In vitro antiplasmodial activity of an optimised series of alkylated (bis)urea and (bis)thiourea polyamine analogs

by

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SUMMARY

The malaria parasite, *Plasmodium falciparum,* exhibits genetic plasticity such that it increasingly develops resistance to current antimalarial drugs, especially in Southeast Asia where multi-drug resistance (MDR) threatens the last line of antimalarial drugs (*1*, *2*). This obstructs the effectiveness of most of the current antimalarial drugs including the quinolines, antifolates and artemisinins (*3*). Therefore, there is an urgent need for innovative strategies to develop novel antimalarial drugs to eradicate the disease. Recently, (bis)urea and (bis)thiourea symmetrical, terminally alkylated polyamine analogs were shown to have potent antimalarial activities against chloroquine sensitive (3D7), chloroquine resistant (W2) and antifolate resistant (HB3) strains of *P. falciparum* parasites with antiplasmodial activities (IC_{50}) as low as 88 ± 7 nM (*4*) and high selectivity to malaria parasites (>7000 fold lower IC_{50} against *P. falciparum*). These polyamine analogs had either 3-4-3, 3-6-3 or 3-7-3 carbon backbones. In the study reported here, 3-5-3 backbone analogs were analyzed for their antiplasmodial activity. Within this series of analogs, IC_{50} values as low as 28 ± 4 nM were obtained against 3D7 *P. falciparum* parasites and these compounds were equally as active against drug resistant strains of the parasite (17 ± 2 nM against W2 *P. falciparum* and 40 ± 3 nM against HB3 *P. falciparum*). These compounds were also found to have selectivity of >5000 fold against the parasite. The combination of the lead compounds with the polyamine biosynthesis inhibitor, α-difluoromethylornithine (DFMO), both resulted in additive interactions against *P. falciparum* 3D7 parasites. The analogs arrested parasitic growth after 48 h of exposure by blocking nuclear division and DNA replication, confining the parasites to the 1N stage (rings and early trophozoites). Compound 6 also led to irreversible parasite cytotoxicity over a 48 h period after a 12 h treatment with IC_{90} drug concentrations. Therefore, terminally alkylated (bis)urea and (bis)thiourea polyamine analogs of the 3-5-3 carbon backbone, pose an enticing structurally novel and distinct class of potential antimalarials with potent activities in the low nanomolar range and high selectivity ranges against *P. falciparum* parasites. Further mechanistic studies and *in vivo* activity determinations are currently underway.

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AdoMetDC	*S*-adenosylmethionine decarboxylase
AIDS	Aquired Immunodeficiency syndrome
BE-4×4	1,20-(ethylamino)-5,10,15-triazanona-decane
BEHSpm	Bis(ethyl)homospermine
BENSpm	Bis(ethyl)norspermine
BESpm	Bis(ethyl)spermine
CHENSpm	N^1-ethyl-N^1-[(cycloheptyl)-methyl]-4,8-diazaundecane
CPENSpm	N^1-ethyl-N^{11}-(cyclo-propyl)methyl-4,8-diazaundecane
DFMO	α-Difluoromethylornithine
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthetase
DMSO	Dimethylsulfoxide
IC_{50}	Concentration at which the compounds resulted in a 50% inhibition of parasite proliferation
IC_{90}	Concentration at which the compounds resulted in a 90% inhibition of parasite proliferation
IDC	Intra-erythrocytic developmental cycle
ELISA	Enzyme-linked immunosorbent assay
IPENSpm	N^1-ethyl-N^{11}-((isopropyl)methyl)-4,8-diazaundecane
LSD1	Lysine specific demethylase 1
MC	Maurer's cleft
MTA	5'-methylthioadenosine
ODC	Ornithine decarboxylase
*Pf*EMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
SAR	Structure-activity relationship
SERA	Serine repeat antigen
SP	Sulfadoxine-pyrimethamine
TB	Tuberculosis
TVN	Tubulovesicular network
WHO	World Health Organisation

Chapter 1: Introduction

1.1 Malaria

Malaria is a deadly parasitic disease with the first cases of malaria occurring around 10 000 years ago. The battle against malaria started in 1898 when Ross and Grassi discovered that malaria is transmitted by an infected female *Anopheles* mosquito (*5, 6*). Malaria infection occurs through unicellular, eukaryotic protozoan parasites of the genus *Plasmodium.* There are five different *Plasmodium* species that can infect humans. *P. falciparum* is the most pathogenic form of human malaria, causing the highest mortality associated with malaria (*7*). The remaining four species are *P. vivax, P. ovale, P. malariae* and *P. knowlesi,* which are arranged in decreasing order of rates of infection and mortality that they each cause. *P. knowlesi* conducts a zoonotic transfer of the parasite between humans and primates. According to the World Health Organization Report of 2011, malaria is currently still found in the tropical and sub-tropical parts of the world and causes approximately 600 000 deaths each year – mostly of African children under 5 years of age and pregnant women (*8*). Along with tuberculosis and acquired immunodeficiency syndrome, malaria is one of the top health challenges that stem the development of some of the poorest countries in the world. The *P. falciparum* parasite has a complex life cycle and for the eradication of the disease, every stage of the life cycle must be considered for treatment (*9*).

1.2 Life cycle of *P. falciparum* parasites

P. falciparum parasites are transferred through the female *Anopheles* mosquito's salivary glands during a blood meal on a human, where sporozoites, which are the infectious form of the parasite, are released into the bloodstream (Figure 1.1) (*10, 11*). These sporozoites then migrate to the liver, where remaining parasites not killed by Kupffer cells infect hepatocytes within 30 min. The multiplication inside the liver occurs in an asexual, exo-erythrocytic cycle lasting approximately 2 weeks. *P. vivax* and *P. ovale* can remain dormant in the liver for years after the initial infection causing relapses (*9*). Drugs targeting the liver stage are important to prevent development of the disease. After this exo-erythrocytic development cycle, the liver cells burst and the parasites are released back into the bloodstream in the

merozoite form to invade erythrocytes and initiate the intra-erythrocytic developmental cycle. The number of merozoites formed and released into the blood is species specific. Upon infection of erythrocytes, the parasite initiates the asexual blood stage of the life cycle as illustrated in Figure 1.1, c-g. (*10, 11*). The asexual stage takes about 44-48 h with successive release of merozoites from the erythrocytes and subsequent re-invasion. This cycle of development is apparent in the host as recurring waves of fever when erythrocytes cyclically release merozoites into the bloodstream (*12, 13*).

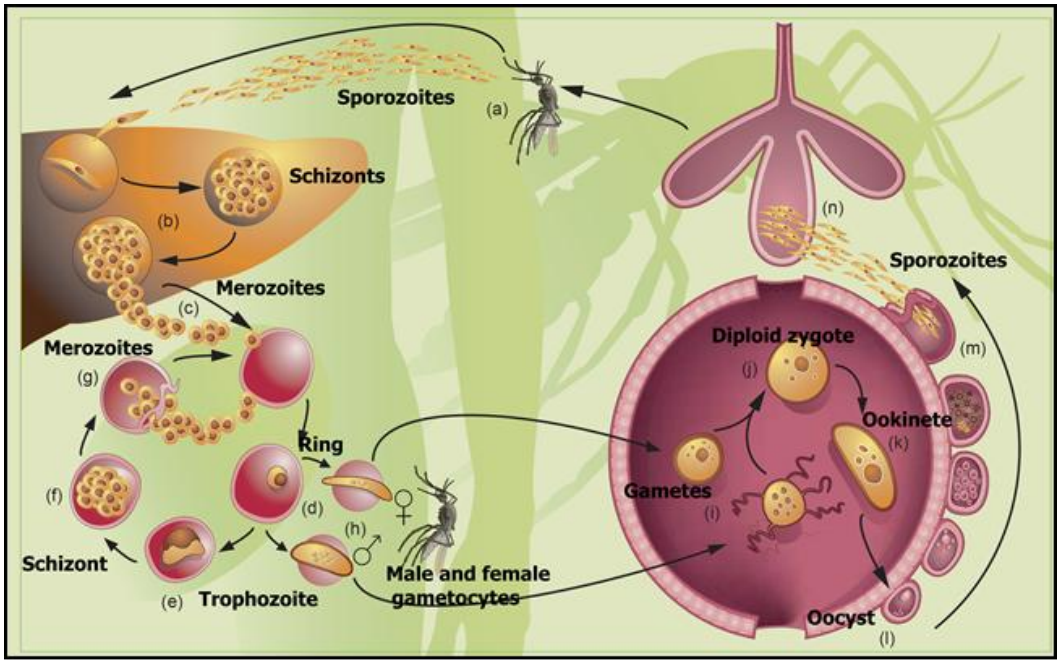


Figure 1.1: Life cycle of *P. falciparum* parasites. a) Transmission of sporozoites from a feeding mosquito to humans, targeted to invasion of the liver. b) Schizogony in the liver. c) Merozoites released into the blood infecting erythrocytes. d) Ring stage parasites. e) Trophozoite stage parasites. f) Schizogony. g) Rupturing of erythrocyte releasing merozoites. h-n) Sexual stage (*11*).

The parasite is now detectable microscopically, allowing the specific species and disease to be diagnosed. Merozoites enter erythrocytes as small, non-active, ring forms containing major organelles such as the nucleus, mitochondria, plastid, ribosomes and ER (*12*). During the early ring stage, membrane structure development is initiated which includes the tubulovesicular network (TVN) and Maurer's clefts. Maurer's clefts are parasite-derived structures inside the erythrocyte cytoplasm, which act as sorting compartments between the parasite and erythrocyte membrane (*14*). The parasite obtains nutrients from its host's erythrocytes to grow and survive (*12*). Ring stage parasites eventually develop into metabolically active trophozoite stages that have a rounder, more irregular morphology (Figure 1.2). The latter is characterized by bulges and tubular extensions of the parasitophorous vacuole, extensive parasite-derived membrane organelles in the red blood

cell cytoplasm (including maurer's clefts) and the formation of numerous protrusions on the red blood cell surface known as "knobs". Multiple nuclear divisions in infected erythrocytes give rise to multi-nucleated schizonts containing between 16-32 new merozoites, which lead to rupturing of erythrocytes' cell membranes. The rupturing occurs through a rapid increase in intracellular pressure and biochemical changes that destabilizes the cell cytoskeleton (*15*). The mechanism of erythrocyte membrane rupturing followed by the destruction of the erythrocytes is however not quite clear. It does appear as if proteases, for example the serine repeat antigen (SERA) family, play a role in the rupturing of the cells (*16-18*). Drugs targeting the blood stage of the parasite is used to control symptoms associated with the disease as well as mortality (*9*).

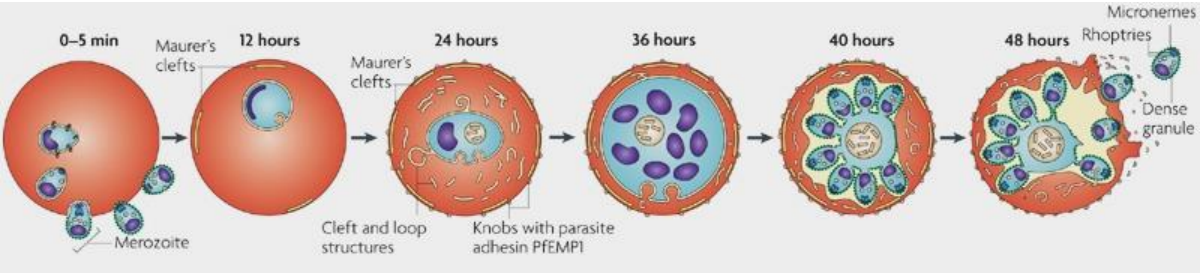


Figure 1.2: Intra-erythrocytic developmental stages of *P. falciparum* parasites. Merozoites enter the erythrocytes as small, ring stage parasites and develop into early trophozoites (±12 h), trophozoites (±24 h) and schizonts (±40 h) before rupturing the erythrocyte (±48 h) releasing up to 32 new daughter merozoites with each rupture (*19*).

Daughter merozoites, containing a set of rhoptries, micronemes and dense granules (*12*, *19*), invade other erythrocytes within seconds due to antigens on the surface of the extracellular parasite (*20*). To enter the erythrocyte, a tight-junction is formed between merozoites and erythrocyte membranes (*10, 21, 22*) to join the apical end of the merozoite with the membrane of the erythrocyte for a closer interaction, leading to the surface of the erythrocyte wrapping around the parasite(*10, 22-25*). As the parasite pushes itself into the erythrocyte, it creates a parasitophorous vacuole to seal itself from the cell's cytoplasm, enabling it to develop efficiently. An important protein transferred to the erythrocyte membrane, via Maurer's clefts, is the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1), which is a transmembrane protein presented at the erythrocyte surface where it allows adhesion of infected erythrocytes to endothelial cells (*26-29*).

Following invasion of merozoites into erythrocytes, sexually committed ring stages progress into gametocytes, which grows and elongates, finally occupying the majority of the

erythrocyte volume (*30*). The process of sexual development of the gametocytes is known as the sporogonic cycle (*31*). Male and female gametocytes are the components of the parasite's life cycle that are taken up from the bloodstream of an infected host by the mosquito, mediating disease transmission. They are morphologically and functionally distinct from their asexual erythrocyte counterparts as reflected in their gene expression, cellular development and metabolism (*30*). Female gametocytes develop into macrogametes whereas the nuclei of male gametocytes divide into eight flagellated microgametes. Flagellation of the microgamete is caused by elements present in the mosquito's midgut and starts about ten minutes after the mosquito's blood meal. The fertilization product is known as the zygote, which develops into an elongated ookinete that ultimately develops into a non-motile oocyst where nuclear division repeatedly occurs. This leads to the formation of as many as 10 000 new individual nuclei, producing a large number of sporozoites. When the mosquito takes a blood meal, the salivary fluid, which has anti-clotting properties, and its content of sporozoites are transferred into the host to start another asexual developmental cycle (*10*). Drugs targeting the transmission and mosquito stages are necessary to prevent infection of other humans and will facilitate in eradication of the disease (*9*).

By understanding the life cycle of the malaria parasite, antimalarial drugs can be developed to target specific stages of the life cycle, particularly in its intra-erythrocytic developmental cycle, and thereby be therapeutic to alleviate malaria pathogenesis (*32*).

1.3 Antimalarial drugs and drug resistance

The fight against malaria is multi-faceted consisting of vector control, potential vaccines as well as antimalarial drug treatment. The majority of vector control is currently based on two strategies namely insecticide-treated mosquito nets and indoor residual spraying. These strategies successfully led to a reduction in disease infections. However, sustainability of the bed nets as well as increased resistance against the insecticides used are threatening the effectiveness of vector control against malaria (*33*). Insecticides used are focused only on a single class of pyrethroids due to its low toxicity in humans, its rapid knock-down effect as well as affordability compared to long term residual spraying. New insecticides are therefore required to preserve the effectiveness of malaria vector control strategies (*33*). The most advanced malaria vaccine currently being analyzed is the RTS,S/AS01 and is being developed by GlaxoSmithKline and the Bill and Melinda Gates Foundation. RTS,S/AS01 is

currently in Phase III clinical trials and has been found to reduce the incidence of clinical malaria by 55% (*33*). At present, the primary component of parasite control is the use of antimalarial drugs; however, the main problem with the use of drugs is the rapid development of drug-resistant parasite strains.

In 1955, the WHO began a program developed to eradicate malaria with clinical treatment using chloroquine, which is a synthetic quinoline compound: 4-aminoquinoline (*34*), and controlling the malaria mosquito using dichlorodiphenyl-trichloroethane (DDT) in bed nets. Chloroquine was found to be active against intra-erythrocytic parasites degrading hemoglobin. It was also found in the lysosome where it is responsible for disrupting the polymerization and detoxification of the heme molecules, stored as hemozoin, which are released after hemoglobin digestion (*2, 35*). In the 1960's, there were recurrences of malaria in many countries through-out the world, indicating that even though chloroquine decreased the number of infections, the parasite had become more resistant to it (*36*). Resistance to chloroquine is now widespread with up to 100% resistance in some parts of the world (*3*). Chloroquine resistance in *P. falciparum* was later found to be as a result of a multi-drug resistance gene, *Pfmdr*1, and a single point mutation in the chloroquine resistance transporter gene, *Pfcrt* (*2, 33*).

Antifolate drugs have an important role in the treatment of *P. falciparum* and are currently among the most widely available drugs. Antifolates target the *de novo* folate synthesis pathway of the parasite, inhibiting the activities of two key enzymes, dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS), thereby interrupting DNA synthesis (*37*). A combination drug consisting of the antifolate pyrimethamine (inhibitor of *Plasmodium* DHFR enzyme) and sulfadoxine (inhibiting DHPS enzyme), sulfadoxine-pyrimethamine (SP), was the most commonly used antifolate for treatment of malaria, but due to resistance, its efficacy varies extensively. Antifolate drug resistance results from polymorphisms in the genes encoding DHFR and DHPS enzymes, which are currently the only two activities being targeted in the folate pathway (*37*).

Artemisinin, derived from a weed *Artemisia annua*, was subsequently used as it had proven antimalarial activity as a Chinese herbal medication (*38*). Artemisinin is a 15-C sesquiterpene lactone with two important structural components, a 6-lactone and an endoperoxide group (essential for antimalarial activity) (*39*). The *in vitro* mechanism of

action of artemisinin involves conversion of artemisinin to a free radical form when the endoperoxide bridge is reduced by an electron from a ferrous ion (Fe^{2+}) to a ferric ion (Fe^{3+}) (*39*). The free radicals generated alkylate and oxidize proteins and lipids within infected erythrocytes leading to parasite death (*39*). Artemisinin-combination therapies (ACTs) constitute the last line of defense against the increasing problem of antimalarial resistance. Artemether-lumefantrine (AL) is the most widely used ACT followed by artesunate-amodiaquine with no absolute cases of treatment failure. However, clear evidence shows emergence of resistance to artemisinin components of combination therapies, most evident in Cambodia, with recurring infections occurring in nearly 30% of patients treated with artesunate monotherapy and 5% when treated with the artesunate-mefloquine combination drug (*40*).

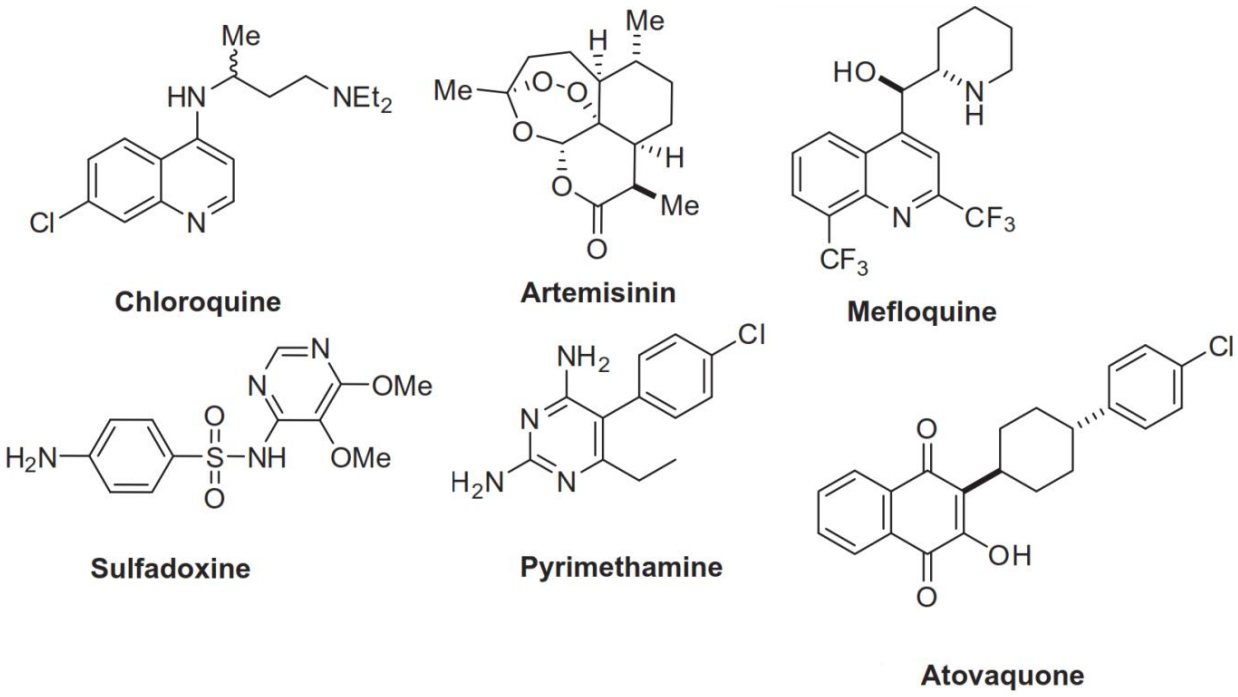


Figure 1.3: Structures of previously used antimalarial drugs (*9*).

Resistance has emerged against most of the currently and previously used antimalarials indicated in Table 1.1. The first signs of resistance against artemisinins are not a question of whether resistance will occur, but rather a question of how much time we have left until it starts spreading (*41*).

Significant departure from the chemical classes and structures of compounds (Figure 1.3), which were effective in the past, with a novel mechanism of action, is required to circumvent problems of parasite resistance to a single drug and cross-resistance between drugs (*42*).

Table 1.1: Antimalarial drugs (*33, 43, 44*)

Name	Chemical class	Other drugs in this family	Mode of action	Resistance emerged	Resistance mechanism
Chloroquine	4-Amino-quinoine	Amodiaquine	Interference with heme crystallisation	1957	*Pf*CRT mutations prevents accumulation of chloroquine in the acidic food vacuole (*45*)
Mefloquine	Amino Alcohol	Quinine, halofantrine, lumefantrine	Interference with heme crystallisation	1982	Point mutations in *Pf*MDR1 allows for export of drugs from food vacuole. Increased resistance with increased copy number of *pfmdr1*(*46*)
Quinine	Amino alcohol		Interference with heme crystallisation	1910	Mutations in *Pf*CRT and *Pf*MDR1. (*47*)
Pyri-methamine	Diamino pyridine	Cycloguanil	DHFR inhibitor (synergistic with sulphadoxine)		
Sulfadoxine	Aminosul-phonamide	Dapsone	DHPS inhibitor (synergistic with pyrimethamine)		
Sulfadoxine-Pyrimetha-mine			Affects folate metabolism (DHPS and DHFR) and the blood stage	1967	Mutations in DHFR and DHPS
Artemisinin derivatives	Endo-peroxides	Artesunate, artemether, arteether, dihyro-artemisinin	Unknown	Signs of resistance on the Thailand Cambodian border	Increased resistance with increased *pf*mdr1 copy number (*48*)
Atovaquone	Naphtho-quinone	None	Mitochondrial cytochrome *bc*1 complex inhibitor	1996	Point mutation in cytochrome c reductase I
Primaquine	8-Amino-quinoline	Pamaquine, bulaquine	Unknown (only structural class active against liver hypnozoites and gametocytes)	None	
Proguanil	Bi-guanidine	None	Metabolized *in vivo* to cycloguanil, a DHFR inhibitor	1949	Mutations in DHFR

In Figure 1.4, resistance to previously used antimalarial drugs across the world is illustrated, indicating the necessity in obtaining novel antimalarials. Two major problems greatly reduce the therapeutic value of the current antimalarials: side effects of drugs and resistance. Current efforts in drug development and discovery are being coordinated through several research networks including the Medicines of Malaria Ventures (MMV, www.mmv.org) and the South African Malaria Initiative (www.sami.org.za). The goal of these organizations is to reduce the burden of malaria by discovering, developing and facilitating delivery of new, effective and affordable antimalarial drugs for disease-endemic countries (*49*).

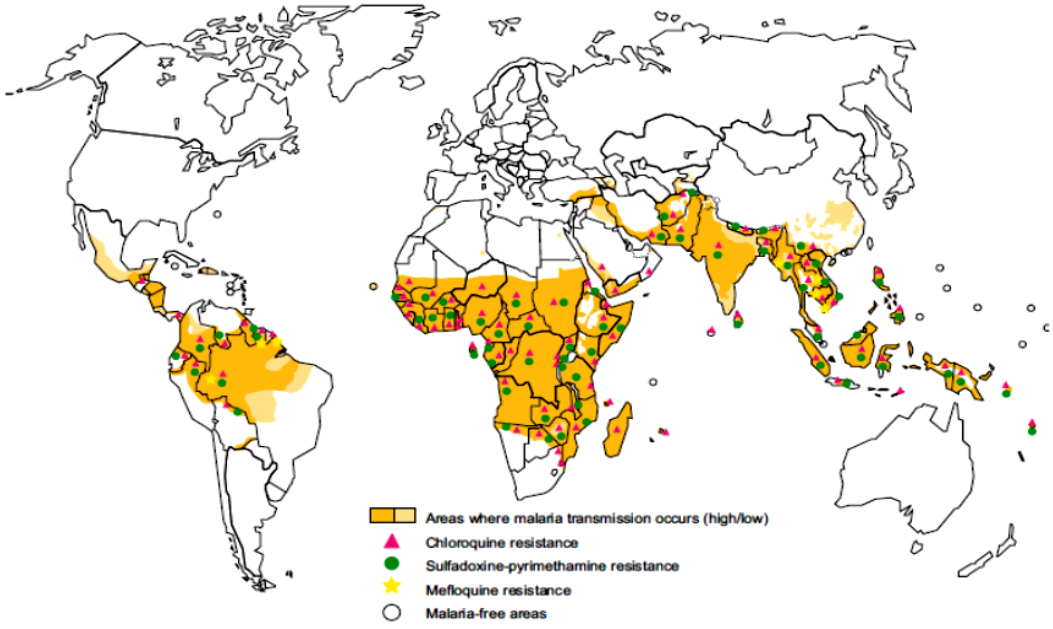


Figure 1.4: The global distribution of malaria parasite transmission and drug resistance of *P. falciparum* antimalarials. Resistance is shown for currently used antimalarials including chloroquine, mefloquine and sulfadoxine-pyrimethamine (World Malaria Report 2005).

Novartis identified spiroindolones as a novel chemotype and optimized the series to deliver NITD-609, currently in Phase II trials (*50*, *51*). The target for NITD-609 was determined to be the cation channel *Pf*ATPase4 (*50*) with an excellent activity against *P. falciparum* 3D7 (IC_{50} = 0.7 nM) and is 100% orally bioavailable in mouse and rat. In mice, NITD-609 has an ED_{50} of 1.2 mg/kg and is therefore more potent than artesunate (ED_{50} = 6.2 mg/kg) and chloroquine (ED_{50} = 1.9 mg/kg). A single dose of 100 mg/kg of NITD-609 afforded a complete cure against *P. berghei* mouse models. It was also found to be active against gametocytes and blocks transmission to mosquitoes, with no cardiotoxicity or genotoxicity liabilities (*52*, *53*). The MMV selected the spiroindolones project as project of the year in

2009. Another example was from Actelion, delivering ACT-21615 (*54*). This compound completely cured *P. berghei* infections after 3 consecutive oral daily doses of 750 mg/kg (ED_{90} = 54 mg/kg/day), and was found to be successful in recently established SCID mice of *P. falciparum* (ED_{90} = 8.4 mg/kg/day). No acute toxicity was observed. The primaquine derivative tefanoquine is currently in Phase III clinical trials and has proven activity against hypnozoites. Tafenoquine has the same G6DP deficiency liability as primaquine, but has the advantage of being a single-dose drug (*9*). In order to provide novel 8-aminoquinoline drugs without the G6DP deficiency a screen was done and imidazolopiperazines were found as a new hit series optimized to give GNF-179 currently in Phase I clinical trials (*55*). GNF-179 has an EC_{50} = 6 nM against 3D7 and an EC_{50} = 5 nM against the liver stage of murine *P. yoelli*. GNF-179 is also orally bioavailable in mice and reduces *P. berghei* parasitemia by 99.7% at 100 mg/kg. A single dose of 15 mg/kg of GNF-179 was found to be completely protective in mice infected with *P. berghei* sporozoites.

Due to artemisinin resistance a quest for fully synthetic peroxides with better pharmacokinetics was started by a research team from the University of Nebraska Medical Center, the Swiss Tropical and Public Health Institute, and the Centre for Drug Candidate Optimisation in Melbourne, which found over 700 adamantane-based ozonides all with low nanomolar activities and striking antiplasmodial selectivity *in vitro (51, 56).* An ozonide compound, OZ439, was found to have excellent activity as well as a longer half-life of elimination. OZ439 was found to be more effective than the semisynthetic artemisinin-artesunate and other comparable drugs, resulting in a single-dose cure at 20 mg/kg against *P. berghei (56).* The potent efficiency of OZ439 is thought to be due to its prolonged plasma exposure demonstrated in both preclinical animal models as well as human volunteers. OZ439 recently finished Phase I clinical trials with results showing no toxicity. Apart from the peroxide bond, OZ439 has no similarities to artemisinin, increasing hope that it may be active against artemisinin-resistant parasites. Currently OZ439 is in Phase II clinical trials and has demonstrated equally well in both *P. falciparum* and *P. vivax* patients. This compound has the potential to meet the MMV's criteria of a single-dose oral drug (Figure 1.5) (*51*).

Further requirements in the target product profile of new antimalarials are oral bioavailability, affordability, activity against *P. vivax* and other human-pathogenic species of *Plasmodium*, and ideally cure with a single-dose treatment. The ability to block the transmission stages is another

important point in line with the malaria eradication agenda. Therefore, more compounds with novel structures and mechanisms of actions are required.

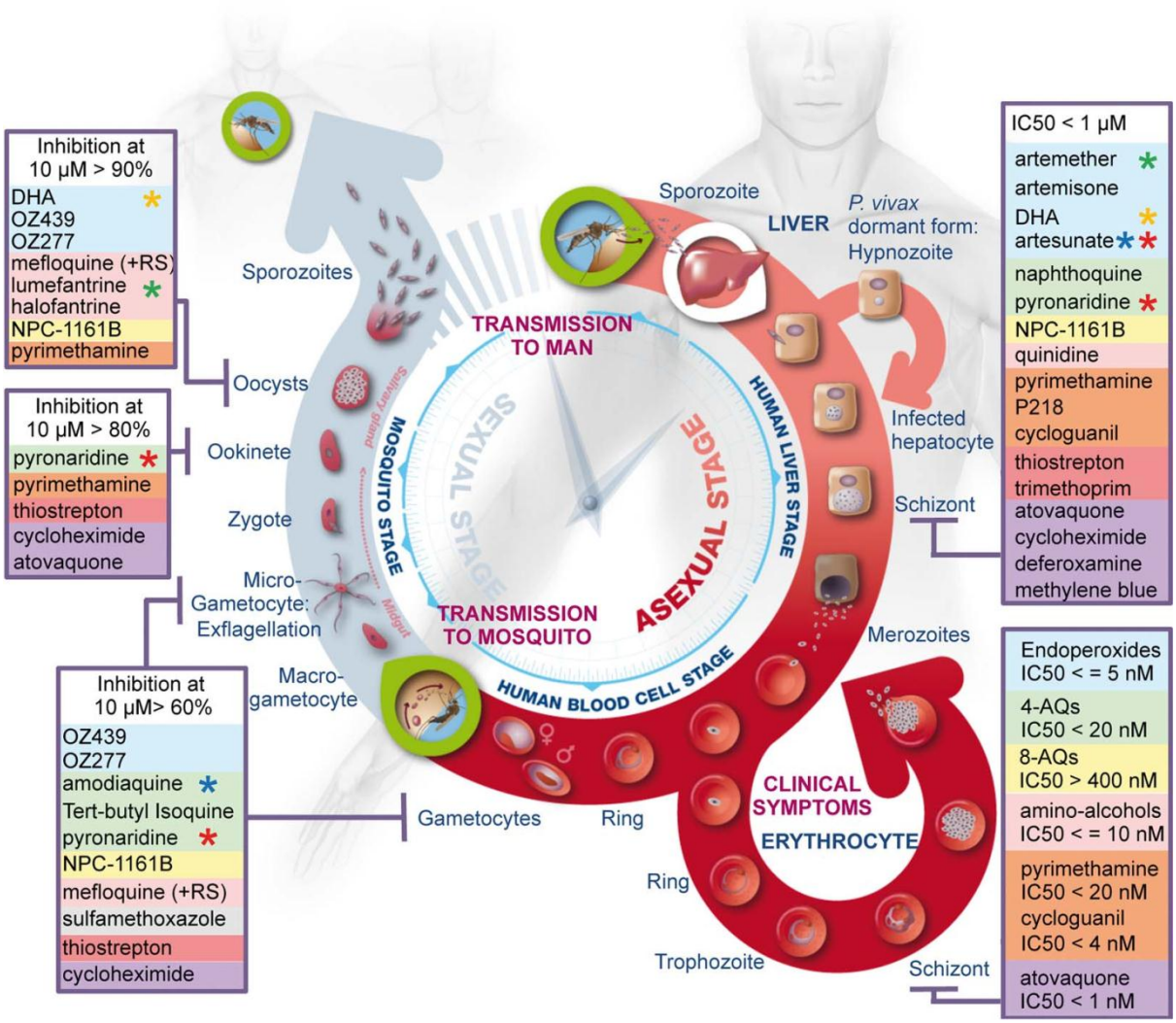


Figure 1.5: Summary of antimalarials used during all stages of *P. falciparum*'s life cycle (*57*)

Malaria (*Plasmodium* species), African trypanosomiasis (*T. brucei* and *T. cruzi*), and leishmaniasis (*Leishmania donovani*), amongst others, are parasitic diseases that affect people globally and in all these organisms polyamine biosynthesis has been identified as drug targets (*58*). This is due to the fact that depletion of polyamines results in the disruption of a variety of cellular functions and in specific cases result in cytotoxicity (*59*). One class of promising, novel antimalarial agents include inhibitors of polyamine biosynthesis as well as polyamine analogs (*4*, *60*)

1.4 Polyamine properties and functions

In 1678, Antonie van Leeuwenhoek isolated crystals from human semen, thereby discovering polyamines. However, the empirical formula for these crystals was only deduced in 1924 followed by chemical synthesis two years later (*61*). Polyamines are aliphatic, low-molecular weight nitrogenous bases, which carry a positive charge on each nitrogen atom at physiological pH. (*61*). The natural polyamines putrescine (1,4-butane diamine), spermidine (*N*-(3-aminopropyl)-1,4-butane diamine) and spermine (*N,N'*-bis(3-aminopropyl)-1,4-butane diamine) (Figure 1.6) are organic polycations found in milimolar concentrations in both eukaryotic and prokaryotic cells. Putrescine is divalent, spermidine is trivalent and spermine, tetravalent (*62*). They interact with the majority of negatively charged components of membranes, both electrostatically and covalently, and therefore have a variety of cellular functions including: stabilization of DNA, RNA, phospholipids and other proteins; altering the structure and function of DNA; transcription; RNA modification; protein synthesis; modulation of enzyme activities; anti-oxidant, anti-apoptotic and metabolic regulatory functions and inhibitory properties in the cell cycle (*61, 63, 64*).

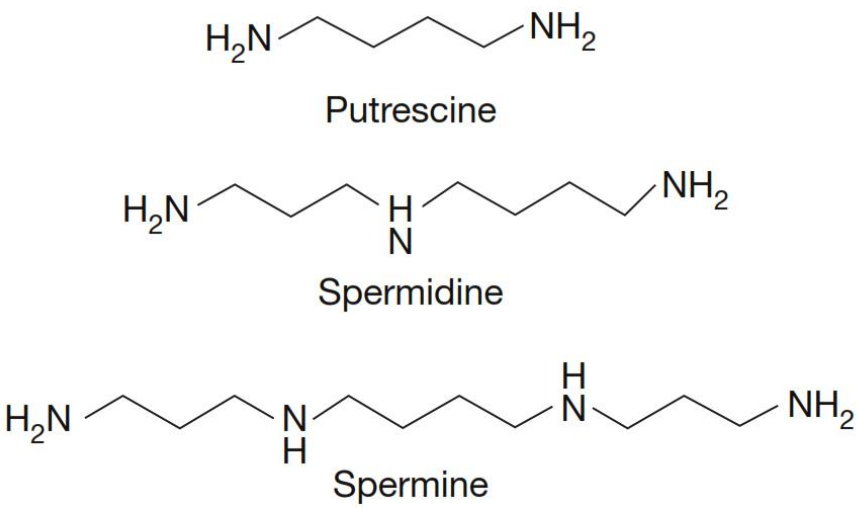


Figure 1.6: Structures of putrescine, spermidine and spermine (*65*).

In proliferating cells, for example cancerous cells and parasitic organisms, polyamines and their biosynthetic enzymes are observed in higher concentrations and are essential metabolites for cell survival. Therefore, polyamine metabolism has been reported to be a potential drug target (*66*). Polyamines have also been shown to be a major metabolite present in malaria parasites, constituting about 14% of the total *P. falciparum* parasite's metabolome

(*67*). Human erythrocytes, on the other hand, only contain trace amounts of polyamines with concentrations ranging between 5-50 µM. During the development of trophozoite stage parasites in *P. falciparum*, there is a significant increase in polyamine levels proportional to an increased parasitemia. Spermidine is the major metabolite present at all stages in the malaria parasite at a maximal concentration of about 6 mM. After spermidine, putrescine is found at a concentration of about 3 mM and only trace amounts of spermine can be seen with a concentration of about 0.5 mM within the parasite (*67*, *68*).

1.5 Polyamine metabolism

There are important interspecies differences in polyamine biosynthesis amongst eukaryotic cells, bacteria and protozoa. Some pathogenic organisms possess enzymes involved in biosynthesis, which are not present in the host and therefore can be valuable targets for the design of therapeutic drugs. The primary precursors of polyamine metabolism in mammalian cells are two amino acids, L-ornithine and L-methionine. L-ornithine is taken up by the diet and decarboxylated by the rate-limiting enzyme ornithine decarboxylase (ODC) to form putrescine. L-methionine is converted to *S*-adenosyl-L-methionine (AdoMet) and decarboxylated by another rate-limiting enzyme, AdoMet decarboxylase (AdoMetDC) to form decarboxylated *S*-adenosylmethionine (dcAdoMet). dcAdoMet is then used by spermidine synthase, donating its aminopropyl to form spermidine and 5' methylthioadenosine (MTA). A second aminopropyltransferase reaction transfers an aminopropyl to spermidine to form spermine and MTA catalyzed by spermine synthase. During the process of inter-conversion and degradation in mammalian cells, spermidine and spermine are acetylated by spermidine/spermine N^1-acetyltransferase (SSAT) using acetyl-CoA to form N^1-acetylspermidine and –spermine (Figure 1.7). These compounds are oxidized by N^1-acetylpolyamine oxidase (APAO) to spermidine and putrecine in a reaction producing H_2O_2 and 3-acetamidopropanal. Spermine can also be converted back into spermidine using spermine oxidase (SMO), producing *N*-acetyl-3aminopropanaldehyde and H_2O_2 (*69*).

The blockade of polyamine biosynthesis by α-difluoromethylornithine (DFMO) targeting ODC activity was successfully used against trypanosomes (causing West African sleeping sickness) due to the rapid proliferation of protozoan parasites during infections, together with the high demand of polyamines for cell proliferation (*70, 71*). Due to the fact that

trypanosomes depend on a unique trypanothione system, they are particularly susceptible to the blockage of polyamine biosynthesis. This system consists of a spermidine glutathione conjugate essential for the removal of toxic hydrogen peroxides. The success of DFMO in the polyamine biosynthesis pathway of trypanosomes has encouraged polyamine research in various other protozoan pathogens (*72*) including the human malaria parasite. During the last three decades polyamine metabolism has gained much attention as a target for antimalarial drug design (*73*).

Polyamine biosynthesis in the *Plasmodium* parasite has been found to exhibit several unique aspects, and is much simpler compared to mammalian cells. In *P. falciparum*, a single open reading frame encodes a bifunctional protein with the AdoMetDC domain on the N-terminal connected to the C-terminal domain (ODC) with a hinge region (*74*). The AdoMetDC/ODC bifunctional protein differs from the mammalian counter parts in the response to putrescine. The ODC activity of *P. falciparum* is more strongly feedback regulated by putrescine than in mammalian cells and AdoMetDC activity is not stimulated by putrescine like the mammalian enzyme. Another difference between the enzymes is the half-life, where the bifunctional protein has a half-life of about 2 h with the mammalian enzymes having very short half-lives of 15 and 30 min for ODC and AdoMetDC, respectively (*72*).

The bifunctional protein, AdoMetDC/ODC, occupies the central position of the polyamine pathway, where two branches starting from L-arginine and L-methionine are combined. This combination produces putrescine using arginase, it produces spermidine using spermidine synthase (SPDS), which then produces spermine. Spermine synthase, however, is lacking but SPDS is able to add another aminopropyl group to form spermine, although at a much lower rate (Figure 1.7). dcAdoMet is also formed from AdoMet using the bifunctional enzyme, AdoMetDC/ODC. Furthermore, MTA is recycled into the purine pool in a parasite unique pathway through adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) (*73*, *75*). The lack of a polyamine oxidase, spermidine/spermine acetyltransferase and an inter-conversion pathway is important to state. The unique bifunctional protein found only in plasmodia has a unique advantage to coordinated transcription and translation. Protein structure, organization and regulation of both activities clearly distinguish the plasmodial AdoMetDC/ODC from the mammalian proteins, which may lead to parasite specific inhibitors.

Although DFMO is used against sleeping sickness, it was found to be clinically ineffective against *Leishmania* and *Plasmodium*. However, some new ODC inhibitors have been shown to be more potent than DFMO against the AdoMetDC/ODC bifunctional protein of *P. falciparum.* A distinct class of 3-aminooxy-1-propane (APA) and its analogs were screened and found to have 1000 fold stronger antiplasmodial activity compared to DFMO. On the enzyme level APA also exhibits a much higher affinity towards the plasmodial ODC than DFMO with K_i values in the low nanomolar range (*72*). However, these drugs failed as antimalarial drugs as they could not decrease parasitemia in *P. berghei* infected mice after 4 days of treatment at a dosage of 100 mg/kg/day.

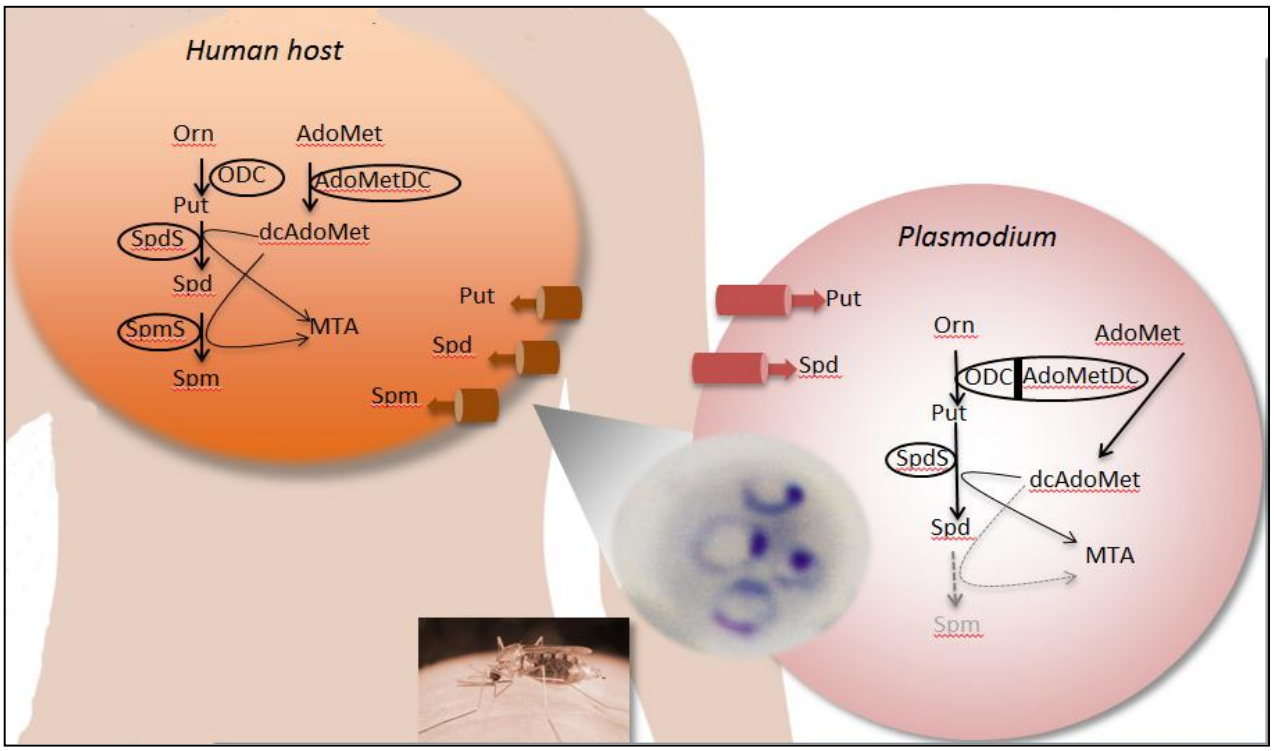


Figure 1.7: Polyamine biosynthesis in *P. falciparum* parasites *(76)*. During polyamine biosynthesis the bifunctional *P. falciparum* AdoMetDC/ODC enzyme catalyzes the production of putrescine and dcAdoMet. From this the production of spermidine and spermine is catalyzed by the rate-limiting enzyme, spermidine synthase. MTA, which is also formed by this reaction, then enters the methionine recycling pathway. The malaria-causing *Plasmodium* parasites illustrated above are transmitted by the female *Anopheles* mosquito (*73, 76*).

Encouraging results were also found with the AdoMetDC inhibitors, CGP 40215A and CGP 48664A, designed by Ciba-Geigi (now Novartis). Both were found to be active against African trypanosomiasis, however, CGP 40215A failed as a drug as it failed to cross the blood brain barrier and CPG 48664A cured *T. brucei* infection in mice and monkeys and underwent further drug candidate studies (*68, 73*). When these compounds were analyzed for antimalarial properties, both revealed potent inhibition of the plasmodial bifunctional enzyme

AdoMetDC/ODC (K_i values in the low nanomolar and low micromolar ranges respectively) as well as *P. falciparum in vitro* growth. When applied against *P. berghei* infected mice, a single dose of 50 mg/kg reduced parasitemia by 50%. A change in the schedule to 25 mg/kg twice daily for 2-3 days lead to a reduced parasitemia of 90-99% and resulted in a curative effect by prolonged survival of mice (*73*). Another candidate CGP 48664, now SAM 486, underwent clinical trials in multiple cancers as single and combination drugs. SAM 486 has been found to inhibit HIV-1 replication through depletion of spermidine levels, compromising hypusine modifications and thus the activation of eukaryotic initiation factor 5A. Reduction in spermidine levels has also been tested for novel antimalarial targets (*73*). 4MCHA, a highly selective SPDS inhibitor, showed good activity on target and whole parasite level, with an IC_{50} of 35 µM against *P. falciparum in vitro*. It has been reported to irreversibly lower the spermidine concentration in treated HTC cells and rat tissues. At a dosage of 25 mg/kg daily for 4 days, 4MCHA had no effect on the parasitemia or the survival of the infected mice (*77*).

To strengthen the curative potential of DFMO, strategies of attacking additional targets in polyamine metabolism by using combination therapies were investigated. DFMO was used in combination with bis(benzyl) polyamine analogs, which are able to displace natural polyamines from their binding sites. This is triggered by the depletion of intracellular polyamines due to the inhibition of the polyamine synthesis pathway with DFMO. Co-administration of DFMO with the polyamine analog, MDL 27695, cured *P. berghei* infected mice. In addition, these mice were found to be immune upon re-infection with the parasites (*42*). These results therefore yielded the most promising to date and initiated a strong focus in polyamine analogs as antimalarial compounds.

1.6 Polyamine analogs

The development of polyamine analogs was pioneered by Porter, Bergeron and colleagues in the 1980's with the generation of symmetrically substituted analogs like *N,N*-bis(ethyl)polyamines (*61, 78*). In the 1990's, polyamine analogs were further synthesized by Woster's group, bringing about a second generation, unsymmetrically substituted compounds like N^1-ethyl-N^1-[(cycloheptyl)-methyl]-4,8-diazaundecane (CHENSpm) (*79, 80*).

Polyamine analogs, which are multi-site inhibitors of the polyamine pathway, are sufficiently similar in structure to natural polyamines to allow recognition and uptake by the polyamine transporter and negative regulation of ODC and AdoMetDC activities (*61*). However, they are usually dissimilar enough from the natural polyamines not to functionally substitute for the latter (*79, 81, 82*). Polyamine analogs usually have terminal primary amine groups, with length variations in the intermediate carbon chains. Terminal nitrogens of linear polyamines are most important for conserving the biological function of the natural polyamines. Two categories of polyamine analogs exist, both of which are taken up into the cells by the polyamine transport process: polyamine anti-metabolites and polyamine mimetics. The anti-metabolites result in significant reduction of the natural polyamine pool through decreased synthesis and increased catabolism and export, subsequently leading to decreased cell growth. Polyamine anti-metabolites can be designed with altered pK_a values at terminal nitrogens to disrupt polyamine biosynthesis and therefore such agents are investigated for potential therapeutics *in vivo* and *in vitro* (*83*). The mimetics decrease cell growth without significant polyamine pool reduction, rather displacing the natural polyamines from their binding sites without substituting their cellular functions and thereby leading to cytotoxicity and cell death (*83, 84*). Further attempts to develop polyamine analogs as modulators of polyamine function focused on synthesis of symmetrical, terminally substituted bis(alkyl) polyamines.

The most successful symmetrically substituted polyamine analogs to date acting as potential anti-tumor agents are the *N*-*N*-bis(ethyl)polyamines, which are terminally alkylated analogs of either spermine or spermidine, namely bis(ethyl)norspermine (BENSpm), bis(ethyl)spermine (BESpm), bis(ethyl)homo-spermine (BEHSpm) and 1,20-(ethylamino)-5,10,15-triazanona-decane (BE-4×4) (Figure 1.8) (*79*). These compounds were tested for their ability to inhibit tumor cell growth, change polyamine content and inhibit ODC and AdoMetDC. BENSpm, which is perhaps the most successful symmetrical alkylpolyamine, causes down-regulation of ODC and depletion of cellular polyamines acting as an antimetabolite. This then results in cytotoxicity in cancer cells. BENSpm is currently undergoing Phase II clinical trials for use against various human solid tumors. BENSpm is metabolized by *N*-deethylation and the removal of aminopropyl equivalents, and has a half-life of approximately 73 min (*85*). BEHSpm is currently being examined as treatment for AIDS-related diarrhea (ARD) (*86*). Polyamines are known to reduce gastrointestinal motility

and BEHSpm has been tested in several animal models and human clinical trials involving ARD. BEHSpm is metabolized almost entirely to homospermine with a half-life of 15.4 days (*87*). Chronic use of BEHSpm could result in accumulation of the analog in the tissue leading to the disruption of normal polyamine metabolism. Therefore, a strategy was developed to increase the clearance rate or half-life of the analog *in vivo*. Molecular modeling showed that the 3,12-dihydroxy derivative of BEHSpm has similar effects to BEHSpm regarding ADR activity but exhibit a significantly reduced tissue half-life due to the hydroxyl groups. BE-4×4 was designed based on the hypothesis that analogs with a different chain length from spermine can increase DNA binding and therefore display antiproliferative effects. BE-4×4 has been shown to be effective against various cancer cell lines including: U-251, MG, SF-126 and SF-188 brain tumor cells at a concentration of 5 µM (*88*) as well as DU-145, LNCaP and PC-3 prostate cancer cells (*79*).

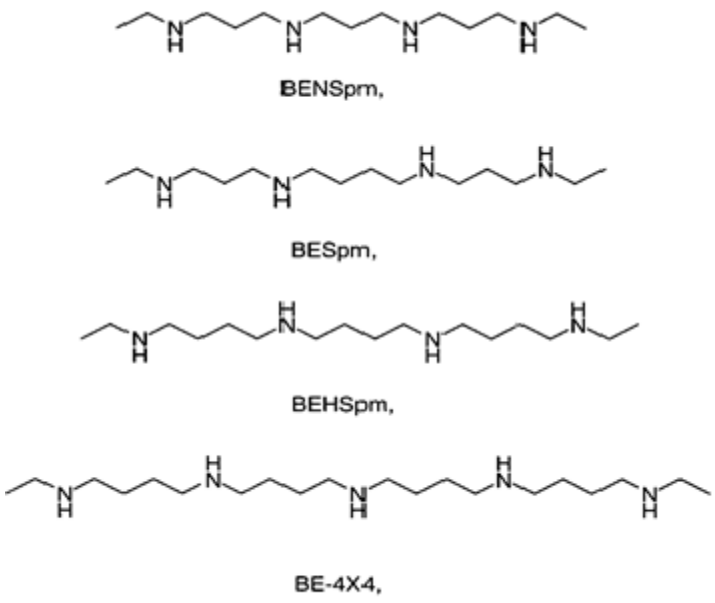


Figure 1.8: Structures of symmetrically substituted bis(alkyl)- and bis(aralkyl)polyamine analogs. BE-4×4 is also amongst the most effective compounds with four aminobutyl moieties and has been found active against brain tumors at a concentration of 5 µM. Adapted from: (*79*).

The second generation polyamine analogs are unsymmetrically substituted analogs, N^1-ethyl-N^{11}-((cycloheptyl)methyl)-4,8-diazaundecane (CHENSpm), N^1-ethyl-N^{11}-((iso-propyl)methyl)-4,8-diazaundecane (IPENSpm) and N^1-ethyl-N^{11}-(cyclo-propyl)methyl-4,8-diazaundecane (CPENSpm) (*79, 89*). These polyamines are mostly spermine analogs with an alkyl group added to the C-terminus and a larger substituent added to the N-terminus. However, synthesis of these analogs requires selective protection and

deprotection of the internal and external nitrogens and is therefore much more difficult to obtain. The latest generation of analogs includes conformationally-restricted, cyclic and long-chain oligoamine analogs, based on the first successful spermine analogs, BENSpm, BEHSpm and BESpm.

These symmetrical and unsymmetrical polyamine compounds illustrate the fact that small structural differences in alkyl polyamine analogs can lead to significant changes in biological activity (*79*). Conformational restriction of the central carbon chain of alkyl polyamines or insertion of a central dimethylsilane group results in significant decrease in growth inhibition compared to the bis(ethyl)-polyamine analogs (*90, 91*). Structure-activity relationship (SAR) correlations have suggested that monoalkylation at both terminal nitrogens of spermine and spermidine is important for antiproliferative activity and alkylation at internal nitrogen reduces *in vitro* activity (*79, 80*). The presence of terminal amines were found to be critical for transport of the analogs (*81*). Analogs substituted with terminal nitrogen bis(alkyl) substituents larger than ethyl led to a reduction in antitumor activity. Moreover, 3-3-3 carbon backbones and spermine-like compounds were more effective against tumor cells than 3-4-3 backbones and spermidine-like compounds, but were inactive against cultured trypanosomas (*81*). Analogs with a 3-7-3 carbon backbone were found inactive against tumors with promising antitrypanosomal and antiplasmodial activities *in vitro* in the μM range. Therefore, it was found that it is possible to synthesize alkyl polyamine analogs of high potency that specifically target parasitic cells (*92*)

1.7 Polyamine analogs against malaria parasites

A series of bis(benzyl) polyamine analogs containing aromatic terminal substitutions were developed for and tested against malaria parasites. It was found that they had marked antimalarial activity and appear to act by regulation of the polyamine biosynthetic pathway (*92*). An aromatic structured compound (MDL 27695) emerged as a promising lead in the search for novel antiparasitic agents (Figure 1.9)(*79, 92*). MDL 27695 combined with DFMO against cultured *P. falciparum* D6 strain showed an additive effect, inhibiting erythrocytic schizogony in *P. falciparum in vitro* and *P. berghei in vivo*. Mice cured with this combination therapy were re-infected with *P. berghei* after 4 months and were found to be immune to re-infection with only temporary parasitemia obtained.

MLD 27695 was also combined with chloroquine, resulting in an additive effect against *P. falciparum*, reducing the parasitemia by 80% (*93*).

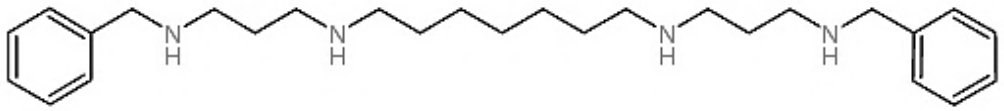


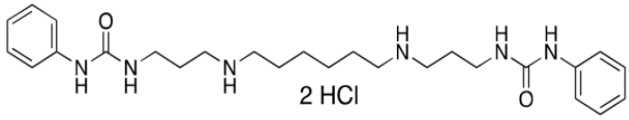
Figure 1.9: Structure of MDL 27695 (*79*).

Based on the success of the terminally (bis)alkylated polyamine analogs against cancer and other parasites, a new generation (bis)urea and (bis)thiourea alkylated isosteres were synthesized and evaluated for their antimalarial properties (*4*). These analogs vary in their carbon backbones and their terminal urea/thiourea substituents are symmetrically substituted aralkyl substituents. When tested for their possible growth inhibitory effect against intra-erythrocytic *P. falciparum* parasites, most showed *in vitro* inhibitory activity at concentrations below 3 μM (*4*). This was tested on both drug resistant (W2, chloroquine and HB3, antifolate) and drug sensitive (3D7) strains of *P. falciparum* parasites. One compound, containing a terminal (bis)diphenylpropyl-guanidine moiety, had inhibitory activity against *P. falciparum* (3D7) at a concentration of 298 nM (*4*). Data analysis on the first generation of (bis)urea and (bis)thiourea alkylated polyamine analogs suggested that the most active compounds had either 3-7-3 or 3-4-3 carbon backbones, with 3-7-3 compounds having the best activity against the parasite. (Bis)urea compounds had the best antimalarial activities, and diphenylpropyl substituents were more effective than the diphenylethyl analogs. It was also found that 3-3-3 and 3-4-3 carbon backbones were not effective against the parasite, whereas 3-7-3 carbon backbones were effective in the nM range, particularly when containing a terminal diphenylpropyl substituent (*4*).

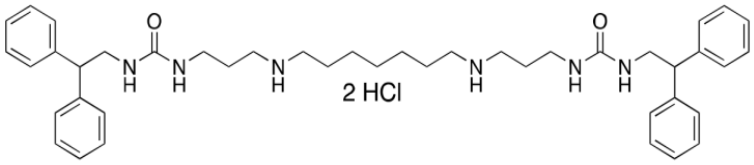
Based on these criteria, second generation symmetrical, terminally alkylated compounds were synthesized containing 3-6-3, 3-7-3 and 3-4-3 carbon backbones with a variety of terminal substituents. These polyamine analogs showed IC_{50} values ranging between 88-846 nM. The most active compounds against *P. falciparum* parasites (3D7), with IC_{50} values of 88-210 nM were all compounds with 3-6-3 and 3-7-3 carbon backbones (Figure 1.10), the majority with (bis)urea and terminal aralkyl substituents (*4*). Selection of terminal substituents of these compounds is very important in enhancing their

antimalarial activity. Compounds with terminal phenyl rings were the most active, followed by diphenylethyl groups, benzyl rings and lastly diphenylmethyl groups. Analogs without terminal aralkyl groups were the least effective, illustrating the importance of bulky terminal substituents.

Polyamines were found to have an important role in DNA replication and therefore implied life cycle development in *Plasmodia* (*94*). The effects of polyamine analogs on the parasite's ability to replicate its DNA were monitored and it was found to induce arrests in schizogony and associated nuclear division (*4*). Polyamine analogs with 3-7-3 carbon backbones had the greatest inhibitory effect on DNA replication and nuclear division with the parasites being confined to the ring stage (*4*).



3-6-3 (bis)urea



3-7-3 (bis)urea

Figure 1.10: Symmetrical, terminally alkylated polyamine analogs. Illustrated in this figure are two second generation polyamine analogs with the best antimalarial activities (lowest IC_{50} values) (*4*).

The cytotoxicity of these analogs were found to be independent of changes in the polyamine pool, which may be due to the ability of the analogs to block intracellular binding sites of the natural polyamines or displace intracellular polyamines from their binding sites, therefore acting as polyamine mimetics. Therefore the mode of action of these compounds may be independent of the polyamine pathway. This series of polyamine analogs were therefore implicated as highly selective antimalarial agents (>7000 fold lower IC_{50} against *P. falciparum* compared to mammalian liver carcinoma cells) with the majority showing parasite IC_{50} values <500 nM (Figure 1.10). In most instances the first- and second-generation analogs elicited a significant irreversible, cytotoxic response in the parasite measured by a decrease in cell viability.

In the above series of compounds, 3-5-3 backbone polyamine analogs were not included. However, 3-5-3 symmetrically alkylated polyamine analogs were found to have good antiparasitic activity against human African trypanosomiasis caused by the parasites *Trypanosoma brucei gambiense* (West African trypanosomiasis) and *Trypanosoma brucei rhodesiense* (East African trypanosomiasis) with IC_{50} values of 90 nM (*95*). Based on this activity as well as the above mentioned success of both first generation and second generation polyamine analogs, a third generation, symmetrically alkylated (bis)thioruea and (bis)urea polyamine analogs with 3-5-3 carbon backbones were synthesized to determine the antimalarial properties of these analogs on *P. falciparum* parasites.

1.7 Hypothesis

Symmetrical, terminally alkylated, 3-5-3 carbon backbone (bis)urea and (bis)thiourea polyamine analogs will have potent *in vitro* antiplasmodial activity against the human malaria parasite, *P. falciparum.*

1.8 Research Objectives

1. Determining the *in vitro* antiplasmodial activity of alkylated polyamine analogs on the malaria parasite, *P. falciparum.*
2. Elucidating the mode of action of the alkylated polyamine analogs on *P. falciparum* parasites.

The following manuscript resulted from this study:

1. Marna de Beer, Bernice Barnard, Bianca Verlinden, Warren Andayi, Colin Dubick, Ethan Marrow, Boobalan Pachaiyappan**,** Lubbe Wiesner, Timothy Egan, Kiplin Guy**,** Patrick M. Woster and Lyn-Marie Birkholtz. (2013). Novel symmetrical, terminally alkylated polyamine analogs with potent antimalarial activity against *Plasmodium falciparum.* In preparation for: Journal of Medicinal Chemistry.

Research findings were also presented at the following international conferences:

1. M. de Beer, B. Verlinden, P. Boobalan, P. Woster and L. Birkholtz. Anti-malarial properties of novel symmetrical, terminally alkylated polyamine analogs against *P. falciparum.* H3D conference: Drug Discovery and Development. Cape-Town, South Africa. October 2012. Presented as a poster presentation.

2. M. de Beer, B. Verlinden, P. Boobalan, P. Woster and L. Birkholtz. Anti-malarial properties of novel symmetrical, terminally alkylated polyamine analogs against *P. falciparum.* Malaria Gordon Research Conference: Molecular and Cellular Biology of Malaria. Tuscany, Italy. August 2013. Presented as a poster presentation.

3. M. de Beer, B. Verlinden, P. Boobalan, P. Woster and L. Birkholtz. Anti-malarial properties of novel symmetrical, terminally alkylated polyamine analogs against *P. falciparum.* 6th MIM PAN-African Conference. Durban, South-Africa. October 2013. Presented as a poster presentation.

Chapter 2: Materials and Methods

2.1 *In vitro* culturing of *P. falciparum* parasites

All culturing procedures were performed under aseptic conditions. *P. falciparum* parasites (strains 3D7, W2 and HB3) were cultivated in synthetic culture media containing 0.5 % Albumax II (Invitrogen), in human erythrocytes (O^+) suspended at 5% hematocrit in RPMI-1640 medium (Sigma-Aldrich) supplemented with 25 mM HEPES buffer (pH 7.5), 200 µM hypoxanthine, 24 µL/mL gentamycin, albumax, 0.2% sodium bicarbonate and 20 mM D-glucose to ensure optimal parasitic growth (*96*). Cryopreserved parasites were thawed at 37°C to which 0.2 mL of freshly made 12% (w/v) NaCl was added and mixed well. This was transferred to a 15 mL tube and incubated at room temperature for 5 min. After this, 1.8 mL sterile 1.6% (w/v) NaCl was added, mixed well and centrifuged at 3500g for 5 min. The supernatant was removed and fresh media were added to the culture (7 mL), mixed and transferred to previously prepared culture media in a culture flask. The culture was gassed for ± 30 s with a low oxygen special gas mix (5% O_2, 5% CO_2, 90% N_2) and incubated at 37°C with moderate shaking (~60 rpm). This process produces a 5% hematocrit solution in 30 mL culture. Routine culturing requires a daily change of growth medium and maintenance of a 5% hematocrit through addition of new erythocytes and assessment of parasitic growth and parasitemia. Type O^+ blood was collected in a blood bag (Fenwal Primary container with citrate, phosphate, glucose, adenine as anticoagulant, 70 mL anticoagulant for the collection of ± 500 mL blood, Adcock Ingram) and left overnight at 4°C to separate the erythrocytes from the serum and leukocytes. O^+ blood was taken and the serum layer and leukocytes removed by washing 4× in total with 50 mL of 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7.4) with centrifugation at 3500g for 5 min. An equal volume of complete culture media was added to the washed erythrocytes to obtain a 50% hematocrit. The blood was stored at 4°C until use (*97*).

2.2 Sorbitol synchronization

This method is based upon changes in the buoyant density and membrane permeability of parasitized erythrocytes (*98*). Mature parasitized cells are permeable to sorbitol and can

be selectively lysed when incubated with iso-osmolar sorbitol due to insertion of new permeability pathways. Sorbitol synchronization, therefore, leads to the selective lysis of approximately 90-95% of metabolically active trophozoite stage malaria parasites leaving only intact erythrocytes infected with ring stage parasites. (*99*). *P. falciparum* parasite cultures were centrifuged at 3500g for 5 min to pellet the cells, the supernatant removed and 10 mL 5% (w/v) sorbitol (pre-heated to 37°C) added to the cells. The solution was mixed well and incubated at 37°C for 15 min and centrifuged again at 3500g for 2.5 min. The supernatant was decanted and 10 mL complete media was added and mixed properly with the cells to remove any excess sorbitol, centrifuged at 3500g for 2.5 min, the supernatant decanted, the pellet re-suspended in 30 mL RPMI culture media and gassed for ± 30 s.

2.3 Antimalarial activity determination

The *in vitro* antiproliferative activity of compounds against intra-erythrocytic stages of *P. falciparum* parasites (strains 3D7, W2 and HB3) were determined using SYBR Green I-based fluorescence, which measures intercalation of SYBR Green I to DNA molecules allowing the direct correlation between DNA levels and parasite growth. *In vitro* ring stage *P. falciparum*-infected red blood cells (iRBCs) (2% hematocrit, 1% parasitemia) were treated with specific concentrations of terminally alkylated polyamine analogs (*100*) (dissolved in a nonlethal concentration of DMSO (<0.013%) (*4*) and diluted in complete culture medium), with chloroquine disulfate as positive drug control (0.5 µM) compared to untreated parasites. iRBCs (200 µl) were grown statically at 37˚C for 96 h in 96-well plates under hypoxic conditions, after which the cells were re-suspendend and combined with equal volumes (100 µL) of SYBR Green I lysis buffer (0.2 µL/mL 10 000× SYBR Green I Invitrogen, Inc; 20 mM Tris, pH 7.5; 5 mM EDTA; 0.008% (w/v) saponin; 0.08% (v/v) Triton X-100) and fluorescence measured after a 1 h incubation at 37˚C using a Fluoroskan Ascent FL microplate fluorometer (Thermo Scientific, excitation at 485 nm and emission at 538 nm). The data, after background subtraction, were expressed as percentage of untreated control to determine cell proliferation and are representative of at least three independent biological repeats ($n \geq 3$), each performed in triplicate, standard error of the mean indicated. Dose-response curves were plotted using GraphPad Prism 5. From these curves, IC_{50} values (concentrations of the compound resulting in a 50% inhibition of parasite proliferation) were determined.

2.4 Determination of cytotoxicity

Human hepatocellular liver carcinoma cells (HepG2) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with heat inactivated (45 min at 56°C) fetal bovine serum. *In vitro* cytotoxicity was measured using the lactate dehydrogenase assay (LDH), which measures the levels of extracellular LDH released from damaged cells as an indicator of cytotoxicity. LDH is a cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant or blood when the plasma membrane of cells is damaged. Therefore, it has been used as an indicator of cytotoxicity in HepG2 cells with exposure to cadmium chloride, where the release of intracellular LDH into the culture medium is an indicator of irreversible cell death due to cell membrane damage (*101*). This assay consists of two-steps. During the first step, NAD^+ (nicotinamide adenine dinucleotide) is reduced to NADH and H^+ by oxidation of lactate to pyruvate using LDH. During the second step, the newly formed NADH and H^+, is used by the enzyme diaphorase to catalyze the reduction of a tetrazolium salt to a red formazan salt product (*102*). Therefore, the amount of red formazan salt detected at 450 nm is directly correlated to the amount of NADH and LDH released by damaged cells (*103*).

For the LDH assay, 200 000 cells were seeded per well in 96-well plates and grown for approximately 24 h at 37°C after which it was treated with various concentrations (up to 5000× the IC_{50} of the compounds against *P. falciparum*). After 48 h exposure, cells were collected by centrifugation at 600g for 10 min and LDH activity was measured in the supernatant by adding LDH reaction mix (200 µL WST substrate mix, 10 mL LDH Assay Buffer) (LDH-Cytotoxicity Assay Kit II; BioVision) followed by incubation for 20 min at room temperature. Colorimetric detection of NADH levels were performed, using a spectrophotometer (*4*) (Thermo Labsystems, multiskan).

2.5 Viability determinations

Highly synchronized ring *P. falciparum* cultures (2% parasitaemia; 5% haematocrit) were treated for 12 h, with each of the polyamine analogues at their respective IC_{90} values as well as 10× IC_{90}. After treatment, compounds were washed off once with RPMI 1640 culture medium. A 1:40 dilution was made of the washed parasites to assess proliferation over a 2½ day period, after drug treatment, with samples taken at certain time points (6 h, 12 h, 24 h, 36 h, 48 h and 60 h). After each incubation period, lysis buffer, containing

SYBR Green I was added and fluorescence measured at an excitation of 485 nm and emission of 538 nm using the Fluoroskan Ascent FL Fluorometer (Thermo Labsystems).

2.5 Complementary interactions between inhibitors

To examine if there is *in vitro* interaction between the polyamine biosynthesis inhibitor DFMO and the most active polyamine analogs (compound 5 and 6), fixed-ratio isobole analysis were performed as described by Fivelman *et al.* (*104*) Each assay was initiated using ring stage *P. falciparum* parasites and the parasite viability determined after a 96 h incubation treated with polyamine analogs at 37°C. Each well contained a final parasitemia of 1% and 2% hematocrit in a total of 200 µL including the specific compound diluted in culture medium. The fixed-ratios combinations of the compounds used were (compound A: compound B): 100:0; 80:20; 60:40; 50:50; 40:60; 20:80 and 0:100. These combinations were then each serially diluted (2-fold) eight times and incubated for 96 h at 37°C. SYBR Green I fluorescence was determined as a measure of parasite proliferation as described in section 2.3. The fractional inhibitory concentration (FIC) was calculated for each compound as follows: FIC (A) = IC_{50} for (A) in combination/IC_{50} for (A) alone; FIC (B) = IC_{50} for (B) in combination/IC_{50} for (B) alone. Isobolograms were constructed by plotting FIC (A) against FIC (B) for each combination using SigmaPlot 11.0. Three independent biological repeats were each done in triplicate, standard error indicated. This method is based on the Loewe additivity zero-interaction theory described by Berenbaum in 1989 (*105*).

2.6 Determination of parasite DNA replication and nuclear division

The effects of the polyamine analogs on the *P. falciparum* parasite's replication of DNA and nuclear division were measured using flow cytometric analysis. Parasites (2% parasitemia, 2% hematocrit) were treated with the two leading polyamine analogs (compound 5 and 6) at 2× IC_{90} in duplicate and samples (50 µL) were taken at specific time intervals (24, 48, 72 and 96 h), fixed with 1 mL 0.025% gluteraldehyde (nontoxic) for 45 min and stored at 4°C until needed. Gluteraldehyde (900 µL) was removed and 100 µL fixed cells were placed into a 96-well plate, washed at least twice with 1× PBS, re-suspended in 100 µL of 1× PBS and stained using a 1:1000 dilution of SYBR Green I for 30 min in the dark at room temperature. The plate was centrifuged at 500g for 5 min and the cells were re-suspended in 1× PBS in 15 mL tubes. SYBR Green I fluorescence

bound to DNA was measured with a BD FACS Aria I flow cytometer, analyzing 10^6 cells for each sample. Fluorescence emission was collected at an excitation wavelength of 488 nm with 502 nm long-band-pass and 530 nm band-pass emission filters. An uninfected erythrocyte control was used, both stained with SYBR Green I and unstained for gating purposes. Data were analyzed using FlowJo 7.6.5 and Cyflogic.

For extra synchronization during DNA replication studies, the parasites were also run through the VarioMACS CS column during the trophozoite stage. The VarioMACS CS column was developed for depletion of magnetically labeled human and animal cells out of a heterogeneous cell suspension. It has a hydrophilic coating allowing most buffers to be used. By passing the trophozoite stage parasites containing hemozoin-, iron-containing crystals through the column, the suspension is depleted of the magnetically labeled trophozoite parasites and only the ring stage parasites flow through. The column was assembled as described in the manual from MACS Miltenyi Biotec. The column was washed with 50 mL 100% EtOH without running dry and making sure there is no air bubbles left in either the column or the syringe, after which it was also washed with 50 mL $dddH_20$ as well as 50 mL incomplete culture media. A flow resistor (needle) was then placed at the bottom of the column. The trophozoite culture was centrifuged at 3500g for 5 min and the supernatant discarded. The pellet was re-suspended in 15 mL incomplete media and transferred to the column. After the cultured cells were run through the column, the column was washed with incomplete media until the flow through was completely clear. The column was removed from the magnetic field and again washed with 50 mL incomplete media retrieving the trophozoites bound to the magnetic column. After isolation the column was washed again with 50 mL $dddH_2O$ and 50 mL 100% EtOH. Blood slides were made of both the isolated trophozoite and the flow through and stained with Giemsa.

2.7 Inhibition of β-hematin formation

The parasite rids itself of toxic levels of heme released during the degradation of hemoglobin through the lipid-mediated production of hemozoin. The water-lipid interface can be mimicked *in vitro* to measure the formation of β-hematin (a synthetic analog of hemozoin). β-Hematin formation was assayed as described previously (*106*). Briefly, NP-40 detergent (31 μM) was added to polyamine analogs at various concentrations (0-1 M). The samples were then incubated at 37˚C for 5 h with 100 μM

hematin in a 1 M acetate buffer (pH 4.8) with occasional shaking. β-hematin formation was measured using the pyridine-ferrichrome method (*107*, *108*).

2.8 Statistical analysis

Statistical significance was assessed with a two-tailed t-test, carried out using the GraphPad Instat (version 3.06) program. Non-linear regression was performed with SigmaPlot (version 11) or GraphPad Prism (version 5). IC_{90} and IC_{99} values were calculated using ICEstimator (*109*). The t-test investigates the likelihood that the difference between the means of the two groups could have been caused by chance at a 95% confidence interval.

2.9 Determination of drug-likeness

The physiochemical properties and Absorption, Digestion, Metabolism and Excretion (ADME) descriptors were determined *in silico* using the Discovery Studio Modeling Environment (Accelrys Software Inc., release 3.0), which is a comprehensive suite of modeling and simulation solutions for life science researchers, using the default parameters. ADME descriptors in Discovery Studio include models for intestinal absorption, aqueous solubility, blood brain barrier penetration, plasma protein binding, cytochrome P450 2D6 inhibition, and hepatotoxicity. These descriptors perform computational prediction based solely on the chemical structure of the molecule.

Lipinski's rule of 5 was determined using four ADME descriptors: molecular weight, log *P*, hydrogen bond donors and hydrogen bond acceptors to determine whether these compounds are orally bioavailable or not. Violations of Lipinski's rules may not result in poor absorption, but the likelihood of poor absorption increases with the number of rules broken and the extent to which they are exceeded.

The Quantitative Estimate of Drug-likeness (QED) (*110*) was also determined *in silico* using Silicos-it QED (Chemoinformatics Services and Software; Biscu-it). QED values range from zero (unfavorable) to one (favorable). Its functions are based on the underlying distribution data of drug properties and, unlike rule-based metrics, can identify cases in which a generally unfavorable property may be tolerated when other properties are close to ideal.

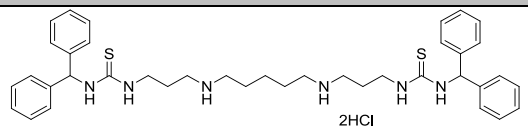
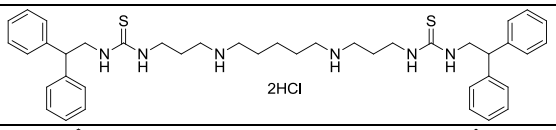
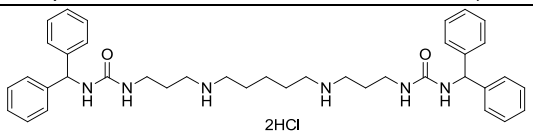
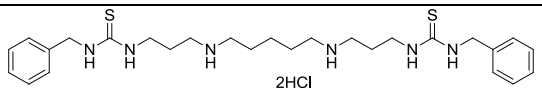
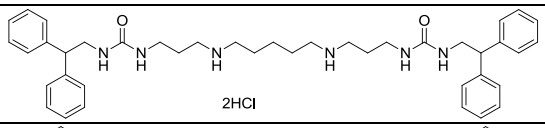
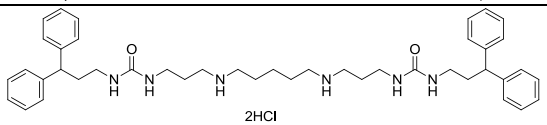
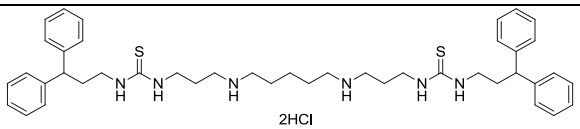
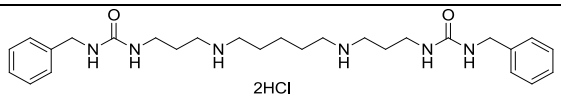
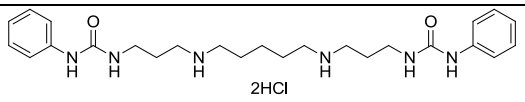
Chapter 3: Results

Based on previously obtained results from the first and second generation symmetrical, terminally alkylated (bis)urea and (bis)thiourea polyamine analogs (*4*), as well as the potent activity obtained against human African trypanosomiasis with the novel 3-5-3 polyamine analog class (Table 3.1) (*95*), the antimalarial properties of this class of polyamine analogs were determined against *P. falciparum* parasites *in vitro* (Compounds obtained from collaborator, Prof. P. Woster, Medical University of South Carolina, USA).

Both (bis)urea and (bis)thiourea polyamine analogs were synthesized by varying the alkylation groups between (bis)diphenyl and (bis)phenyl groups as seen in the second generation polyamine analogs from Verlinden *et al. (4)*. The size, type and flexibility of the aryl group was thought to influence antiplasmodial activity of the compounds, with the (bis)phenyl being most active in the second generation against *P. falciparum* parasites *(4)*. These polyamine analogs from the second generation had good antimalarial activities with IC_{50} values as low as 88 nM, more than 7000 fold selectivity towards the parasite and irreversible cytotoxicity against *P. falciparum* 3D7 (*4*).

Therefore, experiments were performed to determine the antimalarial properties of the third generation polyamine analogs with similar structures to the second generation, modifying the terminal groups accordingly without change in the central carbon-backbone.

Table 3.1: Alkylated 3-5-3 carbon backbone polyamine analogs

Compound No	Compound ID	Chemical Structure	Alkylation	Type
1	BP-107-3		(Bis)diphenyl	Thiourea
2	BP-107-5		(Bis)diphenyl	Thiourea
3	BP-107-7		(Bis)diphenyl	Urea
4	BP-107-9		(Bis)phenyl	Thiourea
5	BP-107-11		(Bis)diphenyl	Urea
6	BP-107-13		(Bis)diphenyl	Urea
7	BP-107-15		(Bis)diphenyl	Thiourea
8	BP-107-17		(Bis)phenyl	Urea
9	BP-107-19		(Bis)phenyl	Urea

1. BP-107-3: 1,1'-((pentane-1,5-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-benzhydrylthiourea)
2. BP-107-5: 1,1'-((pentane-1,5-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-(2,2-diphenylethyl)thiourea)
3. BP-107-7: 1,1'-((pentane-1,5-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-benzhydrylurea)
4. BP-107-9: 1,1'-((pentane-1,5-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-benzylthiourea)
5. BP-107-11: 1,1'-((pentane-1,5-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-(2,2-diphenylethyl)urea)
6. BP-107-13: 1,1'-((pentane-1,5-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-(3,3-diphenylpropyl)urea)
7. BP-107-15: 1,1'-((pentane-1,5-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-(3,3-diphenylpropyl)thiourea)
8. BP-107-17: 1,1'-((pentane-1,5-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-benzylurea)
9. BP-107-19: 1,1'-((pentane-1,5-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-phenylurea)

3.1 Antiplasmodial activity determination

The 3-5-3 series of symmetrical, terminally alkylated polyamine analogs was subsequently tested for their ability to inhibit the *in vitro* proliferation of *P. falciparum* 3D7 parasites (Figure 3.1; Table 3.2). Dose-responses were obtained for all compounds tested against *P. falciparum* 3D7 parasites, with compounds 4, 8 and 9 necessitating high concentrations of compound, ranging between 246–793 µM, before inhibition of parasite proliferation was

observed. Dose-response analyzes were performed to determine the *in vitro* IC_{50} values (concentration at which the compounds resulted in a 50% inhibition of parasite proliferation) of each polyamine analog, as summarized in Table 3.2.

Table 3.2: Antiplasmodial activities against *P. falciparum* 3D7 parasites. Data are representative of three independent biological repeats, each performed in triplicate, ± S.E. indicated.

Compounds	*P. falciparum* 3D7		
	Average IC_{50} (nM)	S.E.	CV[a]
1	68.06	13.0	19.0
2	45.0	3.4	7.5
3	46.7	7.0	14.9
4	248.6	29.6	11.9
5	27.6	4.4	15.9
6	30.0	5.21	17.4
7	59.9	7.4	12.3
8	793.4	78.07	9.8
9	246.21	34.22	13.9
CQ[b]	8.0	1.2	14.7

a. CV = Coefficient of variance

b. CQ = Chloroquine

IC_{50} values in the range of 28-800 nM were obtained when tested against *P. falciparum* 3D7 parasites. The coefficient of variance (percentage error) between independent biological experiments should be less than 20%, allowing for biological variance (*111*), which was true for all the compounds tested in the 3-5-3 series (Table 3.2). The IC_{50} value obtained for chloroquine as control also conforms to literature (*112*). From the series tested, compounds 5 and 6 were the most active against *P. falciparum* 3D7 with the lowest IC_{50} values of 27.6 ± 4.4 nM and 30.0 ± 5.21 nM, respectively. However for the 3-5-3 series the (bis)diphenyl compounds had the most potent antiplasmodial activities, confirming previous results of increased activity with a more bulky terminal end (*79*). However, this is contrasting with findings from the second generation polyamine analogs (*4*).

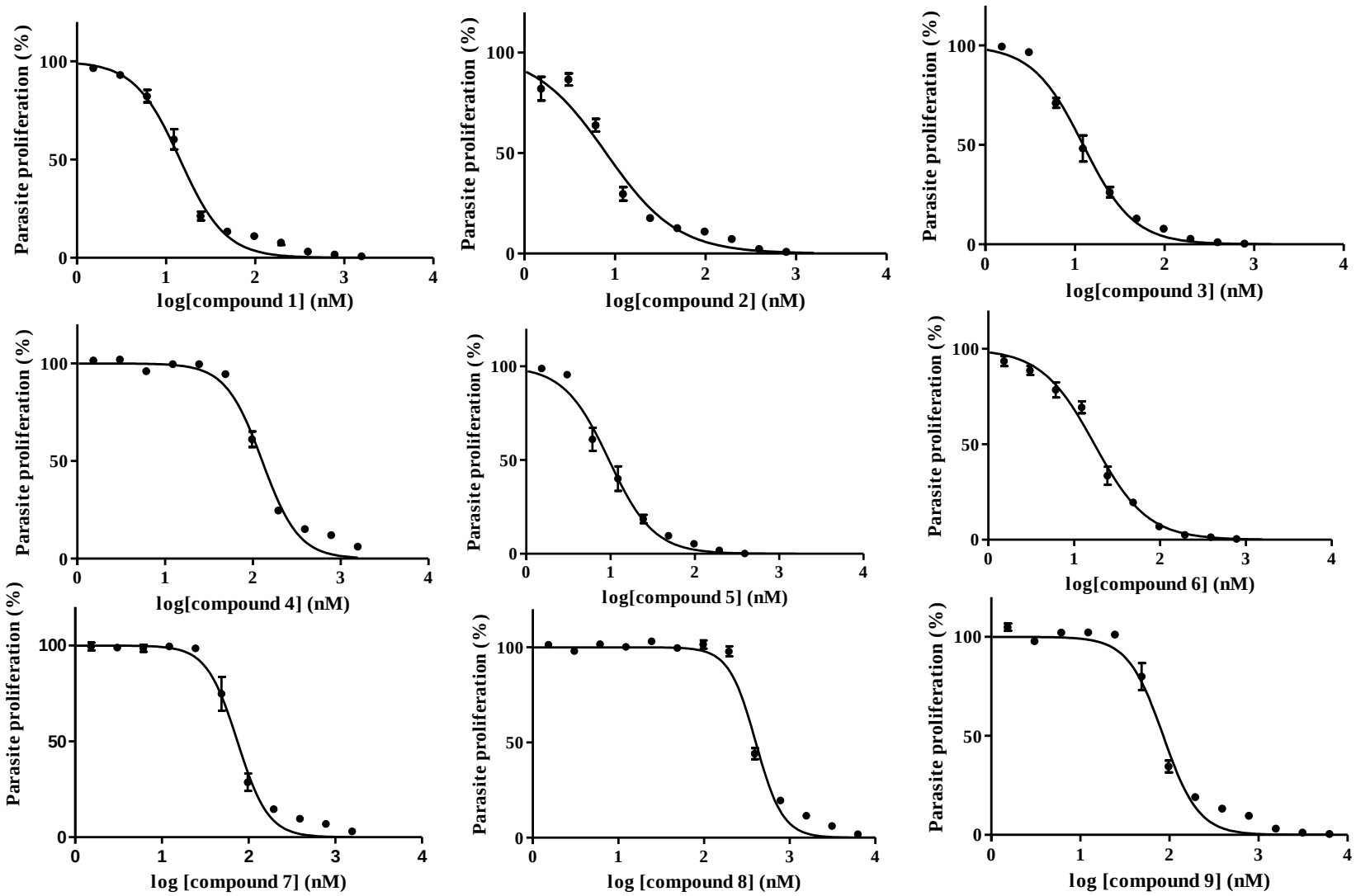


Figure 3.1: Dose-response curves of *P. falciparum* 3D7 parasites after treatment with nine polyamine analogs. Ring stage parasites were treated with each compound series and incubated for 96 h at 37°C, after which parasite proliferation was measured with SYBR Green I, using a fluorometer (excitation at 485 nm and emission at 538 nm). Data representative of at least three independent biological experiments each performed in triplicate. Error bars present ± S.E.

Effective antiplasmodial compounds need to be able to inhibit the proliferation of both drug sensitive and resistant strains of *P. falciparum* parasites *in vitro*. The polyamine analogs were subsequently tested for their ability to inhibit the proliferation of drug resistant *P. falciparum* parasite strains, W2 (chloroquine resistant) and HB3 (antifolate resistant) (Table 3.3). Individual dose-response curves for each compound against these two strains are provided in Appendix 1 (*P. falciparum* W2) and Appendix 2 (*P. falciparum* HB3), respectively.

Table 3.3: Antiplasmodial activities against *P. falciparum* W2 and HB3 strains. Data are representative of four and three independent biological repeats respectively, each performed in triplicate, ± S.E. indicated.

Compounds	*P. falciparum* W2			*P. falciparum* HB3		
	Average IC_{50} (nM)	S.E.	CV	Average IC_{50} (nM)	S.E.	CV
1	34.4	2.0	5.9	74.6	8.7	11.7
2	45.0	1.2	2.7	56.3	3.33	5.9
3	21.2	2.0	9.6	65.0	2.3	3.5
4	36.8	4.5	12.3	178.1	1.5	0.9
5	17.4	2.1	12.1	39.6	2.6	6.5
6	29.8	5.2	17.5	56.3	6.7	11.9
7	50.0	4.6	9.3	61.6	7.1	11.5
8	142.7	20.8	14.6	593.2	20.3	3.4
9	42.1	8.6	20.4	176.7	4.1	2.3
CQ	59.5	8.0	13.4	17.6	3.0	14.5

IC_{50} values in the range of 17-600 nM were obtained against *P. falciparum* W2 and HB3 strains (Table 3.3), with compound 5 again having the lowest IC_{50} at 17.4 ± 2.1 nM and 39.6 ± 2.6 nM against *P. falciparum* W2 parasites (Figure 3.2 A) and *P. falciparum* HB3 parasites (Figure 3.2 B), respectively. Compound 6 was also highly active against both strains, with IC_{50} values of 29.8 ± 5 nM against *P. falciparum* W2 parasites (Figure 3.2 A) and 56.3 ± 7 nM against *P. falciparum* HB3 parasites (Figure 3.2 B).

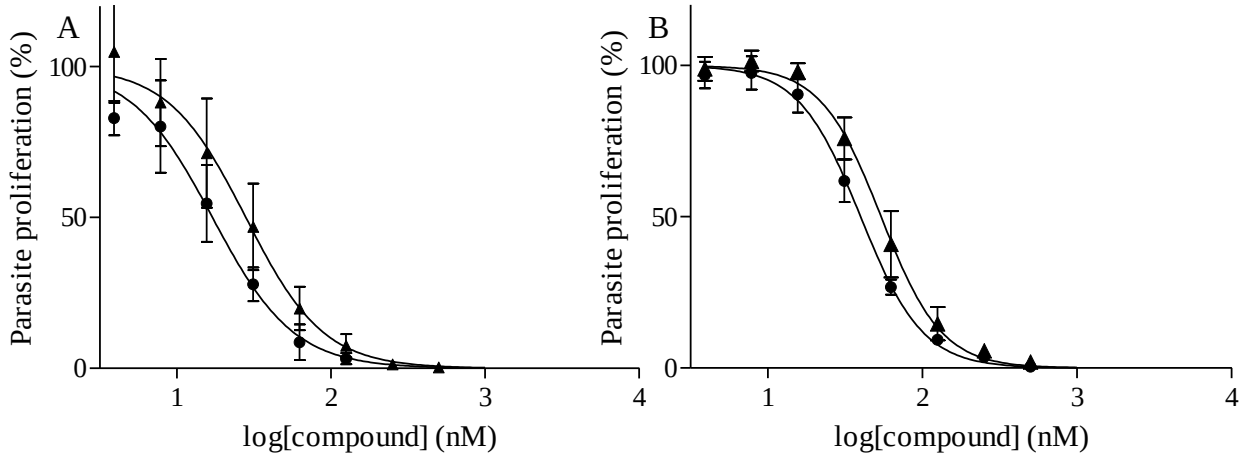


Figure 3.2: Dose-response curves of compounds 5 (circle) and 6 (triangle) against *P. falciparum* W2 (A) and HB3 (B) parasites. Ring stage parasites were treated and incubated at 37°C for 96 h, after which parasite proliferation was measured with SYBR Green I, measuring the DNA content of the parasites, using a fluorometer (excitation at 485 nm and emission at 538 nm). Data represents the mean of 4 (*P. falciparum* W2) and 3 (*P. falciparum* HB3) independent biological experiments all performed in triplicate. Error bars present ± S.E.

The above data allows the determination of resistance indexes (RI) for these polyamine analogs against 3D7 and W2 strains of *P. falciparum* to determine if treatment with these compounds lead to parasites with a shared resistance to parasite strains treated with chloroquine. RI is calculated by dividing the IC_{50} obtained with the *P. falciparum* chloroquine resistant strain (W2) with the IC_{50} obtained with the *P. falciparum* 3D7 sensitive strain. These values ranged from 0.15 to 1.0 (Table 3.4). When these values were compared to the RI of CQ (value of 7.4), the difference in the values indicate that the mechanism that parasites use to generate chloroquine resistance is unlikely to cause resistance to these compounds as well (*113*), and therefore after treatment with these compounds, parasites will not follow the same resistance pathway than after treatment with CQ.

Table 3.4: Resistance index against *P. falciparum* 3D7 and W2 parasite strains.

Compounds	IC_{50} 3D7 (nM)	IC_{50} W2 (nM)	RI[a]
1	68.06	34.4	0.5
2	45.0	45.0	1.0
3	46.7	21.15	0.5
4	248.6	36.8	0.15
5	27.6	17.4	0.6
6	30.0	29.8	1.0
7	59.9	50.02	0.8
8	793.4	142.7	0.18
9	246.21	42.07	0.17
CQ	8.0	59.5	7.4

a) Resistance index where the CQ-resistant IC_{50}'s are divided by CQ-sensitive IC_{50}'s (W2/3D7)

2.6 Cytotoxicity and selectivity

Cytotoxicity and selectivity of the polyamine analogs for *P. falciparum* 3D7 parasites compared to mammalian cells was established by analyzing the effect of these analogs on human HepG2 cell viability *in vitro*. Growth inhibition was determined with the LDH assay as an indicator of viability and to allow determination of the IC_{50} (concentration at which 50% growth inhibition is observed) of each compound using GraphPad Prism 5. Figure 3.3 represents a dose-response curve for the leading compounds (5 and 6) against HepG2 cells, with a IC_{50} value of 94.5 µM and 152.7 µM for compound 5 and 6, respectively.

The polyamine analogs showed poor to marginal activity or cytotoxicity against the mammalian HepG2 cells (Table 3.5) with values all in the µM range, one order of magnitude higher than what was observed for these compounds against *P. falciparum* 3D7 parasites (Appendix 3).

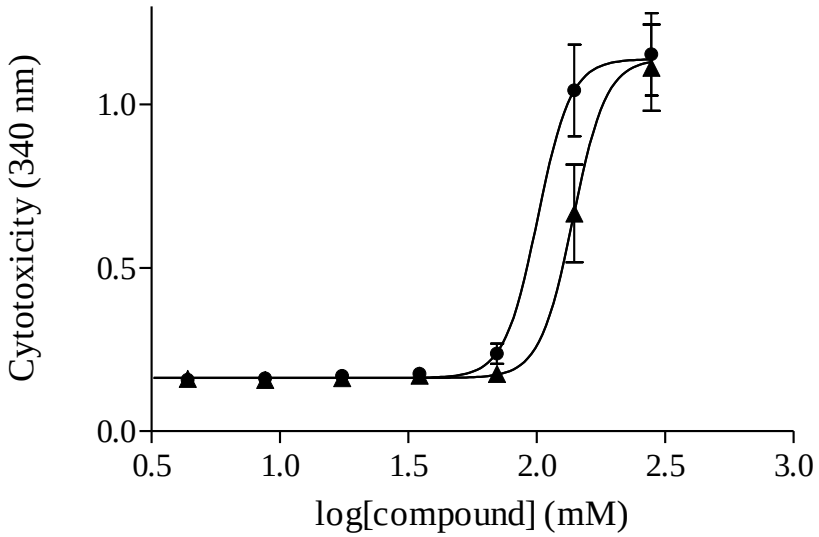


Figure 3.3: Dose-response (IC_{50}) curve of compound 5 (circle) and 6 (triangle) against HepG2 cells. Mammalian cells were treated with up to 5000× IC_{50} concentrations for 48 h, after which the cytotoxicity of the compounds were measured using the LDH assay with colorimetric detection of NADH levels using a spectrophotometer at 340 nm. Data represents the mean of 3 independent biological experiments. Error bars present ± S.E.

The selectivity index (SI) of these compounds was determined by dividing the IC_{50} values of the mammalian HepG2 cells with the IC_{50} values against *P. falciparum* 3D7. These analogs were found to be >5000 fold more selective toward the parasite than mammalian cells (Table 3.5, Appendix 3).

Table 3.5: Cytotoxicity determinations and selectivity of the polyamine analogs against human HepG2 cells. Data are from three independent biological repeats, each performed in triplicate, ± S.E. indicated.

Compounds	Mammalian HepG2 cells			
	Average IC_{50} (μM)	S.E.	*P. falciparum* 3D7 IC_{50} (nM)	SI[a]
1	44.8	0.8	68 ± 13	658.4
2	52.2	7.0	45 ± 3	1 160.0
3	240.3	11.2	47 ± 7	5 112.8
4	2490	-	249 ± 30	>5000
5	94.5	8.6	28 ± 4	3 373.8
6	152.7	7.8	30 ± 5	5 091.11
7	50.6	1.1	60 ± 7	843.33
8	1 947.3	42.6	793 ± 78	2 455.7
9	2460	-	246 ± 34	>5000
CQ	43.09	2.4	8 ± 1	4 787.8

a) Selectivity index – Average IC_{50}/*P. falciparum* IC_{50}

The most active compounds both had poor activity against the mammalian cell line with compound 5 showing >3000 fold and compound 6 showing >5000 fold selectivity towards the *P. falciparum* 3D7 parasite.

2.7 Cell viability based on reversibility

Inhibition of polyamine biosynthesis in *P. falciparum* parasites usually only results in cytostatic, cellular arrest. In order to determine whether *P. falciparum* 3D7 parasites treated with the polyamine analogs could recover or if the growth arrest was a lethal phenotype, parasites were treated for 12 h as was done by Malmquist *et al. (114)* with compounds 5 and 6 at concentrations of 1× and 10× IC_{90}, washed out and the recovery of the parasitic growth measured over a period of 2 days, one complete parasitic life cycle (Figure 3.4). Parasites treated with compound 6 for 12 h only were never able to recover from this pressure compared to untreated parasites as well as parasites treated with compound 5. After treating with compound 5 at both 1× IC_{90} and 10× IC_{90} the parasites were able to recover after drug treatment (Figure 3.4).

Therefore, in the presence of 100 nM (1× IC_{90}) of compound 6, parasite growth increased slightly (2-5%) after 12 h of treatment on the ring stage parasites, but showed no further increase during a consecutive 48 h period**.** In the presence of 100 nM of compound 5 however, the parasites proliferated similar to the untreated parasites, therefore treatment

effects can be completely reversed. The prompt killing effect of parasites in culture with compound 6 is highly desirable for potentially novel antimalarial compounds.

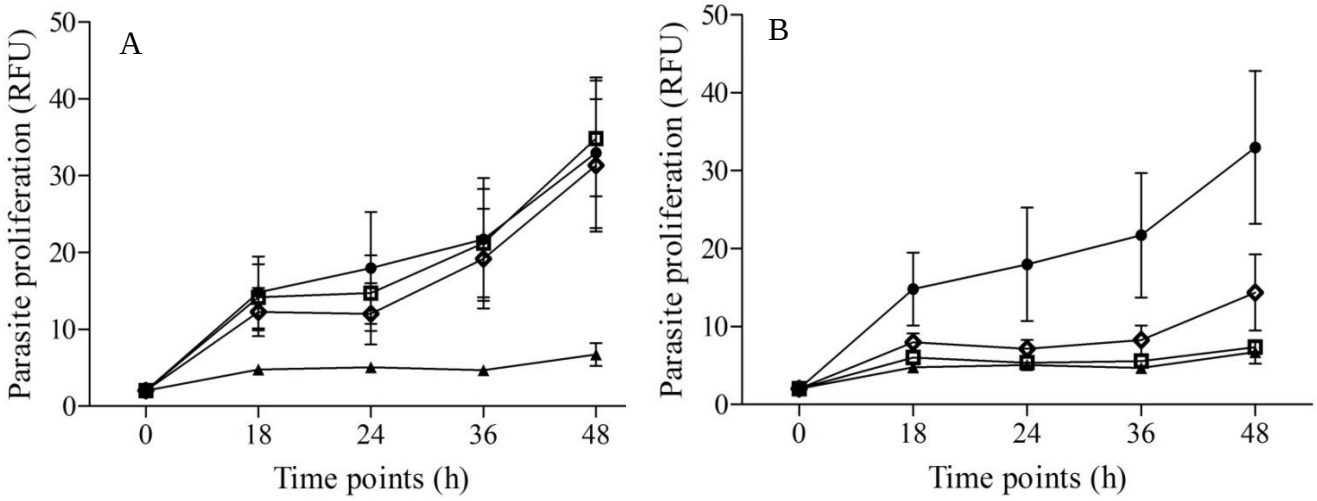


Figure 3.4: Reversibility of treatment of *P. falciparum* 3D7 parasites with polyamine analogs. Parasites (2% parasitemia (0 h)) were treated with 1× and 10× IC_{90} of the polyamine analog compounds 5 (A) and 6 (B) for 12 h (*114*). Drugs were washed out after 12 h and parasites allowed to proliferate for 6 h and measured over time as indicated from 18 h in relative fluorescent units (RFU). Circles illustrate untreated parasites and triangles illustrate 0.5 µM chloroquine (positive drug control, no growth). Squares illustrate 1× IC_{90} and diamonds 10× IC_{90}. Data are from three independent biological experiments, each performed in triplicate, ± S.E.

Morphological images of the *P. falciparum* 3D7 parasites were taken over 24 h after treatment with compound 5 at 100 nM (IC_{90}) (Figure 3.5).

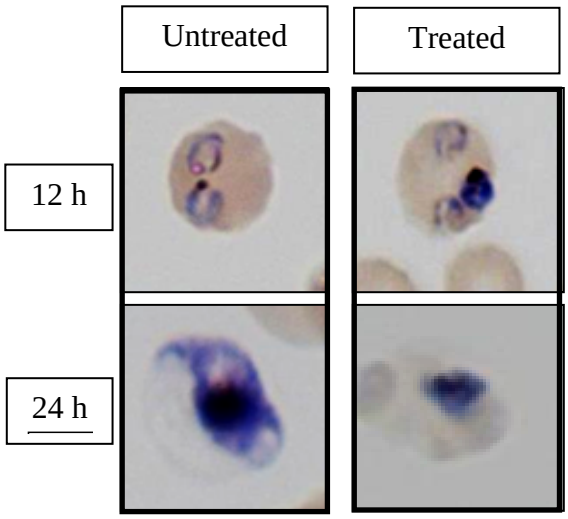


Figure 3.5: Untreated vs. treated *P. falciparum* 3D7 parasites. The parasites were treated with compound 5 for 24 h with 100 nM (IC_{90}), resulting in pyknotic trophozoite stage parasites. This figure illustrates 12 h untreated ring stage parasites as well as 12 h post-treatment rings. It also shows 24 h untreated trophozoite parasites indicating normal progression, with 24 h post-treatment indicating a stressed, pyknotic trophozoite parasite.

Ring stage parasites were still able to proliferate to the trophozoite stage after treatment with compound 5 (Figure 3.5). However, the analogs show some effect on the growth ability of the *P. falciparum* parasites. The trophozoite parasites seem to be pyknotic after drug treatment with 100 nM compound 5 (IC_{90}). Pyknotic parasites can be defined as parasites undergoing either cell shrinkage, membrane blebbing or nuclear fragmentation (*115*). As seen in Figure 3.5 there was cell shrinkage in the trophozoite stage parasites after treatment compared to untreated parasites, indicating pyknotic parasites after drug treatment.

2.8 Complementary interactions between inhibitors

DFMO was previously found to work additively with the parent polyamine analog MDL 27695 against *P. falciparum* parasites with positive inhibitory activity (*42*). This led to the determination of *in vitro* activity of drug interactions between the leading compounds (compounds 5 and 6) and DFMO using a modified fixed-ratio isobologram method (*104*) (Table 3.6). *In vitro* interactions of the two different inhibitors (DFMO and polyamine analogs) were determined on the basis of the fractional inhibitory concentration (FIC) index. Isobole analysis gives an indication of whether the interaction is antagonistic, additive or synergistic. A straight line indicates an additive effect with no implied interaction between the compounds, with a convex curve associated with antagonism where the response of the two compounds in combination is less than their individual effect and a concave curve indicative of synergism, where the combination of the compounds is more effective than alone (*116*) (Figure 3.6).

The shapes of the isobolograms for compounds 5 and 6 differ, which may indicate differential effects of their combination with DFMO (Figure 3.6). However, closer analysis of their ∑FICs indicates that both of these compounds only show additive interaction with DFMO (Table 3.6).

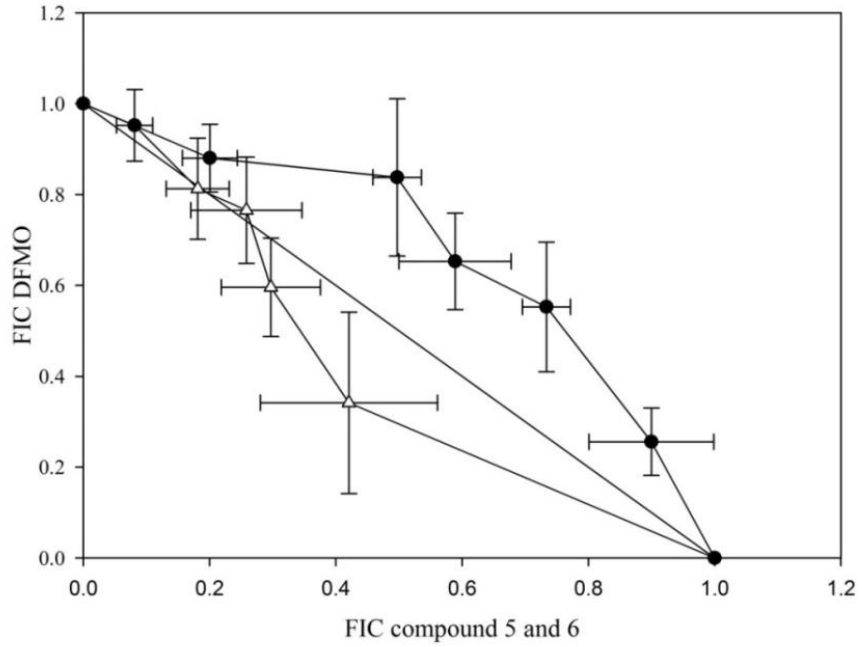


Figure 3.6: Isobologram of interactions between compound 5 (circle) and 6 (triangle) with DFMO performed on *P. falciparum* 3D7 parasites. Axes represent the mean fractional inhibitory concentration (FIC) for these compounds with the straight line representing a hypothetical additive interaction. The FIC values in Table 3.6 were used to plot an isobologram using SigmaPlot 11.0 with a hypothetical linear additive line illustrated. The data are averaged from 3 independent biological experiments each carried out in triplicate, ± S.E.

Although there are no defined breakpoints for antimalarial combinations, a recent study defined antagonism as ∑FICs >2.0, synergism as ∑FICs <0.5 and additive interactions as ∑FICs between 0.5-2.0 (*117*). The average ∑FIC values from three independent biological experiments were found to be between 1 and 1.4 for compound 5 and between 0.6 and 1.05 for compound 6 (Table 3.6). These values remain well within the cut-off range for an additive interaction between the polyamine analogs and DFMO as seen previously with MDL 27965 (*118-120*).

Table 3.6: ∑FIC values of DFMO with compound 5 and 6. Data are representative of three independent biological repeats, each performed in triplicate, ± S.E. indicated.

∑FIC	DFMO and Compound 5			DFMO and Compound 6		
	Average	S.E.	CV	Average	S.E.	CV
∑FIC 100	1.0	0	0	1.0	0	0
∑FIC 80	1.19	0.09	7.28	0.6	0.22	28.5
∑FIC 60	1.3	0.06	4.4	0.6	0.1	11.11
∑FIC 50	1.3	0	0	1.0	0.12	11.5
∑FIC 40	1.4	0.09	6.5	1.0	0.05	4.9
∑FIC 20	1.1	0.01	1.13	1.05	0.05	4.5
∑FIC 0	1.0	0	0	1.0	0	0

2.9 DNA replication and nuclear division studies

Polyamines play an important role in the DNA replication of *P. falciparum* parasites and are therefore important for life cycle development (*94*). Life cycle development can be monitored with flow cytometry, measuring the difference in nuclear content as the parasite matures during schizogony. During the ring stage, parasites contain mostly only DNA in a single nucleated state (1N) where-as in the early trophozoite stage they will begin to accumulate RNA as well. As the parasites' DNA replication continues, the late trophozoites (2 nuclei (2N)) and schizonts (≥2 nuclei (>2N)) increase in both DNA and RNA content. When the parasites reach the pre-lytic segmenter stage, DNA synthesis has reached its maximum and nuclear division occurs resulting in multinucleated parasites (>2N)

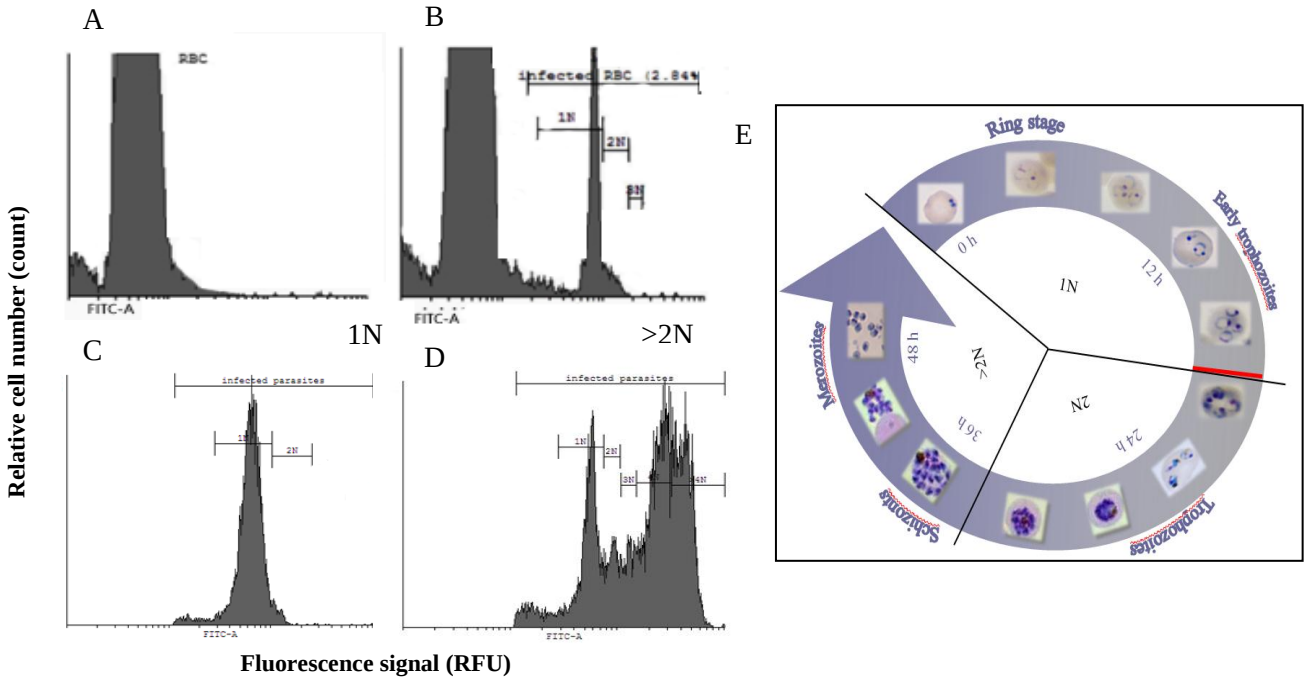


Figure 3.7: *P. falciparum* 3D7 parasite population gating used during flow cytometric analysis. Parasite populations were gated using Cyflogic after counting 100 000 events using a FITC-A channel. (A) Uninfected erythrocyte control. (B) Parasitemia of 2.84% 1N stage parasites gating for infected erythrocytes. (C) Infected erythrocytes gated using Cyflogic indicating normal parasite progression starting at 1N proliferating to. (D) >2N (E) *P. falciparum* life cycle illustrating parasites progressing from 1N to >2N over 48 h. The red line indicates halting of parasites to 1N stage after treatment with compounds 5 and 6 (2× IC_{90}).

(*121*). The parasites' progression can therefore be analyzed through flow cytometric monitoring of nuclear division (via DNA content) in the intra-erythrocytic cycle. Figure 3.7 describes the gating strategy used during this study to identify parasites as well as classify

them based on their DNA content (as a function of fluorescence detected by SYBR Green I staining).

Using this strategy, uninfected erythrocytes can be separated from infected erythrocytes and the parasitemia can be accurately determined (% infected erythrocytes, Figure 3.7 A vs. B). Moreover, within the infected erythrocyte fraction, different parasite populations were gated separately. The intensity of SYBR Green I, used in this study, is proportional to the number of nuclei present and therefore three peaks can be clearly defined: 1N indicates ring stage and early trophozoite stage parasites consisting of only 1 nucleus, 2N demonstrates late trophozoites and young schizonts with 2 nuclei and >2N demonstrates mature schizonts and segmenters containing >2 nuclei (*122*). Therefore, nuclear number is equivalent to the peak number (*123*).

Population gating was subsequently performed on *P. falciparum* 3D7 parasites treated with compounds 5 and 6 (2× IC_{90}) to determine how these compounds influence the parasite population distribution and therefore progression through their cell cycle. Images illustrated in Appendix 4 were used to produce Figure 3.8, illustrating DNA proliferation. Compounds 5 and 6 inhibit parasite DNA proliferation and nuclear division after 48 h (during onset of the second life cycle of the parasite) and effectively prevent maturation beyond the 1N stage (Figure 3.8). This indicates stage-specific drug activity against the parasites, preventing parasites to proliferate into the 2N and >2N stage. This might also be that due to an absence or a significant decrease of functional polyamines after drug treatment, inhibiting the parasites to proliferate from the 1N to 2N and >2N state. Thus increasing and confining the parasites to 1N parasites.

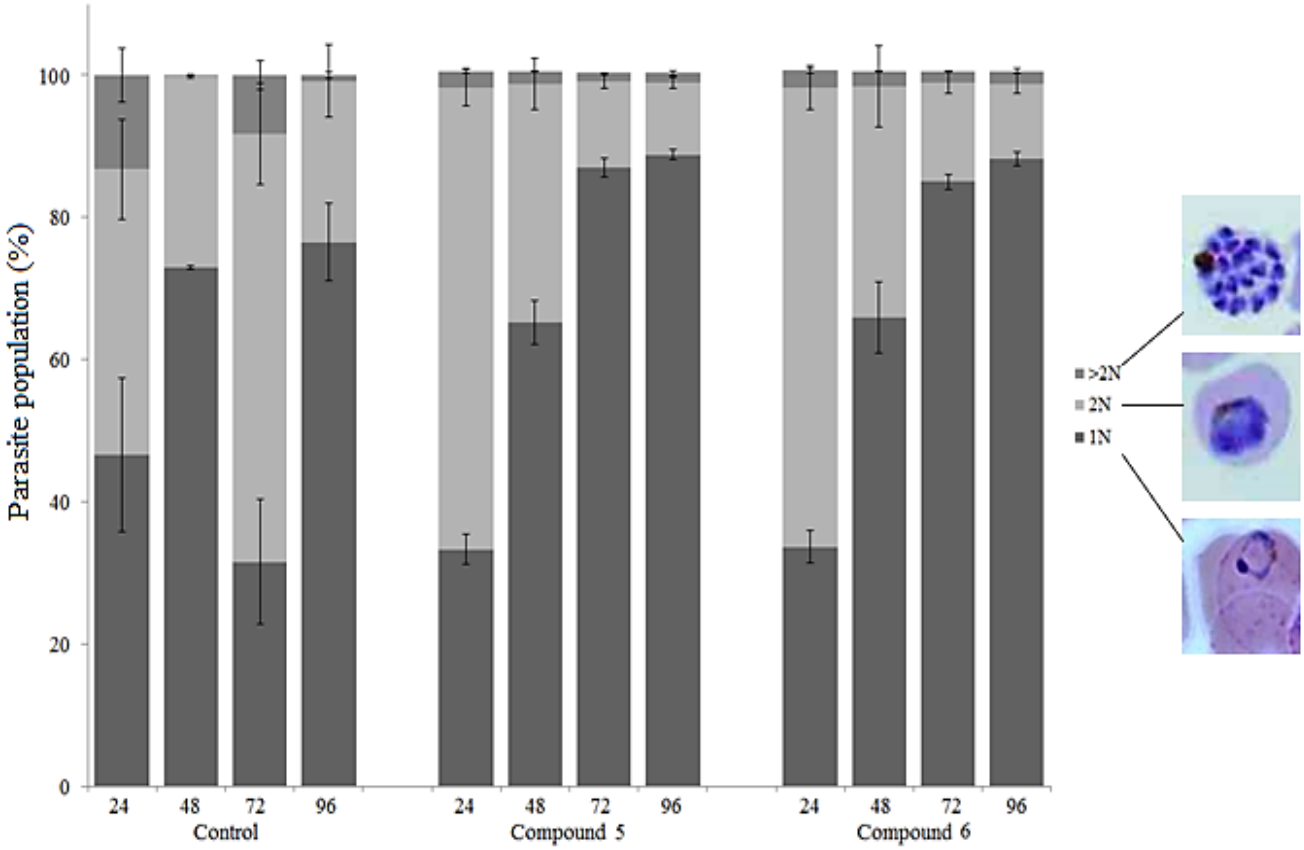


Figure 3.8: Determination of *P. falciparum* 3D7 parasite DNA replication with VarioMACS synchronisation. Flow cytometric analysis of nuclear division of ring stage *P. falciparum* 3D7 parasites (2% parasitemia) treated with polyamine analogs 5 and 6 (2× IC_{90}) over 96 h. Parasite population (%) illustrates rings and early trophozoite stage parasites that contain 1N (1 nucleus). After nuclear division the trophozoites contain 2N (2 nuclei) and the multi-nucleated schizonts contain >2N (>2 nuclei). Nuclear content was measured with flow cytometric analysis using SYBR Green I in the FITC channel after 100 000 events. Data represents the mean of three independent biological experiments performed in triplicate ± S.E. Data was analyzed with Cyflogic.

The polyamine analogs showed a dramatic halt in schizogony and nuclear division with parasites containing up to 89% rings/trophozoites (1N), ± 11% 2N (late trophozoite stage) and ± 2% >2N schizonts at 72 h after treatment, confining the parasites to the 1N stage.

2.10 β-hematin inhibition

Due to the heterocyclic nature of the compounds, these compounds could display a similar mechanism of action compared to chloroquine causing inhibition of β-hematin crystal formation. The latter was tested with compounds 2 ((bis)thiourea); IC_{50} = 45 nM), 6 (bis(urea); IC_{50} = 30 nM) and 8 (bis(urea); IC_{50} = 793 nM), using a chemical reaction of heme forming β-hematin crystals. (Due to insufficient amounts of compound 5, it could not be tested).

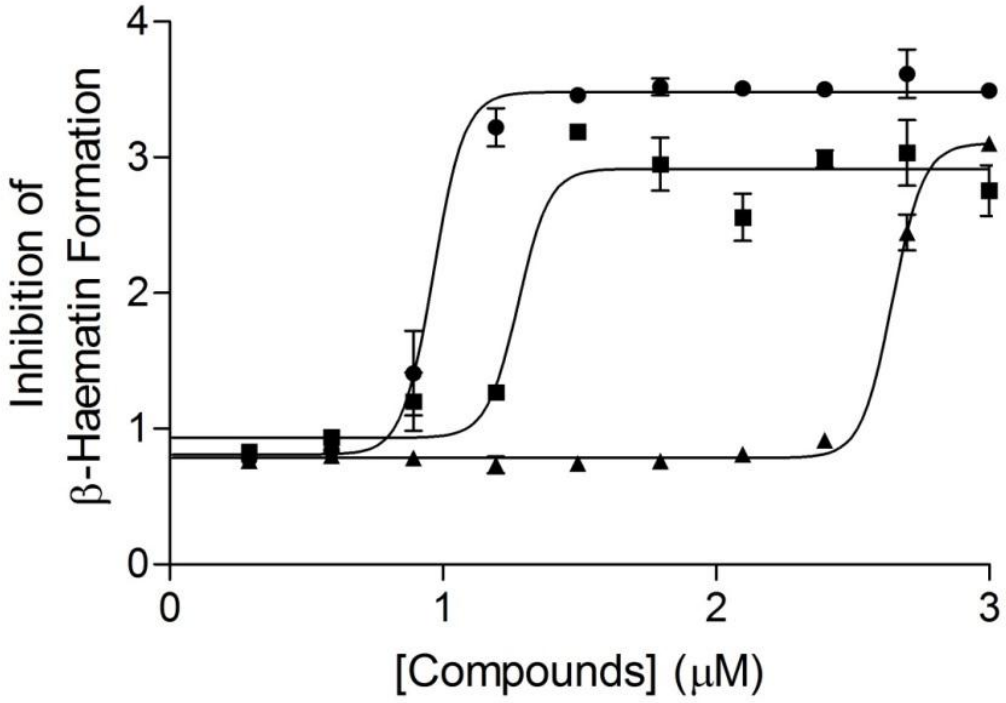


Figure 3.9: NP-40 mediated β-hematin formation assay. Dose-response curves of chloroquine (circle), compound 2 (square) and compound 6 (triangle) with a NP-40 mediated β-hematin formation assay was evaluated for inhibition activities. Absorbance was measured at 405 nm.

Compound 2 showed potent inhibition of hemozoin formation at an IC_{50} of 16.7 ± 0.07 µM, which is comparable to CQ's IC_{50} of 10.1 ± 1.2 µM (Figure 3.9). Compound 6 showed activity at >400 µM and compound 8 showed no activity at 1 mM. Therefore it might indicate primary mode of action for compound 2 ((bis)thiourea).

2.11 Determination of drug-likeness

Lipinski's rule of 5 was determined using four ADME descriptors, molecular weight, log *P*, hydrogen bond donors and hydrogen bond acceptors, to determine whether these compounds are orally bioavailable or not. The rules state that the molecular weight should be <500 g/mole, log *P* should be <5, hydrogen bond donors should be <5 and hydrogen bond acceptors should be <10 (*124*).

Table 3.7: ADME prediction on (bis)urea and (bis)thiourea 3-5-3 polyamine analogs

Properties	Polyamine analogs (Compound number)								
	1	2	3	4	5	6	7	8	9
Human Intestinal Absorption (HIA)[a]	0	0	0	0	0	0	0	0	0
Aqueous Solubility	0.07	0.96	1.63	0.98	2.53	2.95	1.38	2.55	2.03
Aqueous Solubility level [b]	5	5	5	5	5	5	5	5	5
Blood Brain Barrier (BBB)	0.49	0.53	-0.61	-0.5	-0.57	-0.33	0.77	-1.61	-1.61
BBB level [c]	1(High)	1(High)	3(Low)	2(Medium)	3(Low)	2(Medium)	0(Very high)	3(Low)	3(Low)
Cytochrome P450 2D6 (CYP2D6)	-0.27	0.26	-1.11	0.31	-1.21	0.45	0.86	-3.61	-7.74
CYP2D6 prediction	FALSE	TRUE	FALSE	TRUE	FALSE	TRUE	TRUE	FALSE	FALSE
Hepatotoxicity	-4.11	-3.82	-2.9	-4.08	-2.66	-2.45	-3.82	-2.95	0.56
Hepatotoxicity Prediction	FALSE	TRUE	TRUE	TRUE	TRUE	TRUE	TRUE	TRUE	TRUE
Plasma Protein Binding (PPB)	-5.5	-4.8	-5.1	-4.8	-4.58	-3.39	-3.92	-7.19	-4.3
PPB Prediction	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE

[a] 0 = Good absorption, 1 = Moderate absorption, 2 = Low absorption, 3 = Very low absorption.
[b] **Drug-likeness:** 0 = Extremely low, 1 = No, very low, but possible, 2 = Yes, low , 3 = Yes, good, 4 = Yes, optimal, 5 = No, too soluble.
[c] **Blood brain penetration:** 0 = very high penetrant (Brain-Blood ratio greater than 5:1), 1 = High (Brain-Blood ratio between 1:1 and 5:1), 2 = (Medium Brain-Blood ratio between 0.3:1 to 1:1), 3 = Low (Brain-Blood ratio less than 0.3:1), 4 = Undefined (Outside 99% confidence ellipse)

Looking at these criteria, seven of the nine polyamine analogs violated more than one of Lipinski's rules (Table 3.7 and 3.8) and therefore fail, according to these rules, as possible drug candidates. Compound 8 and 9 only violated one of Lipinski's rules, however, they were not very active against *P. falciparum* 3D7 with IC_{50} values of 793 and 246 nM respectively. Although Lipinski's rule of 5 is predictive of oral bioavailability, 16% of oral drugs violate at least one of the criteria and 6% fail two or more (*110*). Therefore another method developed by Hopkins *et al.*, determining the QED of these compounds, was also done.

An advantage of determining the QED value of drugs, is that even though the drug may fail Lipinski's rule of 5 it will still be ranked over a wide range from about 0 – 0.8 on its potential to become a drug. QED values of 0 is seen as unfavorable and therefore not drug-like where a value of 1 is drug-like (*110*). QED-values of between 0.03 and 0.17 were obtained for these polyamine analogs, illustrating the unfavorable potential for drug-likeness of these compounds (Table 3.8) based on both Lipinski's rule of five as well as QED values.

Table 3.8: Quantitative estimate of drug-likeness

Compounds	MW[a]	LOG *P*[b]	HBD[c]	HBA[d]	ROTB[e]	AROM[f]	Lipinski's Violations (≤1)[g]	QED[h]
1	669.02	5.49	6	2	24	4	3	0.05
2	697.08	5.61	6	2	26	4	3	0.04
3	636.89	2.92	6	2	20	4	2	0.06
4	516.83	2.27	6	2	22	2	2	0.10
5	664.94	3.04	6	2	22	4	2	0.05
6	692.99	3.82	6	2	24	4	2	0.05
7	725.13	6.39	6	2	28	4	3	0.03
8	484.69	-0.31	6	2	18	2	1	0.14
9	456.64	-0.32	6	2	16	2	1	0.17

a) MW – Molecular Weight (g/mole); b) LOG *P* – log *P* (lipophilicity); c) HBD - Hydrogen Bond Donors; d) HBA – Hydrogen Bond Acceptors; e) ROTB – Rotatable Bonds; f) AROM – Aromatic rings; g)Violations of Lipinski's rules should be ≤1; h) Quantitative estimate of drug-likeness (QED) – 0 = unfavorable; 1 = favorable.

Chapter 4: Discussion

Malaria, caused by *Plasmodium* parasites, results in up to 800 000 deaths each year (*44*). Even though resistance to currently used antimalarial drugs has spread around the world, chemotherapy is still the leading treatment used for the disease (*33*). Polyamines are able to bind with the majority of poly-anionic molecules in cells due to their cationic nature. With this they are able to influence various cellular processes like cell differentiation and proliferation (*61*). Due to increased concentrations of polyamines in rapidly proliferating cells like cancerous and parasitic cells, the interference of polyamine metabolism has been proposed and analyzed as a potent antimalarial target. This results in the inhibition of cell proliferation, either by preventing intracellular polyamine biosynthesis or by displacing the functional polyamine pool within cells with non-functional polyamine analogs (*60, 73*). Thus far polyamine depletion has not been successful in treating *P. falciparum* infections, however, further systematic investigations of the polyamine biosynthesis pathway occurring in intra-erythrocytic *P. falciparum* parasites showed that the polyamine biosynthesis pathway in this parasite consist of various unique aspects that can be used for treatment. For instance the bifunctional rate-limiting enzyme AdoMetDC/ODC, unique to *P. falciparum*, is considered to be a highly druggable antimalarial target (*74, 125*). It was also found that spermidine synthase is distinctively responsible for production of both spermidine and spermine in the *P. falciparum* parasite, making that a possible antimalarial target as well.

Based on the significant activity and selectivity of the previous generations of polyamine analogs against *P. falciparum* 3D7, as well as the potent activities found against human African trypanosomiasis (*95*), more potent, third generation, symmetrically alkylated (bis)thiourea and (bis)urea polyamine analogs with 3-5-3 carbon backbones were synthesized. The antiplasmodial properties of these analogs on *P. falciparum*, including their effects on DNA replication and nuclear division and drug-likeness, were determined in this study. This novel series of polyamine analogs all had potent antiplasmodial activities against *P. falciparum* parasites in the nanomolar range (Table 3.2). The two most active 3-5-3 compounds, (compounds 5 and 6 both consisting of (bis)urea structures), had IC_{50} values of 28 and 30 nM, respectively, against drug sensitive *P. falciparum* parasites. These compounds

were also active against antifolate resistant (HB3) and chloroquine resistant (W2) strains of *P. falciparum* parasites, illustrating a broad antiplasmodial activity.

The diphenylethyl 3-5-3 and diphenylpropyl 3-5-3 (bis)urea analogs had the highest antimalarial activities against the parasite, whereas phenyl, phenylmethyl (bis)urea and phenylmethyl (bis)thiourea compounds were the least active. This is comparable to results obtained indicating that the selection of terminal substituents of these compounds is very important in enhancing their antimalarial activity (*4*, *79*, *90*). Analogs without terminal aralkyl groups were the least effective, illustrating the importance of bulky terminal substituents. When the 3-5-3 polyamine analogs were compared to the previous generation analogs (3-6-3 and 3-7-3), it was found that the new, strategically optimized series of polyamine analogs with a (bis)diphenyl group had an increase of about 3 fold in antiplasmodial activity against *P. falciparum* 3D7, even though a (bis)phenyl analog in the second generation was found to be the leading compound of that series. Resistance indexes (RI) for these polyamine analogs indicated that the resistance activities of parasites treated with these compounds will not be similar to the CQ-sensitive 3D7 or CQ-resistant W2 parasite strains after treatment with chloroquine (*113*), illustrating that these compounds would not produce parasites following the same resistance mechanism as chloroquine treated parasites. Bisquinolines as well as a series of chalcone derivatives were also found to have lower resistance indexes than chloroquine, illustrating that these compounds are more potent to the chloroquine resistant strain and that it will not act on the same resistance pathway (*126-128*).

Selectivity indexes, which determine the specificity of the compound to the *P. falciparum* parasites compared to mammalian cells, are an important drug property to evaluate. This allows determination of whether the compound will be a cytotoxic drug when administered to the human host cells and therefore kills the normal mammalian cells together with the parasite. Besides affecting a number of women at childbearing age in endemic areas, malaria is also extremely lethal and severe when infection occurs during pregnancy. Malaria is also responsible for stillbirth, preterm labor, low birth weight and intrauterine death (*129*). Antimalarial drug therapy during pregnancy therefore has to be promptly initiated. This demands that new drugs and combinations for mothers and their babies should be particularly safe as well as selective (*129*). Limited data of drug safety is available for the first trimester during pregnancy, with mefloquine being used during the second and third trimesters and

chloroquine throughout the pregnancy. However, chloroquine resistance has spread world-wide and this antimalarial has therefore lost its clinical relevance. Therefore, no antimalarials are currently safe and effective for use in the first trimester (*130*). The amount of antimalarials transferred through breast milk is not sufficient and therefore children still require adequate treatment or chemoprophylaxis (*130*). Subsequent screening of both infected pregnant woman and children born from malaria infected mothers can be employed to determine the selectivity of the drug. A selective compound can mitigate the potential host toxicity, however, selective compounds are rare due to conserved target homology, target essentiality and compound permeability. It can also be difficult to predict the required selectivity index for low toxicity which is safe for use in man (*131*).

A chemical series acting on parasites needs to display SAR which reflects good inhibitory activity of target enzymes as well as selectivity to the host targeted in addition to showing good cellular inhibitory activity. Excellent safety and tolerability is essential for drug development for next generation antimalarials and therefore achieving optimal host target selectivity early on will alleviate potential adverse effects (*131*). Antimalarial toxicity is viewed on two different bases, either for drug treatment or prophylaxis (*130*). However, all drugs cause toxicity, including antimalarials. The third generation polyamine analogs were found to be highly selective for *P. falciparum* 3D7 and *in vitro* cytotoxicity was determined against mammalian HepG2 cells to evaluate whether their antimalarial activities against the parasite were due to the general toxicity of the compounds. These compounds all showed >600 fold selectivity towards the parasite with some even having >5000 fold selectivity toward the parasite. The leading compound of the second generation polyamine analogs had a selectivity of >7000 fold towards the *P. falciparum* parasite, indicating that the selectivity decreased from the second generation but still had a much higher selectivity towards *P. falciparum* than mammalian cells. Compared to other antimalarial compounds such as CQ with a selectivity index of >4500 fold against *P. falciparum* 3D7, these compounds can be classified as having good selectivity towards the parasite (*132*). A compound consisting of the potential to be an antimalarial drug should have a low resistance index and a high selectivity index as determined for these symmetrical, terminally alkylated polyamine analogs (*133*).

It has become important to use antimalarial drugs in combination with other drugs to prevent or delay the development of drug resistance. Therefore, studies on complementary

interactions between compounds that are potential antimalarial drugs are required for combination therapy against malaria. Interaction between antimalarial drugs occurs when the potency of two or more combined drugs is higher (synergism), the same (additivity) or lower (antagonism) than individual activities put together. This interaction might be due to physical contact between the drugs in combination, however, more often the activity of the one drug is affected by the other drug and/or vice versa due to molecular interactions of these drugs with components of the target cell and the biochemical or physiological consequences of these interactions (*134*). Analysis of interactions based on IC_{50} and IC_{90} values can produce different results, and variation between studies is expected (*105, 118-120, 133*). Synergism can be defined as a combination of agents, more effective than expected from the effectiveness of its constituents, antagonism is less effective than expected from the effectiveness of its constituents and additive interactions are similarly effective (*135*). Synergism is favored in some cases as the doses of the drugs may be reduced, maintaining the antimalarial effect while minimizing side-effects (*134*). Synergistic reactions may indicate a possible mechanism of action, with compounds showing synergistic interactions working on the same biological pathway (*93*). However, combination regimens relying on synergy might not give as much protection against resistance as was expected, as resistance to the components of the combination therapy could lead to a marked loss of efficacy (*118, 134*).

Current recommendations for combination therapies are to use artemisinin combination therapies (ACT's), which is currently the cornerstone of malaria control programs. Replacing ineffective, failing treatments such as chloroquine and sulfadoxine-pyrimethamine with ACT has reduced the morbidity and mortality associated with malaria (*40*). However, resistance to artemisinin is emerging in Cambodia, Southeast Asia, with decreased sensitivity of the ring stage parasites to artemisinin (*136*) and therefore there are concerns that the therapeutic lifespans of artemisinin-based combination drugs might get compromized (*137*). Artemisinin compounds are metabolized within hours *in vivo* and therefore recrudescence or relapse of parasites treated with these compounds are believed to occur due to its short half-life because it is not able to eliminate all the parasites during treatment (*138, 139*). Artemisinin should therefore be used in combination with other antimalarial compounds as parasite recrudescence commonly follows artemisinin monotherapy. The contributions of resistance to artemisinin, the partner drug used in combination or other factors are still unclear. However, in a study done in Cambodia, an increased copy number of the *pfmdr1* gene was

found which is a well-known cause of mefloquine resistance, and was associated with treatment failure of artemisinin-mefloquine combination therapies, which is currently being used on the Thai-Cambodia border.

It has been suggested for some time that combinations should be used for antimalarial treatment to decrease the emergence of resistance occurring (*140*). The combination of artesunate with mefloquine has been shown to be effective even in Thailand which consists of mostly mefloquine resistant parasites, with cure rates still well above 90%. It was also seen that this combination decreases parasite recrudescence *in vitro* due to the longer half-life of mefloquine which slowly eliminates the residual, artesunate treated parasites (*138*). Another combination of currently used antimalarials is the combination of chloroquine and artemisinin. This combination had an additive interaction against *P. falciparum* parasites leading to approximately 80% reduction in parasitemia. However, due to parasite resistance to both chloroquine and artemisinin, other combination therapies are a necessity as the usage of these combination therapies is quickly running out due to increased resistance.

Bitonti *et al.* previously analyzed DFMO in drinking water of mice infected with *P. berghei*, and found that it suppressed the parasitemia with 50%, but only after day 4. The compound DFMO is an irreversible inhibitor of ornithine decarboxylase, which is the first enzyme in the polyamine biosynthesis pathway, with no antimalarial resistance to date. DFMO was tested in combination with MDL 27695 *in vitro* against *P. falciparum* D6 and an additive inhibitory effect was observed. MDL 27695 is thought to act by inhibiting DNA and RNA synthesis and may bind to DNA similar to natural polyamines or act as a bifunctional intercalating agent (*42, 93*). When used in combination *in vivo* against *P. berghei*, MDL27695 and DFMO also displayed an additive interaction, resulting in 95% suppression of parasitemia after day 4 with a 100% cure rate (*42*). Because of the interactions between MDL 27695 and DFMO obtained previously (*42, 93*), the 3-5-3 polyamine analogs were tested in combination with DFMO for possible combination therapy against *P. falciparum*. From our studies, compound 5 and 6 showed additive interactions with DFMO. Previous results also showed an additive effect against combinations of chloroquine and MDL 27695 as well as chloroquine with artemisinin (*93*). The additive interaction between chloroquine and MDL 27695 as well as chloroquine and DFMO illustrates that these compounds work on entirely different pathways in the parasite (*93*). Therefore, it can be concluded that the (bis)aralkyl polyamine analogs work on different pathways in the parasite than DFMO, and therefore it will not target the

polyamine pathway in the *P. falciparum* parasite. However, further combination studies need to be done with the polyamine analogs combined with MDL 27695 and chloroquine to conclude similar mechanism of action pathways or not. Therefore, the presence and strength of drug interactions may lead to a preliminary mode of action of individual drugs to cells. The purpose of using polyamine inhibitors together with a known antimalarial compound is that the effective dose can be lowered into a more realistic range. Also, by using more than one polyamine inhibitor, acting synergistically, near to total blockade of the polyamine pathway might be achieved. Additive interactions however, do not determine the mode of action or effectiveness of other compounds *in vivo.* Therefore, an additive interaction with DFMO does not necessarily mean that these polyamine analogs will have the same effect *in vivo* as was seen between DFMO and MDL 27695. Recent studies show that MDL 27695 has potential for the oral or parenteral treatment of visceral leishmaniasis (*141*) indicating the positive bioavailability potential of the compound.

In vivo studies for these polyamine analogs are still underway and therefore it is important to first determine their effectiveness *in vivo* separately before determining combination therapies. Previously, the leading compound from the second generation (bis)urea and (bis)thiourea alkylated polyamine analogues was tested for *in vivo* antimalarial activity. However this compound performed poorly and was unable to show appreciative decrease in *P. berghei* parasitemia in the murine malaria model. Most of the concentrations of this compound were below the limit of quantification during the *in vivo* pharmacokinetic evaluation, indicative of low oral bioavailability with the dosing scheme used. The cause of the low bioavailability is unclear, but may be due in part to low absorption of the compound or rapid metabolism in the gastrointestinal tract of the mice (Verlinden *et al.* unpublished). These poor results obtained *in vivo* predict poor drug-like properties of these polyamine compounds and medicinal chemistry is needed to obtain more bioavailable, metabolically stable, drug-like compounds. As seen in Table 3.7 and 3.8, the 3-5-3 polyamine analogs were also predicted *in silico* to have poor drug-like properties.

Some artemisinin-treated parasites enter a state of hibernation or dormancy, where they are protected from the lethal effects of drugs by temporarily arresting development of ring stage parasites and are able to recover at a later stage to continue its normal growth (*142*). Only ring stages enter a state of dormancy as this is the stage of the parasite that is not susceptible to drug treatment (*143*). Cell cycle arrest or delay upon treatment is commonly seen in

cancerous cells (*144, 145*). Recently such delays were also seen when *P. falciparum* was treated with antimalarials such as artemisinin (*139*), mefloquine (*143, 146*), atovaquine (*138*) and pyrimethamine (*147*). When cultures were treated with pyrimethamine it resulted in complete disappearance of detectable parasites. However, several days after removal of the drug a reappearance of parasites was observed (*138, 139, 147*). Artemisinin sensitive parasites have been seen to stay dormant for up to 20 days after a single treatment with approximately 1.3% of the parasites recovering from this. This may be due to the compound disrupting the parasites availability of nutrients or energy, and therefore the parasite cannot continue its developmental cycle (*148*). Dormancy has also been hypothesized to be a mechanism the parasite uses to survive drug concentrations that may cause damage to the parasite and that it is triggered only when parasite development is significantly inhibited. This allows the parasite to await the elimination of the compounds from the circulatory system in the host (*148*). One example is 5-fluorouracil, which is a chemotherapeutic agent specifically targeting the S phase of the eukaryotic cell cycle. DNA replication and mitosis is divided into four phases, G1, S, G2 and M. Cancer cells resistant to this compound are known to arrest in the G1 phase of the cell cycle after treatment and therefore prevent the drug from targeting the S phase. The parasites becoming dormant however, may still be receptive to other drugs, implying that they still have a basic metabolism while in the dormant phase. This, however, still needs more evaluation and investigation. Also, parasite delay is not a generalized response to the presence of any antimalarial compound. However, decreased sensitivity to other quinolone drugs were also found related to dormancy, and therefore further studies are still required to determine whether this delay is due to a certain structural class of antimalarial compounds (*149, 150*). Recently it has been hypothesized that *P. falciparum* parasites might obtain dormancy by reducing the rate of hemoglobin digestion or degradation in ring stage parasites. This would reduce the amount of hemoglobin products available to potentiate drug activity and enable stalled ring stage parasites to survive drug treatment (*150*).

An important aim in antimalarial drug discovery is to achieve irreversible, rapid parasite death, killing parasites at all stages of the life cycle (*114*). An irreversible arrest in parasite viability was seen with compound 6 (Figure 3.4) over 48 h with no exogenous polyamines added to the reaction. IC_{90}, concentration at which the compounds resulted in a 90% inhibition of parasite proliferation, was used for the viability tests as this is close to the lethal dose of the parasites and therefore recovery should not occur. However, after treatment with

compound 5, the parasites recovered completely with removal of drug pressure. After treatment with compound 6, the parasite growth remained constant without further increase in parasitemia. When morphological images (Figure 3.5) were taken of this time, pyknotic trophozoite stage parasites were seen after 24 h (*115*). Pyknotic parasites can be classified as showing cell shrinkage, membrane blebbing nuclear or DNA fragmentation, chromatin condensation, oxidative stress and cleavage of substrates (*151*). This might be due to the drug inhibiting proper parasite proliferation, inhibiting further DNA replication and decreasing the nutrients and energy parasites require for proliferation. These pyknotic trophozoite parasites might still be viable enough to proceed to the ring stage, however, due to drug pressure and parasitic stress, when the parasites reach the ring stages they may go into a dormant state to survive drug pressure (*139*), therefore keeping the parasitemia, DNA content, constant as the number of parasites stay constant. The cytotoxicity of the second generation polyamine analogs was found to be independent of change in the polyamine pool, meaning that the addition of polyamines to drug treated parasites did not rescue parasite growth (*4*). This may be due to the ability of the analogs to block intracellular binding sites of the natural polyamines or displace intracellular polyamines from their binding sites, therefore acting as polyamine mimetics. Therefore the mode of action of these compounds may be independent of the polyamine pathway and therefore polyamine biosynthesis.

Polyamines have been found to play an important role in DNA replication of *P. falciparum*, by regulation of DNA synthesis (*152*), and are therefore important for life cycle development (*94*). Polyamines were found to be elevated in rapidly proliferating tissues and were increased when growth or differentiation was induced (*153*). Cell proliferation consists of two major processes such as cell growth and cell division. The main event in cell growth is DNA replication, as this is important for successful cell division. Polyamines belong to the category of molecules whose synthesis is strongly activated during the first, G1, phase. The cell division cycle and mitosis of intra-erythrocytic stages of *P. falciparum* is still poorly understood, especially DNA synthesis occurring during the S phase or chromosome separation in the M phase (*154*). Cellular division occurring in *P. falciparum* was described as rings and early trophozoites having a single interphase nucleus (1N) in the G0 stage. Older trophozoites (after cytoplasmic growth, and hematin deposits within the cytoplasm) initiate the preparation for chromosome replication in G1 and induce DNA synthesis (2N) during the S phase. The schizont stage commence when the trophozoite nucleus begins to divide into two or more (>2N) daughter nuclei during the M phase (*154*).

With interference of the polyamine biosynthesis pathway cell cycle progression is affected mainly in the S phase, with effects occurring only later in the G1 and G2/M phases. When cancerous cells were treated with DFMO an effect on the cell cycle progression was seen with fewer cells in the S phase than in the control. These cells were also treated with a spermine analog, N^1,N^{11}-diethylnorspermine, with results showing a depletion of the polyamine pool by stimulation of polyamine catabolism and ODC and AdoMetDC inhibition (*155*). Therefore, unperturbed cell cycle progress, requires unperturbed polyamine biosynthesis. When cells stop proliferating due to polyamine deficiency, cells may accumulate in any of the cell cycle phases . Importantly, cell cycle effects are seen before the onset of growth inhibition and therefore cell cycle progression is affected one cell cycle after drug treatment depleting the polyamine pools (*155*).

From flow cytometric analysis done on the 3-5-3 polyamine analogs it could be seen that these compounds inhibit parasite DNA proliferation and nuclear division during the first 72 h after treatment inhibiting the asexual development of *P. falciparum* 3D7 (Figure. 3.8). Due to reversible cytotoxicity of compound 5 against *P. falciparum* 3D7, parasites were treated for 24 h with both compound 5 and 6 to ensure no recovery. The polyamine analogs showed a dramatic halt in schizogony and nuclear division in the G1/S phase before DNA replication is initiated. This might be due to the fact that these polyamine analogs prevent further nuclear division of the 1N stage due to an absence or lack of functional polyamines. The parasites, therefore, start nuclear division forming approximately 11% 2N and 2% >2N stage parasites, but cannot produce more parasites due to a lack of functional polyamines required for this task. Therefore the compounds could possibly act as polyamine mimetics. As polyamine mimetics, polyamine analogs replace the polyamines from their normal cellular binding sites including DNA and RNA and as such disrupt DNA synthesis, replication and nuclear division (*4*, *83*, *84*, *94*).

Another explanation for the dramatic increase in ring stages after drug treatment might be the parasites entering a dormant state, as mentioned above. The parasites were treated during the ring stages; however, they did proceed to the first cycle trophozoite stage and again to the following ring stage. From this stage (48 h), they did not mature into trophozoite stages but were halted as ring stage, with early trophozoites (1N) (Figure 3.8). Therefore, parasites in the ring stage might enter a dormant stage to survive drug pressure and thus progression still

occurs into the second ring stage but these parasites become dormant for survival. From this explanation this might not be entirely due to drug treatment, but also based indirectly on the ability of the parasite to hibernate to avoid fatal drug stress. However, sufficient drug concentrations are required for the parasite to induce this dormancy and therefore the parasite might only have undergone stress at the start of the second life cycle. This possibility also correlates with results obtained earlier illustrating that this, rather than reducing functional polyamines, might lead to the cell cycle inhibition.

Similar results were found with other antimalarial compounds either confining the parasite to the ring stage, inducing dormancy in the parasites or inhibiting further parasite proliferation from trophozoites to schizonts and merozoites. Mefloquine treated *P. falciparum* parasites were confined to the ring stage where these parasites entered a dormant stage when treated during the ring stage, and delayed parasite maturation at the ring stage (*148*). Synchronized 3D7, FBC and Dd2 parasite strains were all delayed in maturation, occurring already after 6 h treatment and became more pronounced after 12 h (*148*). When mefloquine was added to synchronized trophozoite stage parasites, the parasites proceeded normally until the ring stage 24 h later at which point the maturation again halted indicating a specific effect on the ring stages (*148*). Aphidicolin, a DNA polymerase inhibitor in erythrocytes, were also found to reversibly inhibit DNA synthesis and the asexual development of *P. falciparum* trophozoites and schizonts. This drug, similar to the polyamine analogs, allowed the formation of the ring stage parasites, but blocked further development of ring stages into trophozoite stage parasites (*156*). MDL 73811, an enzyme activated inhibitor of AdoMetDC developed by Marion-Merrel-Dow in the 1980's, were also found to arrest parasite development to the early trophozoite stage (*157*). This data, however, may lead to the conclusion that inhibition of parasites to one specific life stage may not be specific to polyamine analogs as this has been shown previously with other antimalarial compounds.

The anti-proliferative effect of the polyamine analogs may, in addition to their polyamine functionality also influence other metabolic activities of the parasite. During *P. falciparum* infection in erythrocytes, the parasite catabolizes hemoglobin inside erythrocytes, to obtain essential amino acids and osmotic control for parasite survival. During this process they become exposed to cytotoxic oxidative stress caused by free heme formation (*108*). In order to avoid this, the parasite isolates the heme into aggregates of hemozoin, and interference with this process has been ascribed as the mode of action of quinoline type antimalarials. Due

to increasing resistance to quinine and artemisinin families novel classes of drugs are a necessity and one approach to discover new antimalarials is through the screening of compounds against the formation of hemozoin (*108*). Based on the heterocyclic nature of these polyamine analogs compared to chloroquine, a known β-hematin inhibitor, three compounds of the 3-5-3 polyamine analog test set were screened, compound 2, 6 and 8**.** Compound 6 was chosen as the most active compound, with a (bis)urea structure and diphenyl terminal groups. Compound 8 was chosen as the least active compound of the series consisting of phenyl terminal groups and compound 2 was chosen as the most active (bis)thiourea compound with diphenyl terminal groups, with structural similarities to either compound 5 or 6. Compound 2 showed inhibition against hemozoin formation similar to chloroquine. With not much structural similarity between chloroquine and the diphenyl (bis)thiourea compound this mode of action result cannot be explained based on structural similarities. This can however indicate that that β-hematin inhibition may be the primary target of compound 2 in the parasite. With resistance indexes already determined (Table 3.4), indicating different resistance pathways between these compounds, compound 2 can be seen as a potentially, novel inhibitor against hemozoin formation. Further tests to confirm this is needed as was done by Combrinck *et al. (158)* making use of cell fractionation and measuring Fe(III)heme-pyridine in cultured *P. falciparum* parasites. Cell fractionation has previously shown other antimalarial compounds also inhibiting hemozoin formation, such as artesunate, amodiaquine, lumefantrine, mefloquine and quinine (*158*). When comparing compound 2 to these antimalarial compounds, they are only comparable based on the heterocyclic nature of the structures. However, pyrimethamine and sulfadoxine-pyrimethamine, antifolate inhibitors, do not inhibit hemozoin formation, which also consist of heterocyclic structures (*158*). Compound 6 showed activity but only at >400 µM and therefore it would not be classified as the primary target for compound 6 (Fig. 3.12). Compound 8 showed no activity at a 1 mM concentration. This mechanism of action can therefore not be classified as the primary target for these (bis)urea compounds (Figure 3.).

To determine the drug-likeness of these analogs the physiochemical properties and ADME descriptors were calculated and Lipinski's rule of 5 was used to determine whether these compounds had good oral bioavailability and permeability. It states that the compound should have <5 hydrogen bond donors, it should have <10 hydrogen bond acceptors, the molecular weight should be <500 g/mole and the log P value should be <5. According to

Lipinski (*124, 159*), higher molecular weight compounds are generally less likely to produce good bioavailability than smaller molecular weight compounds. They also state that too many hydrogen bond acceptor groups as well as too many hydrogen bond donor groups impair permeability across the membrane bilayer. The log *P* value is used to determine how hydrophilic or hydrophobic a compound is and values less than 5 but more than 0.8 (*160*) shows good oral bioavailability and permeability, thus indicating that the drug is not too hydrophobic or too hydrophilic. Although violation of Lipinski's rules may not result in poor absorption, the likelihood of poor absorption increases with the number of rules broken and the extent to which they are exceeded. These polyamine analogs however mostly had molecular weights >500 g/mole with the exception of compounds 8 and 9. This generation compounds all exceeded the amount of <5 hydrogen bond donors and three compounds (1, 2 and 7) exceeded the <5 *Log P* value. The hydrogen bond acceptors were all well under 10 with most compounds also having log *P* values <5. However, these compounds still violate ≥1 of Lipinski's rules, and therefore fail as potential drug-like candidates.

Hydrogen bonds increase solubility in water and must be broken in order for the compound to permeate into and through the lipid bilayer membrane. An increase in the number of hydrogen bonds reduces partitioning from the aqueous phase into the lipid bilayer membrane for permeation by passive diffusion. As the molecular weight of a compound increases, a larger cavity must be formed in water in order to solubilize the compound and therefore solubility decreases. An increasing molecular weight also reduces the compound concentration at the surface of the intestinal epithelium, thus reducing absorption and impedes passive diffusion through the aliphatic side chains of the bilayer membrane. An increasing log *P* value above 5 leads to a decrease in aqueous solubility, which also reduces absorption. However, in lead discovery optimization target binding is often increased by adding hydrogen bonds and lipophilicity. Therefore, activity optimization can reduce the drug-like properties of a compound series. To obtain favorable biological data from modern *in vitro* biology techniques, a compound need not have significant aqueous solubility (*159*). An additional Veber rule were proposed by Veber *et al.*(*161*), which studies the structural properties that increase oral bioavailability in rats. They concluded that molecular flexibility, polar surface area (PSA) and hydrogen bond count are important determinations of oral bioavailability. Rotatable bonds should be ≤10, the PSA should be ≤140 $Å^2$ or ≤12 total hydrogen bonds (acceptors and donors).

Using the Discovery Studio Modelling Environment various models for drug-likeness were tested. These include models for intestinal absorption, aqueous solubility, blood brain barrier penetration, plasma protein binding, cytochrome P450 2D6 inhibition and hepatotoxicity. Intestinal absorption is defined as percentage absorbed, and a well absorbed compound is absorbed at least 90% into the bloodstream in humans. At level 0 the compound indicates good absorption where level of 3 has very poor absorption. The third generation polyamine analogs exhibit good intestinal absorption all falling in the absorption level 0. The aqueous solubility model uses linear regression to predict the solubility of the compounds in water at 25°C. In this case the level 0 indicates extremely low solubility, level 1 indicates possible solubility, level 2 indicates low solubility, level 3 shows good solubility, level 4 is optimal solubility and level 5 indicates that the compounds are too soluble. When a compound is too soluble it stays in its aqueous environment and does not break the hydrogen bonds to bind the specific target and therefore does not target the required targets. Unfortunately, all nine compounds of the third generation polyamine analogs had a solubility level at 5 indicating that they are too soluble for drug use.

Blood brain barrier penetration is important, especially with cerebral malaria. There are four prediction levels with level 0 being a very high penetrant and level 3 a low penetrant. These results varied between the compounds with compound 7 being a very high penetrant, compounds 1 and 2 high penetrants, compounds 4 and 6 medium penetrants and compounds 3, 5, 8 and 9 low penetrants. Cytochrome P450 2D6 predicts CYP2D6 enzyme inhibition using the 2D chemical structure. CYP2D6 is involved in the metabolism of a wide range of substrates in the liver and its inhibition by a drug creates many drug-drug interactions. When tested the polyamine analogs again showed varied results with compounds 1, 3, 5, 8 and 9 not inhibiting CYP2D6, and compounds 2, 4, 6 and 7 inhibiting CYP2D6 and therefore these four compounds (2, 4, 6 and 7) may lead to an abnormal increase in concentrations of other compounds, thus potentially causing toxic side effects due to other drugs taken that the enzyme CYP2D6 should have metabolized. This may affect the efficiency of the drug. The last hepatotoxicity model predicts the occurrence of dose-dependent human hepatotoxicity. All the polyamine analogs of the third generation were found to be positive for hepatotoxicity, except compound 1. Therefore these compounds are too soluble, lead to hepatotoxicity and the two leading compounds had only moderate blood brain barrier penetration. However, they do not bind carrier proteins in the blood, have good absorption levels and have reasonably good lypophilicity.

Although Lipinski's rule of 5 is predictive of oral bioavailability, 16% of oral drugs violate at least one of the criteria and 6% fail two or more. Therefore, methods to quantify drug-likeness are required. Hopkins *et al.*(*110*) provided a quantitative metric for assessing drug-likeness, the quantitative estimate of drug-likeness (QED). QED values range from zero (unfavorable) to one (favorable). They found that QED outperforms the Rule of 5 and Ghose rules and performs marginally better than the Veber rule (*110*). An advantage of QED is its ability to rank compounds whether they fail the Rule of 5 or not. Oral drugs that fail the rule of 5 show QED values over a very wide range from 0 to 0.8. QED offers a richer, more nuanced view of drug-likeness. Its functions are based on the underlying distribution data of drug properties and, unlike rule-based metrics, can identify cases in which a generally unfavorable property may be tolerated when other properties are close to ideal. The polyamine analogs all had QED values between 0.03 and 0.17 also indicating that these compounds are not favorable for being potential drugs.

Based on these results described, medicinal chemistry is needed to obtain more orally bioavailable as well as metabolically stable compounds compared to the second generation analogs which had low bioavailability *in vivo* (Verlinden *et al.* unpublished). Fluorinated derivatives were seen to have an increased activity (2 fold) compared to the second generation analogs against *P. falciparum* (3D7), however, *in vivo* data to determine metabolic stability is still underway (Barnard *et al.* unpublished). The 3-5-3 polyamine series are still currently the leading test series consisting of the best antiplasmodial activities. Therefore, *in vivo* studies determining metabolic stability for these compounds, testing the transmission blocking properties on the sexual stages (gametocytes) of *P. falciparum* as well as mechanism of action studies are the necessary next steps to be taken to determine and validate these compounds as antimalarial drugs.

Chapter 5: Conclusion

The aim of this study was to determine the *in vitro* antiplasmodial activity as well as a preliminary mode of action of these terminally alkylated (bis)urea and (bis)thiourea polyamine analogs against *P. falciparum* malaria parasites. These polyamine analogs were found to pose as an enticing structurally novel and distinct class of potential antimalarials with potent activities and selectivities against *P. falciparum* parasites as well as being irreversibly toxic against these parasites. Preliminary mode of action studies show that they work additively with the polyamine inhibitor, DFMO, therefore independent to the polyamine pathway, with further studies revealing that (bis)thiourea polyamine analogs inhibit hemozoin formation, whereas the (bis)urea polyamine analogs have a primary target for parasite DNA proliferation and block nuclear division. It was also seen that these compounds induce dormancy in the ring stage parasites. Further mode of action studies is still required to understand the exact mechanism of action of these compounds and this would also include medicinal chemistry changes to enable more drug-like compounds with *in vivo* antimalarial activities. These compounds therefore, pose a novel chemical scaffold with potent *in vitro* antiproliferative action against *P. falciparum* parasites.

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APPENDIX 1

Antimalarial dose-response curves (nM) for *P. falciparum* W2 (n=4)

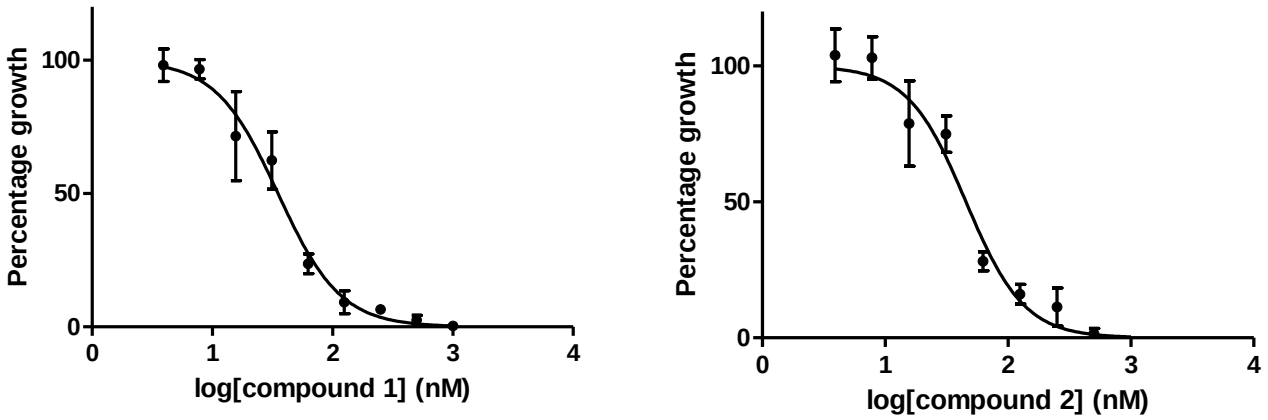

Figure 1: Dose-response curves for compound 1 and 2 against *P. falciparum* W2.

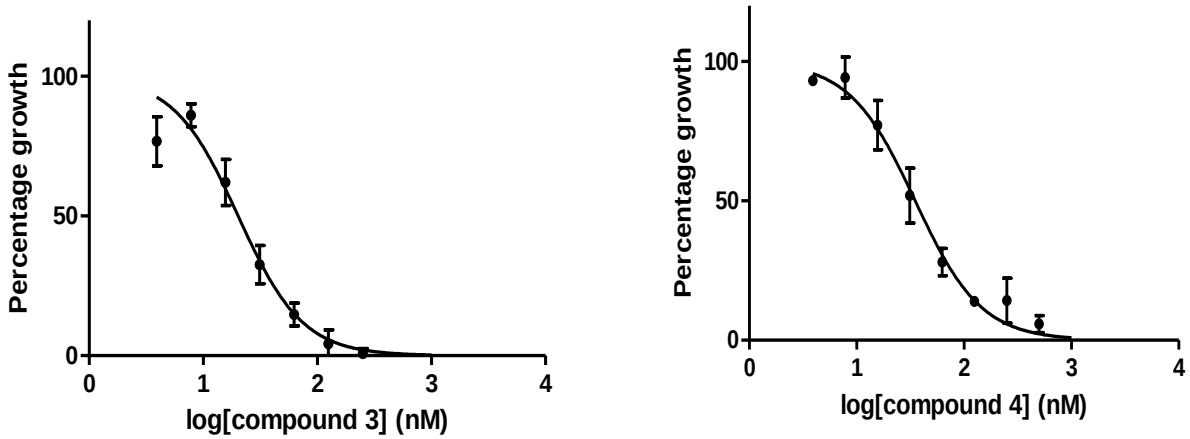

Figure 2: Dose-response curves for compound 3 and 4 against *P. falciparum* W2.

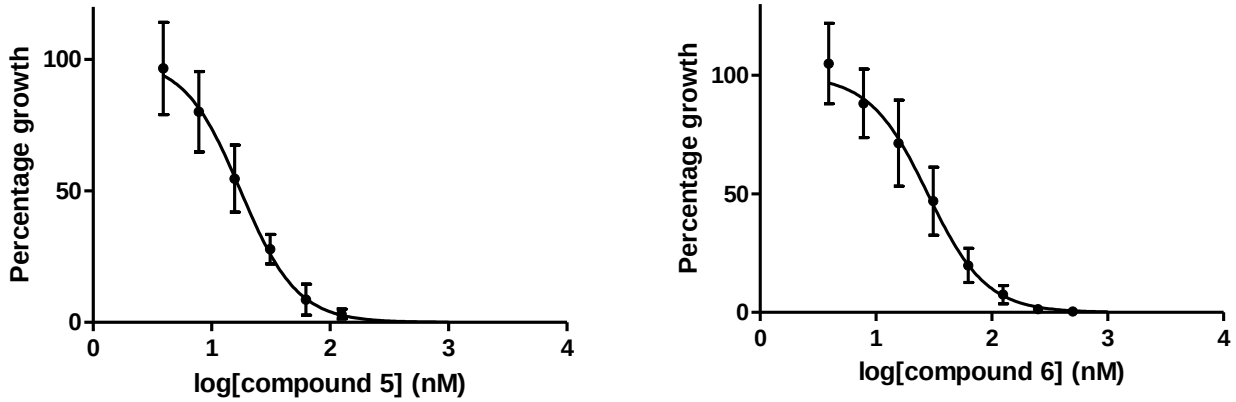

Figure 3: Dose-response curves for compound 5 and 6 against *P. falciparum* W2.

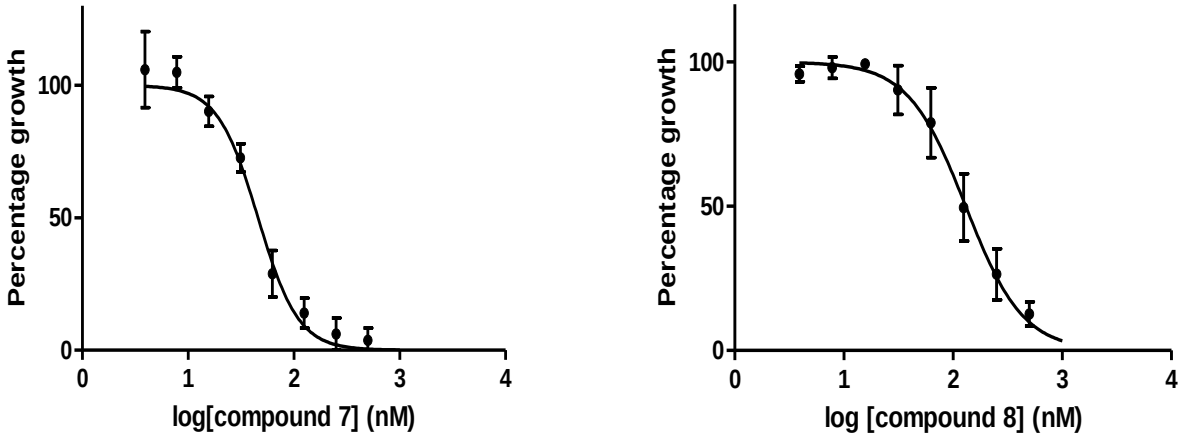

Figure 4: Dose-response curves for compound 7 and 8 against *P. falciparum* W2.

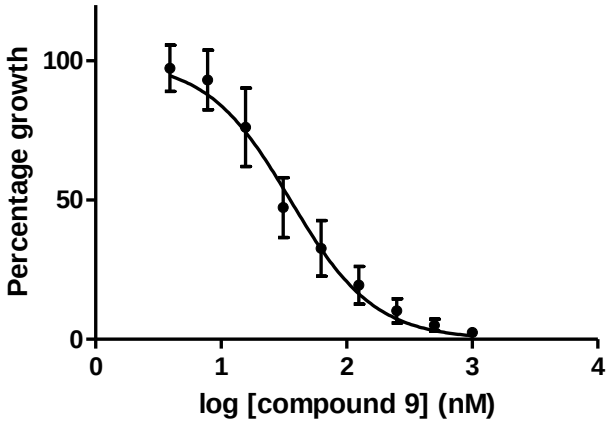

Figure 5: Dose-response curves for compound 9 against *P. falciparum* W2.

APPENDIX 2

Antimalarial dose-response curves (nM) for *P. falciparum* HB3 (n=3)

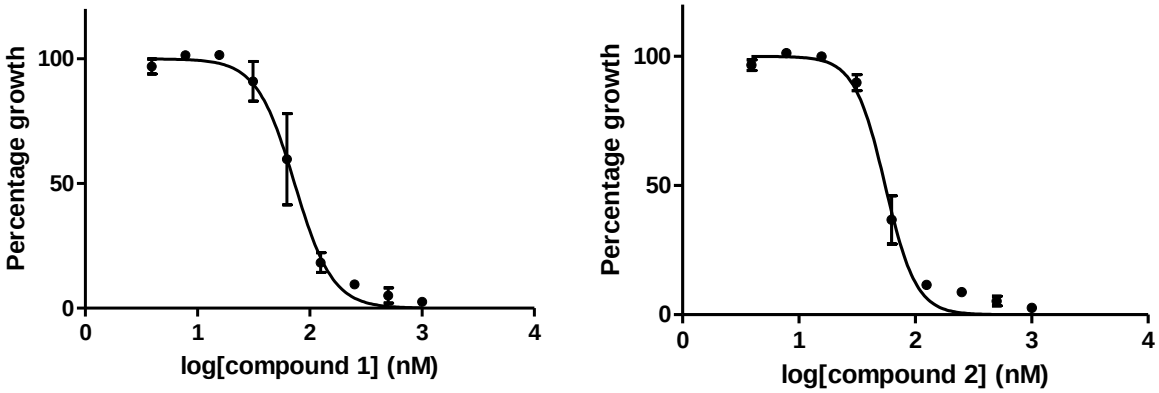

Figure 1: Dose-response curves for compound 1 and 2 against *P. falciparum* HB3.

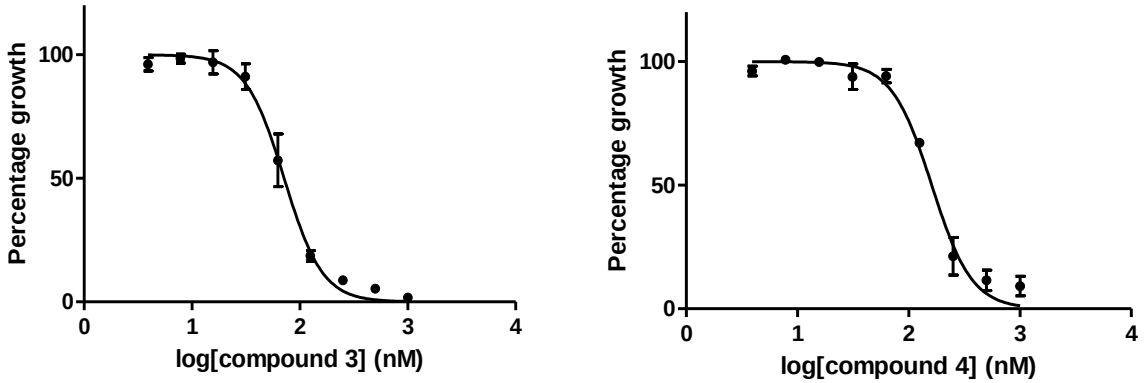

Figure 2: Dose-response curves for compound 3 and 4 against *P. falciparum* HB3.

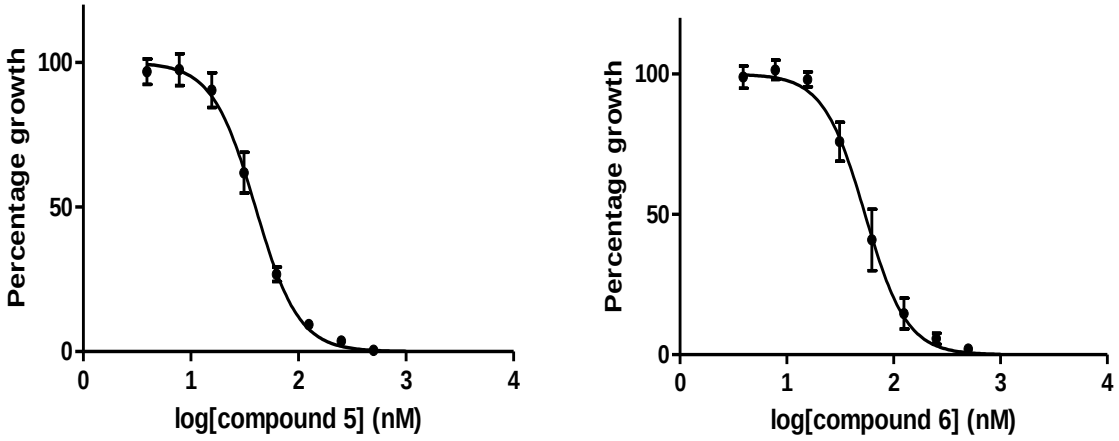

Figure 3: Dose-response curves for compound 5 and 6 against *P. falciparum* HB3.

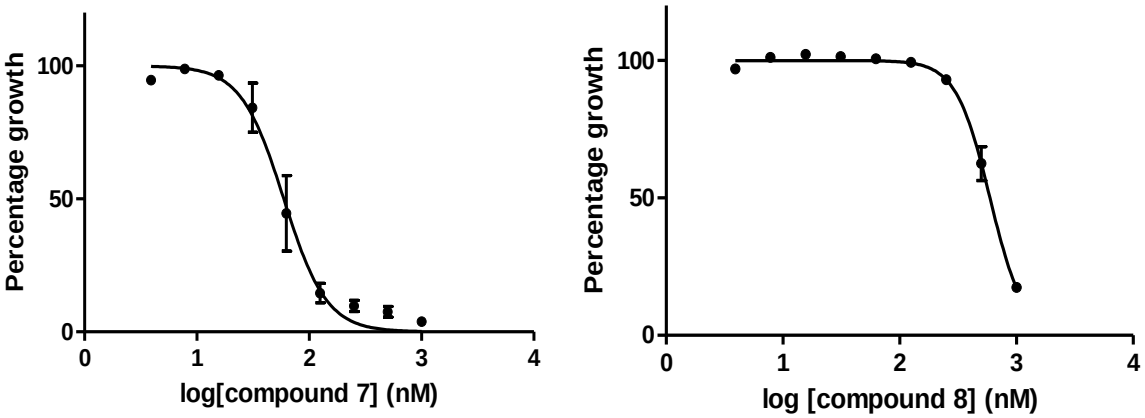

Figure 4: Dose-response curves for compound 7 and 8 against *P. falciparum* HB3.

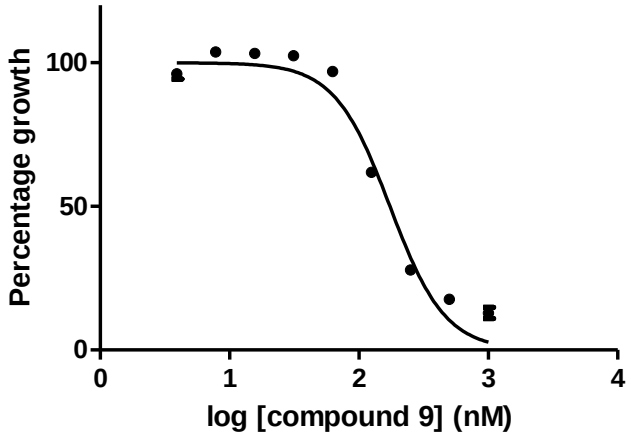

Figure 5: Dose-response curves for compound 9 against *P. falciparum* HB3.

APPENDIX 3

HepG2 mammalian cells cytotoxicity (µM) graphs (n = 3)

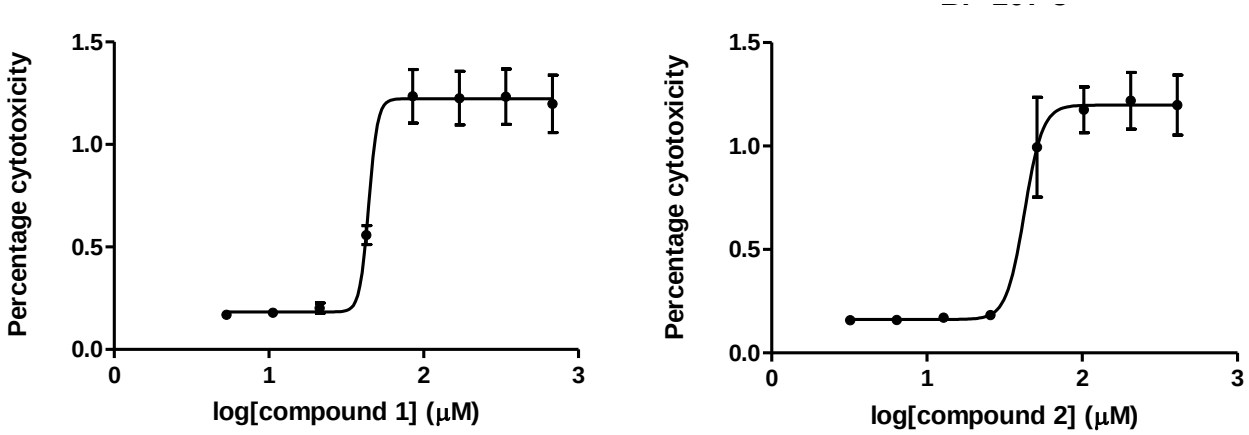

Figure 1: Dose-response curves for compound 1 and 2 against mammalian HepG2 cells

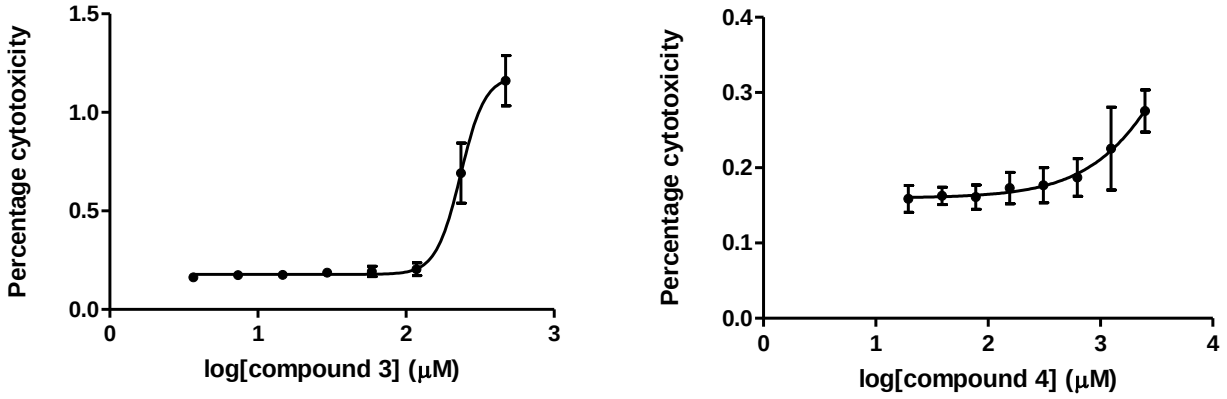

Figure 2: Dose-response curves for compound 3 and 4 against mammalian HepG2 cells

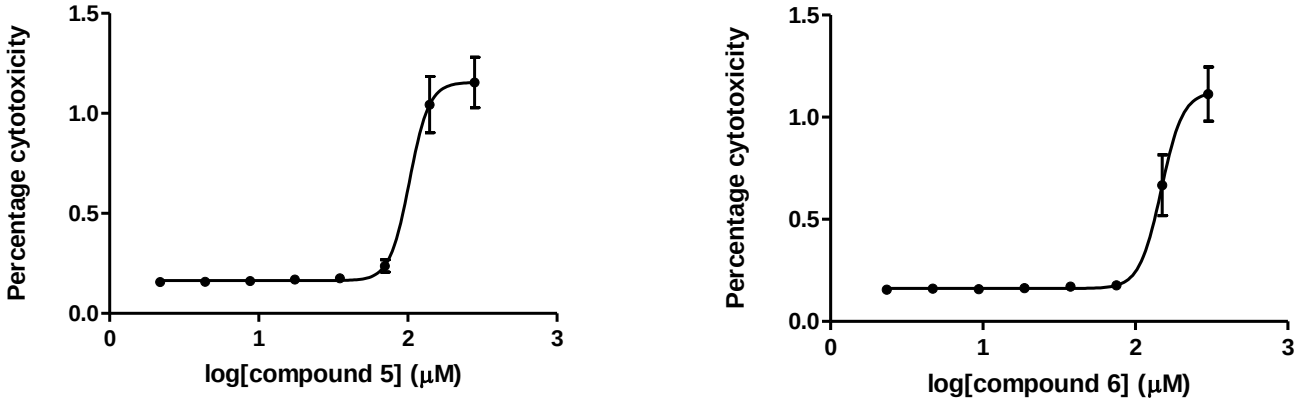

Figure 3: Dose-response curves for compound 5 and 6 against mammalian HepG2 cells

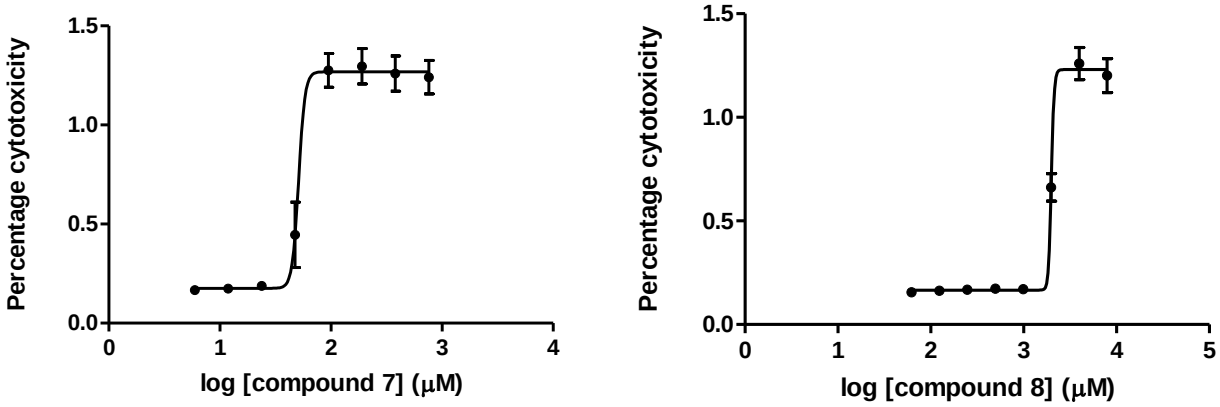

Figure 4: Dose-response curves for compound 7 and 8 against mammalian HepG2 cells

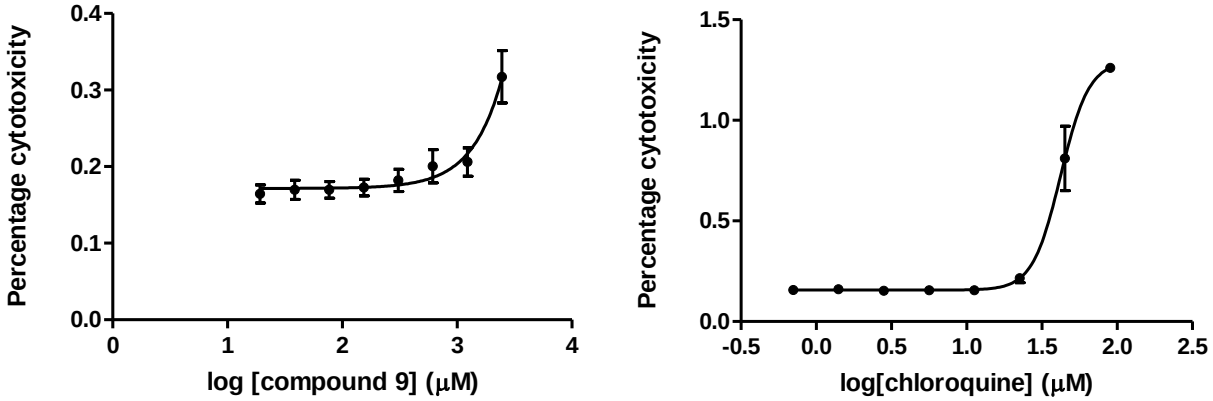

Figure 5: Dose-response curves for compound 9 and chloroquine against mammalian HepG2 cells

Appendix 4

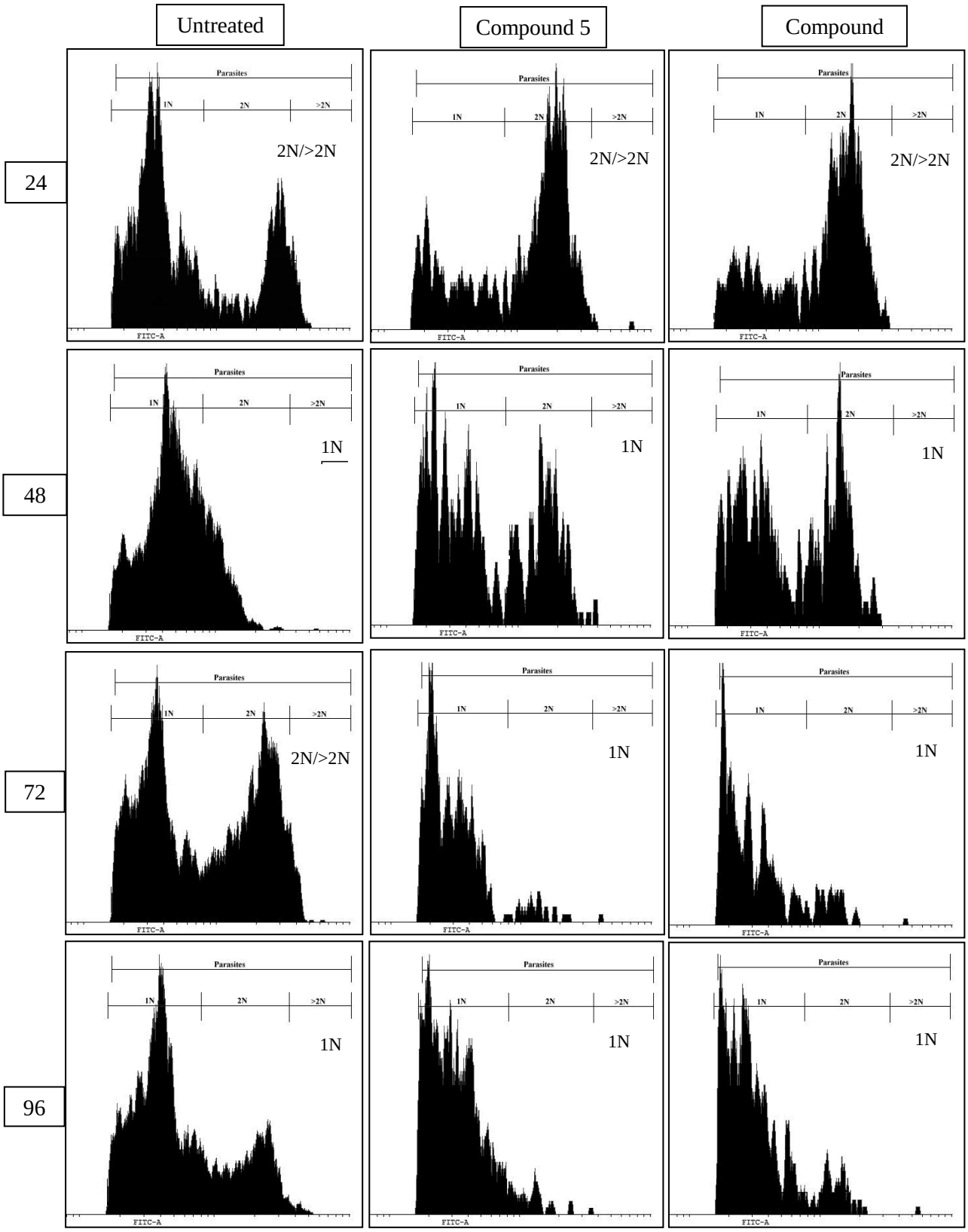

Figure 1: Parasite population gating. Parasite population gating was done using Cyflogic to determine the percentage parasite DNA proliferation over 96 h.