Chapter 2: Literature review

2.1 Introduction

Malaria is a vector borne infectious disease caused by protozoan parasites of the genus *Plasmodium* and is transmitted by an infected female *Anopheles* mosquito. Until the late 19th Century, the contributory agent for malaria was largely unknown. The medieval Italian term, *mala aria*, meaning "bad air", or commonly known as march fever (Reiter, 2000), was used to describe the flu-like symptoms, such as headaches, fever, shivering, joint pain, vomiting, haemolytic anaemia, jaundice, haemoglobin in the urine, retinal damage, and convulsions of patients infected with malaria (Beare *et al.*, 2006). It was not until 1880 that the true cause of malaria was discovered (ANON, Nobel Media 2013).

Currently, there are five known species of *Plasmodium* that are capable of infecting humans, *viz*. *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae and P. knowlesi*. The latter causes malaria in macaques, but is also capable of infecting humans (van Hellemond *et al.*, 2009). Of these species, *P. falciparum* and *P. vivax* are the most virulent and were responsible for an estimated 660 000 deaths in 2011. *P. ovale* and *P. malariae* cause generally less life threatening illnesses. Combined, these parasites had contributed to over 200 million reported cases of malaria in 2011 (WHO, 2012). Global prevalence is concentrated around the equator in tropical and subtropical regions (**Fig. 1.1**), because of ample rainfall, warm temperatures and stagnant waters that provide ideal habitats for *Anopheles* mosquito larvae. Malaria is considered by the WHO to be endemic in 106 countries worldwide, with 10 countries in the eliminating phase (WHO, 2012).

In this chapter, an overview is given of the life cycle and pathogenesis of the *Plasmodium* parasite, including the haemoglobin degradation pathway, leading the reader to the signs, symptoms and diagnosis of this deadly disease. Vector control and prevention strategies to ease the burden of malaria are also discussed. An overview of evolutionary pressures that this parasite had on the human genome in terms of natural immunity, as well as advances in the development of vaccines, is provided. Although important, all of these aspects of malaria were beyond the scope of this text and are only briefly discussed. Finally, comprehensive discussions of chemotherapeutic agents that have been in use over the years are discussed.

2.2 Life cycle and pathogenesis

The most important species in the phylum Apicomplexa are members of the class Sporozoea. Members within this class, including *Plasmodium* and coccidians, cause a variety of diseases in domestic animals and humans. The eukaryotic *Plasmodium* parasite has a very complex life cycle (Fujioka and Aikawa, 2002), which is partly spent in the vertebrate (human) host and partly in the female *Anopheles* mosquito (**Fig. 2.1**). In this section, the different life stages of the *Plasmodium* parasite are discussed in detail.

The life cycle of the *Plasmodium* parasite starts when the human host is infected by plasmodial sporozoites, when a female *Anopheles* mosquito takes a blood meal. The mosquito injects saliva, containing the sporozoites, into the humans' bloodstream. Some of the sporozoites are blocked by the human immune system, i.e. antibodies, but most of them invade the liver. This process is also known as the pre-erythrocytic or exoerythrocytic stage (Plebanski and Hill, 2000). The sporozoites are transferred into the liver *via* the bloodstream, where they replicate and become enlarged, to form a schizont. The schizont fragments into a number of small cells, called merozoites, which are liberated from the liver into the bloodstream. Each schizont produces about 32 merozoites and generally take between 1 - 2 weeks to develop, depending on the species (Ashley *et al.*, 2006). *P. vivax* and *P. ovale* have a dormant stage, called hypnozoites, which can remain in the liver for years before developing into schizonts. This is commonly associated with relapses in malaria, years after infection (Fujioka and Aikawa, 2002).

After lysis of the hepatocytes, infection of human erythrocytes proceeds exponentially, signalling the start of the erythrocytic schizogony stage. Once inside the erythrocyte, the merozoites develop into a ring from where they catabolise haemoglobin. The ring stage develops into trophozoites and finally into schizonts again, which ruptures the erythrocytic cell to release thousands of merozoites that can further infect erythrocytes, typically leading to clinical symptoms (Ashley *et al.*, 2006). The mechanism by which egress proceeds from their host cells is not well understood. Strong genetic evidence exists that malarial cysteine proteases are necessary for this exodus of these invasive stages from their intracellular compartments from liver stage and blood stage schizonts (Aly and Matuschewski, 2005). The selective inhibition of this protein might be a key component for eradicating malaria.

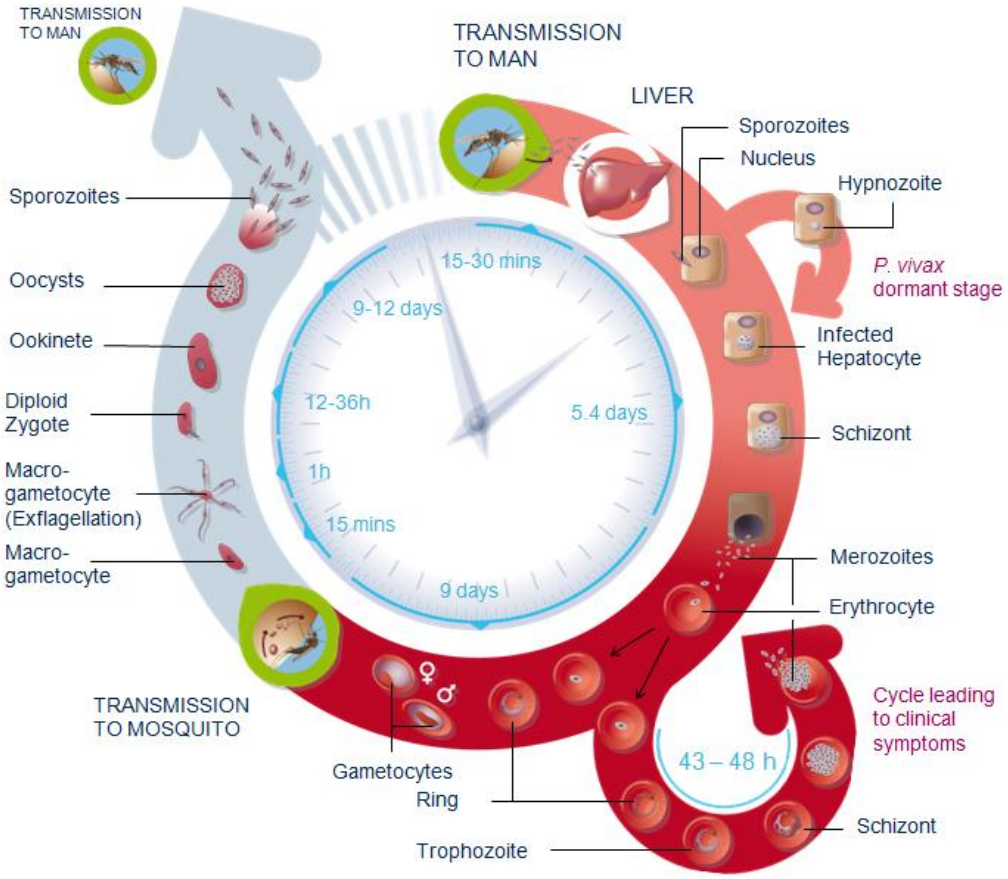

Figure 2.1: The complex life cycle of the *Plasmodium* spp. (ANON, 2012).

Not all merozoites that are liberated from the erythrocytes are capable of invading other erythrocytes. These merozoites develop into gametocytes, which signal the start of the sexual stage, following a number of asexual life cycles. The catalyst for the production of macrogametocytes (female) and microgametocytes (male) are unknown. Mature gametocytes inside erythrocytes are taken up by a female *Anopheles* mosquito after a blood meal and are then transferred into the midgut, where they escape the cells. Microgametocytes exflagellation produces eight haploid motile microgametes, which move rapidly to fertilise macrogametocytes, resulting in zygotes formation.

After about one day, these zygotes transform into ookinetes. The ookinetes then travel to the extracellular space between the midgut epithelium and overlaying basal lamina, where they develop into oocysts. Nine to twelve days later the oocysts rupture, releasing thousands of sporozoites, which invade the salivary gland epithelium of the mosquito. When the mosquito takes a blood meal from an inclined human host, the life cycle begins anew (Fujioka and Aikawa, 2002).

2.3 Haemoglobin degradation

The nutritional needs of the parasite are met by ingestion of the host's haemoglobin and through degradation in the digestive vacuole by a number of enzymes, including cysteine protease (Egan, 2008). This pathway offers an unique target for chemotherapy, which is exploited by a number of compounds, such as CQ and artemisinin (Bray, 2005). It is thus important that an overview is discussed to assist in the understanding of the mode of actions of these compounds.

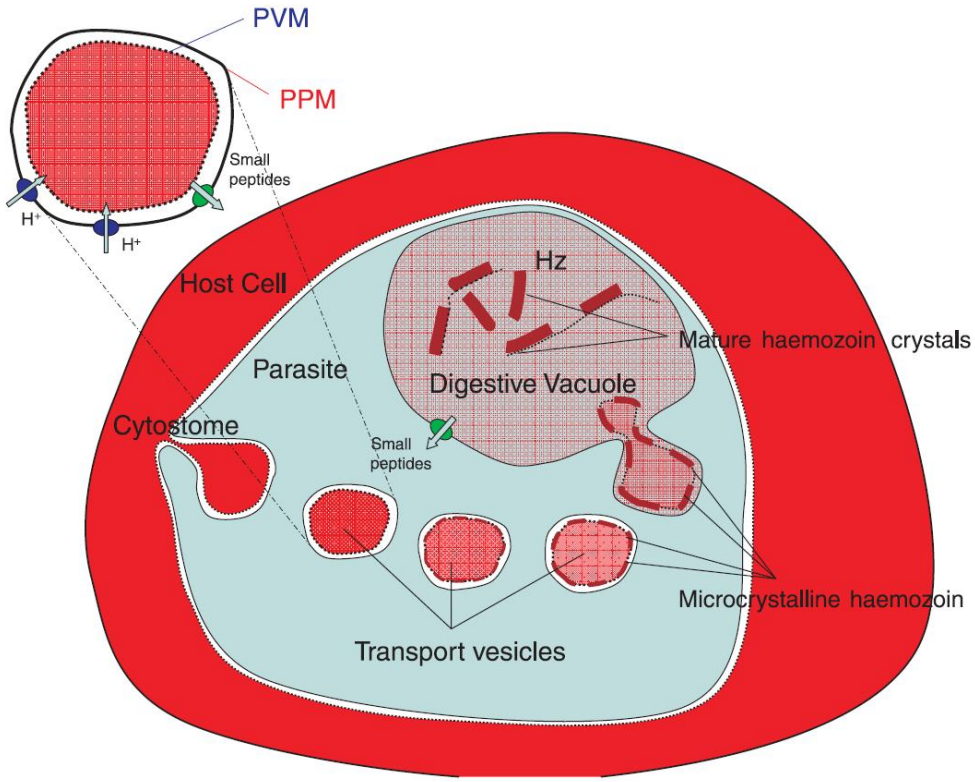

Figure 2.2: Graphic representation of how the *Plasmodium* parasite acquires haemoglobin (Bray, 2005).

During the intra-erythrocytic stage, the malaria parasite ingests between 60 - 80% of the available haemoglobin (Egan, 2008) through the endolysosomal system, called a cytostome (**Fig. 2.2**). The haemoglobin is transported to the digestive vacuole (Bray, 2005), where it is then digested for the biosynthesis of the parasite's own proteins (Krugliak *et al.*, 2002). This is brought about in a semi-concerted fashion (**Fig. 2.3**), i.e. firstly the native haemoglobin is cleaved by a family of proteases, called plasmepsins, to degrade haemoglobin into haem and globin fragments. The haem is toxic and is subjected to haem-detoxification by the formation of hemozoin through biocrystallization. The remaining globin fragments are then

further degraded into peptide fragments of around ten to fifteen amino acid subunits by cysteine proteases, specifically falcipain-2 and -3 (Rosenthal, 2004). These fragments are then catabolised by the metalloprotease, called falcilysin, into oligomers (6 - 8 amino acid subunits) and transported to the cytosol. These oligomers are then further degraded into free amino acids by aminopeptidase, such as serine protease (Ettari *et al.*, 2009).

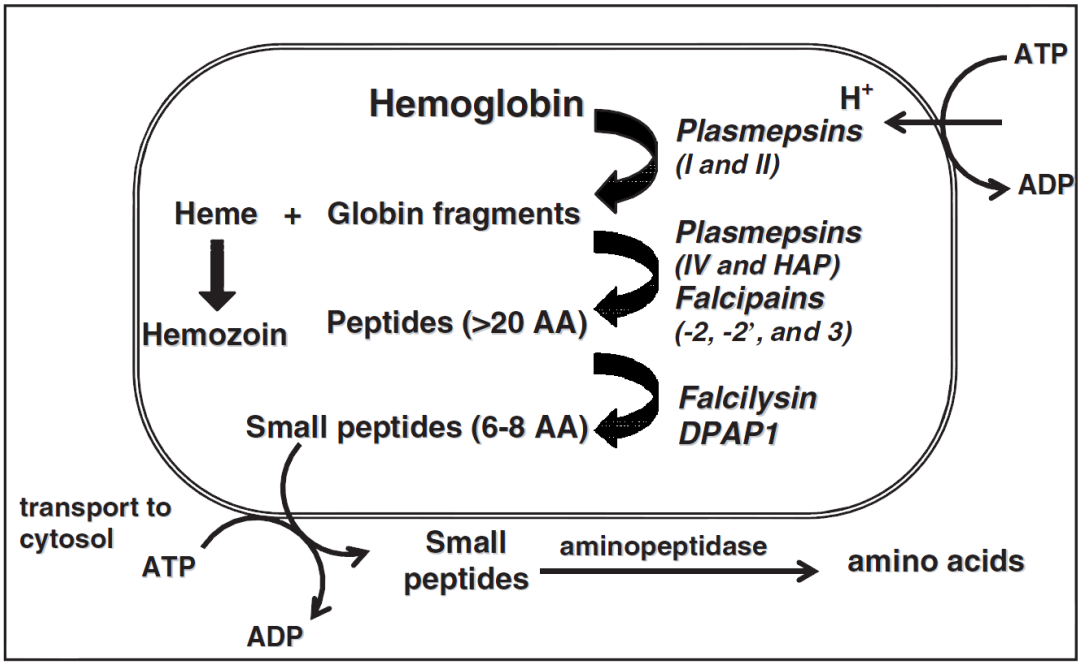

Figure 2.3: Haemoglobin degradation inside the digestive vacuole (Ettari *et al.*, 2009).

2.4 Signs and symptoms

The initial signs and symptoms (**Fig. 2.4**) of *Plasmodium* infection are non-specific and commonly associated with flu-like symptoms, such as fever, chills, headaches, loss of appetite, nausea and vomiting (Ashley *et al.*, 2006). If these symptoms are left untreated, especially with *P. falciparum* infection, it would rapidly result in severe complications, such as renal and multi-organ failure, hypoglycaemia, metabolic acidosis, acute anaemia, seizures, mental confusion, coma, cerebral malaria and eventually death. Most of these symptomatic expressions are, however, still not well understood. Despite the fact that severe malaria is uncommon (less than 1%), it leads to a substantial number of mortalities each year (Idro *et al.*, 2010).

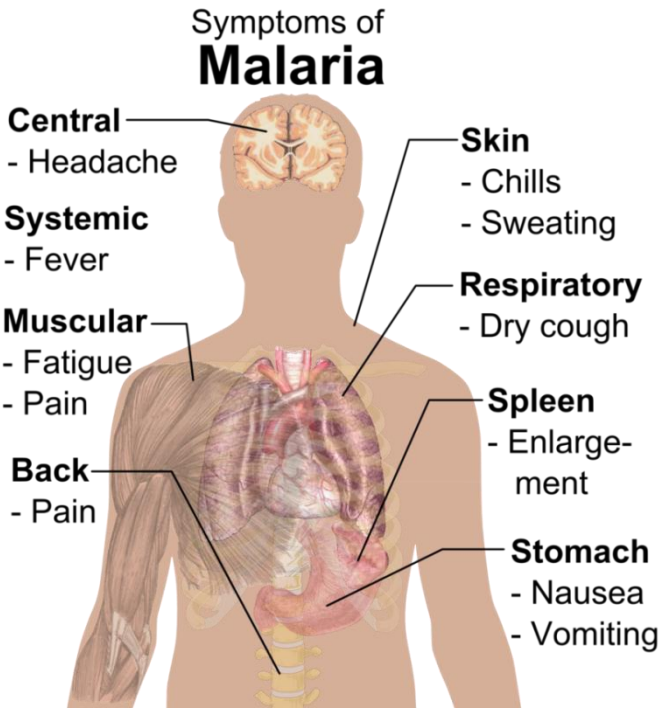

Figure 2.4: Common symptoms and organs affected by malaria.

Acute renal failure (ARF) complicates *P. falciparum* malaria in less than 5% of patients who are inhabitants of endemic areas, but is it much more frequent among visitors from non-endemic countries to these endemic areas (up to 30%), with a mortality rate of up to 45% (Barsoum, 2000). ARF typically presents with electrolyte abnormalities, increased urinary protein excretion, pain, nausea and vomiting. This is most likely caused by dehydration, cytoadherence of parasitized erythrocytes, intravascular haemolysis, intravascular coagulation and sepsis, although the precise mechanism of malarial ARF is unknown (Das, 2008). The prevalence of hypoglycaemia, on the other hand, varies depending on area and age groups, ranging from 8% in South-East Asian adults to 30% in African children (Osonuga *et al.*, 2011). Reduction of glucose levels due to starvation, parasite consumption of glucose and cytokine induced impairment of gluconeogenesis have all been frequently implicated in the complication of hypoglycaemia (Ogetii *et al.*, 2010).

Metabolic acidosis has emerged as a fundamental feature of severe malaria, and is widely implicated as a fatal outcome in both adults and children. The most common manifestations of acidosis are the increased production and impaired metabolism of lactate and ketoacids, although the aetiology of malarial acidosis is not well understood (Maitland and Newton, 2005). Acidosis is generally associated with cerebral malaria and of all the complications of severe malaria, the causative agent for most of the deaths (English *et al.*, 1997; Sasi *et al.*, 2007).

Cerebral malaria (CM) is characterised by impaired consciousness, coma and asexual forms of the parasite on peripheral blood smears, with African children bearing the majority of this burden (Maitland and Newton, 2005; English *et al.*, 1997). Despite the fact that CM is the best studied complication of severe malaria, it is still incompletely understood. CM has a high mortality rate, even with active treatment and causes sustained brain injury, which manifests as long-term neuro-cognitive impairments in some surviving patients (Idro *et al.*, 2010).

The histopathological characteristic of cerebral malaria is the enlargement of cerebral capillaries and venules with parasitized red blood cells (pRBCs) and non-parasitized RBCs (npRBCs), causing intracranial pressure and swelling of the brain (Newton *et al.*, 2000). An increased cerebral blood volume (CBV) has been suggested to be the likely cause of intracranial pressure. Increases in the CBV could result from the sequestration of pRBCs in the cerebral venules, impaired venous return, or increased cerebral blood flow caused by seizure activity and anaemia (Maitland and Newton, 2005). Sequestration is caused by the adherence of pRBCs to capillary endothelial cells by means of a specific interaction between a parasite derived molecule present at the surface of the pRBCs and specific host cell receptors (Franke-Fayard *et al.*, 2010).

2.5 Diagnosis

Owing to the non-specific symptomatic manifestations of *Plasmodium* infection, accurate and rapid diagnosis is of paramount importance to reduce associated morbidity and mortality (Hopkins, 2013). A positive clinical diagnosis of malaria through a physical examination should always be confirmed by means of pathological tests (Mubi *et al.*, 2013) in order to inform malaria control strategies and to prevent the indiscriminate treatment of non-infected patients, which could contribute to resistance (WHO, 2010).

Several methods, such as microscopy and antigen based rapid diagnostic tests (RDTs) are routinely used to diagnose malaria. Microscopy of both thick and thin blood smears remains the golden standard for diagnosing malaria, since it is accurate and reliable under operational conditions (Moody and Chiodini, 2000). Thick blood smears are used for diagnoses, while thin blood smears are used to determine the parasitaemia. Limitations of microscopy include specialised equipment and trained personnel to operate it.

RDTs detect parasite specific antigens or enzymes, with some even incorporating the ability to differentiate between species (Wongsrichanalai *et al.*, 2007; WHO, 2010). RDTs are overall more sensitive than traditional microscopy, and are faster and do not require specialised equipment, nor trained personnel to operate it (Moody and Chiodini, 2000).

2.6 Control and prevention

Control and prevention of the both the vector (*Anopheles*) and *Plasmodia* are vital strategies against malaria infection. In this section, the most important control and prevention strategies are discussed, which include insecticide treated nets (ITN), indoor residual spraying (IRS), vaccination, adaptive immunity and education. Chemoprophylaxis, on the other hand, is discussed under the chemotherapy section, 2.7.

Two of the most important vector control methods include ITNs and IRS. According to the WHO, 11% of people at risk for contracting malaria were protected by ITNs in 2011, while approximately 53% of people in endemic areas had access to an ITN. Unfortunately, there was a shortage of roughly 90 million nets in 2012. With the life span of an ITN of no more than five years (WHO, 2012), the distribution of these nets are regrettably unsustainable.

IRS is the treatment of the inner surfaces of dwellings where many vector species tend to rest after taking a blood meal, specifically at dawn and dust when they are most active. Resistance of mosquitoes to insecticides had been found in sixty-four countries around the world, which hamper effective vector control (WHO, 2012).

Alternatively, vaccination has largely reduced the burden of infectious diseases. Vaccination promotes economic growth and leads to decreased morbidity and mortality. Vaccination is therefore a proficient tool to level wealth and inequities in health (Andre *et al.*, 2008). Despite research efforts over the last four decades, no licensed malaria vaccines are currently available (Targett *et al.*, 2013). A number of potential candidate vaccines are currently undergoing clinical trials, with one candidate (RTS,S/AS01) having reached Phase 3 trials, while approximately twenty others are in Phases 1 or 2 (WHO, 2012). However, should any of these potential vaccines be approved, it will still take a number of years before they are viable to administer. Vaccination may in the future protect the majority of high risk individuals, like children under the age of five, but the vital role that adaptive immunity plays in the prevention of malaria should also be considered (Doolan *et al.*, 2009).

The evolutionary pressure that this parasite has had on the human genome can be observed from the pre-disposition of certain individuals, residing in endemic areas, prone to having certain genetic traits that are indicative of adaptive immunity (Plebanski and Hill, 2000). These include Sickle cell anaemia, Duffy antigens and Interleukin-4.

The most significant genetic disorder for consideration is Sickle cell anaemia, with around 300 000 children born each year with this condition (Oniyangi and Omari, 2006). Sickle cell anaemia is caused by a change of a single nucleotide in the HBB gene, on chromosome 11 coding for β-haemoglobin subunits, known as Sickle cell trait (SCT). Sickle cell disease (SCD), on the other hand, is homozygous haemoglobin SS (HbSS), characterized by red blood cells that assume an abnormal, rigid, sickle shape, which results in abnormal blood flow and deprived oxygen levels (Oniyangi and Omari, 2006; Makani *et al.*, 2010). People with SCD normally have a shortened life span, but it is believed that this condition protects these individuals from contracting malaria (Wambua *et al.*, 2006). The prevalence of both SCD and SCT are more common among people inhabiting malaria endemic areas (Oniyangi and Omari, 2006; Makani *et al.*, 2010).

The Duffy antigen is located on the surface of red blood cells and is the receptor of both *P. vivax* and *P. knowlesi* (Neote *et al.*, 1994). Individuals, who lack this antigen are therefore resistant towards *P. vivax* and *P. knowlesi* infections (Miller *et al.*, 1976).

Interleukin-4 (IL-4) is a cytokine that induces differentiation of naive helper T cells to Th2 cells, with multiple immune-modulating functions on a variety of cell types. IL-4 has been shown to be involved in the regulation of antimalarial antibody responses against *P. falciparum*, including antimalarial IgE, which awards additional immunity (Gyan *et al.*, 2004). It is thus evident that malaria immensely impacts on the evolution of human genes.

Health education strategies are central in enabling communities to support malaria control measures (MRC, 2008). Suitable awareness campaigns to teach inhabitants of endemic areas about what malaria is, how it is transmitted and how it can be prevented may prove successful in addition to the control and prevention strategies set out by the Roll Back Malaria (RBM) initiative. Additionally, education on the proper use of ITNs may further help to decrease the global burden of that malaria causes.

2.7 Chemotherapy

Since no effective vaccination is currently available against malaria infection, chemotherapy remains the only feasible tool for the prevention of and treatment against malaria infections. Chemotherapy falls within one of seven drug classes, *viz*: antifolates (pyrimethamine, sulphadoxine and proguanil), hydroxynaphthoquinones (atovaquone), antimicrobials (doxycycline and clindamycin), 8-aminoquinolines (primaquine), aryl-amino alcohols (quinine, quinidine, mefloquine and halofrantrine), 4-aminoquinolines (chloroquine, amodiaquine and piperaquine) and artemisinins (artemisinin, DHA, artemether and artesunate) (Schlitzer, 2008). In this section the different classes of antimalarial drugs are discussed in detail, including their modes of action and resistance. Additionally, an overview of chalcone- and ferrocenyl based compounds are also discussed.

2.7.1 Antifolates and hydroxynaphthoquinones

Folate biosynthesis is exceptionally species specific and renders a unique target for chemotherapy (Hyde, 2005). Folate is an essential vitamin and is composed of the aromatic pteridine ring linked to para-aminobenzoic acid (PABA) and one or more glutamate residues. It is a co-factor involved in the production of purines and pyrimidines for DNA replication, as well as in the synthesis and/or catabolism of several amino acids (Stanger *et al.*, 2009).

The antifolates comprise a class of antimalarials that inhibit specific enzymes in the biosynthetic pathway of folate metabolism (**Fig. 2.5**), namely dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) (Schlitzer, 2008). DHPS produces dihydropteroate from pteridine pyrophosphate and PABA, but it is not expressed in humans, while DHFR reduces dihydrofolate (DHF) into tetrahydrofolate (THF). The efficacy of antifolates had been demonstrated as early as in the 1930's, while better pharmacokinetic profiles and partner drugs had already been developed in the 1940's. Antifolates were, however, not widely used at the time, because of the availability of highly effective quinoline derivatives (Hobbs and Duffy, 2011).

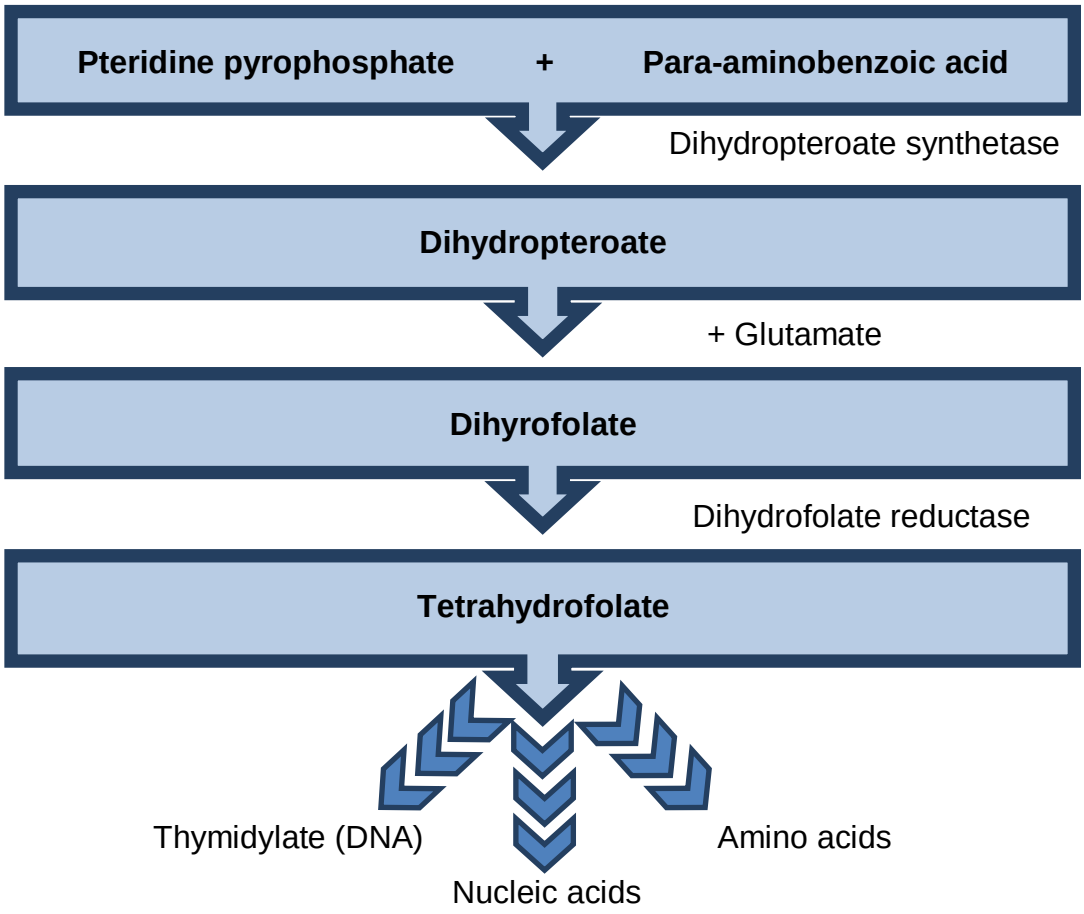

Figure 2.5: Biosynthetic pathway of tetrahydrofolate.

The antifolates can be divided into two sub-classes, based on the specific enzyme being targeted. Sub-class I inhibitors (**Fig. 2.6**) compete with DHPS and include sulphonamides (**1** and **2**). Sub-class I exhibits its activity by competing with PABA for the active site of DHFR and results in a decreased formation of dihydropteroate, consequently blocking the synthetic pathway. Sub-class II antifolates (**Fig. 2.7**) inhibit DHFR and include pyrimethamine (**3**) and proguanil (**4**) (Nzila, 2006). Sub-class II antifolates on the other hand exert their activity by preventing the NADPH dependent reduction of DHF, leading to the disruption of DNA synthesis (Navarro-Martínez *et al.*, 2005).

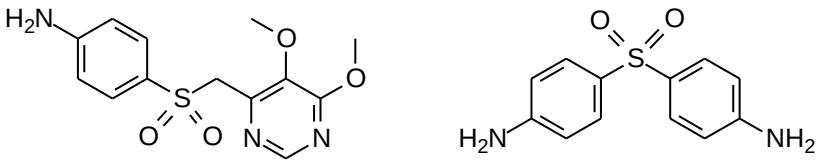

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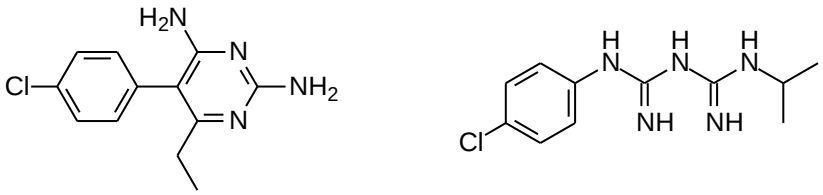
Figure 2.6: Sub -class I antifolates: sulfadoxine (**1**) and dapsone (**2**).



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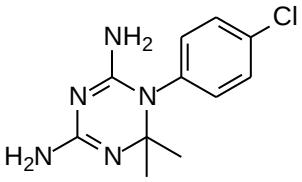

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Figure 2.7: Sub-class II antifolates: pyrimethamine (**3**), proguanil (**4**) and its active metabolite cycloguanil (**5**).

Attempts to use the DHPS inhibitors alone as antimalarial agents were abandoned, due to their low efficacy and unacceptable toxicity {Nzila, 2012 #294}. However, combination of antifolates from the two sub-classes results in a synergistic effect, which increases their therapeutic uses (Hyde, 2005). The sulfadoxine (**1**) and pyrimethamine (**3**) combination, marketed as Fansidar®, is such an example. WHO recommend the use of the sulfadoxine/pyrimethamine (SP) combination as intermittent preventative treatment (IPT) for both infants and pregnant woman (WHO, 2012; Gutman *et al.*, 2013). Sulfadoxine (**1**) is a sulfonamide based compound with a very long half-life and a class I inhibitor, while pyrimethamine (**3**) is a slow acting 2,4-diaminopyrimidine based compound.

Fansidar® antimalarial tablets each contains 500 mg of sulfadoxine and 25 mg of pyrimethamine and is active against the asexual erythrocytic stages of *P. falciparum*. It had been indicated for the successful treatment of acute, uncomplicated *P. falciparum* malaria for those patients in whom CQ resistance is suspected (WHO, 2010). Prophylactic use is,

however, not recommended. Side effects of prolonged administration may cause dyserythropoiesis, by interfering with folic acid metabolism, skin rashes, hypersensitivity and in extreme cases, Johnson syndrome (Ashley *et al.*, 2006).

Another combination of sub class I and II inhibitors is dapsone (**2**) with proguanil (**4**), which is used as both prophylaxis and treatment and is marketed as Maloprim® (Nzila, 2006). Dapsone is an antibacterial agent belonging to the sulphonamide class of compounds, while proguanil is an open chain biguanid prodrug, which is transformed into the active metabolite, cycloguanil (**5**) *via* oxidative ring closure (Schlitzer, 2008). However, these combinations have been abandoned because of the reduced efficacy of cycloguanil, as well as the toxicity associated with dapsone (Nzila, 2012).

Proguanil had also been associated with atovaquone (**6**, **Fig. 2.8**) in a combination drug marketed as Malarone®, although atovaquone falls within the hydroxynaphthoquinones class and is used as treatment and prophylaxis (Nakato *et al.*, 2007). Atovaquone binds to cytochrome bc_1 (Cyt bc_1), leading to the inhibition of mitochondrial electron transfer. It has been demonstrated that the drug exerts its activity by inhibiting the movement of the Rieske protein, as well as by decreasing the electropotential across the inner mitochondrial membrane of the parasite (Vaidya, 2012). Additionally, mutation around the Qo site of the Cyt *b* gene (Mather *et al.*, 2005) had been associated with atovaquone resistance *in vivo* (Srivastava *et al.*, 1999; Nzila and Mwai, 2010). Interestingly, in areas with a high occurrence of pyrimethamine resistance, resistance to Malarone® is also observed (Nzila, 2012).

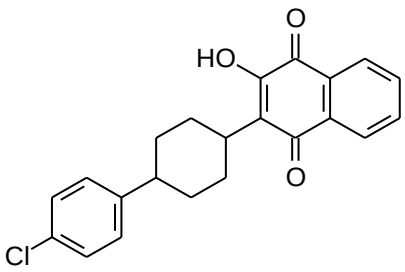

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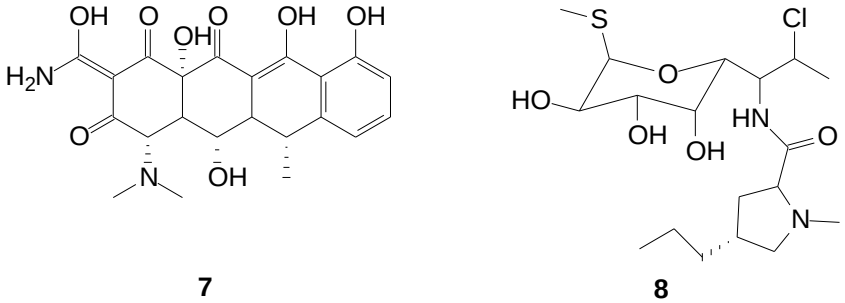
Figure 2.8: Chemical structure of the hydroxynaphthoquinone atovaquone (**6**).

Humans do not have the ability to synthesize folic acid. The nutritional need of folic acid must therefore be taken up through diet. *P. falciparum* does have this ability, as well as the capacity to salvage folic acid from the host, which might be a possible mechanism by which

Plasmodium acquires resistance to antifolates (Hyde, 2005). Some results, however, indicate that the salvage acquisition path may not be satisfactory for the nutritional requirements of the parasite. Conversely, antifolate resistance results from the stepwise accumulation of point mutations in the *dhfr* domain of the *dhfr-ts* gene, which reduces antifolate binding affinity (Gregson and Plowe, 2005; Takimoto, 1996). Acquisition of four *dhfr* mutations renders available antifolates completely ineffective (Ashley *et al.*, 2006).

2.7.2 Antimicrobials

Several antibiotics have been reported as having antiplasmodial activity, such as doxycycline (**7**) and clindamycin (**8**). The use as mono-therapy to treat malaria is not recommended, since these drugs are weak antimalarials and slow acting (Ashley *et al.*, 2006).



7 **8**

Figure 2.9: Chemical structures of the antibiotics doxycycline (**7**) and clindamycin (**8**).

Doxycycline is synthetically derived from oxytetracycline, a broad-spectrum antibiotic. The tetracyclines are mainly bacteriostatic and are thought to exert their antimicrobial effects through the inhibition of protein synthesis. Doxycycline has a similar antimicrobial spectrum of activity against a wide range of gram-positive and gram-negative organisms, including the *Plasmodium* parasite (Ashley *et al.*, 2006). Doxycycline has been found active against the asexual erythrocytic forms of *P. falciparum*, but not against the gametocytes. The precise mode of action of the drug is still unknown. Its most common side effect is diarrhoea (Tan *et al.*, 2011).

Clindamycin is also a bacteriostatic antibiotic, belonging to the group of lincozamides that is also safe for use in children (Obonyo and Juma, 2012). It inhibits the protein synthesis of bacteria at the ribosomal subunit 50S. The most frequently encountered adverse effects include nausea, vomiting and diarrhoea (Kremsner *et al.*, 1995).

2.7.3 8-Aminoquinolines

There are several synthetic 8-aminoquinolines known, but only primaquine (**9**) is currently used (Schlitzer, 2008). Primaquine is the only antimalarial that is active against both the liver and the sexual blood stages of the parasite, although activity against the latter is observed only at doses that are too toxic for general use (Grewal, 1981), making its therapeutic use limited. It is used for both chemoprophylaxis as well as in the treatment of the dormant hypnozoites of *P. vivax* (Pybus *et al.*, 2012). Primaquine has been used for over 60 years now (Waters and Edstei, 2012).

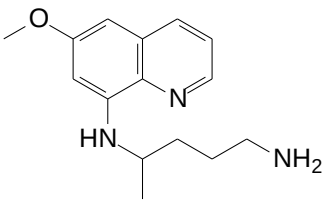


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Figure 2.10: Chemical structure of primaquine (**9**).

As with most antiplasmodial agents, the mechanism of action of primaquine is largely unknown, but is it believed to involve the mitochondria (Waters and Edstei, 2012). Primaquine seems to accumulate within the mitochondria, resulting in swelling and structural changes within the inner membranes, thus destroying mitochondrial function. Primaquine metabolites produce reactive intermediates that eventually result in the build-up of free radicals, such as hydrogen peroxides and superoxides, which may be responsible for antimalarial activity (Pybus *et al.*, 2012).

To date, limited resistance to primaquine exisits, with treatment failures having been attributed to patient non-compliance, or to inadequate weight based doses being employed (Waters and Edstei, 2012). Experimental resistant strains of *P. berghei* and *P. knowlesi* do exist, but little evidence subsists for resistant field strains. The lack of resistance might be explained by the fact that the therapeutic use of primaquine is relatively narrow, as well as by the fact that it is normally not used in isolation (Fernando *et al.*, 2011).

Haemolytic anaemia is the most serious adverse effect of primaquine in glucose-6-phosphate dehydrogenase (G6PD) deficient individuals. G6PD deficient individuals have inadequate levels of erythrocytic NADPH, resulting in a compromised antioxidant system and ultimately cyanosis (Pybus *et al.*, 2012). Methaemoglobinaemia (an oxidised form of

haemoglobin that cannot bind to and transport oxygen) is also a common toxicity associated with primaquine (Waters and Edstei, 2012).

2.7.4 Aryl-amino alcohols

Several natural and synthetic compounds within the class of aryl-amino alcohols exist, such as quinine (**10**), quinidine (**11**), mefloquine (**12**) and halofantrine (**13**). Quinine (**10**) and quinidine (**11**) are natural alkaloids extracted from the cinchona tree's bark and had been of the first antimalarial drugs used to treat malaria, as early as in 1630 (Bray, 2005; Foley and Tilley, 1998).

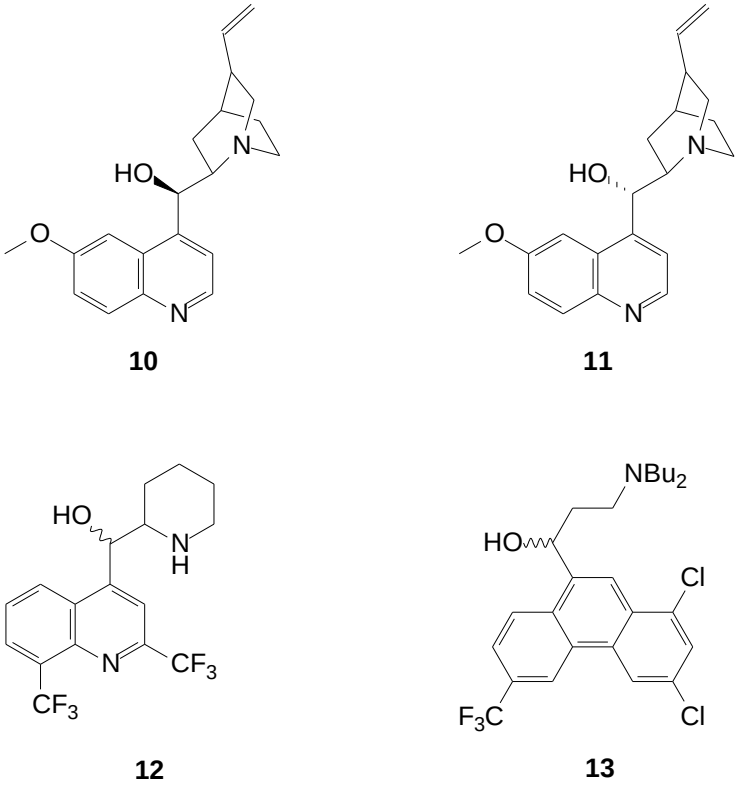


Figure 2.11: Chemical structures of quinine (**10**), quinidine (**11**), mefloquine (**12**) and halofantrine (**13**).

Quinine (**10**) and quinidine (**11**) are diastereomers of each other, with quinine being the preferred drug, because of quinidine's cardiotoxicity, despite its higher intrinsic antiplasmodial activity (Ashley *et al.*, 2006). Until the discovery of CQ, quinine was the mainstay for malaria treatment. Quinine acts rapidly on blood stage parasites and is also gametocytocidal towards *P. vivax* and *P. malariae* (Achan *et al.*, 2011). It is today used in ACT with artemether in the treatment of severe *P. falciparum* malaria (WHO, 2010). In areas

with reduced sensitivity towards quinine, it is combined with antibiotics, such as doxycycline (**7**) (Foley and Tilley, 1998). Adverse side effects include tinnitus, nausea, headaches, abdominal pain and visual defects, which often lead to poor patient compliance and the recrudescence of malaria (Achan *et al.*, 2011). Quinine also stimulates insulin production and may cause hypoglycaemia, which is a particular problem to pregnant woman.

Mefloquine (**12**) is a synthetic analogue of quinine, marketed under the name Larium®, used as a racemic mixture. It is a potent long-acting blood schizonticide, active against asexual intraerythrocytic forms of all *Plasmodium* spp. Mefloquine is used for both treatment and prophylaxis in Africa and has recently been used for intermittent preventive treatment (IPTi) in infants and as a constituent of ACT together with artesunate (Schlagenhauf *et al.*, 2011). It is especially useful as chemoprophylaxis in travellers from non-endemic counties. Adverse effects include dizziness, mild to moderate nausea, vomiting, diarrhoea and abdominal pain, while neuropsychiatric adverse reactions may also be noticed (Schlagenhauf, 1999).

Halofantrine (**13**), a phenanthrene-methanol, is an orally administered schizonticidal drug, with no apparent action on the sporozoite, gametocyte, or hepatic stages of the infection. It is, however, effective against both chloroquine sensitive and resistant strains of *Plasmodia* (Bilolikar *et al.*, 1994). It had first been identified as a potential antimalarial during World War II and was rediscovered as an effective drug against CQR strains in the 1960s by the Walter Reed Army Institute of Research (Nothdurft *et al.*, 1993). The mechanism of action of halofantrine is still unknown, but it seems to function in the haem detoxification process. Adverse side effects include abdominal pain, diarrhoea, vomiting, rash, headaches, itching, elevated liver enzymes and associated cardiotoxicity (Bouchaud *et al.*, 2009).

The exact mode of action of aryl-amino alcohols are unknown, but they act rapidly on blood stage parasites (Sullivan, 2012). However, based on the ultra-structural observation that aryl-amino alcohols cause morphological changes similar to that of chloroquine, some authors suggest that the mode of action of this class of drugs is similar to that of 4-aminoquinolines (Section 2.7.5), i.e. inhibition of hemozoin formation. It is still not clear if this is the only, or major mechanism by which aryl-amino alcohols exert their activity though (Foley and Tilley, 1998). It is considered to inhibit the process of haem crystal formation in the digestive vacuole through reversible π-π interactions (Sullivan, 2012).

The first reported cases of reduced sensitivity against quinine had been observed as early as 1910, but due to its less intensive use, resistance had slowed down towards this group. Polymorphism of the *pf*mdr1 gene appeared as the main factor in aryl-aminoalcohol

resistance (Wongsrichanalai *et al.*, 2002; Schlitzer, 2008). Resistance to mefloquine, on the other hand, was only a few years after its introduction in 1970 (Foley and Tilley, 1998), as a result of its long elimination half-life, thus compromising its therapeutic use. Susceptibility to resistance of all structurally related compounds to quinine seemed to have involved the *pf*mdr1 gene (Wongsrichanalai *et al.*, 2002).

2.7.5 4-Aminoquinolines

The 4-aminoquinolines entail a class of compounds with the quinoline scaffold, which is prevalent in a variety of pharmacologically active synthetic and natural compounds (Kaur *et al.*, 2010). Included in this class are chloroquine (CQ, **14**), amodiaquine (**15**) and piperaquine (**16**). The extensive use of this class of compounds results from their excellent clinical efficacy, limited host toxicity, ease of use and uncomplicated, cost-effective synthesis (Yadav *et al.*, 2012; Bray, 2005). Widespread resistance, however, especially towards CQ, have rendered these compounds ineffective for chemotherapy.

The mechanistic mode of action of this class is not well understood either. It is, however, widely accepted that the accumulation of CQ by ion trapping in the digestive vacuole might be one mechanism through which CQ exerts its action (O'Neill *et al.*, 2012a). The enzymes in the digestive vacuole act at a pH optimum range of 4.5 – 5.0, which also promotes biocrystallization. Because all compounds within this class exhibit a basic nitrogen, accumulation results in an increase in pH, causing the metabolism of these parasites to be impaired (Ettari *et al.*, 2009). Additionally, it is also believed that these compounds inhibit biocrystallization, which results in the build-up of toxic haem (ferriprotoporphyrin IX), which ultimately leads to oxidative cell death (Bray, 2005). Given that biocrystallization is promoted through acidic media, the inhibition of biocrystallization might also be a result of the increased pH. Despite evidence of these inhibiting mechanisms, the debate continues regarding the exact mode of action of this class of antimalarials.

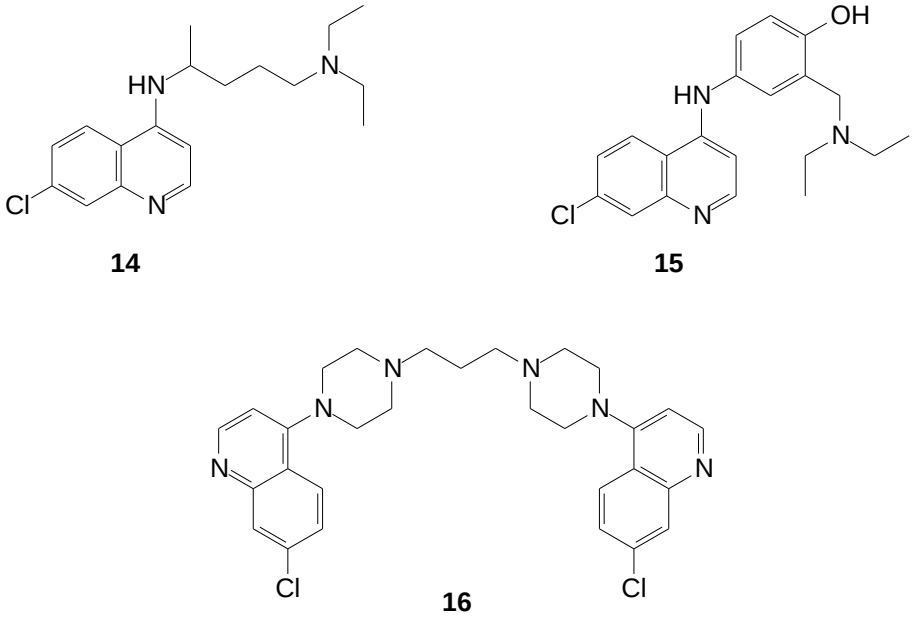


Figure 2.12: Chemical structures of 4-aminoquinolines: chloroquine (CQ, **14**), amodiaquine (**15**) and piperaquine (**16**).

Chloroquine (CQ, **14**) is one of the most extensively used compounds within this class and has been around for more than 75 years (Jensen and Mehlhorn, 2009). CQ is active against asexual erythrocytic stages of *Plasmodium*, but due to widespread resistance, CQ does not have much therapeutic value against CQR *P. falciparum*. It is, however, still used against CQ sensitive *P. malariae* and *P. ovale* species, which are almost completely sensitive towards CQ (Jensen and Mehlhorn, 2009). CQ is used as chemotherapy and as prophylaxis and is marketed under the trade name, Aralen®. Side effects include pruritis, rash, headaches, gastrointestinal disturbance and rarely bone marrow suppression, hair loss and convulsions (Ashley *et al.*, 2006).

Amodiaquine (**15**) is a 4-aminoquinoline mannich base, which is more active than chloroquine against resistant parasites. The incorporation of the *p*-aminophenol moiety has resulted in increased hepatotoxicity and life threatening agranulocytosis, which renders its therapeutic value low (Schlitzer, 2008).

Piperaquine (**16**) is a bisquinoline compound, related to chloroquine, and had been developed in the 1960's when resistance towards CQ had emerged. It is, however, not an FDA approved drug. Piperaqione is well tolerated, with its most important side effect being increased blood pressure (Schlitzer, 2008). It has been co-formulated with dihydroartemisinin as a fixed dose combination and is used in a number of Asian countries for the treatment of uncomplicated *P. falciparum* malaria.

The development of resistance by *P. falciparum* towards the 4-aminoquinolines is connected to multiple parasite gene mutations that influence the abilities of these drugs to accumulate in the digestive vacuole (Foley and Tilley, 1998). Resistance to CQ (**Fig. 2.13**) is associated with a point mutation K76T in the *P. falciparum* chloroquine resistance transporter (*Pf*crt) protein, which seems to allow movement of drugs out of the digestive vacuole (O'Neill *et al.*, 2012a). This in turn ultimately alters the H^+ efflux, reduces drug accumulation and restores the pH. Additionally, the extrusion of CQ from the digestive vacuole is mediated by an ATP dependent P-glycoprotein efflux pump (Pgh1). The Pgh1 efflux pump is the protein product of the *Pf*MDR1 gene, which is associated with multiple drug resistance (O'Neill *et al.*, 2012a).

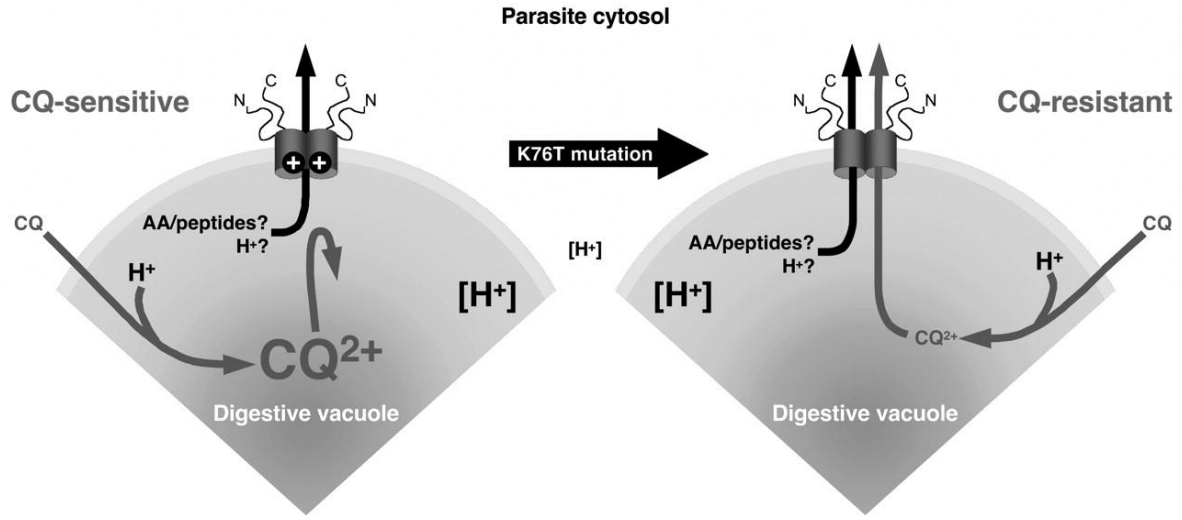


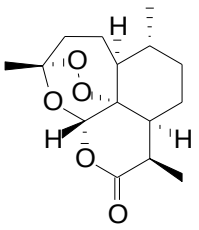
Figure 2.13: Illustration of the *Pf*crt protein that allows the outward movement of CQ (Martin & Kirk, 2004).

2.7.6 Artemisinin and derivatives

Artemisinin (**17**), also called qinghaosu, is a sesquiterpene lactone, consisting of three isoprene units bound to cyclic organic esters with a peroxide bridge, extracted from the leaves of sweet wormwood (*Artemisia annua*) (ANON, 1979). The medicinal herb extract, called qinghao, has been used for about 2000 years in the treatment of fevers. It had first been described in "52 prescriptions", discovered in the tomb of the Mawangdui Han Dynasty. The first recorded treatment of malaria with Qinghao had been in the year 341 AD. The antimalarial activity of qinghao was rediscovered in 1971 (Haynes and Krishna, 2004).

Artemisinin's low water and oil solubilities (Li and Zhou, 2010) have led to the synthesis of several derivatives, such as the oil soluble artemether (**19**) and arteether (**20**) derivatives, as

well as the water soluble artesunate (**21**, AS). The reduction of artemisinin with $NaBH_4$ resulted in dihydroartemisinin (**18**, DHA) (Haynes and Krishna, 2004). Unlike most antimarials that are only effective in destroying the asexual stages of the malaria parasites, artemisinins is effective against nearly all asexual stages and the early sexual stages of the parasites (Li and Zhou, 2010). They do not, however, eradicate the hepatic stages of the parasite. Artemisinins is furthermore, also active against all species of *Plasmodium*.



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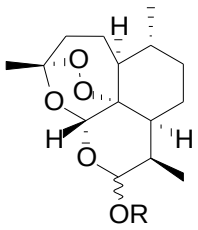


18 R = H
19 R = OCH_3
20 R = OCH_2CH_3
21 R = $CO(CH_2)_2COOH$

Figure 2.14: Clinically used artemisinin (**17**), dihydroartemisinin (**18**), artemether (**19**), arteether (**20**) and artesunate (**21**).

The mechanism of action of artemisinins has been a subject of intense controversy over recent years (Haynes and Krishna, 2004). The initial mode of action stems from the fact that peroxides are good sources of free reactive oxygen species (ROS) and that the Fe^{2+} dependent fenton process enhances the production of ROS, thereby inhibiting the parasite haemoglobin-digestive processes within the digestive vacuole (Li and Zhou, 2010). As Fe^{2+} is the principal element deposited in haemozoin (parasitic pigment deposited within the digestive vacuole), the endoperoxide bridge of artemisinins is proposed to be activated by ferrous iron to generate free radicals that hence overwhelm the antioxidant mechanisms and destroy the parasites (O'Neill *et al.*, 2010; Krishna *et al.*, 2008). Iron mediated cleavage of the artemisinin endoperoxide bridge was first proposed by the Meshnick group (Meshnick *et al*. as quoted by Li & Zhou, 2010), who had isolated haem-artemisinin adducts from artemisinin treated *P. falciparum* (Li and Zhou, 2010). The reported increase in antimalarial activity of artemisinin against *P. falciparum* parasites cultured in the presence of carboxyhaemoglobin, contradicted these earlier findings (Krishna *et al.*, 2008). This increase was unexpected, since carboxyhaemoglobin inhibits haem-Fe^{2+} reactivity, thereby indicating that haemoglobin iron plays no part in activating artemisinin and that another mechanism must be responsible for the observed activity of artemisinins (Krishna *et al.*, 2008).

Furthermore, potent activity against non-pigment-producing parasites had also been observed.

Other studies suggested that artemisinins could also target the parasite *Pf*ATP6, a parasite-encoded sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) (Krishna *et al.*, 2008). This was based on structural similarities between artemisinins and thapsigargin, a known inhibitor of SERCA (Cui and Su, 2009). The pre-incubation of parasites with artemisinin had indicated that labelled parasites, using a fluorescent thapsigargin derivative, had been eliminated, supported the hypothesis that artemisinin and thapsigargin had the same target site. Reports, supporting and disagreeing with this theory, however, exist (Li and Zhou, 2010).

Recently, a third model was proposed, suggesting that artemisinin is activated by malarial mitochondria and free radicals non-specifically damage surrounding organelles. Although this model was demonstrated in yeast, it lacked scientific evidence (Li and Zhou, 2010). Irrespective of the mechanism by which artenisinins eradicate the parasite, evidence that the endoperoxide is involved in the observed activity cannot be disputed.

The emergence of tolerance towards artemisinins could pose serious consequences to the fight against malaria, since this class of drugs is the last line of chemotherapeutic defence against multi-drug resistant strains (WHO, 2012). A decreased sensitivity of malaria parasites towards artemether has been indicated in South-Asia and along the Thai-Cambodian border (WHO, 2013). This observation confers a gene mutation S769N in *Pf*ATP6, but although this may not be considered as resistance yet, it signifies early signs thereof. The reported maximal IC_{50} values are still within the range of achievable maximal human plasma concentrations (WHO, 2013; Cui *et al.*, 2012).

Artemisinin has largely been replaced by DHA, artemether and artesunate. The WHO also recommends artemisinin combined therapy (ACT) instead, as a precautionary attempt to avoid artemisinin resistance development and to address the high recurrence of malaria, associated with the use of artemisinin in mono-therapy. ACT is based upon the combination of a fast acting artemisinin derivate with a slow acting counterpart having a different mode of action (O'Neill *et al.*, 2012b). The WHO recommends one of the following treatment regimes for uncomplicated *P. falciparum* infection: (1) artemether plus lumefantrine, (2) artesunate plus amodiaquine, (3) artesunate plus mefloquine, or (4) artesunate plus sulfadoxine-pyrimethamine (WHO, 2010). The ACT artemether and lumefantrine in a fixed dose combination of 80/480, mg respectively, is marketed under the trade name Coartem®.

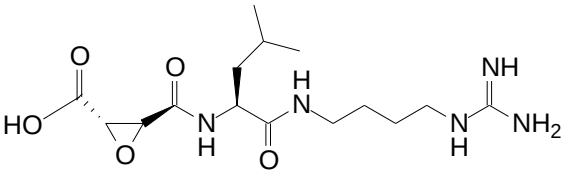
Lumefantrine is an aryl aminoalcohol in the same general group as mefloquine and halofantrine and is active against all *Plasmodium* species (Nosten and White, 2007). Side effects of artemisinin include nausea, vomiting, bowel disturbance, abdominal pain, headaches and dizziness (O'Neill *et al.*, 2012b).

2.7.7 Chalcone compounds

With the advent of sequencing the *Plasmodium* genome, several unique targets have been identified for exploitation with chemotherapy (Rosenthal, 2004). Parasite induced permeation pathways and malarial cysteine proteases are among these targets. Parasite induced permeation pathways are pathways that are induced in infected erythrocytes to become more permeable to essential nutrients, such as amino acids, vitamins and carbohydrates. These pathways are absent in healthy cells and are therefore attractive targets (Baumeister *et al.*, 2003).

Malarial cysteine proteases, on the other hand, are species specific enzymes used in the catabolism of haemoglobin (**Fig. 2.3**). Cysteine proteases, collectively called falcipains, mediate protein hydrolysis *via* nucleophilic attack on a carbonyl of a susceptible peptide bond. The main function of malarial cysteine protease is the hydrolysis of haemoglobin in the food vacuole (Rosenthal, 2004). Sequencing of the genome revealed several clans of falcipains in the genetic code, although only a few of these enzymes' functions had been assigned (Rosenthal, 2004). The best known falcipains are falcipain-2 and -3, which mediate haemoglobin catabolism and may have additional functions, such as being involved in the rupture of the erythrocytic membranes (Aly and Matuschewski, 2005). Reports on genetic knockout experiments state that falcipain-1 is a non-essential enzyme, although its exact function is still unknown (Sijwali *et al.*, 2004).

A well known cysteine protease inhibitor is E64 (**22**), a natural, modified peptide containing an active epoxide functional group. Other inhibitors include naturally based derivatives, such as chalcones (1,3-diaryl propenone) and isatins. Chalcones are biosynthetic precursors of flavonoids (Ettari *et al.*, 2009), consisting of two aromatic rings connected with an α,ß-unsaturated propenone linker, with one ring bound to a vinylic propene Ring A and an aromatic ring, bound to a carbonyl carbon Ring B (**23**). The first reported chalcone with antimalarial activity was Licochalcone A (**24**), a natural product isolated from Chinese liquorice roots, with an IC_{50} of 6.5 µM against 3D7 clones. Licochalcone A also shows *in vivo* activity (Larsen *et al.*, 2005; Go *et al.*, 2004).



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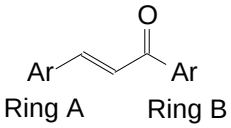

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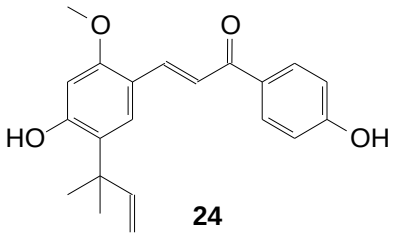

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Figure 2.15: Cysteine protease inhibitor E64 (**22**), general structure of a chalcone (**23**) and Licochalcone A (**24**).

Of the first synthetic chalcones synthesized by Li *et al.* in 1995, are still among the most potent chalcones to date. 1*E*-(2,5-dichlorophenyl)-3(4-quinolinyl)-2-propen-1-one (**25**; **Fig. 2.16**) showed an IC_{50} value of 200 nM against both CQ sensitive (D6) and CQ resistant (W2) strains of *P. falciparum*. The structure activity relationship (SAR) drawn from this study was that the alkene bond was essential for activity, i.e. substitution on this bond diminished the activity, whilst the introduction of halogenated groups on ring A and electron withdrawing groups on ring B increased activity (Li *et al.*, 1995). Several other authors have confirmed these early findings (Go *et al.*, 2004; Liu *et al.*, 2003).

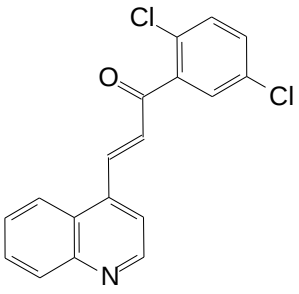

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Figure 2.16: Chemical structure of 1*E*-(2,5-dichlorophenyl)-3(4-quinolinyl)-2-propen-1-one (**25**).

Numerous authors have reported that chalcones, containing basic nitrogen or sulphur groups, possess antimalarial and cytotoxic activities (Kumar *et al.*, 2010; Reddy *et al.*,

2008). Besides antimalarial activity displayed by chalcones, they possess a vast array of biological functions, such as antibacterial-, antifungal-, antiviral-, anti-inflammatory- and antitumour activities (Mishra *et al.*, 2008; Kumar *et al.*, 2010). For these reasons, toghether with its ease of synthesis and the relatively low cost involved, the chalcone moiety could prove an outstanding candidate for possible treatment against malaria. Powers *et al.* (1998) report the automated synthesis of chalcones, demonstrating the ease of synthesis of over 74 000 compounds.

2.7.8 Hybrid drug theory

Although ACTs currently are the preferred treatment method for both uncomplicated and severe malaria (WHO, 2010), all of the shortcomings of the individual compounds remain problematic, including resistance to the parent molecule. With the aid of a prodrug approach, one can stabilize, improve efficacy, increase the half-life of the drug if formed from a parent molecule inside the body, and improve bioavailability and delivery of the drug (Testa, 2004), although this approach may not address resistance. An alternative method that has been proposed to overcome the development of resistance is the incorporation of a second pharmacophore *via* a chemical bond, forming a hybrid drug molecule (**Fig. 2.17**) (Meunier, 2008).

Hybrid molecules combine two drugs in a single molecule, with the goal of creating a chemical entity with two or more structural domains, having different biological functions and dual activity that are medically/therapeutically more effective than its individual components. There are effectively three ways in which the hybrid can interact with the target (Meunier, 2008). In the first scenario (**Fig. 2.17** - **A**), the two targets are close to each other or to related targets and the hybrid interacts with the targets simultaneously. In the second scenario (**Fig. 2.17** - **B**), the two targets are not within the same organelle and thus act independently. In the last (**Fig. 2.17** - **C**) case, the hybrid has the same target with two different pharmacophores, thus resulting in the so-called double edged sword.

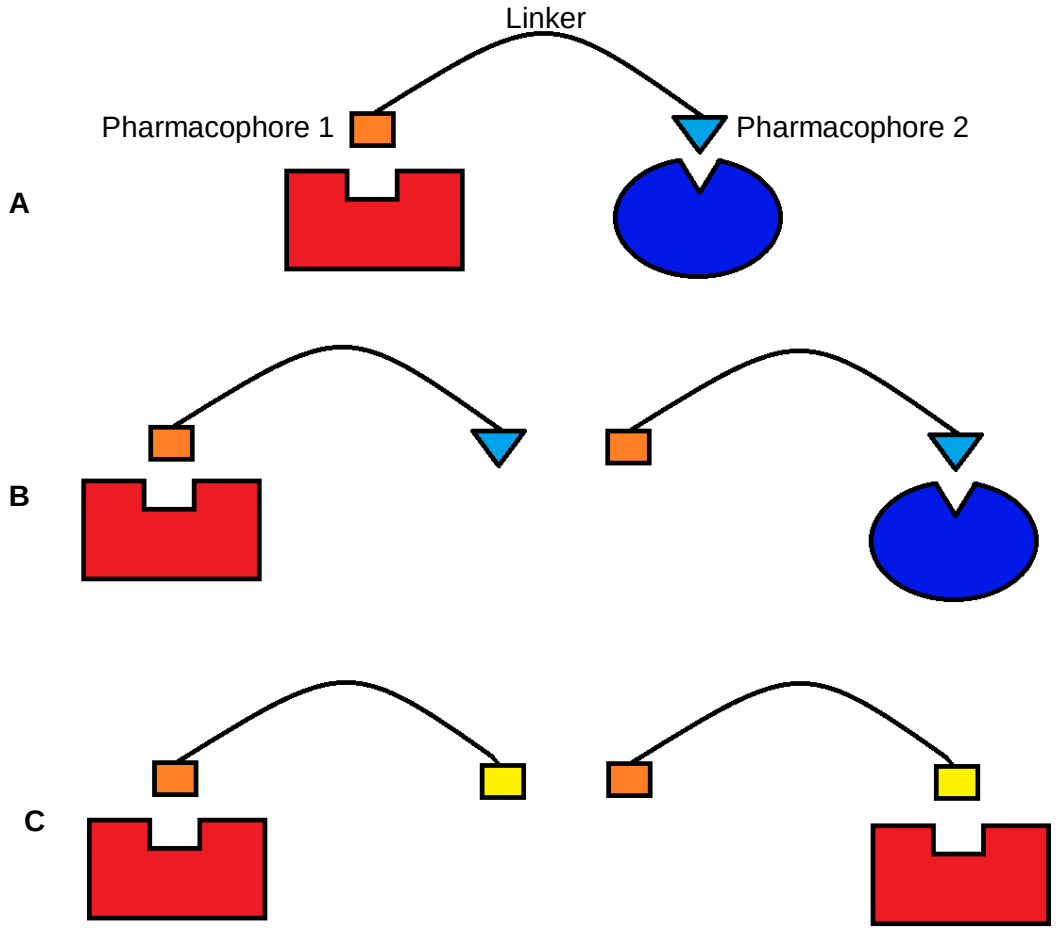

Figure 2.17: Three binding possibilities of hybrid compounds (Meunier, 2008).

Combining drugs into a hybrid molecule may offer several advantages over combination therapy, such as that the two components may act synergistically, restore the antimalarial activity of the individual compound, impart favourable physicochemical properties, lower toxicity as well as being potentially cheaper to manufacture (Walsh and Bell, 2009).

2.7.9 Chalcone based hybrids

Several chalcone based hybrids are reported containing a number of different pharmacophores, such as thiolactone-, isatin- (Hans *et al.*, 2010), stilbene- (Sharma *et al.*, 2012), quinoline- (Sashidhara *et al.*, 2012a; Sashidhara *et al.*, 2012b; Sharma *et al.*, 2009), ferrocene- (Attar *et al.*, 2011; Wu *et al.*, 2006; Wu *et al.*, 2002) and endoperoxide-chalcone based hybrids (Walsh and Bell, 2009). For the purpose of this text only the quinoline-, ferrocene- and endoperoxide-chalcone based hybrids are discussed.

In 2009, Sharma *et al.*, synthesized some of the first reported substituted quinolinyl-chalcone based hybrids (**26**, **Fig 2.18**), of which the 4-amino linked compounds showed low to no activity against the NF-54 strain of *P. falciparum*. This disappointing outcome corroborated earlier reports by Chibale *et al*. (2000) that the length of the methylene spacer between the two nitrogen groups in the side chain of CQ analogues was a major determinant of their antimalarial activities against CQ resistant *P. falciparum*. This finding *inter alia* formed the rationale underlying the design of the novel quinolinyl-chalcone hybrids during this study (Chapter 3).

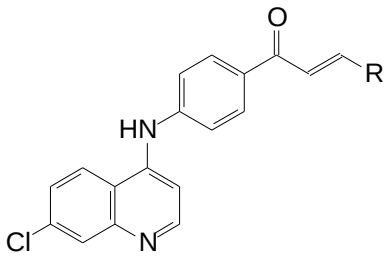

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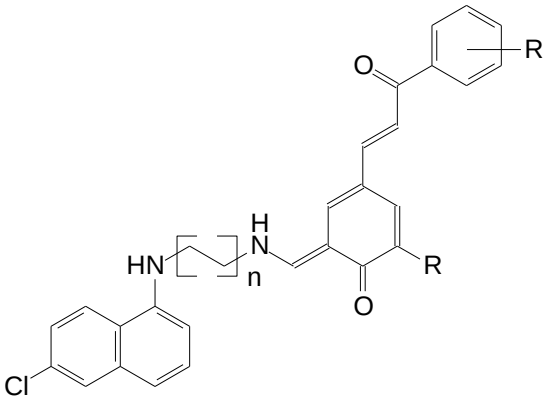
Figure 2.18: General structure of quinolinyl-chalcone based hybrids (**26**) synthesized by Sharma *et al.* (2009).

Sashidhara *et al.* (2012) report the synthesis and biological activity of a number of chloroquine-chalcone based hybrids. Of these keto-enamine chloroquine-chalcone based hybrid compounds (**27**), several were equipotent to CQ against 3D7 strains. These compounds also displayed antimalarial activity *in vivo* against the MDR rodent malaria parasite, *P. yoelii*. Mechanistic studies done by these authors had revealed that these compounds acted through haem polymerisation targets (Sashidhara *et al.*, 2012a; Sashidhara *et al.*, 2012b).



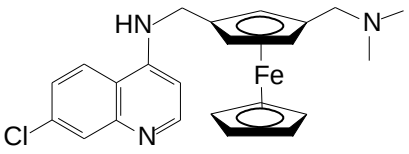
n = 0; 1

27

Figure 2.19: General structure of the keto-enamine chalcone-chloroquine based hybrid (**27**) (Sashidhara *et al.*, 2012b).

These results indicated that the combination of chloroquine-chalcone based hybrids may become leading compounds in the future design of more potent antimalarial drugs. Additionally, since both the chalcone and CQ exert their activities in the digestive vacuole, a synergistic effect might be expected.

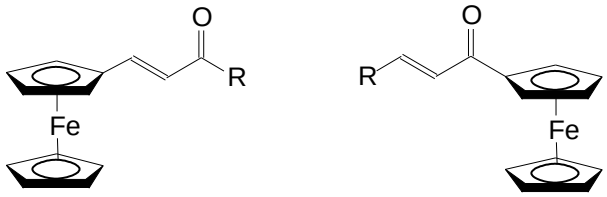
Ever since recent reports on the *in vitro* activity of ferroquine (**28**, **Fig. 2.20**), a ferrocene derivative of CQ, being active against CQR strains of *P. falciparum*, interest into organometallic compounds has attracted much attention (Mathiyalagan *et al.*, 2012; Gimeno *et al.*, 2011). Ferrocene (Fc) has the remarkable ability to be recognised by many biological systems, including amino acids, proteins, DNA and carbohydrates (Nabi & Liu, 2011; Muller *et al.*, 2012). The ferrocene moiety has also been proven to be a successful addition to biological compounds, such as to penicillin, cephalosporine, tamoxifen, or to known malaria therapeutics, thereby increasing their efficacy towards CQ resistant strains of the parasite, such as is the case with FQ (Gimeno *et al.*, 2011). Ferrocene related organometallic compounds have shown to possess antitumour (Muller *et al.*, 2012), antimicrobial (Mathiyalagan *et al.*, 2012) and antimalarial (N'Da *et al.*, 2010; Wu *et al.*, 2006; Wu *et al.*, 2002) activities, making these compounds perfect drug candidates to further investigate for possible use in the fight against malaria.



28

Figure 2.20: Chemical structure of ferroquine (**28**).

The role of ferrocene is still debated. Some authors suggest that it merely is a spacer group in biological systems (Beagley *et al.*, 2003), while others attribute ferrocene a more prominent role, i.e. that of contributing to the overall oxidative stress or cytotoxicity (Wu *et al.*, 2006). Numerous studies have been conducted on ferrocene containing chalcones with limited success. In one such study, the aryl groups were replaced with ferrocene (**Fig. 2.21**) in an attempt to evaluate their biological activity and the function of ferrocene (Attar *et al*.; 2011, Wu *et al.*, 2002). Some of these compounds showed moderate activity (~4 µM against KB3-1 cells), whilst the activity was found to be largely influenced by the position of the ferrocene ring, as well as the type of aromatic ring used as counterpart for these compounds (Wu *et al.*, 2006; Wu *et al.*, 2002). Ferrocenyl-chalcones with the general structure of compound **30**, displayed overall better activities (Wu *et al.*, 2006).



29 **30**

Figure 2.21: General structures of ferrocenyl-chalcones.

Currently, very limited data on ferrocene and chalcone derivatives, utilising methylene spacers between two nitrogens in CQ-like fashion, has been reported. The Fe^{2+} may undergo redox reactions and thus influence the redox cycling of the parasite. The ease of oxidation of Fe^{2+} in the ferrocenyl moiety may be influenced by chemical groups in the vicinity of this moiety and in turn would impact on the electrochemical potential of the compound. Recently, it has been reported that chalcones also possess radical-scavenging properties, resulting in oxidative stress (Nabi and Liu, 2011; Jayasinghe *et al.*, 2004). The combination of ferrocene and chalcones, incorporating methylene spacers, may thus impart favourable properties for potent antimalarial activity.

The combination of chalcones and endoperoxides has been pursued for almost a decade. In 2004, the first reported endoperoxide-chalcone based compounds (**31**) had been synthesized synthesised by O'Neil *et al*. (Walsh and Bell, 2009). Later, Gibbons *et al.* (2010) revealed that these compounds underwent ferrous mediated decomposition into active metabolites, while displaying equipotent activity to artemisinin and arteflene against cultured parasites (Gibbons *et al.*, 2010).

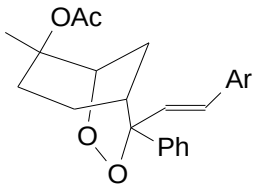


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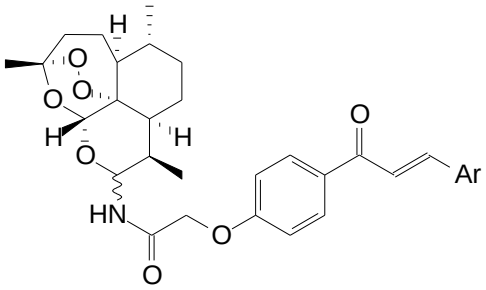
Figure 2.22: General structure of endoperoxide-chalcone based hybrids (**31**) (Gibbons *et al.*, 2010).

In 2009, Bhattacharya *et al.* had investigated the potential of new ACTs with chalcones and observed that most chalcones had either synergistic or additive interactions with the artemisinin (Bhattacharya *et al.*, 2009). Although the chalcones and artemisinin had not been combined in a hybrid molecule, it is noteworthy to mention this interaction. The dihydroartemisinyl-chalcone esters being synthesized during this study contained a hydrolysable ester bond, which could ultimately result in similar interactions. It was not until 2011 that chalcones and DHA had been combined in hybrid (**32**) fashion by means of an amide bond with intended use as antitumour agents. These compounds displayed a ten-fold increased activity, compared to DHA (Xie *et al.*, 2011).



32

Figure 2.23: General structure of dihydroartemisinin-chalcone based hybrids.