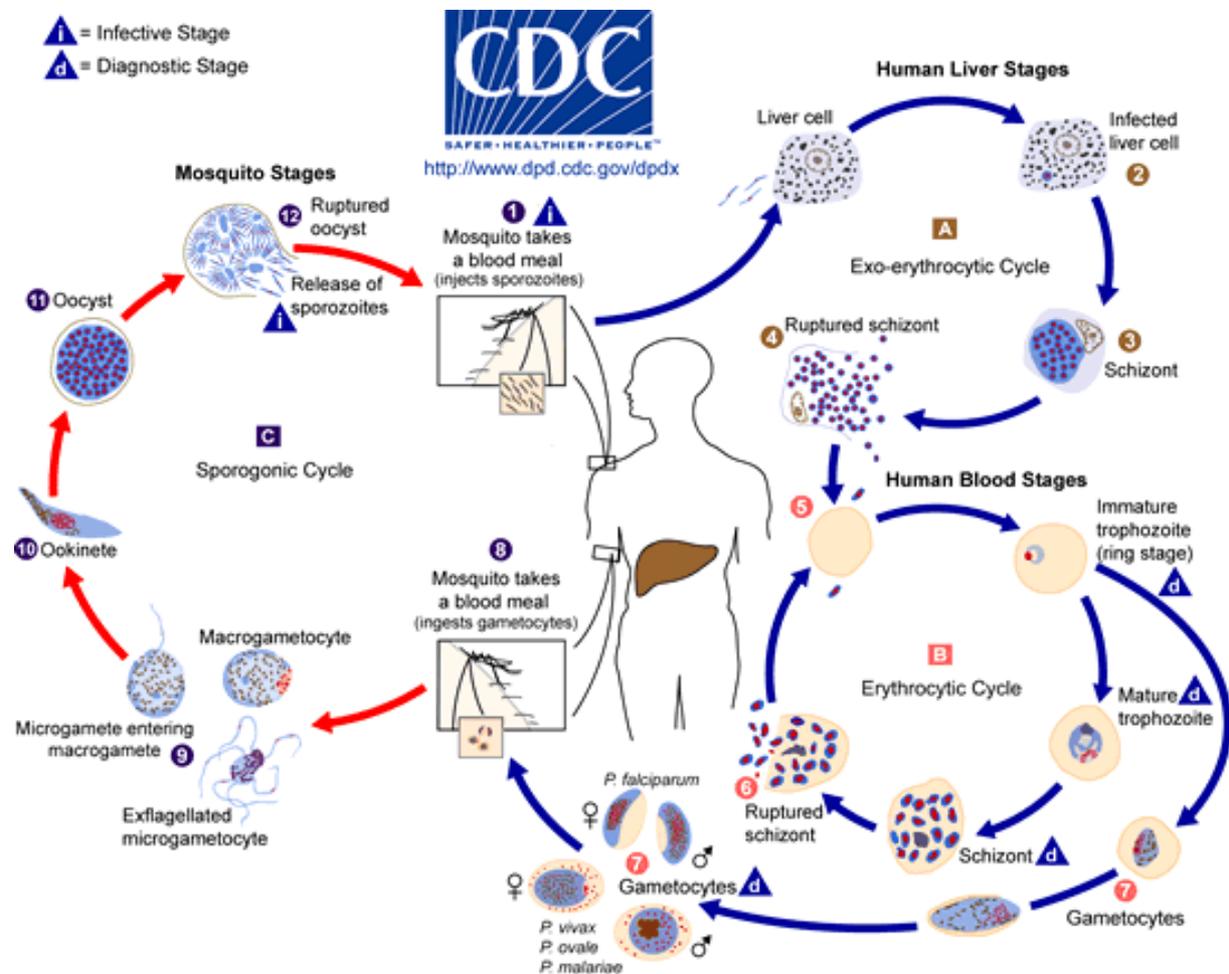


## CHAPTER 2

### 2 LITERATURE REVIEW

#### 2.1 The malaria parasites

Malaria infecting humans is caused by four species of single-celled parasites of the genus *Plasmodium* with *Plasmodium falciparum* being responsible for most human deaths. The other species are *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*. The life cycle of *Plasmodium falciparum* is adapted and specialized to survive different intra- and extra-cellular environments and to evade its hosts' immune responses (Florence *et al.*, 2002). The life cycle is divided into sexual stages in the mosquito and asexual stages in the human host.



**Figure 2** Diagram of *Plasmodium falciparum* life cycle reproduced with permission from the CDC.

## 2.2 *Plasmodium falciparum* life cycle in the human host (Figure 2, stages **A** and **B**)

The pre-erythrocytic development **A** and asexual blood-stage **B** of the parasite life-cycle take place inside the human host. Transmission occurs with intravenous inoculation of malaria sporozoites into the bloodstream during a female mosquito bite. **1** The sporozoites invade the hepatocytes within 45 minutes of inoculation and multiply in the liver, **2** undergoing asexual schizogony. **3** The schizont then ruptures to release thousands of merozoites into the bloodstream that invade erythrocytes and commence the erythrocytic cycle **4** (Miller *et al.*, 1994). Within 30 - 60 seconds the released merozoites penetrate the host's erythrocytes **5** and transform into characteristic ring stage parasites. The immature trophozoites (ring stage) then mature to trophozoites (trophozoite stage). During this stage the parasite prepares the surface of the erythrocyte to mediate cytoadherence and ingests the erythrocyte's cytoplasmic contents, especially haemoglobin. The trophozoites develop into schizonts that undergo nuclear division followed by merozoite formation (Florence *et al.*, 2002). The erythrocyte membrane ruptures to release between 6 - 36 merozoites back into circulation that rapidly re-invade other erythrocytes to continue asexual amplification **6** (White, 2002). Approximately 1% of formed merozoites inside erythrocytes differentiate into sexual forms of male micro-gametocytes and female macro-gametocytes which transmit the infection to the *Anopheles* mosquito **7** (Oh & Chishti, 2005).

## 2.3 *Plasmodium falciparum* life cycle in the mosquito (Figure 2, stage **C**)

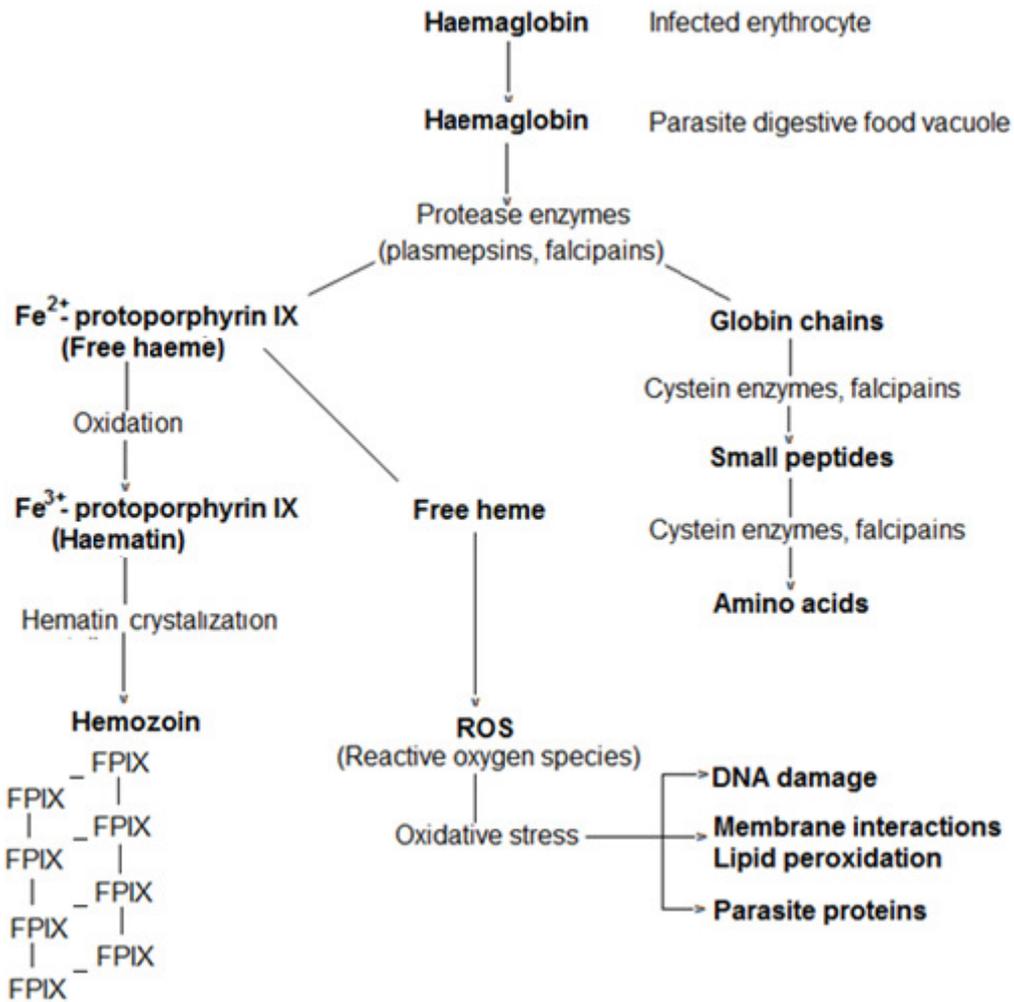
The female *Anopheles* mosquito is the vector of malaria transmission to the human host. The mosquito stage **C** in the parasite life cycle involves the sexual stages in its development and takes between 8 - 35 days. The cycle starts with the ingestion of a blood meal, **8** and requires only one male microgamete and one female macrogamete for infection to occur **9** (White, 2002). The ingested male and female gametocytes undergo gametogenesis in the mosquito's midgut to form a zygote. Within 24 hours the zygote differentiates into an ookinete, **10** which penetrates the wall of the mosquito midgut and develops into an oocyst. **11** The oocyst produces sporozoites that migrate to the salivary glands of the mosquito to invade the gland epithelium. **12** In order for the life cycle to repeat itself, the sporozoites need to be inoculated into a human host during a mosquito blood meal (Oh & Chishti, 2005).

## 2.4 The haemoglobin degradation pathway

### 2.4.1 *Plasmodium* iron metabolism (Figure 3)

During the intra-erythrocytic stage the parasite ingests 75% of its human host's red blood cell content (RBC) (Buller *et al.*, 2002). Haemoglobin is ingested from infected erythrocytes through a cytosome and transported in vesicles to the parasite's digestive food vacuole (Goldberg, 2005). Inside the digestive vacuole the haemoglobin is degraded by the parasite's proteases into globin chains and iron. These chains are enzymatically cleaved into small peptides needed as a source of amino acids for protein synthesis. The parasite uses the iron for nucleotide-, DNA-, pyrimidine- and haem synthesis and electron transport (Mabeza *et al.*, 1999; Scholl *et al.*, 2005). During this degradation process a noted by-product ( $\text{Fe}^{2+}$ ) ferrous-protoporphyrin IX (free haem) is formed that can become toxic. This poses an exploitable problem for the parasite as it lacks haem oxygenase activity or iron storage proteins like ferritin (Scholl *et al.*, 2005). The parasite can only prevent the free haem from accumulating to toxic concentrations by incorporating it into hemozoin. To do this the ( $\text{Fe}^{2+}$ ) ferrous-protoporphyrin IX undergoes one electron oxidation to produce ( $\text{Fe}^{3+}$ ) ferric-protoporphyrin IX (haematin). The haematin rapidly precipitates and forms inert cyclic dimers that crystallize under acidic conditions to form hemozoin (Buller *et al.*, 2002; Fitch *et al.*, 2003; Egan, 2008). This biocrystallisation process is of critical importance to the parasite in order to prevent oxygen radical production, which ideally takes place in the oxygen-rich and acidic environment of the digestive vacuole. In the event of hemozoin formation prevention the molecular oxygen readily accepts the iron electron to initiate a chain of oxygen radical metabolism through the Fenton reaction ( $\text{O}_2^- + \text{H}_2\text{O}_2 \leftrightarrow \text{HO} + \text{O}_2 + \text{HO}^-$ ), which produces damaging free radicals (Pisciotta & Sullivan, 2008; Sullivan, 2002).

If the biocrystallisation process is delayed or inhibited, the reactive oxygen species produced may induce oxidative stress, resulting in parasite death through a cascade of lethal events. These toxic effects include parasite DNA damage, inhibition of proteolytic enzymes, haemoglobin accumulation, ferri-protoporphyrin accumulation, lipid peroxidation of parasite membranes and parasite membrane impairment (Rayenes, 1999; Kumar *et al.*, 2007).



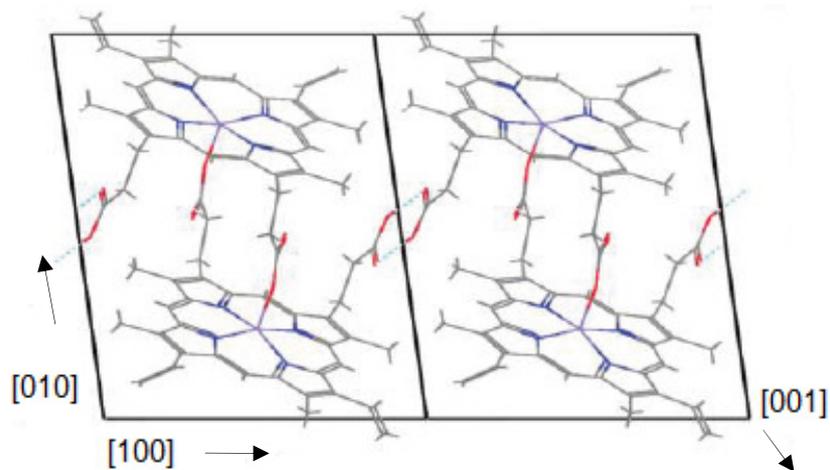
**Figure 3** Haemoglobin degradation pathway and generation of hemozoin (Bray *et al.*, 2005; Buller *et al.*, 2002; Raynes, 1999).

### 2.4.2 Hemozoin structure, formation and function

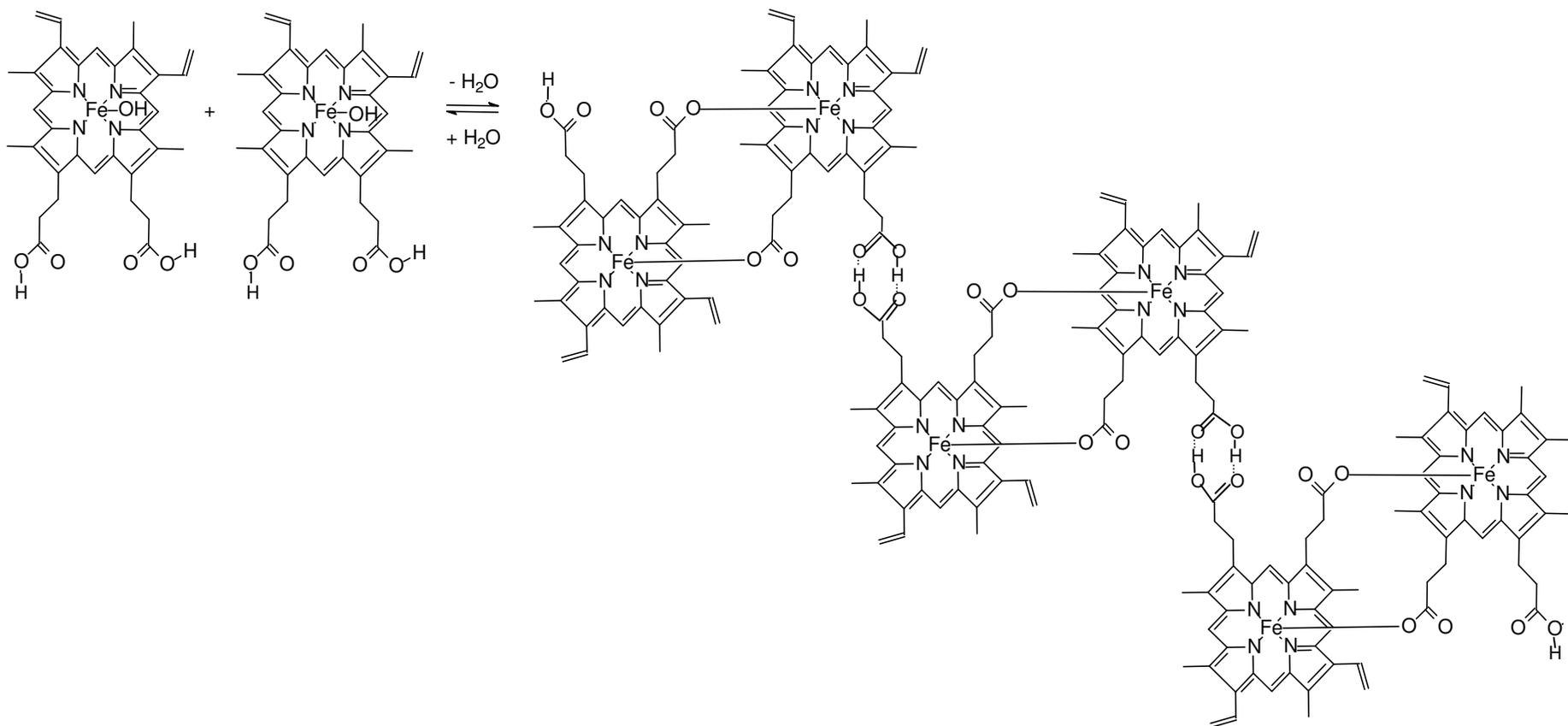
The formation of hemozoin (malaria pigment) is a type of biomineralisation or biocrystallisation process that is initiated by oriented nucleation (Hempelmann *et al.*, 2003; Egan *et al.*, 2002). This nucleation process is induced by the 1-myristoyl-glycerol lipid molecules (MMG) through stereospecific interactions at the {100} crystal face (Figure 4) (de Villiers *et al.*, 2009). The molecular units of the hemozoin crystal consist of ferric-protoporphyrin IX cyclic dimers. These dimers reciprocally link through coordination complexes between the carboxyl group of a propionate side chain of one molecule and the central iron atom of another (Figure 5) (Fitch *et al.*, 2003; Slater *et al.*, 1991). The remaining free propionic acid groups of the dimers interact *via* intermolecular forces to form a hydrogen

bond network, with the head to tail dimers  $\pi$ -stacked along the [010] direction of the crystal face (Figure 4) (Chong & Sullivan, 2003; de Villiers *et al.*, 2009; Fitch, 2004).

Thereafter hemozoin construction takes place inside the digestive vacuole to assemble a membrane impenetrable crystal with 100 nm x 100 nm x 500 nm dimensions, containing over 10 000 000 haemes (Pisciotta & Sullivan, 2008). The crystal has two fast growing faces at the end of the  $\beta$ -haematin needle. This is due to the {001} crystal surface (Figure 4) corrugation and O-H-O hydrogen bonds between the propionic acid groups of neighbouring molecular units (Figure 5) (Egan, 2003; Sullivan, 2002).



**Figure 4** The theoretical growth direction of hemozoin crystal side faces {100} and {010} (Marom *et al.*, 2011).



**Figure 5** Hemozoin formation; a Fe1-O41 head to tail dimer model of hemozoin with coordinating bonds linking dimer pairs and hydrogen bonds linking neighbouring dimer pairs (Egan, 2003; Sullivan, 2002).

## **2.5 The pathology of *Plasmodium falciparum***

Malaria is an acute febrile illness with the clinical manifestation of the disease associated with the development of the asexual parasites in the blood. The onset of pathology is triggered by the rupture of erythrocyte membranes that releases merozoites and erythrocyte material into the circulation (Clark & Schofield, 2000). The released parasite antigens, pigment and malarial toxins induce production of pro-inflammatory cytokines, tumour necrosis factor (TNF $\alpha$ ), interleukin-1 (IL-1) and IL-6 that result in an immune response from the host (Gilles, 1997; Miller *et al.*, 1994). Thus, the first signs and symptoms of malaria are fever followed by headache, chills and vomiting. These symptoms appear 10 - 15 days after an infectious mosquito bite. In uncomplicated malaria the first symptoms are non-specific and resemble those of influenza, which makes the diagnosis of the disease difficult. If the infection is not treated within 24 hours the uncomplicated case can progress to severe illness that often leads to death (WHO 2010). In untreated malaria the infected erythrocytes adhere to the vascular endothelium (cytoadherence) and disappear from circulation. This process is referred to as sequestration and compromises the microcirculation in vital organs. In addition to sequestration, the formation of erythrocyte clumps through rosetting, where uninfected erythrocytes adhere to infected erythrocytes, further compromise blood flow to vital organs (Dondorp; 2005). Cytoadherence, rosetting and autoagglutination lead to microcirculatory obstruction in patients and result in reduced oxygen supply that causes anaerobic glycolysis, lactic acidosis and cellular dysfunction (White, 2002). Other complications of the disease include coma, renal failure, noncardiac pulmonary oedema, anaemia, liver dysfunction, gastrointestinal dysfunction, placental dysfunction, acidosis, hypoglycaemia and bacterial infections. Death in children suffering from severe cases of the disease is often attributed to cerebral malaria, malarial anaemia or metabolic acidosis (Gilles, 1997).

## **2.6 Malaria treatment**

The eradication campaign launched against malaria from 1940 to 1970 was the first attempt at global level to control the disease. The disillusionment that followed the emergence of monodrug- and multidrug resistance in *Plasmodium falciparum*, as well as *Anopheles* mosquito vector resistance to DDT spraying led to the abandonment of the global control efforts (Sachs, 2002). The recent increase in malaria incidence renewed the interest in innovative malaria research and new efforts to control rather than eradicate the disease. New initiatives for drug discovery, vaccine development and malaria research were launched in 1997 to 1999. These initiatives include Roll Back Malaria (RBM), the Medicines for Malaria

Venture (MMV), Multilateral Initiative on Malaria (MIM) and the Malaria Vaccine Initiative (MVI) (Sachs, 2002; Wellems, 2002). The development of vaccines against malaria has made good progress with many potential candidates in clinical trials (WHO 2010). These include pre-erythrocytic vaccines that target sporozoites, asexual stage vaccines that target the merozoites, and a transmission blocking vaccine against the sexual parasite stages in the mosquito vector (Breman *et al.*, 2004). Although promising, there is currently no licensed vaccine available against malaria (WHO 2010). Therefore the mainstay of malaria treatment and prophylaxis still remain antimalarial chemotherapy.

The antimalarial drugs possess selective actions on different stages of the parasite life cycle. These are:

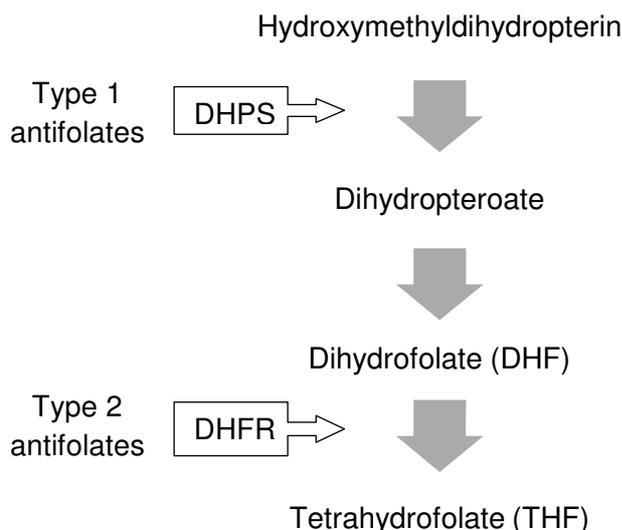
- Blood schizonticides: antimalarial drugs that act on erythrocytic parasites by eliminating blood schizonts in the erythrocytes during the erythrocytic stage.
- Tissue schizonticides: drugs that prevent invasion of malaria parasites in erythrocytes by eliminating developing tissue schizonts or hypnozoites in the liver.
- Gametocides: drugs that destroy the sexual forms of the parasite in the blood and prevent transmission to mosquitoes.
- Sporontocides: antimalarial drugs that prevent the development of oocysts in the mosquito and render gametocytes non-infective (Katzung, 2001).

## **2.7 Antimalarial drugs**

Many of the current antimalarial drugs can be divided into the broad groups of antifolates, artemisinins, quinolines and arylaminoalcohols. These groups are divided according to different pharmacophores, mechanism of action and selective action on different stages of the parasite life cycle.

### **2.7.1 The antifolate drugs**

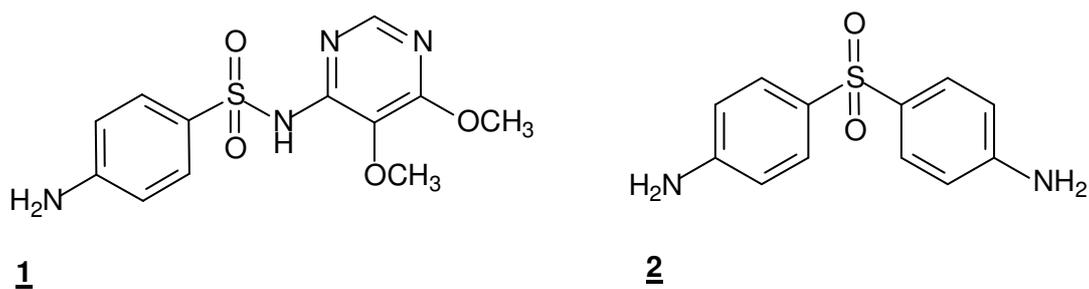
Humans and parasites have the ability to convert folic acid into tetrahydrofolic acid. While humans obtain the needed folic acid from their diet, the parasite has to synthesise its own dihydrofolic acid from dihydropteroic acid. This feature is responsible for the selective toxicity of antifolate antimalarials, which interfere with the parasite's folic acid synthesis (Vangapandu *et al.*, 2006).



**Figure 6** Targets of the antifolate drugs (Olliaro, 2001).

### 2.7.1.1 Type 1 antifolates: sulphonamides and sulphones (Figure 7)

Sulphonamides such as sulphadoxine **1**, and the sulphones such as dapsone **2**, mimic *p*-aminobenzoic acid (PABA) and interfere with folic acid synthesis by preventing the formation of dihydropteroate. These compounds compete with the parasitic enzyme dihydropteroate synthase (DHPS) for its active binding site (Figure 6) (Olliaro, 2001). The drugs mainly act as blood schizonticides and are usually administered with pyrimethamine or other various combinations (Sweetman, 2009).

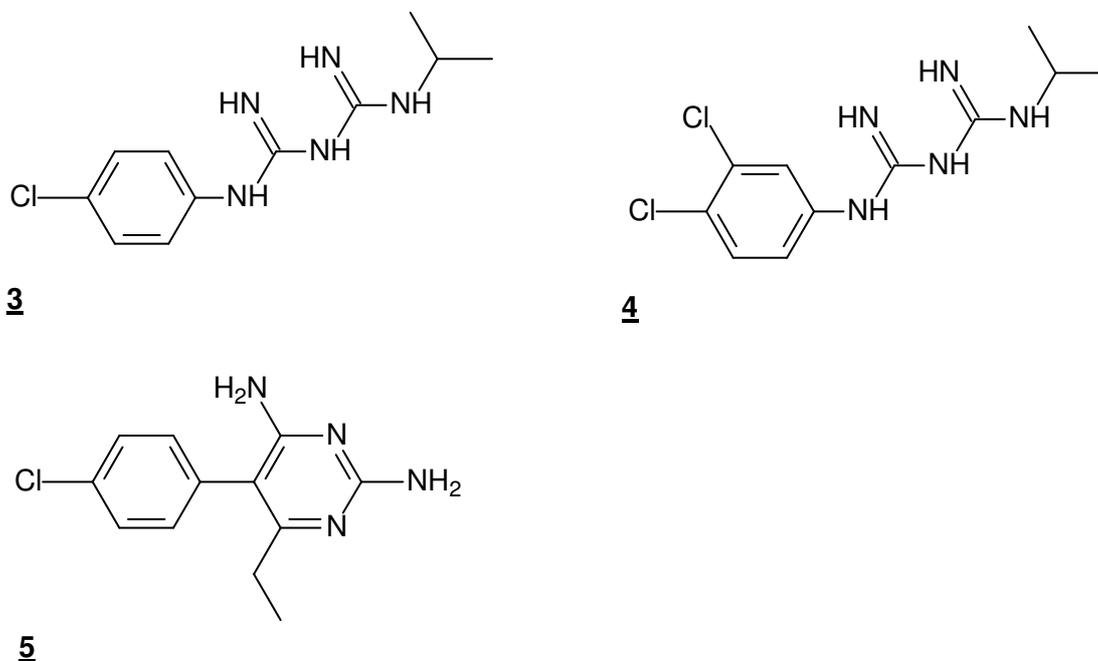


**Figure 7** The dihydropteroate inhibitors (DHPS) sulphadoxine **1** and dapsone **2**.

### 2.7.1.2 Type 2 antifolates: biguanides and diaminopyrimidines (Figure 8)

The biguanides such as proguanil **3** and chlorproguanil **4**, and the diaminopyrimidines such as pyrimethamine **5** inhibit dihydrofolate reductase (DHFR). The type 2 antifolates interfere with folic acid synthesis by preventing the NADPH-dependent reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) (Olliaro, 2001). These types of drugs act as tissue

schizontocides and are slow-acting blood schizontocides but are weak antimalarials. Proguanil is usually administered in combination with chloroquine for chemoprophylaxis or atovaquone for treatment and chemoprophylaxis of *P. falciparum* infection (Katzung, 2001; Sweetman, 2009). Pyrimethamine is combined with the sulpha drugs that provide a synergistic activity (Ashley *et al.*, 2006).



**Figure 8** The dihydrofolate reductase inhibitors (DHFR) proguanil 3, chlorproguanil 4 and pyrimethamine 5.

### 2.7.1.3 Resistance to antifolate drugs

The emergence of resistance in *Plasmodium falciparum* to antifolate drugs results from specific point mutations in DHFR and DHPS malarial enzymes. The folate antagonists that inhibit the folate pathway cannot recognize the mutated enzymes of resistant strains, whereas these enzymes can still bind to substrates and catalyze the formation of DHF and THF (Plowe *et al.*, 1998). Resistance to biguanides and pyrimethamine are associated with DHFR mutation at codons 164, 108, 54, 51 and 16, whereas *Plasmodium falciparum* resistance to sulphonamides and sulphones is related to DHPS mutation at codons 581, 540, 436, 437 and 613 (Plowe, 2005; Olliaro, 2001).

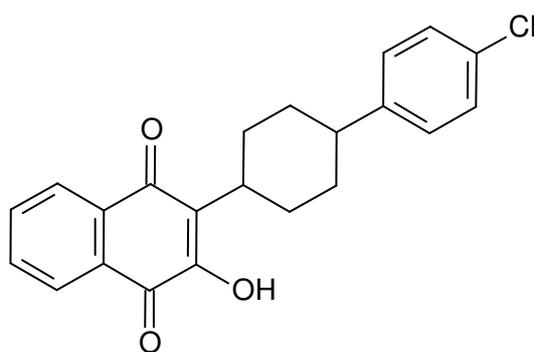
## 2.7.2 The naphthoquinones

### 2.7.2.1 Atovaquone

The combination of the hydroxynaphthoquinone atovaquone and proguanil has a synergistic antimalarial activity effective for treatment and prophylaxis of malaria (Katzung, 2001). Atovaquone exhibits weak antimalarial activity when administered as an individual agent by acting as a blood schizonticide. The drug inhibits the parasite's cellular respiration by interfering with parasite mitochondrial electron transport and also depolarizing the parasite mitochondria (Strivastava & Vaidya, 1999; White, 2002).

### 2.7.2.2 Resistance to atovaquone

Resistance in *P. falciparum* to this drug results from single-point mutations in the cytochrome b gene that changes amino-acids in the co-enzyme Q binding site (Canfield *et al.*, 1995; Olliaro, 2001).



**6**

**Figure 9** The hydroxynaphthoquinone atovaquone **6**.

## 2.7.3 Sesquiterpene lactones

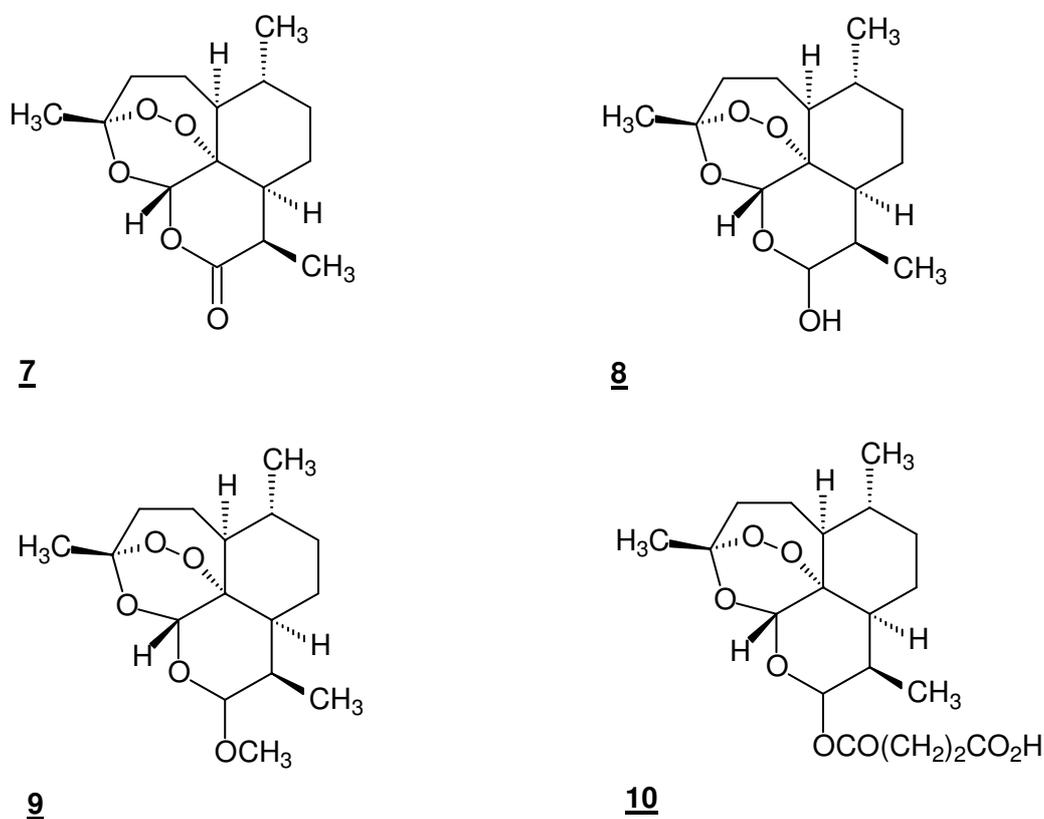
### 2.7.3.1 The artemisinins

The artemisinin compounds include artemisinin **7**, dihydroartemisinin **8**, artemether **9**, arteether and artesunate **10**. These drugs are very rapid acting blood schizontocides with selective action on the young ring stage up until early schizont stage of the parasite erythrocytic cycle (White, 2002). However, the exact mechanism of action of artemisinins remains uncertain. One of the mechanisms that have been proposed involves the reductive cleavage of the intact peroxide bridge by ferrous-protoporphyrin IX to produce reactive free radical intermediates. The oxygen free radicals are rearranged to form carbon centred radicals that alkylate vital parasite proteins (Olliaro, 2001; Biagini *et al.*, 2003). The

artemisinins are co-formulated with other antimalarial drugs such as lumefantrine, amodiaquine, mefloquine, piperazine and pyronaridine as fixed-dose combination called ACT's. Artemisinin combination therapy (ACT) is replacing monotherapies for the treatment of malaria in an effort to prevent the emergence of resistance to artemisinins (White, 2002).

### 2.7.3.2 Resistance to artemisinins

Recently artemisinin based combination therapies' efficacies have declined on the Thai-Cambodian border. Artemisinin resistance in multi-drug resistant parasites are associated with slower parasite clearance *in vivo*; without corresponding reduction on conventional *in vitro* susceptibility testing (Dondorp, 2005). This observed artemisinin-resistant phenotype has a genetic basis that is not yet known and parasites do not contain consistent mutations (Dondorp, 2005).



**Figure 10** Artemisinin 7, dihydroartemisinin 8, artemether 9 and artesunate 10.

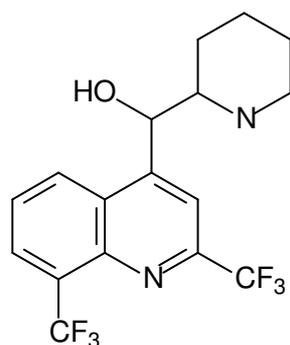
### 2.7.4 The quinolines and arylaminoalcohols

The quinoline antimalarial drugs' efficacy in the treatment of malaria started with the isolation of quinine from the bark of the Cinchona tree by Pelletier and Caventou in 1820 (Foley & Tilley, 1998). Since then, the synthetic quinoline antimalarial drugs represent the leading

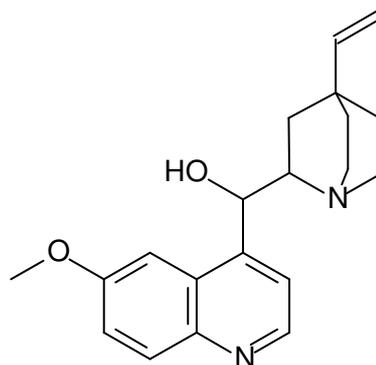
chemotherapy against malaria for much of the last 50 years (Biagini *et al.*, 2003). This broad group of antimalarials are all weak bases, and act on the growing intra-erythrocytic stages of the parasite life cycle when haemoglobin ingestion takes place.

#### 2.7.4.1 The 4-methanolquinolines

The 4-methanolquinolines such as mefloquine **11** and the cinchona alkaloids such as quinine **12** and quinidine act as blood schizontocides. Intravenous or intramuscular quinine remains the first-line drug for severe malaria treatment (Newton & White, 1999). Mefloquine is structurally related to quinine and inhibits ingestion of host cell haemoglobin by interfering with the parasite's endocytic process (Bray *et al.*, 2005b). The emergence of drug resistance in *P. falciparum* to mefloquine is associated with amplification of the multi-drug resistance (*mdr*) genes that encode for *Plasmodium* p-glycoprotein efflux pump, which reduces drug concentrations within the parasite (Newton & White, 1999).



**11**

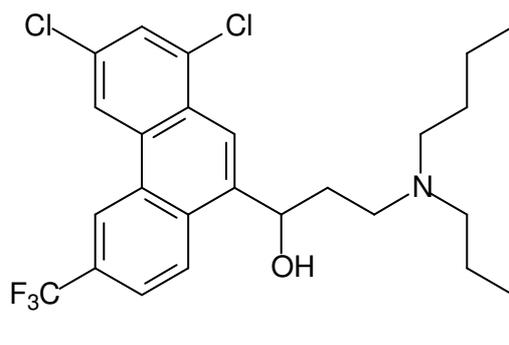


**12**

**Figure 11** The 4-methanolquinoline mefloquine **11** and cinchona alkaloid quinine **12**.

#### 2.7.4.2 The 9-phenanthrenemethanols

The 9-phenanthrenemethanol halofantrine **13** is structurally related to mefloquine and is used as a blood schizontocide (Sweetman, 2009). This drug is effective against erythrocytic stages of all four human malaria species, but its use is limited by irregular absorption and cardiotoxicity (Katzung, 2001). *Plasmodium falciparum* drug resistance to halofantrine is contributed to multiple unlinked mutations (White, 2002).

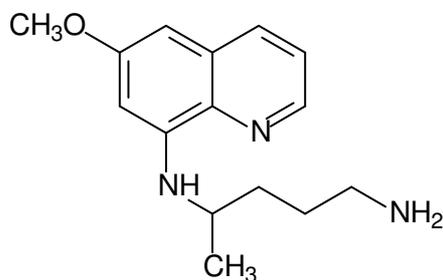


**13**

**Figure 12** Halofantrine **13**.

#### 2.7.4.3 The 8-aminoquinolines

The 8-aminoquinolines such as primaquine **14** are tissue schizontocides. Primaquine is the only drug effective against the dormant hypnozoite stages of *P. vivax* and *P. ovale*. It is also used as a single dose to eradicate *P. falciparum* gametocytes *via* an oxidative stress mechanism (Sweetman, 2009; Grimberg & Mehlotra, 2011; White, 1999). Some strains of *P. vivax* in New Guinea, Southeast Asia are relatively resistant to primaquine. However, these reports of resistance are sporadic (Katzung, 2001; Vangapandu *et al.*, 2006).



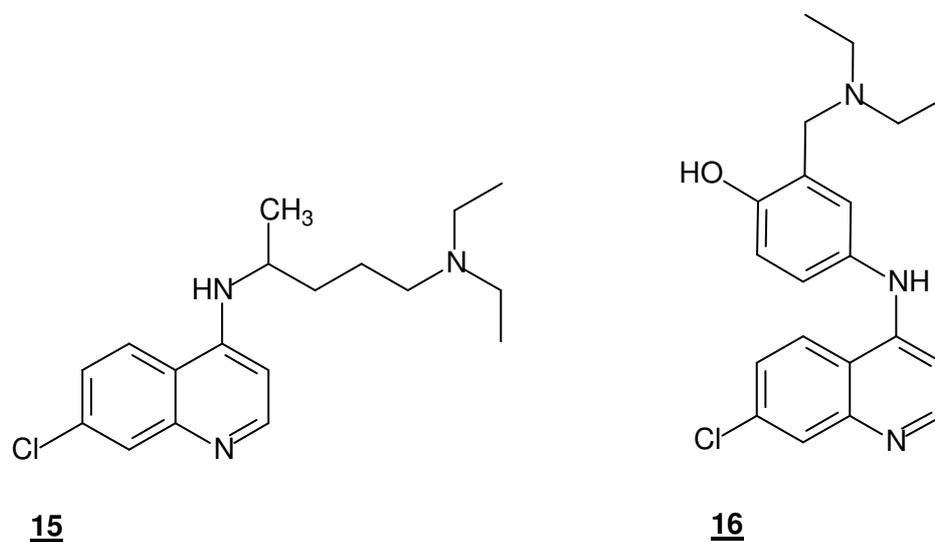
**14**

**Figure 13** Primaquine **14**.

#### 2.7.4.4 The 4-aminoquinolines

The 4-aminoquinolines include chloroquine **15**, hydroxychloroquine and amodiaquine **16**. These drugs are rapid acting blood schizontocides with some gametocidal activity (Sweetman, 2009). The 4-aminoquinolines inhibit the parasite detoxification pathway of free haem released during haemoglobin degradation that results in intraparasitic toxicity. Other effects of these drugs include the inhibition of parasite protein synthesis, impairment of

lysosome function and parasite membrane permeability (White, 2002). Amodiaquine has been widely used to treat malaria because of its low cost, limited toxicities and effectiveness against chloroquine resistant parasites strains (Katzung, 2001).



**Figure 14** The 4-aminoquinolines chloroquine **15** and amodiaquine **16**.

#### 2.7.4.4.1 Development of 4-aminoquinoline resistance

The development of resistance in *Plasmodium falciparum* to the 4-aminoquinolines is connected to multiple parasite gene mutations. Chloroquine resistance is mainly associated with an altered drug accumulation or extrusion mechanism, which reduces drug accumulation inside the digestive food vacuole. The *P. falciparum* chloroquine resistant phenotype is determined by multiple mutations in PfCRT alleles that consistently include mutations of codons A220S and K76T (van Schalkwyk & Egan, 2006). These mutations by the parasite create important physiological changes in the digestive vacuole that are necessary for chloroquine resistance (Fidock et al., 2000). An important protein identified in chloroquine resistance is a 424 amino acid protein (PfCRT), which facilitates the transport of chloroquine out of the digestive vacuole to ultimately reduce drug accumulation (Fidock et al., 2000). Furthermore, the rapid extrusion of drug out of the digestive vacuole is mediated by an ATP-dependant P-glycoprotein efflux pump (Pgh1), which also contributes to reduced drug concentrations. The Pgh1 efflux pump is the protein product of the PfMDR1 gene, which is associated with multiple drug resistance (Plowe, 2005; Grimberg & Mehlotra, 2011). Polymorphisms, copy number variation and point mutations in PfMDR1 or Pgh1 modulate chloroquine resistance in PfCRT mutant parasites and contribute to the parasite's susceptibility to antimalarial drugs.

Other mechanisms that may be responsible for chloroquine resistance include an elevated digestive vacuole pH, a rapid chloroquine efflux multidrug-resistant mechanism, a carrier-mediated chloroquine uptake, and a reduced drug affinity to ferri-protoporphyrin (Gullion *et al.*, 2004).

#### **2.7.4.4.2 Mechanism of action of 4-aminoquinolines**

The efficacy of the aminoquinoline antimalarial drugs is stage specific for parasites actively degrading hemozoin. This was confirmed by findings that quinoline-containing drugs were inactive against *Babesia microti*, a parasite with a similar blood cycle as *P. falciparum* but which does not form hemozoin (Chong & Sullivan, 2003; Egan, 2003). Ward and co-workers also confirmed that the activity of the aminoquinoline-based drugs were (Fe<sup>3+</sup>) ferri-protoporphyrin dependant. Given that, inhibitors of the protease enzymes (plasmepsins) that initiate haemoglobin degradation antagonized their activity (Egan *et al.*, 2000).

Two main theories exist as to how hemozoin crystal formation is inhibited by the aminoquinoline drugs;

- The formation of a drug-ferri-protoporphyrin IX complex that causes substrate depletion,
- Hemozoin crystal “capping” of the fastest growing face.

The formation of a drug-ferri-protoporphyrin IX complex is supported by the ferri-protoporphyrin IX (FPIX) interaction hypothesis of Fitch, (2004). The drug will noncovalently bind to undimerised FPIX or form a drug-FPIX complex that prevents the molecular units from acting as substrates for incorporation into the hemozoin crystal (Dorn *et al.*, 1998). The other mechanism involves the inhibition of hemozoin crystal growth, where adsorption of the drug onto the crystal’s growing face forms a covering layer on the surface that prevents further growth. This interaction would then ultimately block the fastest growing crystal face by “capping” the hemozoin chain. This mechanism may explain the efficacy of chloroquine in the inhibition of hemozoin crystal formation in the presence of a large molar excess of haematin (Egan, 2003; Buller *et al.*, 2002; Sullivan *et al.*, 1996; Scholl *et al.*, 2005).

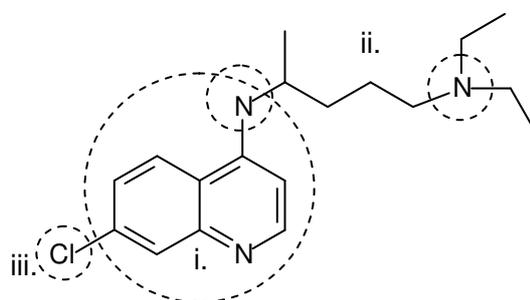
The exact mechanism of the inhibition of hemozoin formation is yet to be fully elucidated. However, the accumulation of undimerised FPIX or drug-FPIX complex exerts its intrinsic toxicities by a similar mechanism as free haem, leading to a cascade of events that ultimately results in parasite death (Pisciotta & Sullivan, 2008). Toxicities include morphological changes in the parasite, haemoglobin accumulation, ferri-protoporphyrin

accumulation, impairment of membrane function, inhibition of proteolytic enzymes, masking of lipids that promote FP IX dimerisation, destruction of glutathione and the release of calcium from acidic stores (Fitch *et al.*, 2003). The most lethal defects induced are membrane impairment caused by lipid peroxidation and the destabilization of membranes through a colloid osmotic mechanism (Egan, 2008).

#### 2.7.4.4.3 The potential binding site for 4-aminoquinoline drugs

The interactions of these drugs with haematin involve neither the formation of chemical bonds nor docking with structurally well defined binding pockets. Consequently these complexes are likely to be highly flexible and capable of assuming many different formations (Kaschula *et al.*, 2002). Buller and co-workers proposed possible binding sites for the 4-aminoquinoline drugs on the  $\beta$ -haematin crystal's corrugated {001} surface (Buller *et al.*, 2002). The fastest growing crystal face contains parallel grooves that expose flexible propionic acid groups, vinyl and methyl groups and aromatic surfaces that are attractive sites for drug interactions (Figure 15).

The structure-activity relationships for chloroquine is describe according to the theoretical binding sites proposed;



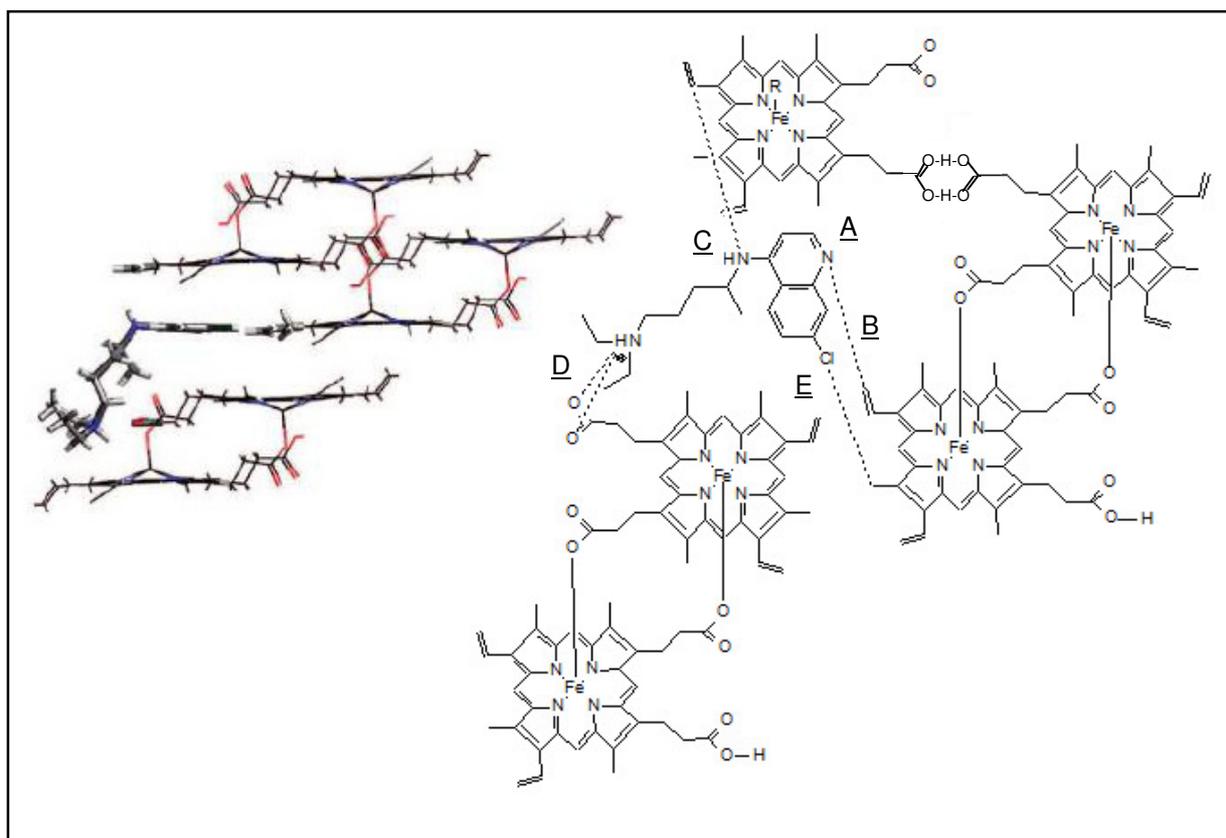
**Figure 15** The structure of 7-chloro-4-aminoquinoline.

##### i. The importance of the aromatic nucleus in haematin binding

The 4-aminoquinoline scaffold is a feature that regularly repeats in malaria chemotherapeutics and is known to be the ( $\text{Fe}^{3+}$ ) ferri-protoporphyrin complex formation template. The stability of the drug-haem complex is determined by the ability of the aromatic quinoline nucleus to intercalate onto the tetrapyrrole surface of ferri-protoporphyrin IX through non covalent  $\pi$ - $\pi$  interactions. In the CQ-FPIX complex the quinoline ring positions itself towards the edge of the tetrapyrrole ring of the iron porphyrin rings rather than above the ferric iron centre (Leed *et al.*, 2002). The quinoline moiety also contributes to the weak

basicity afforded to chloroquine that is responsible for drug accumulation through the weak base effect of the Henderson-Hasselbach model (Raynes, 1999; Sullivan, 2002).

- Diprotonated chloroquine may stereochemically cap onto the surface of the crystal through intercalation of the quinoline rings between the aromatic porphyrin groups **A** (Figure 16).
- The quinoline nitrogen can form a hydrogen bond with the vinyl group of a porphyrin ring **B** (Figure 16).



**Figure 16** Theoretical binding sites for chloroquine within the crystal crevice that allows for interactions with three porphyrin groups of the hemozoin crystal (Buller et al., 2002; Egan, 2003; Weissbuch & Leiserowitz, 2008).

ii. The 4-aminoquinoline drug side chain and haematin binding

The role of the aliphatic side chain in haematin binding is to stabilize the complex with the formation of a salt bridge between the positively charged terminal amino group, and the negatively charged propionate group of the porphyrin molecule (Bray *et al.*, 2005; Egan, 2003). The alteration of the side chain of chloroquine has little influence on the activity of compounds against chloroquine sensitive strains, but enables derivatives to regain activity

against chloroquine resistant strains (Egan *et al.*, 2000). The basic nitrogen atoms attached to the amino-alkyl side chain is a requirement for potent antimalarial activity, since it assists in drug accumulation inside the digestive vacuole through pH-trapping.

- The 4-amino group of the quinoline drugs can interact with the  $\pi$ -system of the vinyl-group of the haematin porphyrins **C** (Figure 16).
- The protonated tertiary amine of the chloroquine side chain may form a stabilizing salt bridge with the carboxyl group of the haematin propionate side chain **D** (Figure 16).

iii. The role of chlorine substitution in haematin binding

The chlorine substituent at the C-7 position of the quinoline ring contributes to the inhibition of hemozoin formation but not the strength of ( $\text{Fe}^{3+}$ ) protoporphyrin-drug association (Egan *et al.*, 2000; Kaschula *et al.*, 2002). Previous studies have shown that a 7-H derivative of chloroquine has a 14 fold lower antimalarial activity than chloroquine itself (Raynes *et al.*, 1996).

- The 7-chloro group of the drug can interact with a methyl-group of a neighbouring porphyrin moiety of haematin **E**.

#### **2.7.4.5 The pH-trapping mechanism of quinoline antimalarials**

The transmembrane proton gradient is a determining factor in chloroquine accumulation (Yayon *et al.*, 1984; 1985). The quinoline drugs are weak bases which traverse down the pH gradient between the extracellular matrix and the acidic food vacuole of the malaria parasite. The unprotonated form of the drug diffuses freely across the parasite membranes and accumulates in the acidic vacuole where it becomes protonated, membrane-impermeable and trapped. The protonation of the drug at the acidic pH results in inflow of more drug until the free base concentrations are equal on both sides. The accumulated drug inside the digestive vacuole binds to hemozoin that reduces free drug concentration inside the vacuole. Subsequently, this drives the equilibrium towards complex formation through Le Chatelier's principle (Raynes, 1999; Egan *et al.*, 2000). Binding to the drug target strongly contributes to the total accumulation of the drug within the digestive vacuole, which may explain the hyper concentration of chloroquine from nanomolar- in the plasma to millimolar concentrations in the digestive vacuole (Raynes, 1999; Sullivan, 2002).

## 2.7.4.6 Advances in quinoline antimalarials: The bisquinolines

### 2.7.4.6.1 Rationale for the synthesis of biscompounds

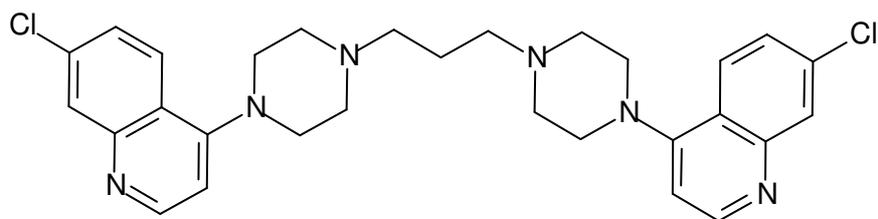
Biscompounds comprise two identical pharmacophores combined through an aliphatic, aromatic or amino alkyl linker. These structures are designed in an effort to increase the concentration of the pharmacophore at the drug target with the aim of avoiding cellular efflux associated with multidrug resistance of the monopharmacophoric drug (Caffrey *et al.*, 2007). The bisquinolines are attractive templates in the design of new drugs, since their potency as hemozoin crystal inhibitors are well correlated with their *in vitro* antimalarial activity against chloroquine sensitive strains (Vennerstrom *et al.*, 1998). It is possible that bisquinolines utilize the pH-trapping mechanism to a greater extent than their monoquinoline forms, seeing that they contain more potential protonation sites. Apart from the advantage of increased accumulation through the pH-trapping mechanism, the bulky structures and their decreased conformational mobility may also make them less efficiently extruded by the PfPgh1 efflux proteins (Gullion *et al.*, 2004). Furthermore, the bisquinolines may overcome parasite resistance through alteration of the chloroquine side chain, which avoids recognition of the structures by efflux proteins (Vippagunta *et al.*, 1999).

### 2.7.4.6.2 Overview of related literature

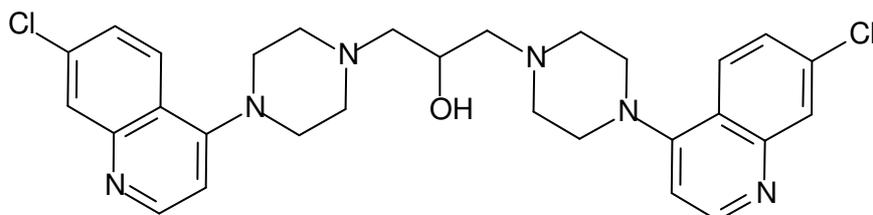
The development of the biscompounds from early bisquinolines such as piperavaquine was aimed at understanding their efficacy against chloroquine resistant strains of *P. falciparum*, clarifying their mechanism of action, overcoming cytotoxicity and optimizing their structure activity relationships. The progress in bisquinolines study can be summarized as follows:

1. Bisquinoline compounds containing a piperazine linker group attached to the 4-position (Figure 17)

The first bisquinoline antimalarial drug was synthesised in 1960 (Bawa *et al.*, 2010). Piperavaquine **17** and its analogue, hydroxylpiperavaquine **18** were more potent *in vitro* than chloroquine against chloroquine sensitive and resistant strains, but further development of the series was temporarily suspended for toxicity reasons (Vangapandu *et al.*, 2006).



**17**

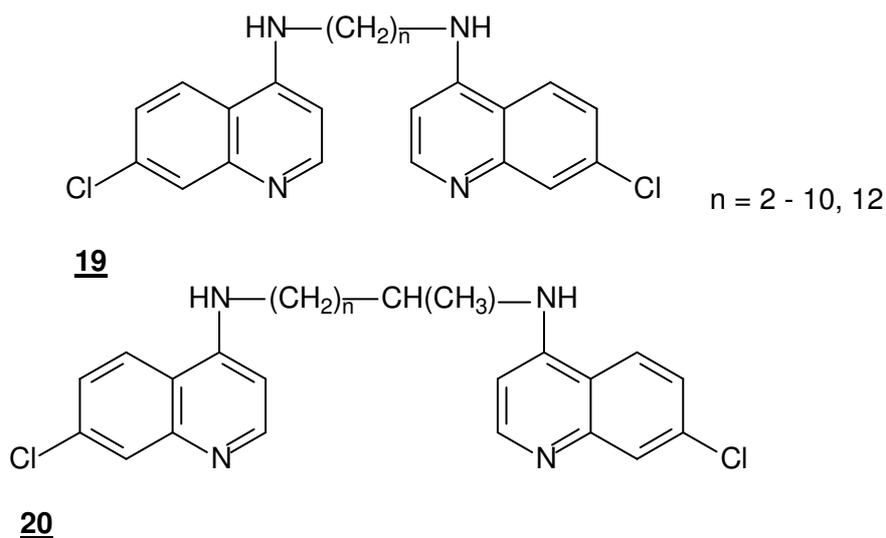


**18**

**Figure 17** Piperavaquine **17** and hydroxypiperavaquine **18**.

2. Bisquinoline compounds linked through an aliphatic diamine attached to the 4-position of the quinoline ring (Figure 18)

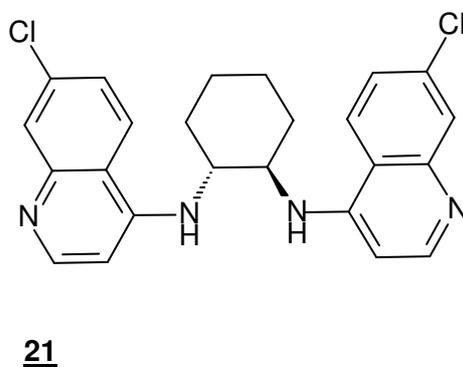
Vennerstrom and co-workers synthesised thirteen *N,N*-Bis(7-chloroquinolin-4-yl)alkanediamines derivatives of which 12 had a significantly lower resistance index than chloroquine (Vennerstrom *et al*, 1992). Seven of these compounds **19** and **20** had  $IC_{50}$ -values of less than 6 nM against *P. falciparum* D-6 and W-2 strains. In their study maximum activity was observed for bisquinolines connected by a bridge of two carbons, where decreased conformational mobility increases antimalarial activity.



**Figure 18** *N,N*-Bis(7-chloroquinolin-4-yl)alkanediamines **19** and **20** (Vennerstrom *et al.*, 1992).

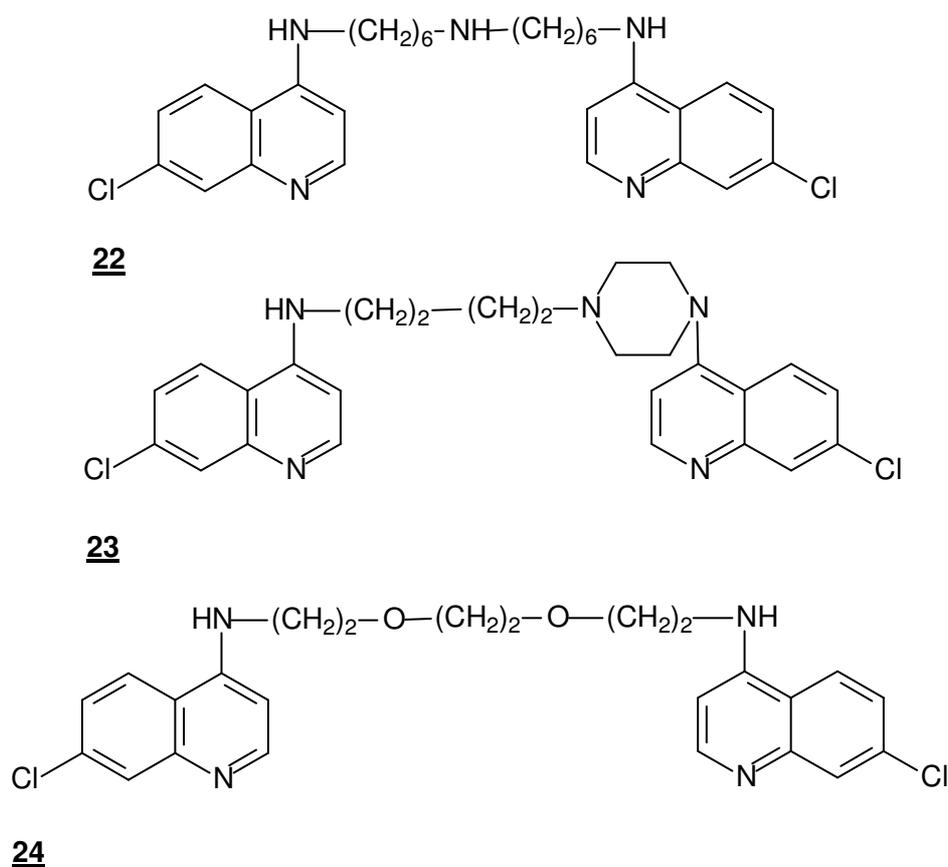
3. Bisquinoline derivatives linked through the 4-position with a cyclohexane diamine linker (Figure 19)

The *trans*-*N*<sup>1</sup>,*N*<sup>2</sup>-bis-(7-chloroquinolin-4-yl)cyclohexane-1,2-diamine, WR 268,668 **21** is a very efficient haematin polymerization inhibitor and showed potent *in vivo* antimalarial activity. The compound underwent preclinical evaluation but further development was stopped due to phototoxicity (Vennerstrom *et al.*, 1998; Vangapandu *et al.*, 2006). Ridley and co-workers extended upon this work and prepared the enantiomeric forms of the bisquinoline *trans*-*N*<sup>1</sup>,*N*<sup>2</sup>-bis(7-chloroquinolin-4-yl)cyclohexane-1,2-diamine (Ridley *et al.*, 1997). The (*S,S*)-enantiomer Ro 47-7737 overcame chloroquine resistance better than the (*R,R*)-enantiomer and was significantly more potent than the previously reported racemate, but remained phototoxic.



**Figure 19** The bisquinoline WR 268,668 (Ro 48-6910), **21** (Vennerstrom *et al.*, 1998).

Vennerstrom and co-workers synthesised a series of bisquinoline heteroalkanediamines (Vennerstrom *et al.*, 1998). These compounds were found to be potent inhibitors of haematin polymerization with IC<sub>50</sub> values in the 5 - 20 μM range. These bisquinolines had IC<sub>50</sub>-values from 1 - 100 nM against *P. falciparum* D-6 and W-2 strains. The highest activity in each series was observed for compound **22** (IC<sub>50</sub> = 9.9 nM), **23** (IC<sub>50</sub> = 5.4 nM), **24** (IC<sub>50</sub> = 9.0 nM) against the chloroquine resistant W2 strain. The incorporation of nitrogen and oxygen atoms in the heteroalkane connecting bridge did not improve antimalarial activity compared to their alkane bridged bisquinoline counterparts.

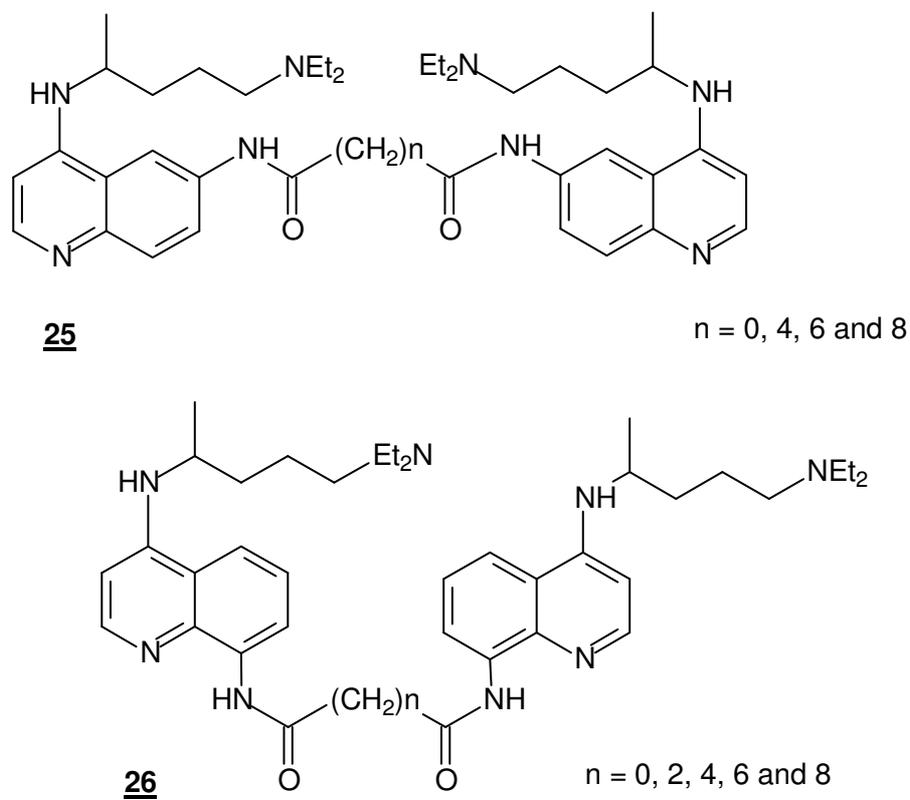


**Figure 20** *N,N*-Bis(7-chloroquinolin-4-yl)heteroalkanediamines **22** - **24**.

4. Bisquinolines connected by bisamide linkers from the 6- and 8-quinoline position (Figure 21)

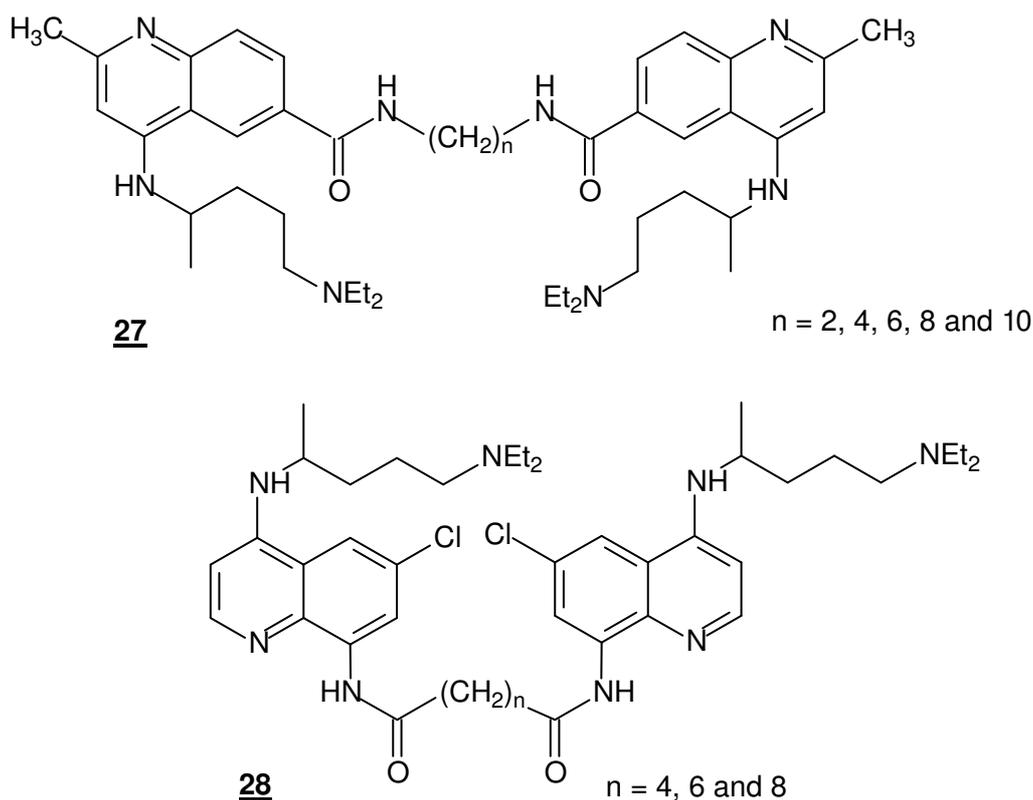
Raynes and co-workers investigated the antimalarial activity of two series of bisquinolines connected by bisamide linkers from the 6- and 8-quinoline ring. The bis-8-aminoquinolines possessed greater activity than the 6-aminoquinoline series. These bisquinolines showed IC<sub>50</sub>-values between 0.13 - 15.9 μM against *P. falciparum* D10 and K1 strains. The lower

activity was attributed to the lack of the 7-chloro group on the quinoline ring (Raynes *et al.*, 1995).



**Figure 21** Bis-4-quinolines linked through the 6-amino 25, and 8-aminoquinoline position 26.

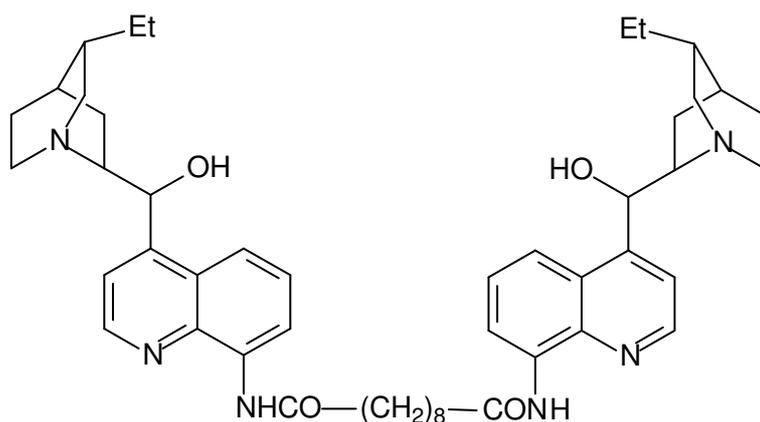
Raynes and co-workers continued their work and synthesised a series of bisquinolines slightly modified from the previously synthesised, containing the chloro-substituent on the quinoline ring. Again the bis-8-aminoquinolines possessed greater activity than the 6-series. The bisquinolines containing the 7-chloro group had similar activity to their previous 7-H counterparts, indicating that the addition of the chlorine substituent did not substantially increase the antimalarial activity. The study concluded that the linkage of the quinoline moieties through the carboxyl group rather than the amino group was associated with the decrease in antimalarial activity (Raynes *et al.*, 1996).



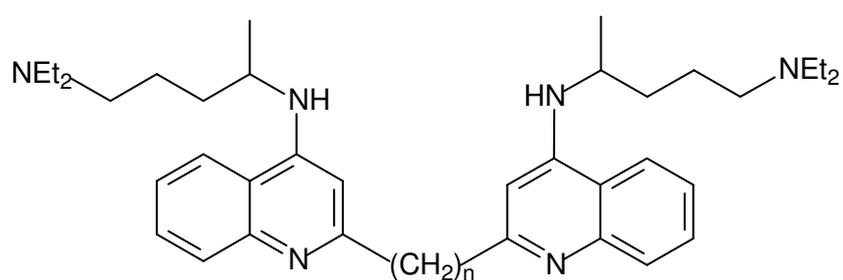
**Figure 22** Bis-4-quinolines linked through the 6- amino **27**, and 8-aminoquinoline position **28**.

5. Bis-4-aminoquinolines, bis-4-quinolinmethanols, linked through an amine substituent in the 8-position of the quinoline ring leaving the basic side chain at the 4-position intact (Figure 23)

In previous work Ayad and co-workers synthesised a cinchonidine-like bisquinoline **29** with activity similar to chloroquine against the D10 strain, and that overcame chloroquine resistance. However, this compound was also cytotoxic (Ayad *et al.*, 2001). They continued their work by synthesising new bisquinolines that retained the chloroquine side chain and was linked by a hydrocarbon linker at position 2 **30**. The most active compound had superior activity compared to chloroquine against the resistant K1 strain with an  $IC_{50}$ -value of 17 nM (43 nM against D10).



**29**



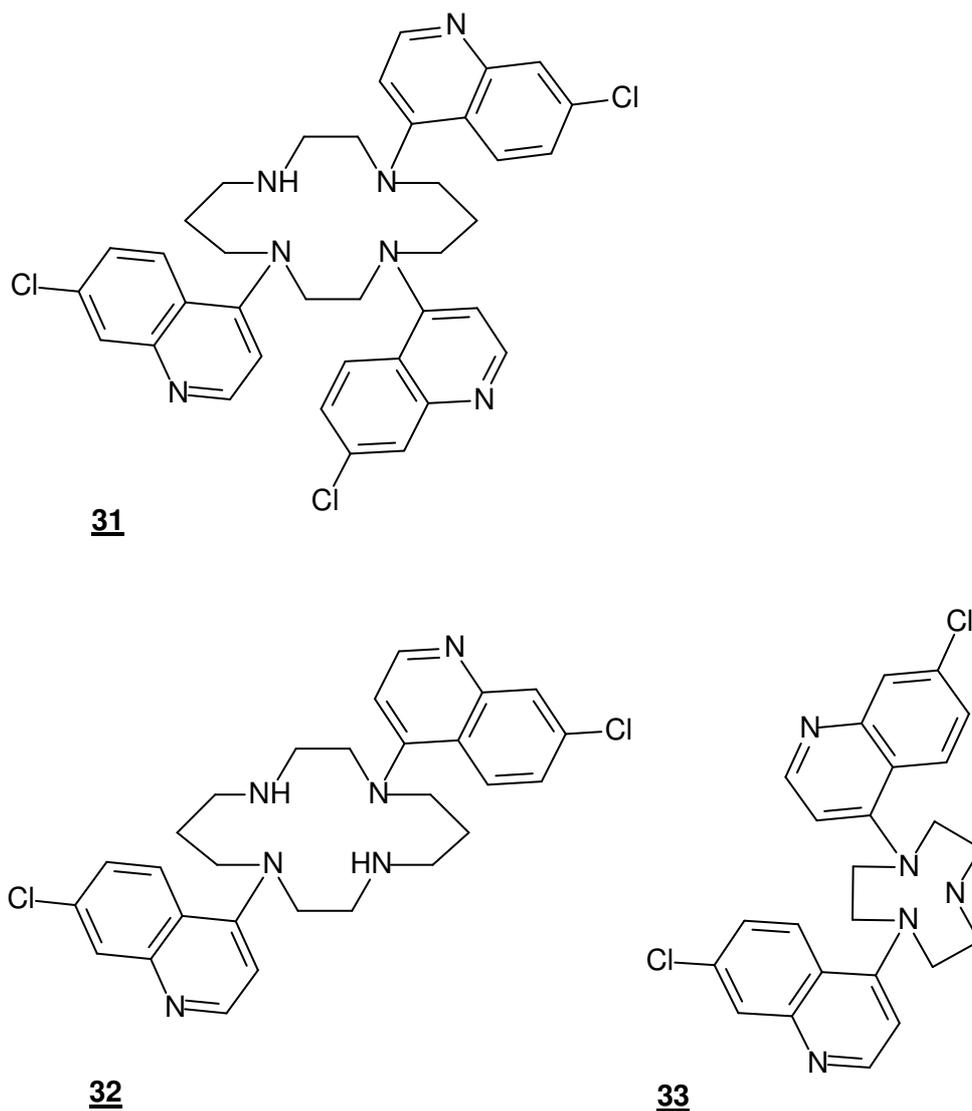
**30**

n = 8, 10 and 12

**Figure 23** The cinchonidine-like bisquinolines **29** and bisquinolines linked at 2-position, **30**.

6. Bis-, tris-, and tetraquinolines with linear or cyclic amino linkers (Figure 24)

Girault and co-workers synthesised a series of bis-, tris-, and tetraquinolines **31** - **33** with increased steric hindrance and enhanced bulkiness. The compounds displayed good activity against resistant *P. falciparum* strains, suggesting that the greater bulkiness results in a weaker efflux by PfCRT. The study concluded that increased rigidity by cyclisation produced compounds that were not more active than their linear counterparts but had less cytotoxic effects (Girault *et al.*, 2001).



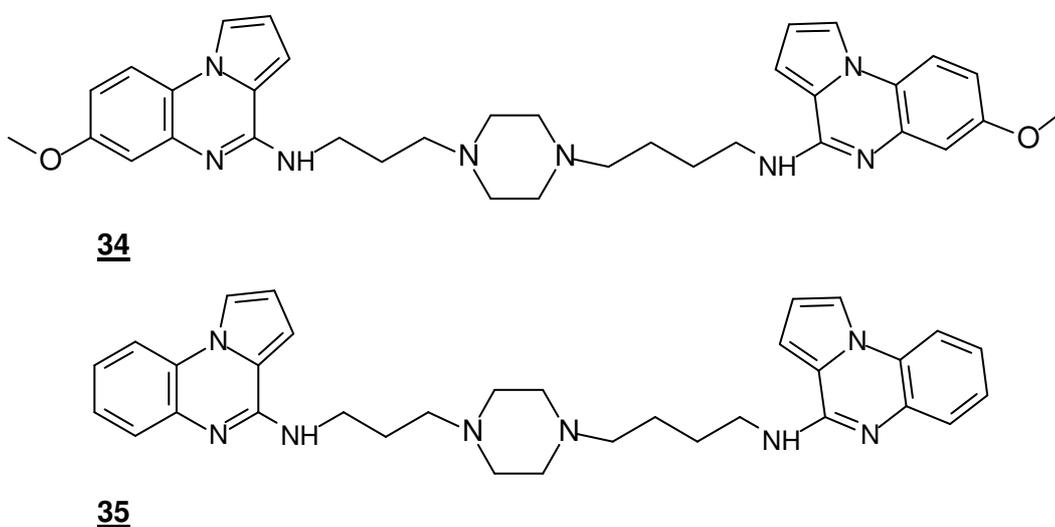
**Figure 24** Trisubstituted **31**, disubstituted **32** and **33** compounds.

### 2.7.5 The Rationale for the synthesis of bispyrroloquinoxalines

Gullion and co-workers suggested that the increased bulkiness of the pyrrolo[1,2a]quinoxaline derivatives deter extrusion by the protienaceous transporter, which results in increased drug concentration at the site of action. The variation of pyrrolo[1,2a]quinoxaline derivatives from the structure of quinolines may also allow them to avoid structural recognition of the parasite drug efflux mechanisms. The planar heterocyclic surface of pyrroloquinoxaline derivatives can intercalate between adjacent nucleic acid bases of parasite DNA as well as between two ferri-protoporphyrin molecular units of hemozoin (Gullion *et al.*, 2004). Thus, the bispyrroloquinoxalines may be capable of inhibiting hemozoin crystal formation in a mechanism similar to that of bisquinolines

### 2.7.5.1 Related literature of bispyrroloquinoxalines

Gullion and co-workers synthesised a series of bispyrrolo[1,2a]quinoxaline compounds that were more active than the monopyrrolo[1,2a]quinoxalines against *P. Falciparum* (Gullion *et al.*, 2004). The derivatives **34** and **35** containing a methoxy-group attached to the quinoxaline moiety and/or linked by a bis(3-aminopropyl)piperazine were the most active in the series. These compounds had IC<sub>50</sub>-values of 0.03 - 0.28 μM against the chloroquine sensitive Thai strain, and 0.13 - 1.09 μM (FcB1) and 0.08 - 0.48 μM (K1) against *P. falciparum* resistant strains (Gullion *et al.*, 2004). The bispyrrolo[1,2-a]quinoxalines featuring a methoxy substituent and bis-(3-aminopropyl)piperazine linker possessed the most potent activity (IC<sub>50</sub> = 80 nM) against CQR (K1).



**Figure 25** Bispyrrolo[1,2a]quinoxalines linked by a piperazine group.

## 2.8 Potential application of quinoline containing compounds in anticancer chemotherapy

### 2.8.1 Cancer chemotherapy

Cancer is a disease of the cells that is characterised by a shift in control mechanisms that govern cell proliferation and differentiation. The cancer cells proliferate excessively and form local tumours that can invade adjacent normal structures. These cells retain the ability to undergo proliferation and can migrate to different sites in the body to colonise various organs through metastasis. Anticancer chemotherapy can be broadly classified as (Katzung, 2001);

- Alkylating agents

Drugs that exert their cytotoxic effects *via* alkylation of DNA and target the nucleotide bases, phosphate atoms and proteins associated with DNA. This results in cross linking of DNA and miscoding through abnormal base-pairing, which leads to DNA breakage.

- Antimetabolites

Anticancer drugs that target the metabolism of proliferating cells, specifically the biochemical pathways that relate to nucleotide and nucleic acid synthesis. These include the purine- and pyrimidine antagonists.

- Plant alkaloids

Anticancer drugs that depolymerises the microtubules, which are an important part of the cytoskeleton and the mitotic spindle. Other plant alkaloids inhibit topoisomerase I and topoisomerase II, which results in DNA damage through strand breakage.

- Antibiotics

Anticancer drugs that bind to DNA through intercalation between specific bases. These drugs block the synthesis of new RNA and DNA, causes DNA strand scission and interfere with cell replication.

- Hormonal agents

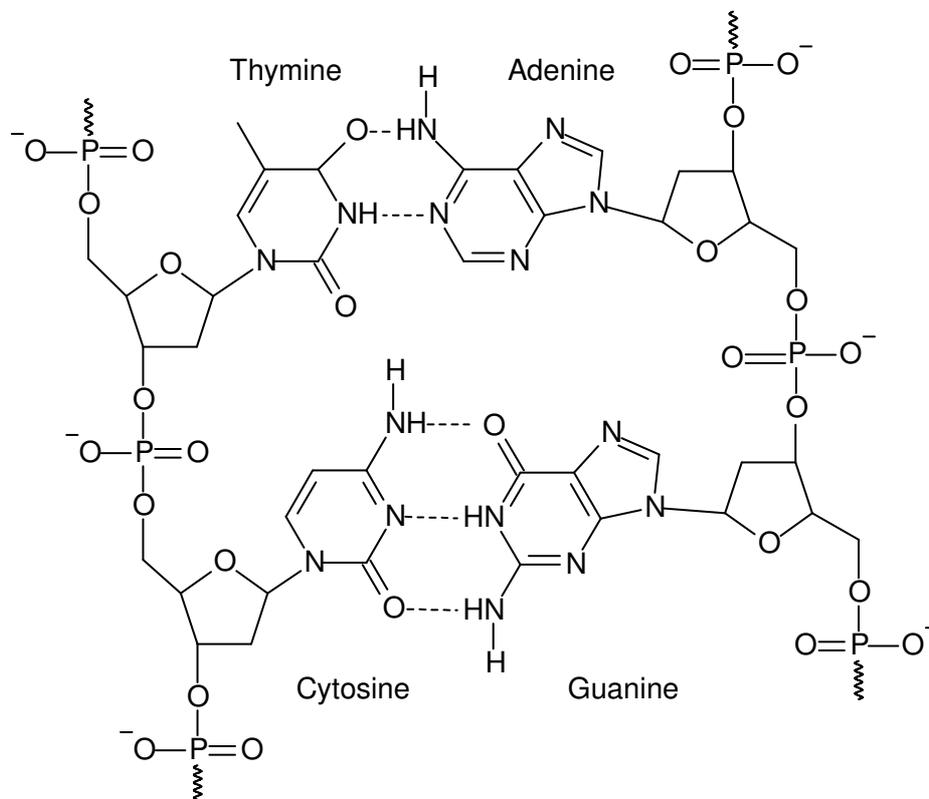
The sex hormones and adrenocortical hormones are employed in cancer therapy since they control the proliferation of certain tissues. Cancer arising from these tissues may be stimulated or inhibited by changes in hormone balance.

- Miscellaneous anticancer drugs

A major concern in anticancer chemotherapy is the emergence of drug resistance in some tumour types. The development of multidrug-resistant cancer cells are associated with an increased expression of the MDR1 gene that produces a cell surface P-glycoprotein involved in drug efflux. In light of the severity of the disease, multidrug-resistance and the absence of anticancer drugs that eradicate cancer cells without harming normal tissues, anticancer drug development remains a major focus area in drug research. The anticancer drug development program has employed *in vitro* assays for measuring the drug sensitivity of a set of human tumour cells to a variety of drugs. These *in vitro* assays shorten the testing program and are currently used as the primary screening tests for new agents by the National Cancer Institute (Katzung, 2001).

## 2.8.2 The molecular structure of DNA

The DNA molecule consists of two antiparallel polymers of nucleotides with a backbone constructed from alternating ribose sugars and phosphate molecules. The deoxyribose sugars are joined at both the 3'- and 5'-hydroxyl group to a phosphate group with phosphodiester bonds. The DNA double helix is stabilized by hydrogen bonds between nucleotides and base-stacking interactions among the aromatic nucleobases. The purine bases in one strand of the double helix form hydrogen bonds with the pyrimidine bases in the other strand, with adenine only bonding to thymine and guanine to cytosine. The bonding of adenine to thymine (AT) involves the formation of two hydrogen bonds, while the bonding of guanine to cytosine (GC) involves the formation of three hydrogen bonds. These hydrogen bonds are not covalent and can easily be broken and rejoined. Thus, short helices with an AT-rich content have weaker interacting strands and are easier to pull apart (Berg *et al.*, 2002). The spaces and grooves between twin helical strands of DNA form another double helix. These grooves are known as the narrow minor- and wide major groove of DNA and provide a possible binding site for drugs. The grooves are adjacent to the base-pairs and make the edges of the bases accessible for interactions (Ghosh & Basal, 2003).

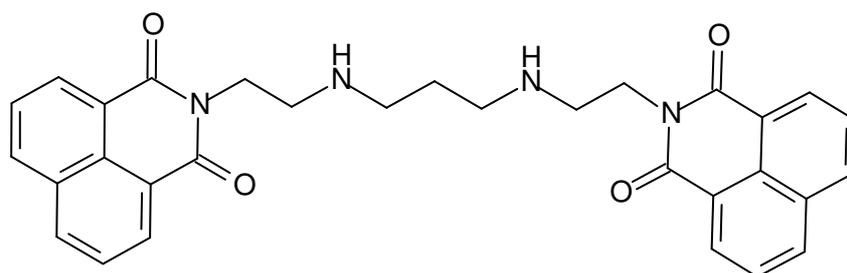


**Figure 26** The DNA backbone and base-pairs (Garrett & Grisham, 1997).

### 2.8.3 The quinolines and anticancer chemotherapy

The quinoline heterocycles that are capable of binding or intercalating with the DNA double helix have a potential application in anticancer chemotherapy (Mahalingam *et al.*, 2010). McFadyen *et al.* (1988) suggested that the 4-aminoquinoline is a weak binding intercalating agent, and that the quinoline moiety binds by external attachment to the DNA duplex. Thus, their anticancer activity may result from the non-intercalating pharmacophore, which interacts favourably with the narrow minor groove of AT-rich sequences that causes DNA strand scission.

A new class of dimeric anticancer drugs that consist of two pharmacophores joined by a flexible linker chain has recently been reported (Deady *et al.*, 2000). The rationale behind the design of these drugs is that the structure may be capable of bisintercalation with the DNA double helix. The bisimidazolides with dicationic linker chains  $(\text{CH}_2)_2\text{NR}(\text{CH}_2)_2\text{NR}(\text{CH}_2)_2$  and  $(\text{CH}_2)_2\text{NR}(\text{CH}_2)_3\text{NR}(\text{CH}_2)_2$  showed extraordinarily high potencies against human Jurkat leukaemia, with a 1000-fold more potent activity than the respective monomers. The bis-(naphthalimide) LU 79553, **36** are undergoing clinical trials (Deady *et al.*, 2000).



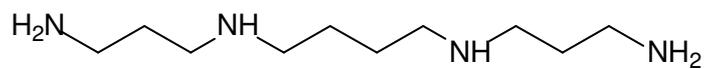
**36**

**Figure 27** LU 79553 bis(naphthalimide) **36** in clinical trials.

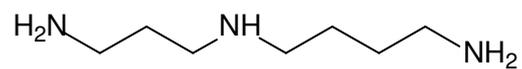
### 2.8.4 The polyamine transporter

The polyamine transporter mediates the uptake of extracellular polyamines like spermine **37** and spermidine **38** into cells, which are needed in various cellular processes such as growth and replication. Rapidly dividing tumour cells require large quantities of polyamines for their excessive proliferation and replication. Consequently, the polyamine transporter is up-regulated in tumour cells. This feature allows for a potential drug uptake mechanism for drugs covalently bound to polyamines with selectivity for cancer cells (Figure 27) (Blagbrough *et al.*, 1997). The increased uptake of the anticancer drug into the cancer cells

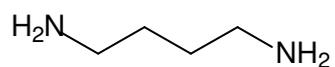
may lower the concentration of drug needed to induce cell death, reducing toxicity towards normal cells.



**37**



**38**



**39**

**Figure 28** Polyamines spermine **37**, spermidine **38** and putrescine **39**.