2.3.12. Antiplasmodial activity against asexual parasites

For the asexual antiplasmodial drug assays, a fluorescence based assay using SYBR Green I dye was used, which binds to double-stranded *Plasmodium* DNA in any erythrocytic stage of *P. falciparum* growth.

All compounds were firstly evaluated on a dual point basis as a primary filter for antiplasmodial activity on the drug sensitive *P. falciparum* 3D7 strain. Each of the compounds were initially tested at 1 and 5 µM in triplicate wells on a 96 well plate using a parasite suspension with 1% haematocrit with 1% parasitaemia. Chloroquine (0.5 µM) was used as positive drug control in addition to a culture medium negative control. The parasites were incubated with drug for 96 h at 37°C under hypoxic conditions as described in Section 2.3.4 (maintained every 24 h by gassing the gas chambers). Subsequently, equal volumes (100 µl each) of the *P. falciparum* parasite cultures was added to SYBR Green I lysis buffer (0.2 µl/ml 10 000x SYBR Green I; 20 mM Tris, pH 7.5; 5 mM EDTA; 0.008% (w/v) saponin; 0.08% (v/v) Triton X-100). The sample was incubated at 37°C for 1 h at room temperature after which fluorescence was measured with a Fluoroskan Ascent FL microplate fluorometer (excitation at 490 nm and emission at 530 nm). The data were expressed as a percentage of untreated control (after subtraction of the background control – Chloroquine treated infected erythrocytes) to determine parasite proliferation. All experiments were performed in technical triplicates and for at least 3 biological replicates.

The compounds that demonstrated effective parasite toxicity during the screening assay were then tested to determine the concentration at which the compounds inhibited parasite proliferation by 50% (IC_{50}) further characterisation using parasites with different antimalarial drug susceptibilities was also performed. Different drug sensitive and resistant *P. falciparum* parasite strains were treated with the compounds in a series of full dose-response assays. Ring stage intra-erythrocytic *P. falciparum* parasite cultures (1% parasitaemia, 1% haematocrit) were exposed to 2-fold serial dilutions of compounds (starting concentration between 11 and 12 µM). Data obtained was normalised and log transformed using GraphPad Prism v5.0. The sigmoidal dose-response curves were plotted from which the IC_{50} was inferred and presented as the mean of three independent biological replicates (each performed in technical triplicates), with a standard error of the mean indicated.

2.3.12.1. Kill kinetics and stage-specificity against asexual parasites

The purpose of the IC_{50} speed assay (kill kinetic evaluation) was to determine if the compounds were fast acting or slowing acting. The activity of the compounds was

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determined with full dose-response evaluation as above but now for three incubation times: 24, 48 and 72 h as per Le Manach *et al.* (Le Manach *et al.*, 2013).

In addition, the stage specificity of each test compound was assessed to determine whether a specific asexual parasite stage is more susceptible to the compounds. Treatment was initiated at different stages of the intra-erythrocytic *P. falciparum* parasite cultures with the test compounds being added at the ring, trophozoite and schizont stages, where each population had been synchronised to approximately 90%. To obtain such tight synchronisation, staggered sorbitol synchronisation was performed with a 6 h window between two consecutive rounds of synchronisation.

The concentration-dependent growth of the ring, trophozoite and schizont forms (4% parasitaemia, 5% haematocrit) was measured under compound exposure ranging from 100x IC_{50} to 1.6x IC_{50}. Figure 11, below is a schematic representation of how the stage specificity assay was conducted. The ring, trophozoite and schizont stages were treated with the compounds at 7 h, 30 h and 42 h after the first sorbitol treatment, respectively (Figure 11).

For both these assays, the fluorescence based SYBR Green 1 assay was performed and analysed as described above in Section 2.3.12. In all cases, data were from three independent biological replicates, each performed in technical triplicates and analysed with either GraphPad Prism v5.0 or Microsoft Excel.
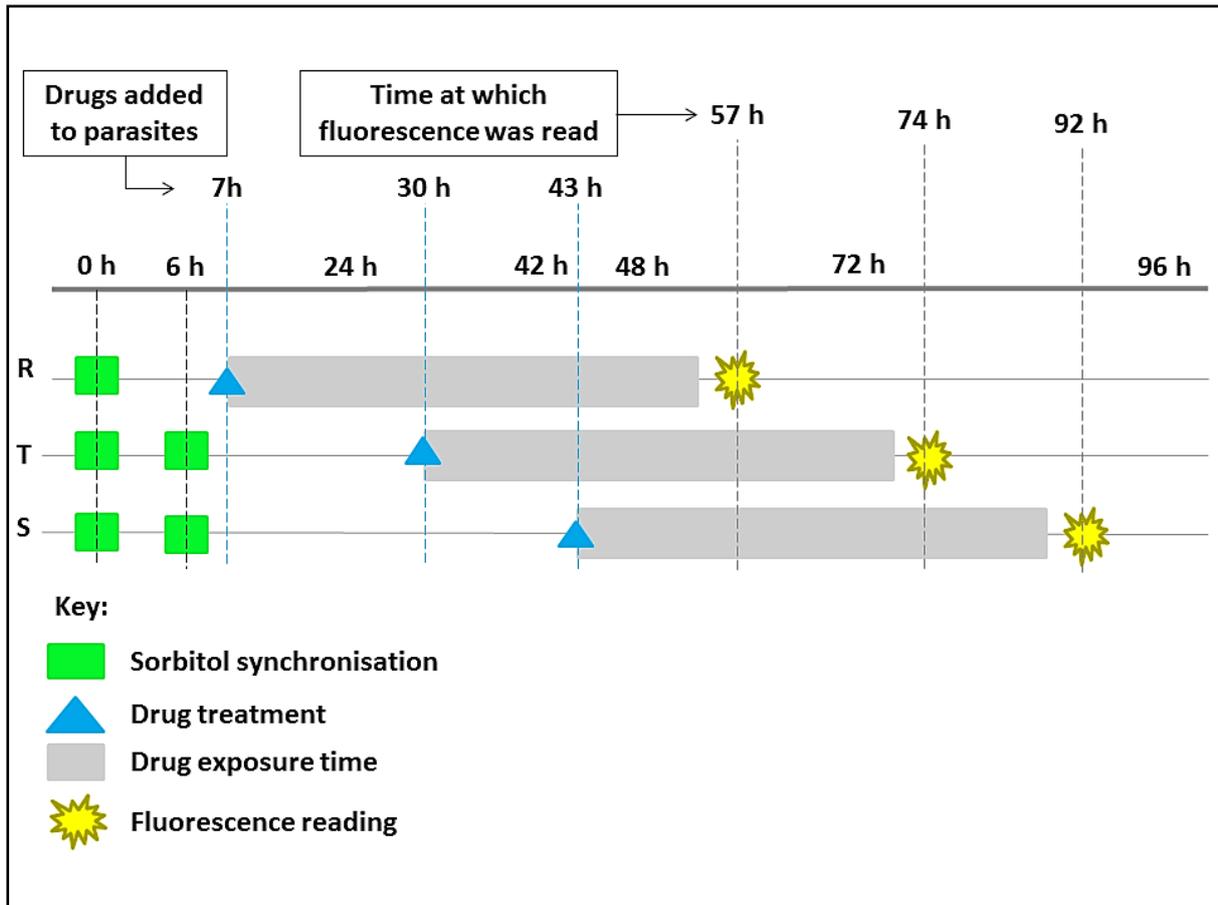
Figure 11: Schematic representation of the stage specificity assay. The assay was performed on synchronised 3D7 parasites on the asexual stages of the parasite. R- Ring, T- Trophozoite, S- Schizont. This diagram was adapted from Le Manach *et al.* (Le Manach *et al.*, 2013) with permission from BioMed Central (Publisher)

2.3.13. Antiplasmodial drug assays for gametocytes (Reader *et al.*, 2015; Verlinden *et al.*, 2015)

A luciferase-based reporter gene assay was performed to test the activity of compounds against the gametocyte stages of the parasite, as described in Reader *et al.* (Reader *et al.*, 2015). Luciferase was produced under either an early stage gametocyte promotor (pfs16-GFP-Luc) in the NF54$_{pfs16}$ cell line or under a late stage gametocyte promotor (mal8p1.16) in the NF54$_{mal8p1.16}$ cell line, enabling the production of bioluminescence.

Gametocytes were produced as described above in Section 2.3.8 and used in the assay either as early stage gametocytes (Day 5; stages – I/II/III) or late stage gametocytes (Day 10, stages – IV, V). Compounds were evaluated again in a primary screening at 1 and 5 µM in triplicate on gametocyte parasite cultures (2% gametocytaemia, 2% haematocrit). Gametocytes were incubated with test compounds for 48 h at 37ºC under hypoxic conditions, without agitation. To determine luciferase activity, 20 µl of the lysis buffer (125

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mM Tris-HCl (pH 7.8), 10 mM DTT, 50% (v/v) Glycerol, 5% (v/v) Triton X-100) were added to 50 µl of the luciferase assay reagent (Promega Luciferase assay system) then transferred to a white 96-well plate, at room temperature. The resultant bioluminescence was detected at an integration constant 10 s with Glomax-Multi+ Detection System with Instinct Software.

Data was analysed using Excel. In each plate, three controls were included (Methylene Blue control, 100% viable parasites control (c+), and a background control with medium only (c-)). The mean (µ) and standard deviation (σ) of the controls were determined. The µ and σ were used to calculate the following to determine assay quality:

1. Signal (S) to background (B)

$$\frac{S}{B} = \frac{\mu_{c+}}{\mu_{c-}} \qquad \textbf{Eq. 3}$$

2. Signal (S) to noise (N)

$$\frac{S}{N} = \frac{(\mu_{c+} - \mu_{c-})}{\sigma_{c-}} \qquad \textbf{Eq. 4}$$

3. Z' factor

$$\textbf{Z' factor = 1 -} \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} - \mu_{c-}|} \qquad \textbf{Eq. 5}$$

The percentage inhibition was calculated by adjusting the mean of the drug treated parasites and adjusting the mean of the 100% viable parasite control. Both means were subtracted from the mean of the background control. The percentage of viable parasites was then calculated as follows:

$$\textbf{Percentage viability =} \frac{\textbf{Adjusted drug treatment mean}}{\textbf{Adjusted 100\% viable parasites mean}} \times \textbf{100} \qquad \textbf{Eq. 6}$$

2.4. Bromodomain protein sequence similarity

Plasmodial and human bromodomain proteins were found on the protein databank www.rcsb.org. The *P. falciparum* bromodomain protein 4NXJ and 3FKM sequences were compared to human bromodomain proteins KAT2A (GCN5), KAT2B (PCAF) and BPTF, with PDB ID 3D7C, 5LVQ and 3UV2, respectively. The protein sequences were aligned and sequence alignment was performed using MUSCLE (Edgar, 2004). Since these proteins have ligands in the binding site, the protein amino acids that interact with the binding site could then be identified. The amino acids that were found to protrude into the binding site were examined using Chimera 1.11.2 (Webb and Sali, 2014). Protein sequence similarity of the binding site amino acids was calculated.

Chapter 3

3.   Results

3.1.   Computer-aided drug design

Work in this section (Section 3.1) was performed by Dr A Stander, Department of Physiology.

3.1.1.   Ensemble docking

Ensemble docking is a method used to model protein flexibility by docking flexible ligands into the rigid structures of multiple protein receptors of the same protein (Totrov and Abagyan, 2008).

A total of 1098 ligands from Gamo *et al.* that satisfied the criteria mentioned in Section 1.4.4 were selected and 3D models of the ligands were generated and docked into homology model receptors (Gamo *et al.*, 2010). Docking the bumped kinase inhibitors (Table 1) back into their respective receptors using Autodock Vina revealed that the BK5 ligand had the lowest binding energy (-10.8 kcal/mol) (Table 2). The RMSD for all the docked bumped kinase inhibitors was <1.5 (Table 2), indicating that the docking software can reproduce the correct binding pose of the ligands into the 3D structure of the proteins. In order to increase the stringency of selecting new potential CDPK1, CDPK4 and FIKK9.1 kinase inhibitors only compounds with binding energies of <-11.5 kcal/mol were considered potential lead compounds.

Table 2: Binding energies and RMSD of docked bumped kinase inhibitors

Receptor	Ligand	Binding energy	RMSD
3MWU	BK3	-10.5	1.074
2WEI	VGG	-9.6	0.215
4MXA	BK7	-9.7	0.981
3N51	BK3	-10.6	0.703
3SXF	BK5	-10.8	0.309
3SX9	BK7	-10.7	1.001

Gamo *et al.* separated the lead compounds into 857 clusters based on the chemical similarity index (Gamo *et al.*, 2010). After docking the selected compounds into the homology model receptors, GeneVenn (Pirooznia *et al.*, 2007) was used to identify compounds from clusters which had a binding energy of <-11.0 kcal/mol. Eleven clusters of compounds were found to have members with good binding energies against all six of the tested homology models (Table 3). Clusters 4, 21, 46, 51, 72, 80, 96 and 125 all contain the benzimidazole scaffold which is known to be an attractive scaffold for designing kinase

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inhibitors (Singla *et al.*, 2014) (Table 3). Clusters 38 and 55 contain a quinolone scaffold (Table 3). Quinolone containing antimalarials are known to kill malaria parasites by interfering with haem detoxification (Foley and Tilley, 1997). Quinolone is also a known scaffold in many kinase inhibitors (Zhang *et al.*, 2002). It was decided to focus on the benzimidazole scaffold since it appeared to be the most enriched scaffold after the initial docking study.

Table 3: Clusters of compounds with a member that has a binding energy of <-11.5 kcal/mol against CDPK1, CDPK4 and FIKK9.1 homology models.

Compound cluster number	Representative motif of cluster	Possible substituents for X	Possible substituents for Y	Possible substituents for Z
4		H, CH3	H, F, CF3	Misc
21		-	-	Misc
38		Misc	Misc	-
46		F, CF3	Misc	-
51		CH3	H, F, CF3	Misc
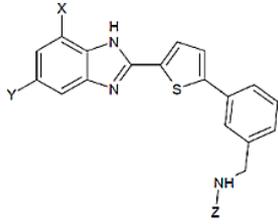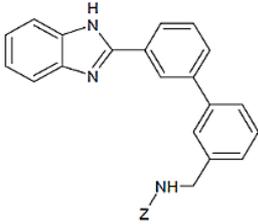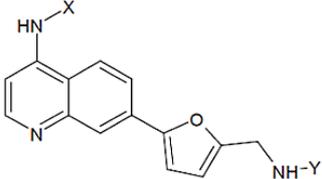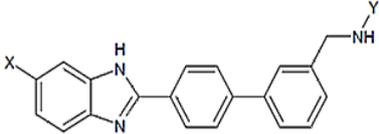
37

No.	Structure	X	Y	Z
55	[structure]	Misc	Misc	-
72	[structure]	H, CH3	H, F, CF3	Misc
80	[structure]	H, CH3	H, F, CF3	Misc
96	[structure]	CH3	F, CF3	Misc
125	[structure]	H, CH3	H, F, CF3	[phenyl, thiophene]
309	[structure]	Misc	-	-

3.1.2. Homology models

X-ray structures of the CDPK1, CDPK4 and the FIKK kinases of *P. falciparum* were not available at the time of this part of the study, therefore homology models of these kinases

were generated. The sequences of CDPK1 (NCBI reference sequence: XP_001349680.1) and CDPK4 (Swiss-Prot: Q8IBS5.3) were obtained from www.pubmed.com. A protein sequence blast search at www.rcsb.org protein databank using *P. falciparum* CDPK1 and CDPK4 protein sequences identified the CDPK1 and CDPK4 proteins from *Cryptosporidium parvum* (3MWU, 2WEI), *Neospora caninum* (4MXA) and *Toxoplasma gondii* (3N51, 3SXF, 3SX9) as the closest proteins in terms of sequence similarity. These proteins were used to generate homology models of the *P. falciparum* CDPK1 and CDPK4 proteins. The reason for using these specific protein templates is that they are complexed with bumped kinase inhibitors that were designed to bind to the hydrophobic pocket of the ATP-binding site near the small gatekeeper amino acid (Murphy *et al.*, 2010; Larson *et al.*, 2012; Ojo *et al.*, 2014). Therefore, the receptors are already in the configuration that can bind to these small-gatekeeper ATP inhibitors and thus useful for identifying other small gatekeeper inhibitors.

Homology modelling of FIKK kinases presented a challenge since FIKK kinases are unique in the sense that to date they have only been found in Apicomplexa (Ward *et al.*, 2004). The name for this group of kinases is derived from an amino acid motif ("FIKK", Figure 12) corresponding to subdomain II of the protein kinase. Hanks and Hunter identified 12 conserved subdomains of kinases, therefore it is important to identify these subdomains in the kinase that is being modelled and then align it properly with the template being used (Hanks and Hunter, 1995). For this study, CDPK proteins of *Toxoplasma gondii, Cryptosporidium parvum*, *Neospora caninum* were used as homology model templates to make the FIKK kinase homology models since all the subdomains of the FIKK kinase align with the conserved amino acids of the CDPK proteins (Figure 12). The 12 subdomains for the FIKK9.1 kinase were identified and they were in turn used to search the www.rcsb.org protein databank for suitable templates. Subdomain I of FIKK is unusual since it does not contain the full GxGxxG motif and instead the best-aligned sequence is DxGxxD. As mentioned, subdomain II is unique to FIKK kinases and this domain also did not yield any suitable templates from the database. Combining subdomains III to V did not yield any suitable candidates (non-kinases were better hits than kinases). When combining subdomains VIa and VIb the best hits from CDPK proteins of *Toxoplasma gondii, Cryptosporidium parvum*, *Neospora caninum* were identified. The CDPK1 and CDPK4 protein templates were therefore used to make the FIKK kinase homology models. All the subdomains of the FIKK kinase aligned well with the conserved amino acids of the CDPK proteins (Figure 12).

```
Subdomain              ---------SD_I-------      --SD_II---
CDPK1_p_falciparum     RKKEGKIGESYFKVRKLGSGAYGEVLLCREKHGHGEKAIKVIK-K
CDPK4_c_parvum         Q---GTFAERYNIVCMLGKGSFGEVLKCKDRITQQEYAVKVIN-K
CDPK4_p_falciparum     QNSNVVFNEQYKGIKILGKGSFGEVILSRDKHTGHEYAIKVIS-K
CDPK1_n_caninum        QHSTAIFSDRYKGQRVLGKGSFGEVILCKDKITGQECAVKVIS-K
CDPK1_t_gondii         QHSTAIFSDRYKGQRVLGKGSFGEVILCKDKITGQECAVKVIS-K
FIKK9.1_p_falciparum   ----------NWKLSPIDFGDMDDISKKKKKNNNTKVFIKKINIK
                                 ::  *   ::       :        :* *   *|

Subdomain                               ---SD_III---
CDPK1_p_falciparum     SQFDKMKYSITNKIECDDKI--HEEIYNEISLLKSLD--HPNII-
CDPK4_c_parvum         ASAK-----NK-D---------TSTILREVELLKKLD--HPNIM-
CDPK4_p_falciparum     KHVK-----RKTD---------KESLLREVELLKMLD--HINIM-
CDPK1_n_caninum        RQVK-----QKTD---------KESLLREVQLLKQLD--HPNUM-
CDPK1_t_gondii         RQVK-----QKTD---------KESLLREVQLLKQLD--HPNIM-
FIKK9.1_p_falciparum   DWLKLFNCMEKYDGEYMYTKDNKDNFVMEAVALSFLEEYHKGITP
                              :         :          :  *: :* *:   *   *

Subdomain              -SD_IV- --SD_V#-                        --
CDPK1_p_falciparum     KLFDVFEDKKYFYLVTEFYEGGELFEQIINRHK--------FDEC
CDPK4_c_parvum         KLFEILEDSSSFYIVGELYTGGELFDEIIKRKR--------FSEH
CDPK4_p_falciparum     KLYEFFEDNNYYYLVSDVYTGGELFDEIISRKR--------FYEI
CDPK1_n_caninum        KLYEFFEDKGYYYLVGEVYTGGELFDEIISRKR--------FSEV
CDPK1_t_gondii         KLYEFFEDKGYFYLVGEVYTGGELFDEIISRKR--------FSEV
FIKK9.1_p_falciparum   KLHKILFEPDNIVIVSELY-GDDIKN---YKKKMRKVYPKVFNKK
                       **   ::  :     :::* ::*  *  ::       :::        :

Subdomain              ----SD_VIa-------       --SD_VIb---    |   -SD
CDPK1_p_falciparum     DAANIMKQILSGICYLHKHNIVHRDIKPENILLENKHS-LLNIKI
CDPK4_c_parvum         DAARIIKQVFSGITYMHKHNIVHRDLKPENILLESKEK-DCDIKI
CDPK4_p_falciparum     DAARIIKQILSGITYMHKNNVVHRDLKPENILLETKNKEDMIIKI
CDPK1_n_caninum        DAARIIRQVLSGITYMHKNKIVHRDLKPENLLLESKSK-DANIRI
CDPK1_t_gondii         DAARIIRQVLSGITYMHKNKIVHRDLKPENLLLESKSK-DANIRI
FIKK9.1_p_falciparum   SKKKILFECLKLIDKLHDTGLSHLDFTPENILI---SD-NFEFRI
                          :*:   :   *  :*   : *:*: ***:*:      ::*

Subdomain              _VII-                           -SD_VIII-
CDPK1_p_falciparum     VDFGLSSFFSKDNKLRDR-------------LGTAYYIAPEVLR
CDPK4_c_parvum         IDFGLSTCFQQNTKMKDR-------------IGTAYYIAPEVLR
CDPK4_p_falciparum     IDFGLSTHFEYSKKMKDK-------------IGTAYYIAPDVLH
CDPK1_n_caninum        IDFGLSTHFEASKKMKDK-------------IGTAYYIAPEVLH
CDPK1_t_gondii         IDFGLSTHFEASKKMKDK-------------IGTAYYIAPEVLH
FIKK9.1_p_falciparum   CDFGKSTPLYTNKIRHIKKVNTMCSFESCSPYVGKVPFIPPECL-
                       :***:*: :        : :          :*  :  *  **:*

Subdomain              --------SD_IX-----------     -----SD-X---
CDPK1_p_falciparum     KKYN-EKCDVWSCGVILYILLCGYPPFGGQND-QDII-KKVEKGK
CDPK4_c_parvum         GTYD-EKCDVWSAGVILYILLSGTPPFYGKNE-YDIL-KRVETGK
CDPK4_p_falciparum     GTYD-EKCDIWSCGVILYILLSGCPPFNGSNE-YDIL-KKVEAGK
CDPK1_n_caninum        GTYD-EKCDVWSTGVILYILLSGCPPFNGANE-YDIL-KKVEKGK
CDPK1_t_gondii         GTYD-EKCDVWSTGVILYILLSGCPPFNGANE-YDIL-KKVEKGK
FIKK9.1_p_falciparum   KLYDVTSADKYMLGILFIWIWNDHHLWECSDPLTDKNYMKFEKNN
                        : *:   :* :  *:::::::         *:    ::*:

Subdomain              --        ------SD_XI-----------
CDPK1_p_falciparum     YYFDFNDWKNIS---EEAKELIKLMLTYDYNKRITAKEALNSKWIK
CDPK4_c_parvum         YAFDLPQWRTIS---DDAKDLIRKMLTFHPSLRITATQCLEHPWIQ
CDPK4_p_falciparum     YTFDLPQFKKIS---DKAKDLIKKMLMYTSAVRISARDALEHEWIK
CDPK1_n_caninum        YTFELPQWKKVS---ESAKDLIRKMLTYVPSMRISARDALDHEWIQ
CDPK1_t_gondii         YTFELPQWKKVS---ESAKDLIRKMLTYVPSMRISARDALDHEWIQ
FIKK9.1_p_falciparum   --MSLDTFRVTKSWPEDIKIMIKNLIDIKYRKNIKLKNLIKHPWWF
                        : :            : :* :*: ::      * ::
```

Figure 12: Amino acid sequence alignment of CDPK and FIKK kinases used to generate homology models. Light grey block indicates the unique subdomain II (SD_II) FIKK sequence found only in the FIKK kinases. The black box and "#" indicate the small gatekeeper amino acids. Sequences were aligned using MUSCLE (Edgar, 2004). * indicates conserved amino acid and **:** indicates amino acids with similar biochemical properties e.g. hydrophobic or hydrophilic or charged.

Homology models were generated using Modeller (Webb and Sali, 2014). Chimera was used to inspect the various homology models and to identify any steric clashes when

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hydrogen atoms were added into the model. Furthermore, the receptors were visually inspected to confirm if key interactions were present in the homology model. For example, the conserved lysine residue in subdomain II is capable of interacting with the aspartic acid of the DFG motif in subdomain VII. Other important criteria were that when the ligand was placed into the receptor, no steric clashes should be present. This was especially important for the FIKK kinases since the Phe (F) residue protruded into the ATP-binding site and can cause a steric clash with the kinase inhibitors. The presence of the F residue in the ATP-binding site is another interesting aspect of FIKK kinases that is not present in other kinases and can be exploited for drug design purposes (e.g. generate ligands that can form a *pi*-bond interacting group with the F residue). Ligands that satisfied the above criteria were chosen for further molecular dynamics simulations. Homology models of FIKK9.1, CDPK1 and CDPK4 generated based on the *Cryptosporidium parvum* (3MWU, 2WEI), *Neospora caninum* (4MXA) and *Toxoplasma gondii* (3N51, 3SXF, 3SX9) CDPK proteins respectively are represented in Figure 13.
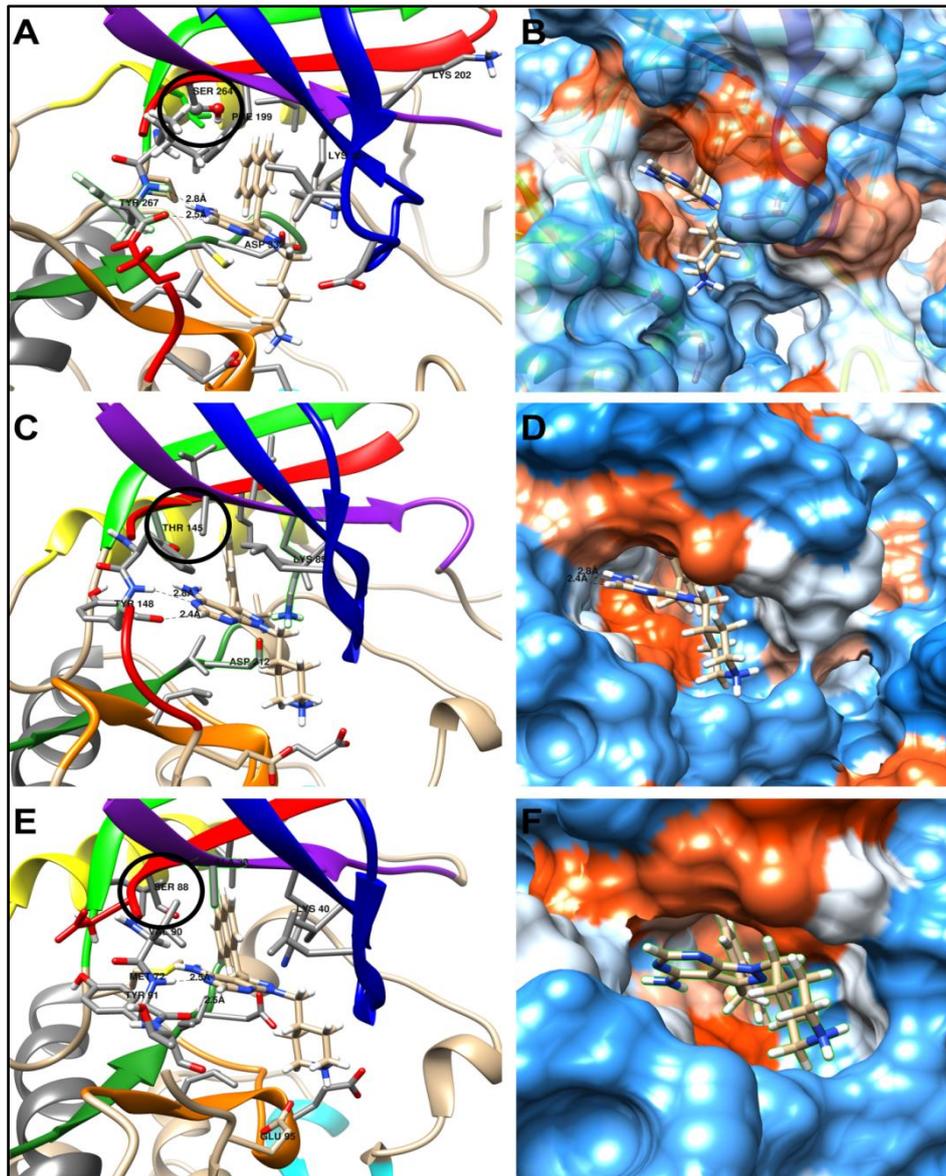
Figure 13: Homology models of *P. falciparum* FIKK9.1 (A and B), CDPK1 (C and D) and CDPK4 (E and F) proteins with ligand BK3 from 3N51 superimposed into the binding site with the hydrophobic surface (B, D and F). The black circle indicates the gatekeeper amino acid residue of FIKK9.1 (A, SER26) CDPK1 (C, THE145) and CDPK4 (E, SER88). Subdomains are coloured as follows: Subdomain I (SD I) = dark blue, SD II = purple, SD II = yellow, SDIV = light green, SD V = red, SD VIa = dark grey, SD VIb = orange, SD VII = dark green, SD VIII = cyan.

Several structures of plasmodial BRD proteins were found on www.rcsb.org including 3FKM, 4NXJ, 4PY6 and 4QNS. These structures were used to dock the compounds from clusters 4, 21, 46, 51, 72, 80, 96 and 125 (Table 3). After the completion of the second round of docking using plasmodial BRD proteins, three out the top five best binding energies contained ligands with the methyl benzimidazole scaffold together with the biphenyl formamide scaffold (Table 4).

Table 4: Structures and properties of the ligands that performed well when docked against CDPK1, CDPK4, and FIKK9.1 kinase and bomodomain proteins. The methyl benzimidazole scaffold together with the biphenyl formamide scaffold is seen in three out of five of the compounds.

Compound number from Gamo *et al.* 2010	SMILES	Cluster	XC$_{50}$ against 3D7 (µM)	Molecular weight	QED score	Autodock Vina binding energy (kcal/mol)				Structure
						CDPK1	CDPK4	FIKK9.1	BRD	
TCMDC-133179	Cc1cccc2nc([nH]c12)-c1cccc(c1)-c1cccc(NC(=O)Nc2ccc(Cl)cc2)c1	50	0.2356	452.934	0.366	-12.5	-11.4	-11.2	-12.0 (4NXJ)	
TCMDC-136936	Cc1cccc2nc([nH]c12)-c1cccc(c1)-c1cccc(NC(=O)c2c[nH]cn2)c1	80	0.2583	393.441	0.453	-12.1	-12.2	-11.0	-11.4 (4NXJ)	
TCMDC-132907	CS(=O)(=O)c1ccc(CNCc2cccc(c2)-c2cccc(c2)-c2nc3cc(F)ccc3[nH]2)cc1	72	0.5587	485.572	0.407	-11.8	-11.3	-12.9	-11.4 (4NXJ)	
TCMDC-133053	Cc1noc(C)c1-c1cccc(CNCc2cccc(c2)-c2cccc(c2)-c2nc3cc(ccc3[nH]2)C(F)(F)F)c1	72	0.456	552.589	0.272	-12.4	-12.6	-13.4	-11.3 (4PY6)	
TCMDC-137063	Cc1cccc2nc([nH]c12)-c1cccc(c1)-c1cccc(NC(=O)c2cncn2C)c1	80	0.167	407.467	0.489	-11.9	-11.0	-12.6	-11.3 (4NXJ)	
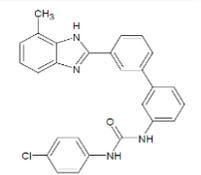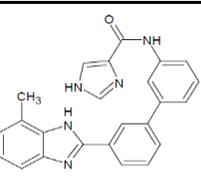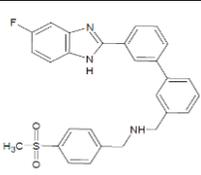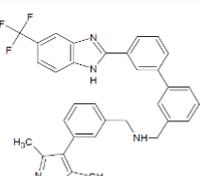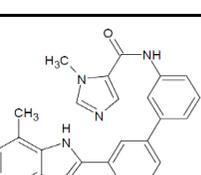
3.1.3.   *In silico* docking of ligands to proteins

Docking studies of 1098 compounds from Gamo *et al.* on the CDPK1, CDPK4 and FIKK9.1 kinases identified four distinct chemical compound clusters (cluster 38, 51, 80 and 125) that had binding energies of <-11.5 kcal/mol when bound to the ATP-binding site of the homology models (Gamo *et al.*, 2010). When docking these four clusters of compounds into the plasmodial BRD proteins, only one cluster (cluster 51) of compounds had binding energies of <-11.0 kcal/mol (Figure 14).
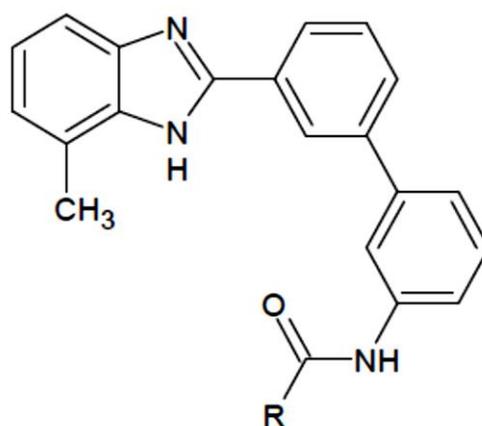
Figure 14: Scaffold structure of compounds from cluster 51 from Gamo *et al*. Compounds from these clusters showed good Autodock Vina binding energy against homology models of *Pf*CDPK1, *Pf*CDPK4 and *Pf*FIKK9.1, as well plasmodial bromodomain proteins. Chemsketch was used to draw the chemical structures.

The R-group from this scaffold was modified in order to improve the drug-like parameters of these compounds. Chemsketch was used to draw the chemical structures. Drug-likeness properties such as Lipinski and QED parameters (Bickerton *et al.*, 2012) were monitored with DruLiTo. QED scores of greater than 0.5 are found in more than 70% of compounds on the market and the aim was to design compounds with a score of as close to 0.5 or higher (Bickerton *et al.*, 2012). A set of compounds with the best drug-likeness parameters, as well as good binding energies (<-11 kcal/mol) against homology models of *Pf*CDPK1, *Pf*CDPK4 and *Pf*FIKK9.1, as well as plasmodial BRD proteins were chosen and only the compounds that were synthetically accessible by WuXi AppTec were synthesized (Table 5). When compound W14 was docked into *P. falciparum* BRD 4NXJ hydrogen bond interactions were observed (Figure 15A) and when the compound was docked into *Pf*FIKK9.1 kinase *pi*-bond interactions were observed (Figure 15B).
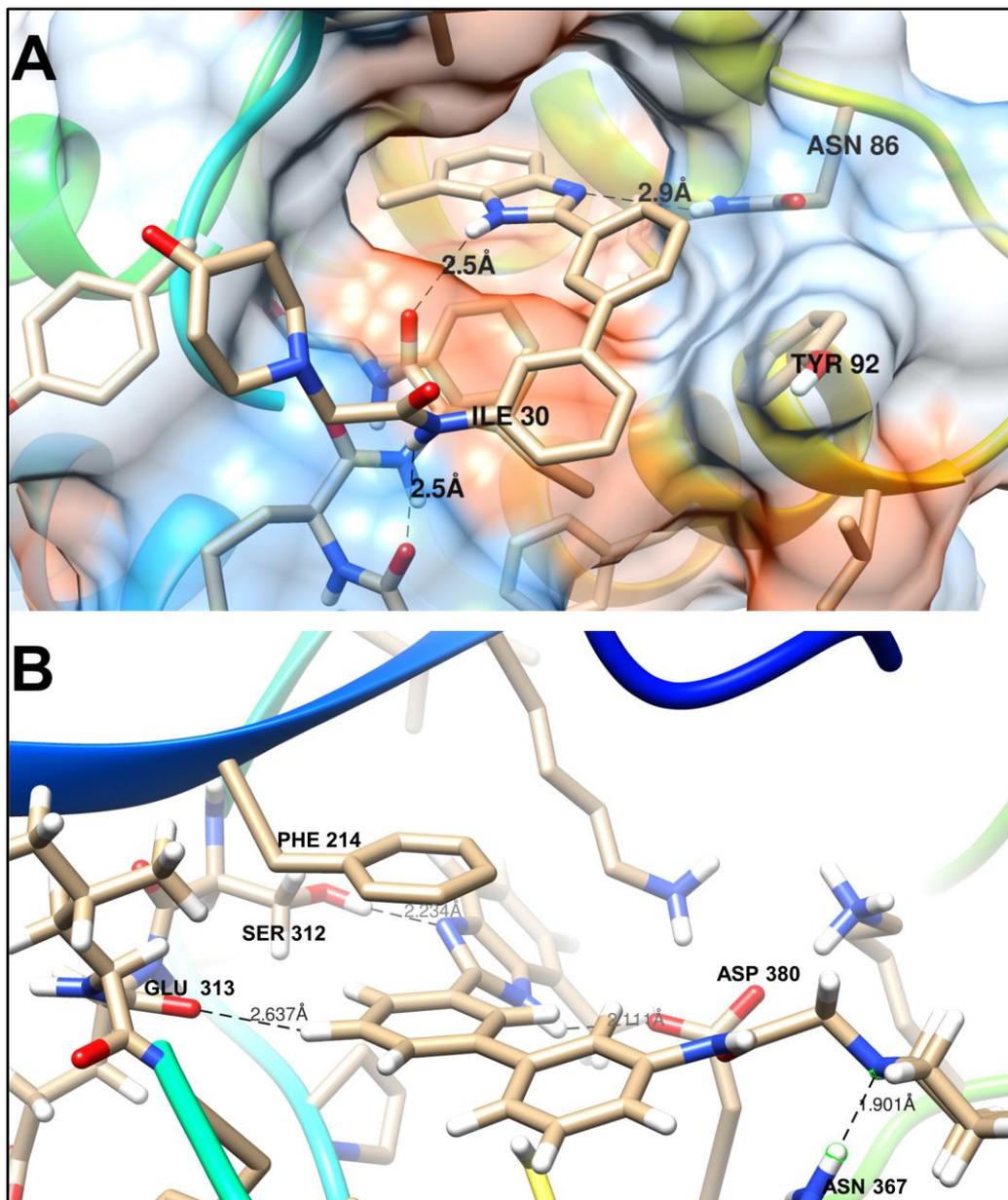
Figure 15: Docked binding pose of compound W14 bound to *P. falciparum* A) bromodomain 4NXJ and B) *Pf*FIKK9.1. In the bromodomain protein, hydrogen bond interactions are observed between the benzimidazole ring of W14 and ASN86, as well as the backbone oxygen of ILE30 of the protein. For the PfFIKK9.1 kinase, a pi-bond interaction between PHE214 ("F", in FIKK kinase) and the biphenyl benzene ring of W14 is expected. Additionally, hydrogen bonds are observed between the benzimidazole ring of W14 and the small gatekeeper amino acid SER312 as well as ASP380

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Table 5: Structures and properties of the prioritised compounds with predicted activity against CDPK1, CDPK4, and FIKK9.1 kinase and bromodomain protein 4NXJ

Compound	SMILES	Molecular weight	cLogP[a]	Autodock Vina binding energy (kcal/mol)				Structure
				CDPK1	CDPK4	FIKK9.1	BRD	
W10	C=C1CC(C1)C(=O)Nc2cccc(c2)c3cccc(c3)c4nc5c(C)cccc5n4	393.48	5.57	-11.9	-11.2	-12.1	-10.3	
W11	FC1CC(C1)C(=O)Nc2cccc(c2)c3cccc(c3)c4nc5c(C)cccc5n4	399.46	4.86	-12.5	-11.9	-11.9	-10.1	
W12	CC1(CCC1)C(=O)Nc2cccc(c2)c3cccc(c3)c4nc5c(C)cccc5n4	395.496	5.80	-11.6	-11.4	-12.0	-10	
W13	O=C(CN1CCN(CC1)C(C)=O)Nc2cc(ccc2)c3cccc(c3)c4nc5c(C)cccc5n4	467.562	4.40	-12.2	-11.2	-12.7	-9.9	
W14	O=C(CN1CCC(=O)CC1)Nc2cc(ccc2)c3cccc(c3)c4nc5c(C)cccc5n4	438.521	4.47	-12.2	-11.3	-12.4	-10.3	
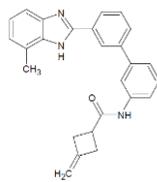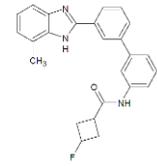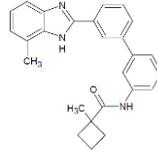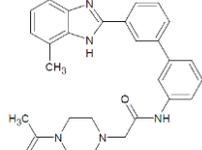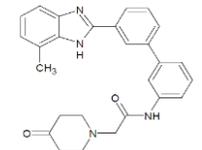
[a]cLogP – Calculated partition coefficient of the compound octanol and water, this is a measure of the compounds' lipophilicity

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3.2. *In vitro* cultivation of *P. falciparum* parasites

3.2.1. Asexual stages

Asexual *P. falciparum* parasites were cultivated in human erythrocytes and evaluated daily for asexual proliferation and development. Figure 16 indicates different stages during cultivation of asexual, intra-erythrocytic *P. falciparum* parasites. Parasites were viable as evaluated morphologically through Giemsa staining and evident from the rapid multiplication rate for the 3D7 parasites at 4 – 6 fold.
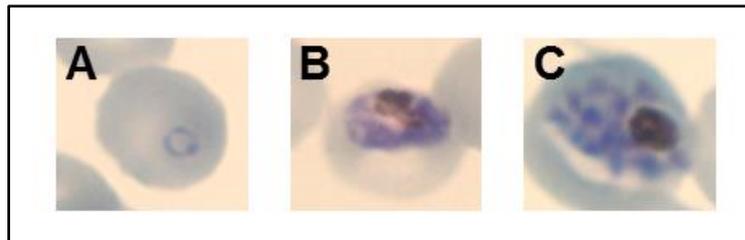
Figure 16: ***In vitro* asexual, intra-erythrocytic forms of *P. falciparum* 3D7 parasites.** The different IDC stages viewed were A) rings, B) trophozoites and C) schizonts. Parasites were viewed after Giemsa staining under a light microscope at 100x magnification, with oil immersion.

3.2.2. Gametocyte development

Gametocytes were produced from *P. falciparum* NF54 parasites. Gametocytogenesis was initiated by a drop in haematocrit and growing the parasites under starvation conditions (glucose free medium) with gametocytogenesis being evaluated for a period of 10-12 days. Figure 17 illustrates the development of the gametocyte stages after induction at Day 1 until Day 10.
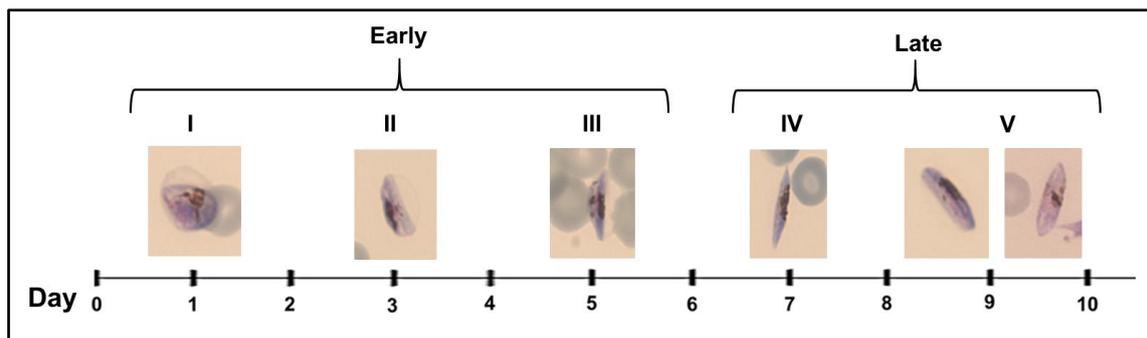
Figure 17: ***P. falciparum* NF54 gametocytes developmental stages in culture.** Stage I – V was seen. Parasites were viewed under a light microscope at 100x magnification, with oil immersion.

Using the description by Dixon *et al.* (Dixon *et al.*, 2012), gametocytes were considered viable during stage II and III if the parasite adopted a D-shaped morphology. For stage IV parasites to be used in subsequent experiments the gametocytes were elongated and stage

V gametocytes had more rounded ends. Gametocytes were not used if they appeared to be curled up or rounded up.

3.3. *In vitro* antiplasmodial drug assays for the asexual stages

3.3.1. Dual point assay

A dual point assay (1 and 5 µM) was used as a primary filter for the antiplasmodial activity of the compounds against asexual intra-erythrocytic *P. falciparum* 3D7 parasites (Figure 18). The test compounds that displayed inhibition of parasite proliferation of >50% at 1 µM and >70% at 5 µM were prioritised for further full dose-response evaluation.
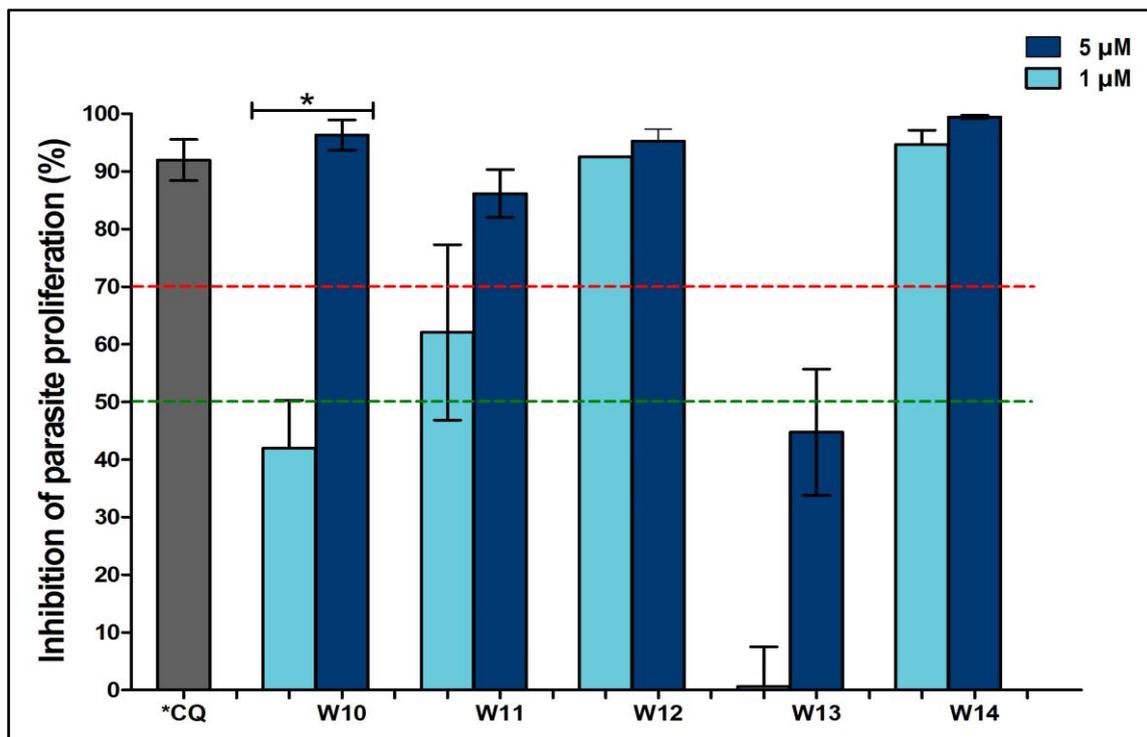
Figure 18: Dual point screening of the test compounds against asexual intra-erythrocytic *P. falciparum* parasites. Compounds were tested at 1 and 5 µM against drug sensitive *P. falciparum* 3D7 strain for 96 h at 37°C before inhibition of parasite proliferation was determined using the fluorescence based SYBR Green 1 assay. The selection was set at >50% inhibition at 1 µM (green dashed line) and >70% inhibition at 5 µM (red dashed line). Results are the mean of three independent biological replicates, each performed in triplicate (SEM indicated). Significance ($P<0.05$) is indicated through a two-tailed Students t-test with asterisk. *CQ- Chloroquine at 0.5 µM

A concentration-dependent response was observed for all compounds, with compound W13 the least active against the asexual *P. falciparum* parasites *in vitro*, achieving less than 45% inhibition of parasite proliferation even at 5 µM. This compound was not tested further. By contrast, compounds W12 and W14 were both highly active (>90% inhibition of parasite proliferation) at both concentrations tested. Compound W10 did show acceptable inhibition at 5 µM, which was significantly different from that at 1 µM ($P<0.05$, n=3). Based on this

primary selection, compounds W10, 11, 12 and 14 were selected for subsequent dose-response evaluation.

3.3.2. Dose-response evaluation of hit compounds

Dose-response evaluation was firstly performed on drug sensitive asexual intra-erythrocytic *P. falciparum* 3D7 parasites to determine the IC_{50} of the selected hit compounds from the primary screening evaluation. Figure 19 indicates the individual sigmoidal dose-response curves for the four compounds. In all cases, a minimum of 8-point evaluation was performed. From the four compounds that were tested, three (W11, W12 and W14) had IC_{50}'s below 1 µM. Compound W14 was the most potent at 0.58 ± 0.11 µM and was statistically different from W10, the least potent at 1.24 ± 0.14 µM (*P*<0.05, n=3). Means for W10 and W12, W11 and W14 were also found to be statistically different when tested using a two-tailed t-test (*P*<0.05, n=3).
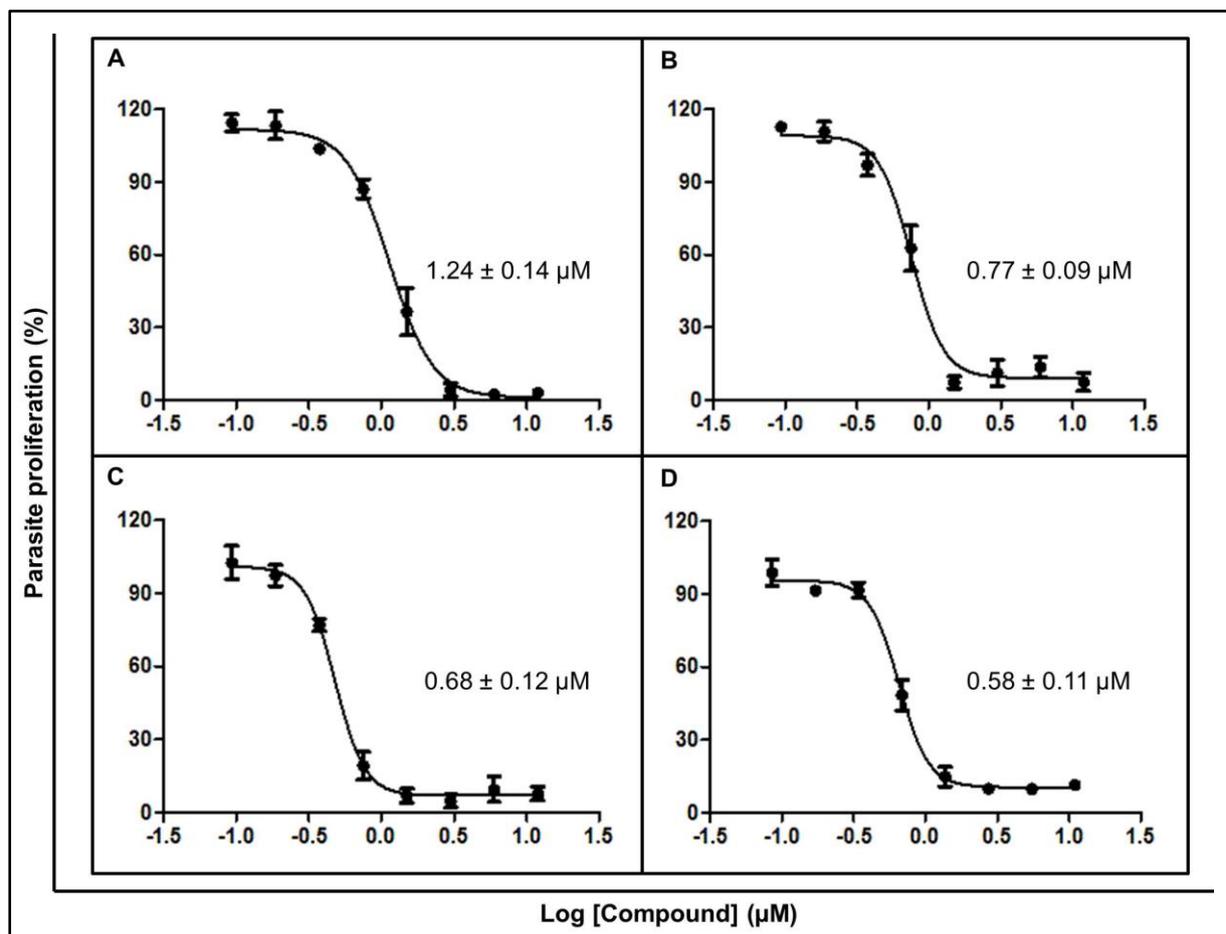
Figure 19: IC_{50} of the test compounds against asexual intra-erythrocytic *P. falciparum* parasites. Compounds were screened against drug sensitive *P. falciparum* 3D7 strain for 96 h at 37°C before inhibition of parasite proliferation was determined using the fluorescence based SYBR Green 1 assay. The graphs A) W10, B) W11, C) W12, D) W14 represent the dose-response relationship of the compounds with the IC_{50} indicated on the graphs. Results are the mean of three independent biological replicates, each performed in triplicate (SEM indicated, n=3).

The compounds were also screened against the drug sensitive *P. falciparum* NF54 strain. This strain is the parent strain for the 3D7 strain and is the preferred strain for gametocyte induction (Reader *et al.*, 2015) and therefore also had to be evaluated. Figure 20 shows the dose-response analysis on NF54 parasites. Comparison of the IC_{50} values of the compounds between the 3D7 and NF54 strains using a two-tailed t-test indicated no statistical variance (*P*>0.05 in all instances).
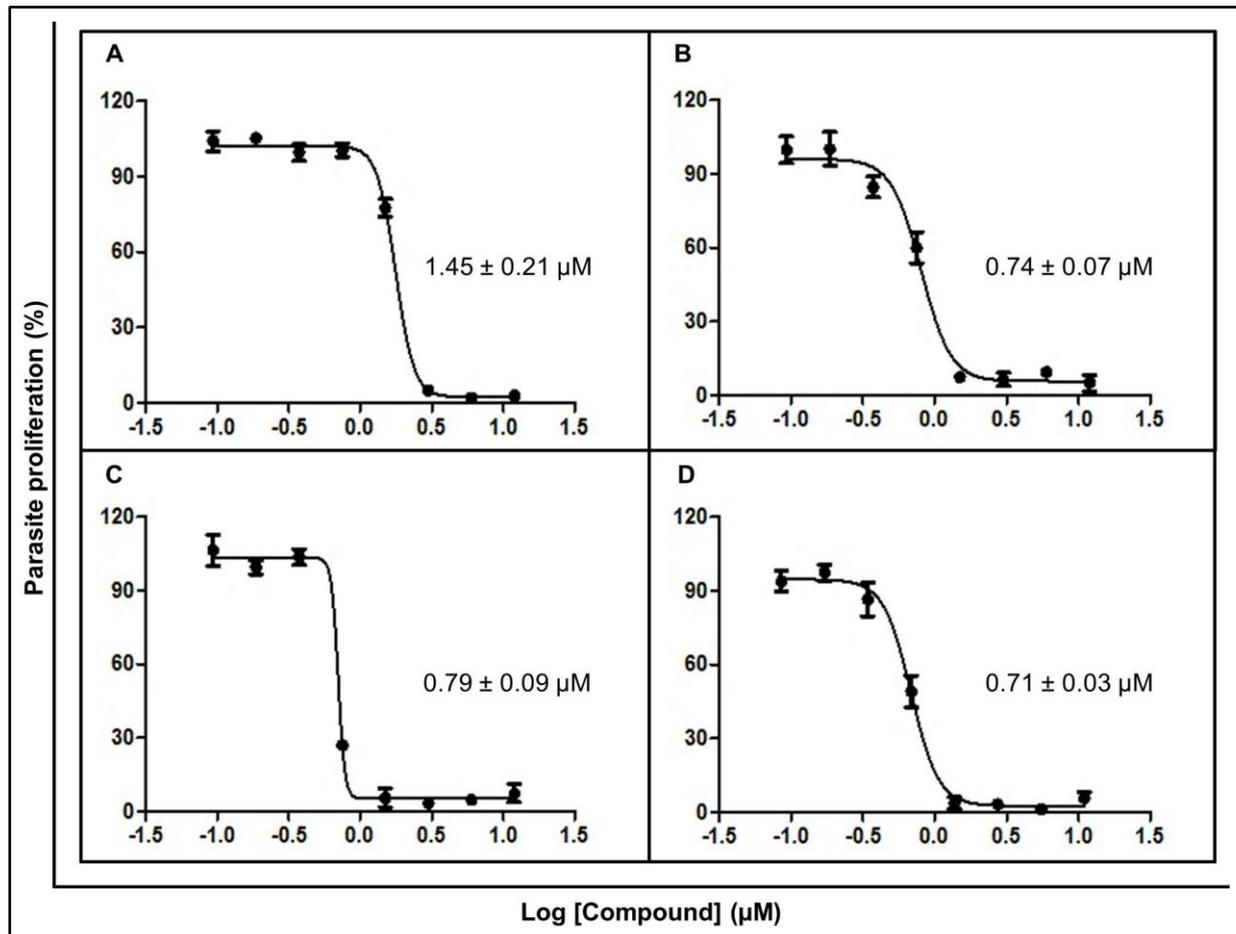
Figure 20: IC_{50} determination of the test compounds against the *P. falciparum* NF54 strain. The parasites were incubated in the presence of the compound for 96 h at 37°C. The fluorescence based SYBR Green 1 assay was used to determine parasite proliferation. Graphs A) W10, B) W11, C) W12, D) W14 represent the dose-response relationship with IC_{50} indicated. The data are expressed as the percentage of parasite proliferation relative to the untreated control and represented as the mean of three independent biological replicates, each performed in triplicate (SEM indicated; n=3).

3.3.3. Cross-resistance evaluation of hit compounds

The four active test compounds were evaluated for their ability to inhibit drug-resistant forms of asexual intra-erythrocytic *P. falciparum* parasites *in vitro*: K1 (multidrug-resistant) and W2 (chloroquine-resistant) *P. falciparum* strains (Figure 21).
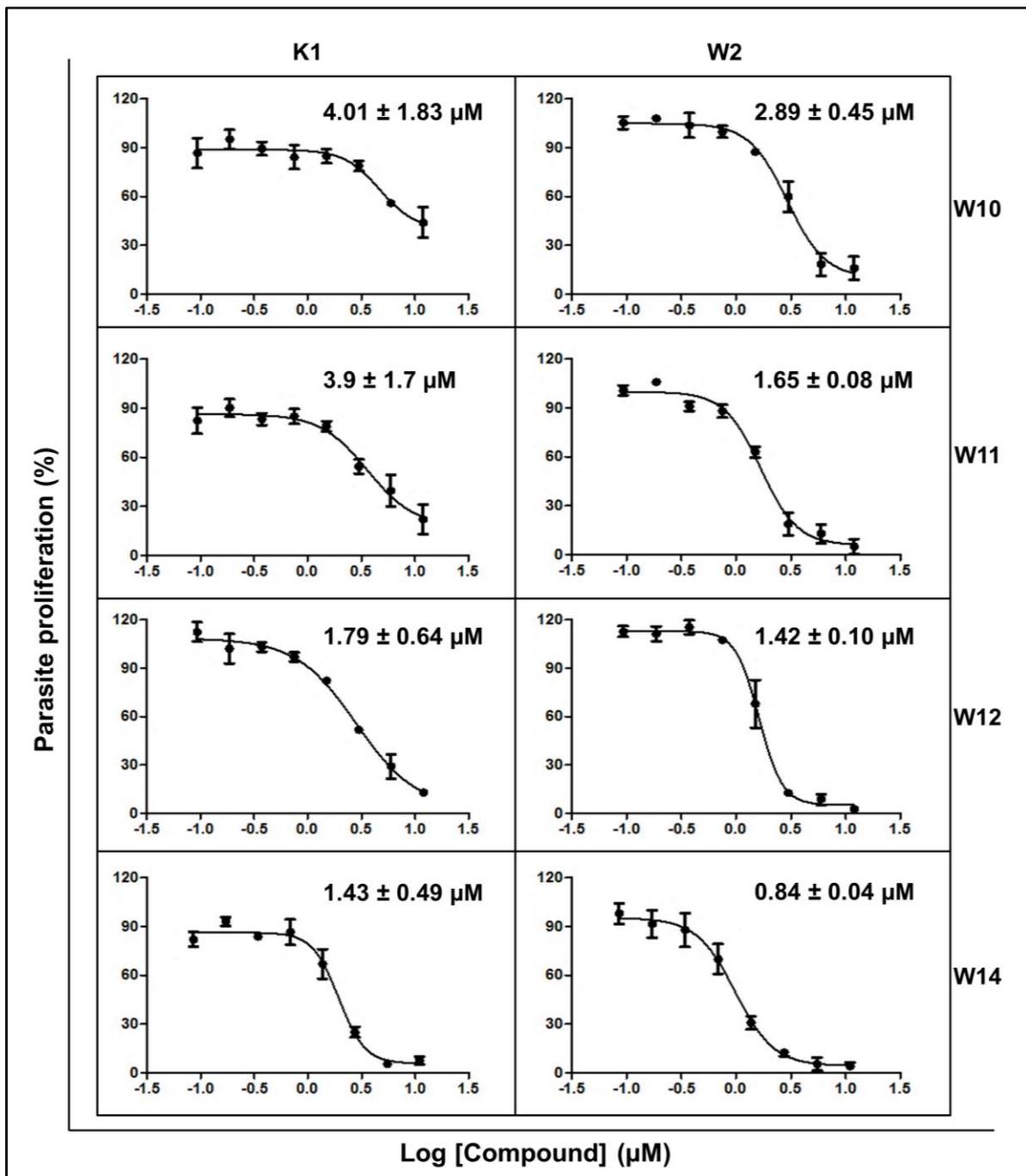
Figure 21: IC$_{50}$ of the test compounds on the *P. falciparum* K1 and W2 strains. The parasites were incubated in the presence of the compound for 96 h at 37°C. The fluorescence based SYBR Green 1 assay was used to determine parasite proliferation. The data are expressed as the percentage of parasite proliferation relative to the untreated control and represented as the mean of three independent biological replicates, each performed in triplicate (SEM indicated; n=3).

To determine potential cross-resistance between the sensitive and resistant strains, the resistance index (RI) was determined for each compound and compared to that of antimalarials (Chloroquine) with known resistance mechanisms. The RI provides a measure of the antiplasmodial activity against the chloroquine resistant strains relative to the

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chloroquine sensitive strain (Iwaniuk *et al.*, 2009). The RI (K1 IC_{50}/NF54 IC_{50} and W2 IC_{50}/NF54 IC_{50}) for all the compounds (Table 6) was <6 (Musonda *et al.*, 2004), whereas chloroquine had an RI of 15 and 23 for the K1 and W2 strains respectively. Compounds W10 and W11 showed the largest loss in activity against the K1 strain, less so against W2. However, none of the RI values obtained were >10 and the compounds can, therefore, be considered to have a low resistance profile against the current strains of the parasite tested (Musonda *et al.*, 2004; Iwaniuk *et al.*, 2009).

Table 6: *In vitro* **antiplasmodial activity of the test compounds, against asexual intra-erythrocytic *P. falciparum* strains (3D7, NF54, K1 and W2).**

Compound	Antiplasmodial activity against *P. falciparum* (IC_{50}, µM)				Cross-resistance	
	3D7	NF54	K1	W2	RI[a]	RI[b]
W10	1.24 ± 0.14	1.45 ± 0.21	4.01 ± 1.83	2.89 ± 0.45	2.8	2
W11	0.77 ± 0.09	0.74 ± 0.07	3.9 ± 1.7	1.65 ± 0.08	5.3	2.2
W12	0.68 ± 0.12	0.79 ± 0.09	1.79 ± 0.64	1.4 ± 0.1	2.3	1.8
W14	0.58 ± 0.11	0.71 ± 0.03	1.43 ± 0.49	0.84 ± 0.04	2	1.2
Chloroquine	0.009 ± 0.004	0.01 ± 0.003	0.154 ± 0.014	0.233 ± 0.049	15	23

[a]Resistance Index (RI) = K1 IC_{50}/NF54 IC_{50}
[b]Resistance Index (RI) = W2 IC_{50}/NF54 IC_{50}

Although all the compounds showed a dose-response relationship when tested against the W2 strain of the parasites, this was not entirely true for the K1 strain. Compounds W10-12 could not completely kill off the multidrug-resistant parasites with 15 – 45% of parasites still viable even at the highest concentrations tested during a two life cycle exposure time. This prompted kinetic and stage-specific evaluation of the action of these compounds against asexual intra-erythrocytic *P. falciparum* parasites.

3.3.4. Kill kinetics evaluation

The IC_{50} speed assay was used to determine whether the compounds are fast or slow acting according to Le Manach *et al.* (Le Manach *et al.*, 2013). Figure 22 shows the shift in the dose-response curves of the compounds at different periods; 24, 48 and 72 h incubations. Interestingly, typical dose-responses could not be obtained for any compound at the 24 h incubation period, questioning the accuracy of the assay at this short period of drug exposure. However, between the 48 and 72 h incubation periods, full dose-response curves were observed.
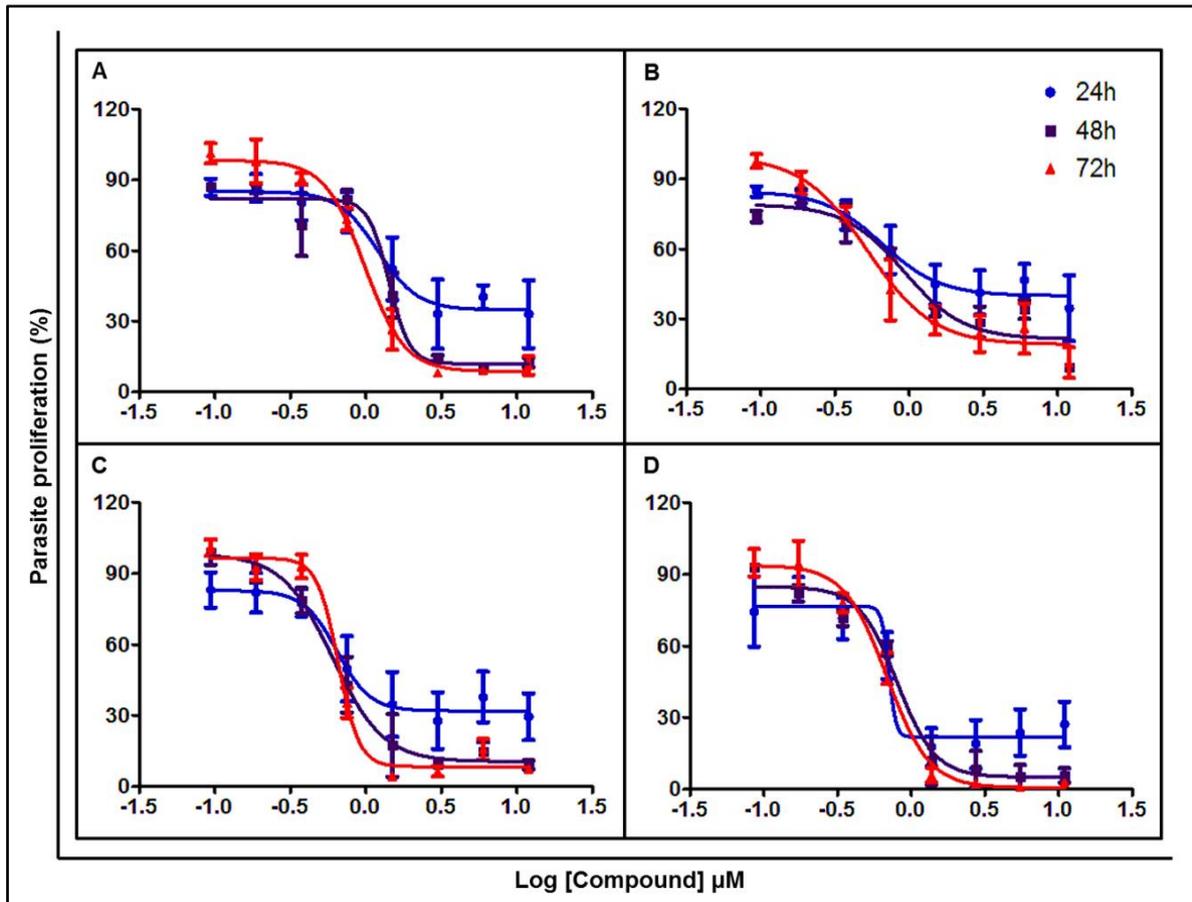
Figure 22: Kinetic evaluation of the test compounds. IC$_{50}$ speed assay of compounds A) W10, B) W11, C) W12 and D) W14. Compounds were screened against drug sensitive *P. falciparum* 3D7 strain for 24, 48 and 72 h at 37°C before inhibition of parasite proliferation was determined using the fluorescence based SYBR Green 1 assay. The graphs A) W10, B) W11, C) W12, D) W14 represent the dose-response relationship of the compounds. The data are expressed as the percentage of parasite proliferation relative to the untreated control and represented as the mean of three independent biological replicates, each performed in triplicate (SEM indicated; n=3).

Figure 23 shows the change in IC$_{50}$ of the compounds following the 48 and 72 h incubation periods. A two-tailed t-test showed that there was a significant difference (*P*-value<0.05) between the 48 h and 72 h IC$_{50}$ for W11 and W14, however, this did not hold true for either W10 or W11.
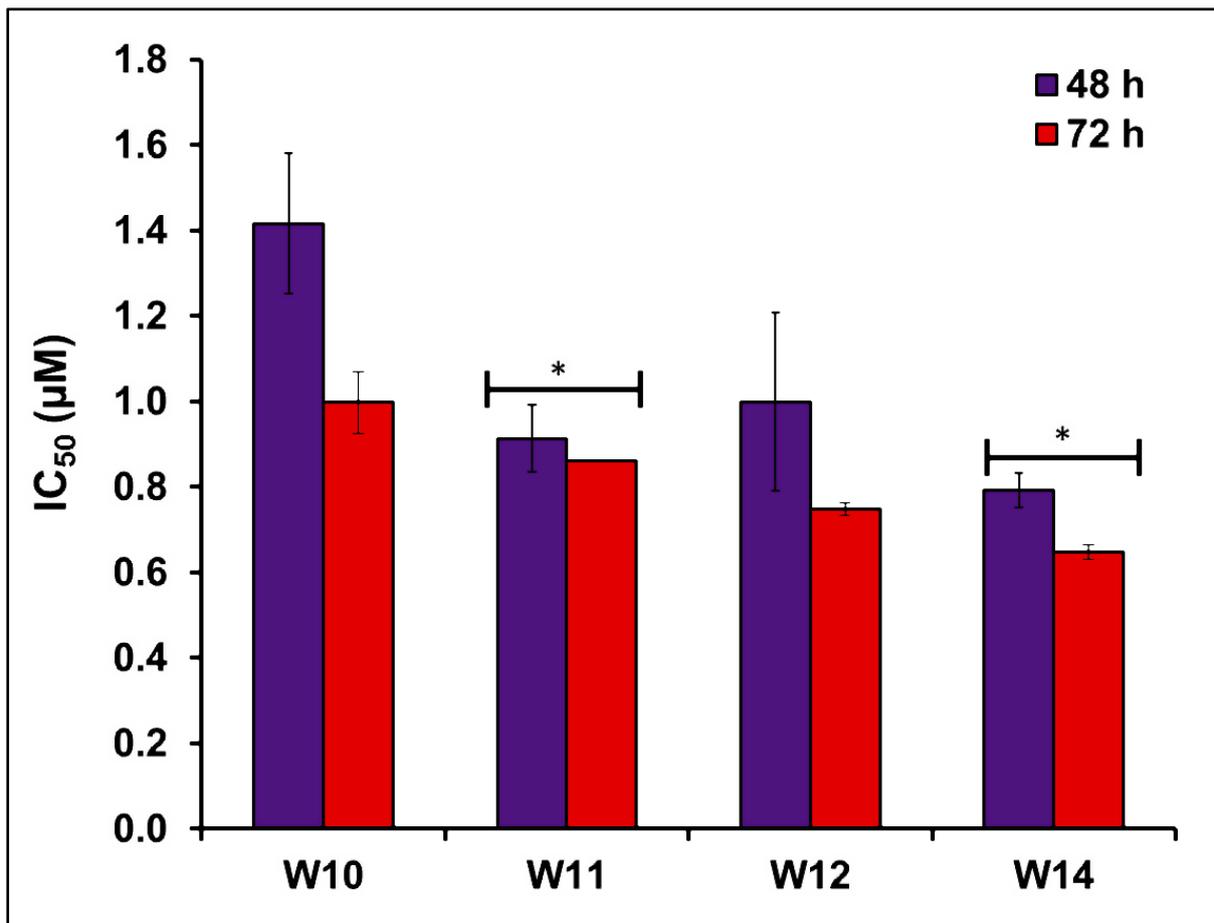
Figure 23: Difference in IC$_{50}$ of the test compounds. IC$_{50}$ is indicated for two incubation periods, 48 and 72 h in the presence of the compound determined using the fluorescence based SYBR Green 1 assay. The data are represented as the mean of three independent biological replicates, each performed in triplicate (SEM indicated; n=3). Significance (P<0.05) is indicated through a two-tailed Students t-test with asterisk.

As an indicator of fast acting or slow acting mechanisms of compounds, Le Manach *et al.* used the ratio of IC$_{50}$ 24 h/IC$_{50}$ 72 h. Compounds were considered as fast acting if the ratio was less than 1.4 and if the ratio was greater than 1.4, the compound was considered as slow acting. To determine whether the compounds were fast or slow acting, the ratio of the IC$_{50}$ of the compounds was determined using the 48 h IC$_{50}$/ 72 h IC$_{50}$ ratio (Table 7). In all cases, the compounds have an IC$_{50}$ ratio that is less than or equal to 1.4, indicating that the compounds were effective within the first 48 h of action.

Table 7: Ratio of the IC$_{50}$ of the test compounds at 48 and 72 h.

Compound	IC$_{50}$ (µM)		IC$_{50}$ 48 h/ IC$_{50}$ 72 h (ratio)
	48 h	72 h	
W10	1.42 ± 0.16	1 ± 0.07	1.4
W11	0.91 ± 0.08	0.86 ± 0.01	1.1
W12	1 ± 0.21	0.75 ± 0.01	1.3
W14	0.79 ± 0.04	0.65 ± 0.02	1.2

3.3.5. Stage-specificity determination

The aim of the stage assay was to determine at which point in the IDC the compounds have a preferred action. The compounds were tested at the ring, trophozoite and schizont stages of the IDC.

The intra-erythrocytic *P. falciparum* 3D7 parasite cultures were synchronously maintained at ≥90% ring stage synchronisation. Table 8 shows the percentage of parasites that were counted when performing the stage specificity assays for the three biological repeats (performed on the ring, trophozoite and schizont stages).

Table 8: Percentage stage specificity of the parasite cultures used for the intra-erythrocytic *P. falciparum* stage specificity assay

Stage	B1* (%)	B2* (%)	B3* (%)
Rings	96	93	89
Trophozoites	82	94	96
Schizonts	90	92	86

*B1-3: Biological replicate 1 to 3

Using these synchronised parasite populations, test compounds were evaluated for their stage-specificity. Figure 24 presents the change in the percentage of parasite proliferation at each stage when the parasites were treated with different concentrations of the compounds.
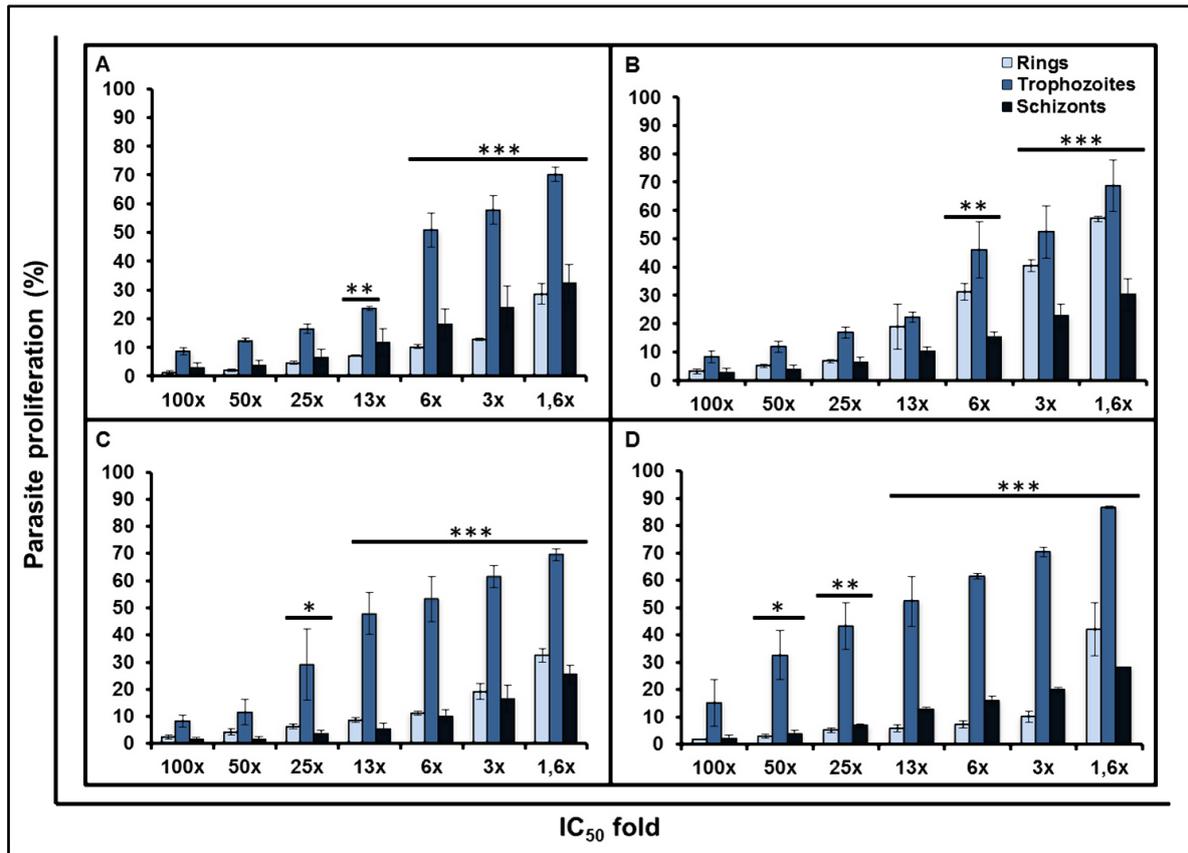
Figure 24: Stage specificity analysis of the test compounds against the asexual intra-erythrocytic *P. falciparum* parasites. Compounds A) W10, B) W11, C) W12 and D) W14 were tested against the drug sensitive *P. falciparum* 3D7 strain at different parasite stages. The parasites were incubated for 96 h at 37°C, thereafter parasite proliferation was determined using the fluorescence based SYBR Green 1 assay. The data are expressed as the percentage of parasite proliferation relative to the untreated control and represented as the mean of three independent biological replicates, each performed in triplicate (n=3; SEM indicated) and analysed using ANOVA with a level of significance of *$P<0.05$, **$P<0.01$, ***$P<0.001$.

A dose-dependent response was observed when the individual intra-erythrocytic stages of the *P. falciparum* 3D7 parasites were treated with the compounds at different concentrations. At the highest compound concentration (100x IC_{50}), there was less parasite proliferation compared to the lowest compound concentration for all the intra-erythrocytic parasite stages. There was a significant difference ($P<0.01$ or $P<0.001$, n=3) between parasite proliferation of the trophozoite and schizont stages at 6x to 1.6x concentrations as well as between the ring and trophozoite stages, for all compounds. There was, however, no significant difference between the mean percentage proliferation of the ring and schizont stages for all the test compounds at any concentration.

3.4. Gametocytocidal activity for the test compounds

Evaluation of a compound's gametocytocidal activity is used as an indicator of the ability of the test compounds to block parasite transmission between humans and mosquitoes

(Birkholtz *et al.*, 2016). To determine the gametocytocidal activity of the test compounds, the compounds were evaluated against the late stage NF54 *P. falciparum* gametocytes by analysing the expression of a luciferase reporter gene. Figure 25 shows the activity of the compounds against the late stages of the parasite when screened at 5 µM. At 5 µM, the compounds showed poor inhibition of the late stages of the gametocyte development. Compound W12 was the only compound that showed marginal inhibition, between 30-40%, of viability (Reader *et al.*, 2015). None of the test compounds were therefore deemed to have transmission-blocking activity and were not evaluated for any further gametocytocidal activity.
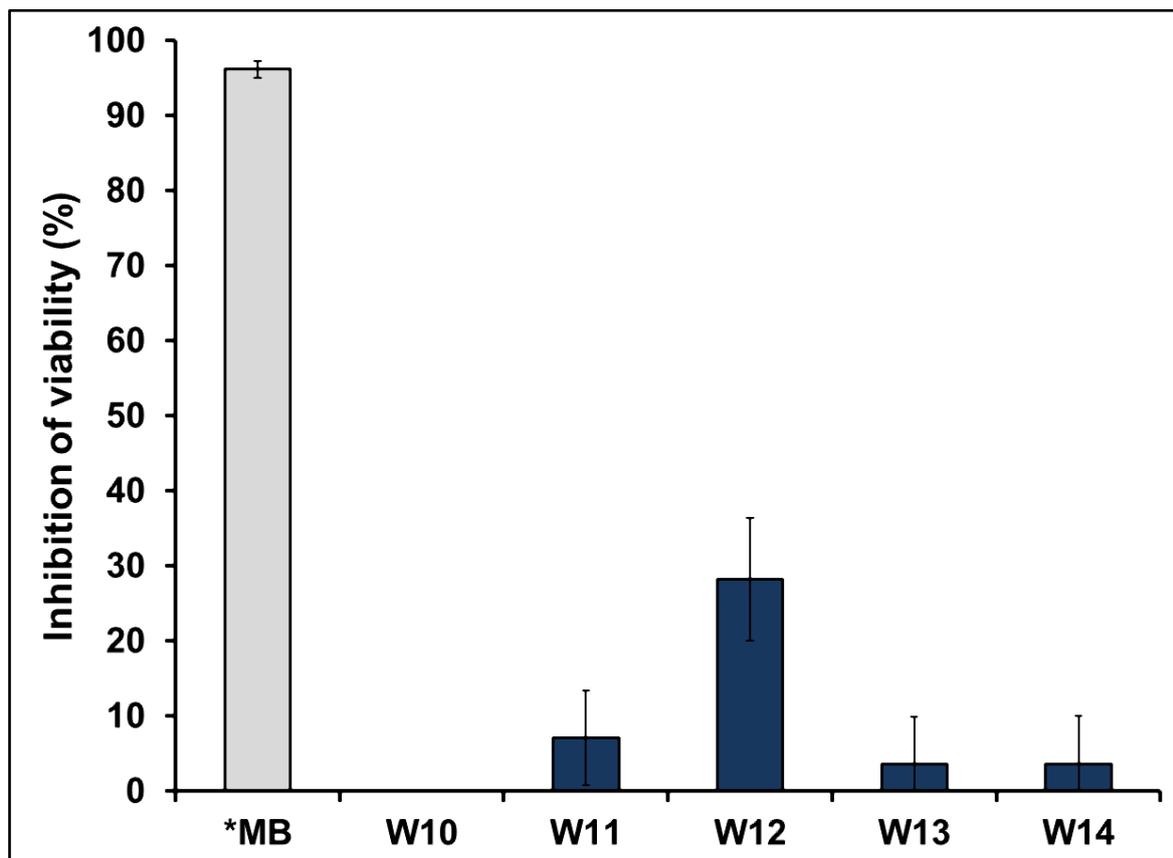
Figure 25: Percentage inhibition of gametocyte viability of the test compounds against the late stages of *P. falciparum* gametocytes. Compounds were screened at 5 µM against the late stage (stage IV to V) NF54 *P. falciparum* gametocytes. The gametocytes were incubated for 48 h at 37⁰C, Bioluminescence was measured using the luciferase assay. Results are the mean of three independent biological replicates, each performed in triplicate (n=3; SEM indicated). *MB – Methylene blue at 10 µM

3.5. Cytotoxicity

The cytotoxicity of test compounds W10, W11, W12 and W14 were tested on a human hepatocellular carcinoma, HepG2 cell line using the LDH assay. The compounds were tested at 100 µM because the compounds should have at least 10-fold less cytotoxicity

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against human cells than for the parasite. When tested at 100 µM, the HepG2 cells still demonstrated at least 70% viability, as determined by taking the colorimetric readings. Table 9 shows the percentage of cells that were viable at 100 µM for the four compounds tested.

Table 9: Viability of HepG2 cells when tested with 100 µM of compounds W10, W11, W12 and W14

Compound	Viable cells (%)
W10	82
W11	86
W12	80
W14	73

Additionally, the selectivity of the compounds was evaluated against a human breast cancer cell line (MDA-MB-231), to determine if the compounds are cytotoxic to another rapidly dividing cancerous human cell line. Figure 26 is a representation of the dose response of the compounds against the MDA-MB-231 cells. The IC_{50} for compounds W10, W11, W12 and W14 against MDA-MB-231 cells were 24.6, 10.2, 44.1 and 83 µM, respectively.
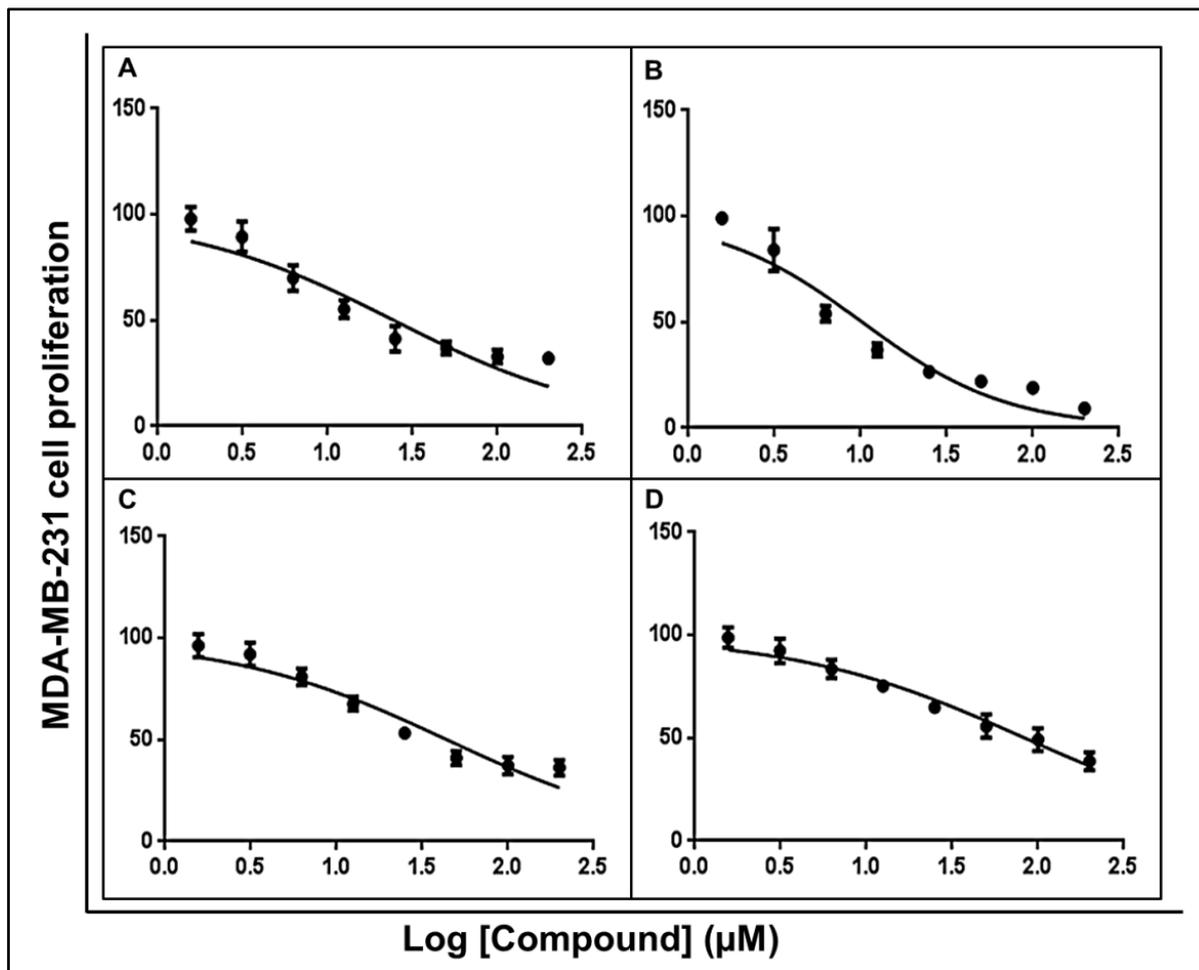
Figure 26: The IC$_{50}$ against MDA-MB-231 human breast cancer cells. The cells were exposed to the compound for 48 h and incubated at 37ºC. Crystal violet was used to quantify fixed monolayer cells using spectrophotometry.

Using these IC$_{50}$ values, selectivity indices (SI) could be determined. Table 10 is indicative of the selectivity of the compounds towards the breast cancer cells calculated using the IC$_{50}$ of the compounds towards the MDA-MB-231 cells divided by the *P. falciparum* 3D7 strain.

Table 10: Selectivity of test compounds for cytotoxicity of *P. falciparum* (3D7) compared to MDA-MB-231 human breast cancer cells

Compounds	MDA-MB-231 cells (IC$_{50}$, µM)	*P. falciparum* 3D7 strain (IC$_{50}$, µM)	SI[a]
W10	24.6	1.24	20
W11	10.2	0.77	13
W12	44.1	0.68	65
W14	83	0.58	143

[a]Selectivity index was determined as the compound IC$_{50}$ mammalian cell/IC$_{50}$ *P. falciparum*

The SI of all the compounds was more than 10, which is the minimum ratio required (Sarr *et al.*, 2011; Guiguemde *et al.*, 2012). W14, the most potent against asexual *P. falciparum* 3D7 parasites, had the lowest selectivity towards the human breast cancer cells.

3.6. Sequence similarity of plasmodial and human bromodomain proteins

The sequences of the plasmodial and human bromodomain proteins were aligned to determine similarity of these proteins. Aligning plasmodial and human bromodomain proteins shows that these proteins have high similarity. The amino acids present in the binding site of these proteins are similar. Although not all the binding site amino acids are the same, 55% of the amino acids are conserved in both Plasmodium and human bromodomain proteins. Figure 27 also shows other amino acids in the protein sequences that have similar biochemical properties. This indicates that the binding site of Plasmodium and human bromodomain proteins are similar and this could have an effect on the selectivity of the test compounds in this study.


```
3d7c.pdb_KAT2A_Human      S.................M........................... 2
3fkm.pdb_PF3D7_1033700    .................MHHHHHHSSGRENLYF............... 16
3uv2.pdb_BPTF_Human       .SMQCQSTEDAMTVLTP............................. 16
4nxj.pdb_PFD7_1475600     ...........................................GYDEI 5
5lvq.pdb_KAT2B_Human      ....................SMGKEKSKEPRD..... 12


3d7c.pdb_KAT2A_Human      ..ED.PDQLYTTLKNLLAQIKSHPSA..WPFMEPVKKSEA.......PD. 39
3fkm.pdb_PF3D7_1033700    .Q.G.NKQWYLLANQLILSLSKYEGG..HIFEKL.V....DAKKQNCPDY 56
3uv2.pdb_BPTF_Human       ....LTEKDYEGLKRVLRSLQAHKMA..WPFLEPVDPNDA.......PD. 52
4nxj.pdb_PFD7_1475600     EE...LKSKNEVLTNLLNKLIAF..DKKRIFLYPVNVQLV.......PD. 42
5lvq.pdb_KAT2B_Human      .P....DQLYSTLKSILQQVKSHQSA..WPFMEPVKRTEA.......PG. 47
                            ..   .  . :: .:  .   .:*         *.

3d7c.pdb_KAT2A_Human      .YYEVIRFPIDLKTMTERLRSRYYVTRKLFVADLQRVIANCREYNPPDSE 88
3fkm.pdb_PF3D7_1033700    YD.V.IKNPMSFSCIKTKLKKGQYAYPSEFVKDVQLIFDNCSLYNTSNSV 104
3uv2.pdb_BPTF_Human       .YYGVIKEPMDLATMEERVQRRYYEKLTEFVADMTKIFDNCRYYNPSDSP 101
4nxj.pdb_PFD7_1475600     .YLNVIKEPMDFTTMKQKLQNFKYKSFQEFEKDVLLIINNCYTYNDPSTI 91
5lvq.pdb_KAT2B_Human      .YYEVIRFPMDLKTMSERLKNRYYVSKKLFMADLQRVFTNCKEYNPPESE 96
                            *: *:.:  :   ::: *    * *: :: ** **  ..:

3d7c.pdb_KAT2A_Human      YCRCASALEKFFYFKLKEGGLI......DK................. 112
3fkm.pdb_PF3D7_1033700    VAITGKNIETYFNNQLIVMGYNN..FI...LKEKKINDMLKLVEEENIKW 149
3uv2.pdb_BPTF_Human       FYQCAEVLESFFVQKLKGFK..ASR..S................. 125
4nxj.pdb_PFD7_1475600     YYKFAEDIETYYKKLNIKI.Q.TKYM.N................. 116
5lvq.pdb_KAT2B_Human      YYKCANILEKFFFSKIKEAGL.I...D................. 119
                            .     .. :*.::

3d7c.pdb_KAT2A_Human      ....................
3fkm.pdb_PF3D7_1033700    SKEKEDQIEEIDELENN.... 166
3uv2.pdb_BPTF_Human       ................H... 126
4nxj.pdb_PFD7_1475600     ................IHL 119
5lvq.pdb_KAT2B_Human      ....................
```

Figure 27: Sequence alignment of plasmodial and human bromodomain proteins. The blue blocks indicate the amino acids that protrude into the binding site of the bromodomain proteins. ***** indicates conserved amino acids, **:** indicates amino acids that are either hydrophobic, hydrophilic or charged and **.** indicates a high degree of similarity between the amino acids with one or two exceptions. The sequences were aligned using MUSCLE (Edgar, 2004).

Chapter 4

4. Discussion

Of all the *Plasmodium* species that affect humans, *P. falciparum* is the most virulent. *P. falciparum* has developed resistance to currently available antimalarial drugs in south eastern Asia. The complex ways in which the parasite mutates and confers resistance to antimalarial drugs is of concern. To address issues such as parasite resistance to antimalarial drugs, new drug targets have to be explored. This study investigated compounds that were designed to have more than one target and this is important in order to avoid drug resistance and human toxicity effects (Jensen *et al.*, 2012). Designing selective inhibitors is, therefore, important. The protein targets of the test compounds were the *P. falciparum* kinases (CDPK1, CDPK4, and FIKK9.1) and Bromodomain proteins (4NXJ and 3FKM). The ATP-binding site of these kinase proteins was exploited (Doerig *et al.*, 2010) for the design of selective inhibitors.

Although the compounds have a common scaffold structure, they do not have identical chemical properties. One compound can be more water soluble than another. The change in the R functional group can make them either more hydrophilic or hydrophobic. The state of ionisation of the compound is also important. Non-ionised compounds can penetrate lipid bilayer membranes, whilst ionised (or polar) compounds cannot passively penetrate the membrane (Harvey *et al.*, 2012). This becomes important when compounds are tested *ex vivo* because the state of ionisation of a compound plays a role in whether the compound can be transported across cell membranes to its target site. The LogP value is the partition coefficient of a compound between octanol and an aqueous phase. This is where all compounds are present in their neutral form and it is a measure of the compounds lipophilicity. Compounds that have a LogP of 3 – 5 or greater are said to have high permeability (Milner *et al.*, 2010). The molecular weight (MW) also influences a compound's permeability. It is suggested that compounds with MW less than 414 g/mol have increased permeability (Molnar and Keseru, 2011). From the compounds investigated in this study, it could be predicted that W10, W11 and W12 should have good permeability. Although compound W13 and W14 have cLogP of less than 5, their MW suggests that their permeability is not that good. In a study by Baragana *et al.*, the hit compound was found to have a cLogP of 4.3; although the compound had high potency the physicochemical properties were poor. The compound was then optimised further resulting in the compound DDD107498 with cLogP of 3.2. DDD107498 had improved physicochemical properties (Baragana *et al.*, 2015).

The main objective of this study was to determine the antiplasmodial activity of the 5 compounds that were designed *in silico*. To do this, a primary filter was performed (dual point screening), whereby only compounds that inhibited parasite proliferation more than 50% at 1 µM were chosen. From the 5 compounds that were screened only 4 (W10, W11, W12 and W14) were successful. With the compounds sharing the same scaffold structure it was interesting to note that the compounds do not all show activity against the parasite. The decreased effect of W13 could be attributed to its lack of permeability through the membrane. Milner *et al.* stated that compounds with high MW become less permeable (Milner *et al.*, 2010). As stated above compound W13 has a MW of more than 414 g/mol and there might be other factors that contribute to the compound not being permeable or inactive against the *P. falciparum* parasite. It might be that this compound does not diffuse through the membranes or does not reach the target site. However, if the compound does reach the target site, the decreased effect could be because the compound does not interact with the protein it is meant to target. The difference in parasite proliferation between W13 and the other compounds was statistically significant. W12, which had the highest cLogP, was active against the parasite; at both 1 and 5 µM. W12 inhibited parasite proliferation to approximately 90 – 100% at these concentrations. These results suggest that although cLogP is an indicator of lipophilicity, another property to take into consideration with compound permeability could be the MW. However, currently approved kinase inhibitors reported by Wu *et al.* are shown to adhere to Lipinski's rule of 5 but some of these inhibitors have MW between 400 – 600 g/mol (Wu *et al.*, 2016).

For the compounds that showed activity against the parasite during the initial screening, an IC_{50} was then determined. The IC_{50} determines at which concentration of a compound is needed to have a half maximal effect on parasite toxicity. Using the chloroquine-sensitive strains, 3D7 and NF54, to determine IC_{50} helps compare the test compounds to the benchmark IC_{50} of chloroquine. However, the MMV has set criteria that potential antimalarial compounds should ideally have an IC_{50} of less than 1 µM. With the four compounds that were screened for $IC_{50,}$ only three had an IC_{50} of less than 1 µM for both the 3D7 and NF54 strains (Figure 19 and Figure 20). Most of the newer potential antiplasmodial compounds have potent activity in the nM range, for example, the PI4K inhibitor MMV048 (Younis *et al.*, 2012) and imidazolopiperazines (from a GNF compound library) (Wu *et al.*, 2011).

In antimalarial drug discovery, it is important to determine compound cytotoxicity early during the drug discovery process. Considering that the liver is the primary site of metabolism of most exogenous compounds, the human hepatocarcinoma cell line (HepG2 cells) was used and a reason for this is to also detect hepatotoxicity. The HepG2 cell line maintained viability

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when tested at concentrations as high as 100 µM (Table 9) for the test compounds. These results suggest that the compounds are selective towards the parasite. This is also validated when the compounds were tested against a rapidly dividing human breast cancer cell line; the selectivity index of the test compounds was more than 10 which indicates an acceptable safety margin. A selectivity index of more than 10 is considered to show selectivity towards the parasites and the compound is minimally cytotoxic to human cells. However, relative to chloroquine's selectivity index of 2462 (Verlinden *et al.*, 2011) as determined by Verlinden *et al.*, a selectivity index of 10 is very low. It would, therefore, be better to compare the selectivity index of potential compounds to that of chloroquine. Although the compounds tested in this study do not show activity at nM ranges, the high selectivity of these compounds is in agreement with other kinase inhibitors that have been tested, such as the bumped kinase inhibitors that target *Pf*CDPK4, that were shown to have potent activity (at nM ranges) and a high selectivity against mammalian fibroblast cells (Ojo *et al.*, 2012). The selectivity of these compounds is also in agreement with other inhibitors that have been tested against *P. falciparum*, that have more than a 10-fold selectivity against mammalian cells (Wu *et al.*, 2011). The compounds did not show cytotoxic activity against the sexual stages (gametocytes) of the parasite. When tested at 1 µM against gametocytes, the compounds did not exhibit any significant cytotoxic activity. This is true for early stage gametocytes (results not shown since no activity was observed at all) as well as late stage gametocytes, indicating that these compounds have no capacity as transmission-blocking drugs.

Burrows *et al.* stated that with the development of parasite resistance to available drugs being a constant challenge, it is important to select molecules that have a low potential for the development of resistance (Burrows *et al.*, 2014). It is, therefore, important to assess the risk of resistance, for the purpose of *in vitro* studies the *P. falciparum* K1 and W2 strains were used. In Figure 21, compounds W10, W11 and W12 for the K1 strain did not kill off 100% of the parasites at any concentrations. A reason for this could be that the compounds take a longer time to inhibit parasite proliferation in this strain or that there is an inherent resistance to the compounds due to the mutations that have given these strains the single or multi-drug resistance. With these strains, cross-resistance had to be determined. A drug that is active against the sensitive strains has to be active against the resistance strains to show low potential for the development of resistance (Burrows *et al.*, 2014; Burrows, 2014). This is to ensure that even the resistant strains can be targeted. To calculate the resistance index, the IC_{50} of the NF54 strain was used (Table 6). The RI can be used as an indicator of the likelihood of the parasite developing resistance to the potential compounds. With the compounds that were tested in this study, the RI indicates that a dose of at least twice as

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much as required for the sensitive strain would be needed to kill the resistant parasite for all the test compounds. This poses a concern for these compounds because in an *in vivo* environment it means that resistance will occur quicker. Even with an RI that is less than that of chloroquine (reported by Verlinden *et al.* as 8 (Verlinden *et al.*, 2011)) the parasite still has a greater chance of developing resistance to these compounds.

The time it takes for a compound to kill the parasite is important, because this will determine if the compound can rapidly kill the parasite. Compounds that are fast acting such as the artemisinins are successful in clearing the parasite within a few hours of contact. This benefits the patient because such drugs are able to prevent aggravation of symptoms. Knowing whether a compound is fast acting or slow acting enables to find appropriate partner compound combinations that will work synergistically or have an additive effect (Sharma and Awasthi, 2015). Clinically when looking at ACTs, these drugs have been successful in clearing the parasites. Where the fast acting artemisinins have not been successful at eliminating the parasite the partner drug is able to clear the slower developing parasites. The compounds investigated in this study work within the first 48 h of the parasite's life cycle. An IC_{50} for the 24 h period could not be determined, this could be as a result of the assay that was used (fluorescence based SYBR Green 1 assay), The fluorescence based SYBR Green 1 assay requires increased DNA content as is observed in schizont stages (Johnson *et al.*, 2007) and requires at least 48 h of incubation to significantly detect this increase in the proliferating parasite (Smilkstein *et al.*, 2004). Le Manach *et al.* used the [3H]hypoxanthine incorporation assay which measures incorporation of radioactively labelled nucleotides and this active DNA synthesis can be accurately detected within 24 h (Chulay *et al.*, 1983; Sanz *et al.*, 2012). Using TCPs to classify the compounds, the compounds fall into TCP 1 as they are active against the intra-erythrocytic stages and not the sexual stages of the parasite.

To determine stage-specificity, specificity studies and kinetics of a test compound can be performed. Stage-specificity will give information on which stage of the parasite's life cycle is most susceptible to the compound. By doing this, the stage can be related to certain pathways in the parasites life cycle. In Figure 23, W11 and W14 appear to have a similar mechanism of action judging from the kinetic effect seen from the bar graphs. Although mechanism of action cannot be determined from the kinetic effect only, Sanz *et al.* found that compounds that have the same targets have a similar way of killing the parasite (Sanz *et al.*, 2012). The test compounds could be showing similar effects as they were designed to target the same proteins. Both compounds W11 and W14 showed no significant difference in the

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IC$_{50}$ between the 48 h and the 72 h. This suggests that at both time points the same amount of compound is required to elicit the same effect.

Determining at which point in the parasite's life cycle the compounds are selective is of importance. This will enable the determination of whether the test compound does target the protein for which it was designed. The stage specificity results of the IDC show that all the compounds inhibit parasite proliferation in a dose-dependent manner at the three IDC stages (ring, trophozoite and schizont). W10 and W14 do, however, show similar inhibition patterns for the different IDC stages. Both these compounds seem to be more selective towards the ring stage (Figure 24A and Figure 24D), than the following two IDC stages. Although speed analysis of the compounds show that the parasite may be responding the same way to W11 and W14, the stage specificity results do not show this. W11 appears to be in the same class as W12, both these compounds are shown to be more selective towards the schizont stage of the IDC. What is interesting is that although the compounds show a dose-dependent relationship during stage analysis, none show a greater selectivity towards the trophozoite stage.

The test compounds were docked against CDPK1 (PlasmoDB gene ID PF3D7_0217500), CDPK4 (PlasmoDB gene ID PF3D7_0717500) and FIKK9.1 (PlasmoDB gene ID PF3D7_0731400) kinases, based on expression profiles from PlasmoDB data (PlasmoDB, 2016), the transcriptomic expression profiles of these kinases showed that: during the IDC, CDPK1 is suppressed throughout the ring and trophozoite stage and only transcribed during the early to the late schizont stages (Bozdech *et al.*, 2003). This finding corresponds with findings from other studies that suggest that this protein is essential for growth at the asexual stage and that inhibiting this protein inhibits parasite proliferation at the schizont stages (Ansell *et al.*, 2014). The fact that CDPK1 is repressed throughout the IDC and only transcribed during the schizont stages suggests that this protein is of importance for the parasite's survival. CDPK4, on the other hand, is transcribed during the early ring stage and the late schizont stages. This protein, although present during the IDC is not transcribed in large amounts during the trophozoite stages.

Transcriptional expression profile of FIKK9.1 (from the PlasmoDB data (PlasmoDB, 2016)) shows that during the IDC this protein is actively transcribed during the trophozoite stage and FIKK9.1 has a similar expression to other FIKK kinases such as FIKK7.1, FIKK7.2, which in general, are transcribed throughout the IDC. FIKK kinases being transcribed during the IDC may be important for parasite survival at specific stages of the IDC. For example, it could be possible that the FIKK kinases that are transcribed during the trophozoite stages

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may be needed for the highly metabolically active parasites during this stage. FIKK kinases such as FIKK12 which are highly transcribed during the ring and late schizont stages have been studied by Nunes *et al.*, they found that this protein may be interacting with the membrane of infected erythrocytes at the trophozoite stage (Nunes *et al.*, 2010). Proteomic data of some of the proteins show that CDPK4 is present in high abundance in the ring stage and protein abundance is not as high during the trophozoite stage. During the schizont stage and stage I-IV of the gametocytes the protein is down-regulated but up-regulated in stage V. Proteomic expression profiles indicate that CDPK4 is highly abundant in the ring stage and is present in low abundance during the trophozoite stage and stage V of the gametocytes. CDPK4 is shown to be down-regulated during the schizont stage and stage I-IV of the gametocytes. CDPK1 protein expression data suggests that this protein is present in high abundance during stage III of the gametocytes. Correlating the stage specificity data with transcriptomic and proteomic data it is apparent that the compounds may be targeting a protein that is expressed during the ring stages and also in the schizont stages. A possible target for these drugs may be CDPK1 as expression of this protein is up-regulated during these stages, and CDPK1 is one of the proteins that the compounds were designed to target. The loss in compound activity during the sexual stages of the parasites could be because CDPK4 protein is up-regulated during stage V of the gametocytes. CDPK4 has previously been shown to be important for male gamete formation and transmission to the mosquito (Billker *et al.*, 2004). This study only determined antiplasmodial activity against the intra-erythrocytic and sexual gametocyte stages, and not against gametes.

The compounds were also designed to target the *Plasmodium* bromodomain proteins. The functions of this proteins remains to be elucidated, however, they share high sequence similarity with a subgroup of human bromodomain proteins KAT2A (GCN5), KAT2B (PCAF) and BPTF (when compared on www.rcsb.org). Furthermore, when the amino acids that protrude into the binding pocket were examined, a similarity of 55% between the plasmodial and human bromodomain proteins was found. These amino acids included those that are conserved in both Plasmodium and humans, and some of the amino acids had similar biochemical properties. In humans, KAT2A, KAT2B and BPTB are important mediators in controlling cell growth (Wang *et al.*, 1997; Ionov *et al.*, 2004). Proteomic and transcriptomic data of the proteins show that 3FKM (PlasmoDB gene ID PF3D7_1033700 – BDP1) is present throughout the IDC, but highly transcribed during the ring and early trophozoite stage. Josling *et al.* investigated BDP1; they found that the protein is expressed throughout differentiation of the parasite's life cycle (Josling *et al.*, 2015). The study by Josling *et al.* found that BDP1 directly regulates the correct expression of invasion genes and knockdown of this protein lead to defects in the invasion process, resulting in decreased parasite growth

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(Josling *et al.*, 2015). This study showed that BDP1 can be targeted for drug development. The protein expression analysis of data from PlasmoDB (PlasmoDB, 2016), *in vitro* data suggests that possible targets for the compounds could be proteins that are transcribed or expressed during the ring and late schizont stages. However, W11 and W12 are shown to be more selective to the schizont stage of the IDC, and the protein that is transcribed only during this stage is CDPK1.

Chapter 5

5. Conclusion

The *in silico* approach used in this study for the design of small molecules to target specific proteins was shown to be an effective way of focussing on compounds that could possibly inhibit parasite proliferation while eliminating compounds that would not be expected to bind to the target proteins. With the *P. falciparum* parasite constantly developing resistance to antimalarial drugs, an alternative approach where molecules with high binding potential to multiple pharmacological targets are designed to reduce the risk of developing drug resistance. In this study, compounds designed *in silico* to have polypharmacological targets were investigated in an *in vitro* setting to assess their antiplasmodial activity during all blood stages of the parasite.

The objectives of this study were to determine whether, *in silico* designed compounds targeting CDPK1, CDPK4, FIKK9.1 and bromodomain proteins in the parasite have antiplasmodial activity. The test compounds, which were designed to have potential polypharmacological targets, were shown to have effective antiplasmodial activity against the intra-erythrocytic stages of *P. falciparum* parasites, at concentrations that are physiologically feasible but not in the low ranges of drugs that have been developed further and which are undergoing non-clinical or clinical trials. With these compounds showing activity during the intra-erythrocytic developmental cycle but not exhibiting gametocytocidal activity they can be classified as TCP1 type candidates. The compounds have a low resistance profile as determined by testing the compounds against drug resistant laboratory strains of *P. falciparum*. With the compounds also showing selectivity towards the parasite, these compounds can, therefore, be deemed to have antiplasmodial activity. The compounds would need to be further optimised to improve the antiplasmodial activity against both the intra-erythrocytic and sexual stages of the parasites.

The effectiveness of these compounds might have been limited by the number of proteins into which it was docked. Further studies can include focussing on one or two target proteins. For example, targeting CDPK1 and CDPK4 only and in the process designing molecules that can be used to achieve TCP1, TCP5 and/or TCP6. This would then allow for the design of compounds that would not only treat malaria symptoms but also serve as transmission-blocking agents. Furthermore, to determine if the compounds have inhibitory effects on the protein kinase and bromodomain proteins biochemical activity assays such as kinase inhibitory assays and thermal shift assays for bromodomain proteins can be

performed. These assays will give information of the binding capacity of the compound to the protein as well as stability of the protein.

The overall aim of this study was to assess the efficacy of a series of *in silico* designed compounds with polypharmacological targets. From the compounds that were assessed four had antiplasmodial activity against the *P. falciparum* 3D7 and NF54 strains, these compounds however showed less sensitivity against the K1 and W2 strains. Stage specificity of the compounds showed that the compounds were more active against the ring and schizont stages and have a rapid kill kinetic. The *P. falcipuram* gametocytes were unaffected by the compounds and no cytotoxicity was observed against the human hepatocytes. The compounds were not able to kill all life cycle stages of the *P. falciparum* parasite, such as the gametocytes which is important for the transmission of malaria. To overcome the ineffectiveness of the compounds on other stages of the parasite further structural modifications can be performed. These modifications could lead to the improvement of the overall potency and efficacy of the compounds.

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Appendix

Ethics approval certificate

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

29/10/2015

Approval Certificate
New Application

Ethics Reference No.: 459/2015

Title: In vitro efficacy assessment of targeted antimalarial drugs synthesized following in silico design

Dear Dikeledi Matlebjane

The **New Application** as supported by documents specified in your cover letter dated 16/10/2015 for your research received on the 19/10/2015, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 28/10/2015.

Please note the following about your ethics approval:
- Ethics Approval is valid for 2 years
- Please remember to use your protocol number (**459/2015**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

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