CHAPTER 1

Literature Overview

1.1 History and overview of malaria

Malaria has plagued mankind since at least 4 BC when Hippocrates first described the manifestations of the disease (Goodwin, 1992). He also linked these to seasonal changes and to the regions where people lived. Since then man has been searching for the cause, treatment and preventative measures. It was however, not until 1880 that Laveran who was working in Algeria at the time, discovered the protozoal nature of the causative agent of malaria. The mode of transmission of the disease was unknown until 1897 when Ronald Ross, a British scientist, identified the mosquito as being the vector for this protozoan parasite (Krettli and Miller, 2001; McKenzie, 2000). Later, workers in Italy specifically linked mosquitoes of the genus *Anopheles*, to human malaria (Krettli and Miller, 2001). It is interesting to note that the first recorded treatment of malaria dates back to 1600 in Peru, where the Peruvian Indians utilized the bitter bark of the cinchona tree. This is the natural source of quinine, a drug still in use today for the treatment of malaria (Krettli and Miller, 2001; McKenzie, 2000).

The malaria parasite belongs to the phylum Apicomplexa, which includes *Plasmodia*, *Toxoplasma gondii* and *Cryptosporidium sarcocystis*. Four different species of *Plasmodia*

infect humans, namely, *falciparum*, *vivax*, *ovale* and *malariae*. The most virulent of the four is *Plasmodium falciparum* and its life cycle is shown in Figure 1.1.

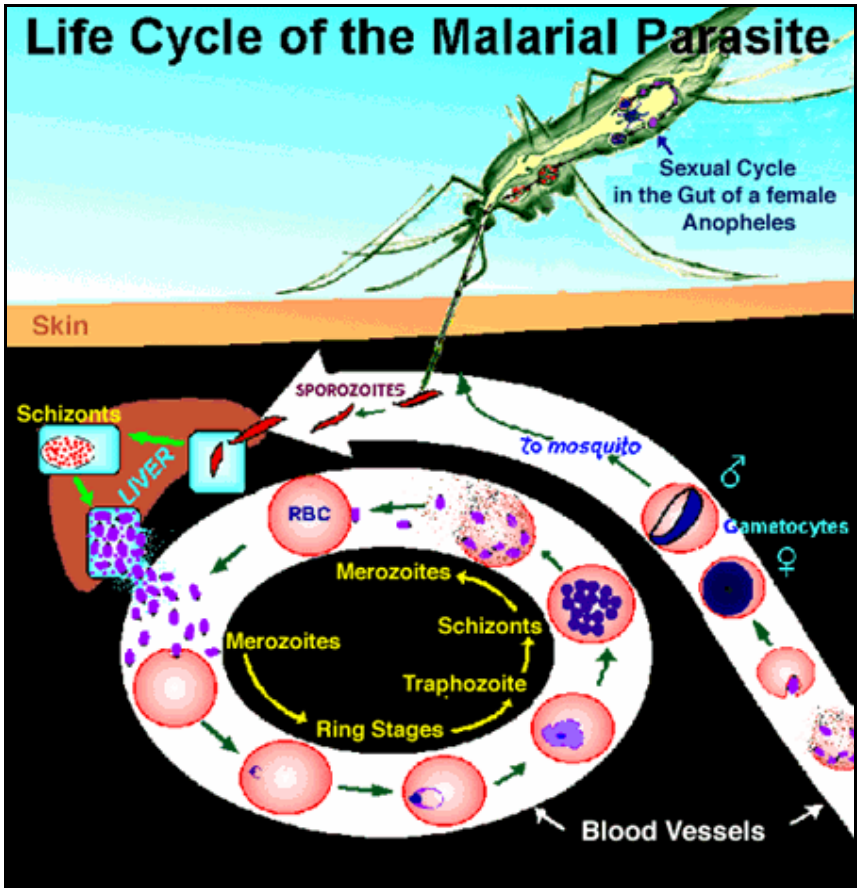


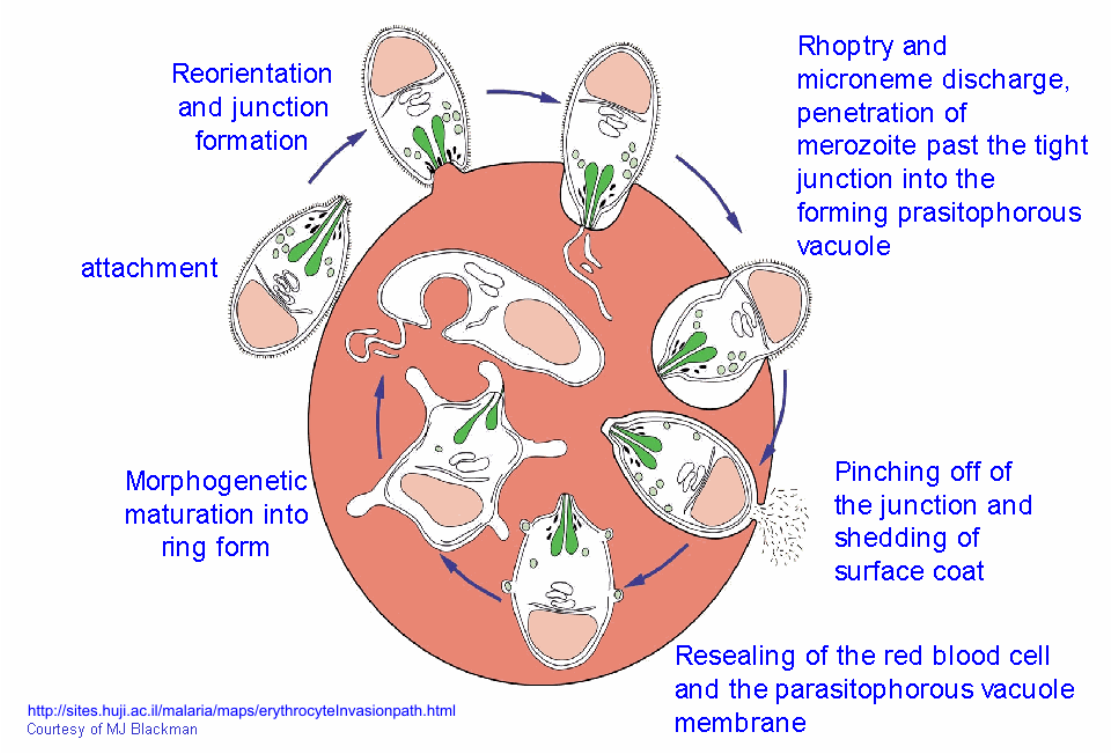
Figure 1.1: The complete life cycle of the human malaria parasite, *P.falciparum*. (Malaria Foundation International (www.malaria.org/lifecycle.html).

There are three distinct parasite stages that invade host cells, namely the merozoites (erythrocytes), ookinetes (mosquito gut) and sporozoites (liver). The cycle begins with sporozoites, being transmitted to the human host from the mosquito salivary gland. The sporozoites enter hepatocytes where they develop into schizonts. Merozoites are then released from the infected hepatocytes from where they enter the circulating blood and infect host erythrocytes, within 1 –2 minutes. Once inside the erythrocyte the parasite

develops into the ring phase and then into the feeding trophozoite stage. The trophozoite-phase parasites then undergo division to form schizonts. The schizont-containing erythrocytes rupture, releasing new merozoites. Some of the merozoites develop into micro - and macro - gametocytes, while others go on to re-infect new erythrocytes. Gametocytes are ingested by feeding female *Anopheles* mosquitoes and develop into zygotes after fertilization in the mosquito's gut. The zygote develops into an ookinete, which then permeates the midgut lining and forms an oocyst on the outer gut wall. The oocytes eventually rupture, releasing sporozoites. These then migrate to the mosquitoe's salivary glands from where the human host is infected when the mosquitoes ingest a blood meal. The approximate number of merozoites produced per liver schizont in *P. falciparum* is 30 000, compared with over 10 000 for *P. vivax*, 15 000 for *P. ovale* and 15 000 for *P. malariae* (Gilles, 1993).

The maximum parasitaemia (per 1 mm^3 blood) for *P. falciparum* is 2 000 000, compared with 50 000 for *P. vivax*, 30 000 for *P. ovale* and 20 000 for *P. malariae*. Invasion of erythrocytes by merozoites is assisted by specialized secretory organelles at their apical ends namely, rhoptry organelles, micronemes and dense granules which all function at specific steps in the invasion process as illustrated in Figure 1.2 (Bannister *et. al*., 2000).

Microneme content is released first and occurs with the initial contact between the parasite and host cell. The content of the rhoptry organelles is released directly afterwards and is associated with the formation of the parasitophorous vacuole. Dense granule content is the next to be released and is associated with modifications to the host cell



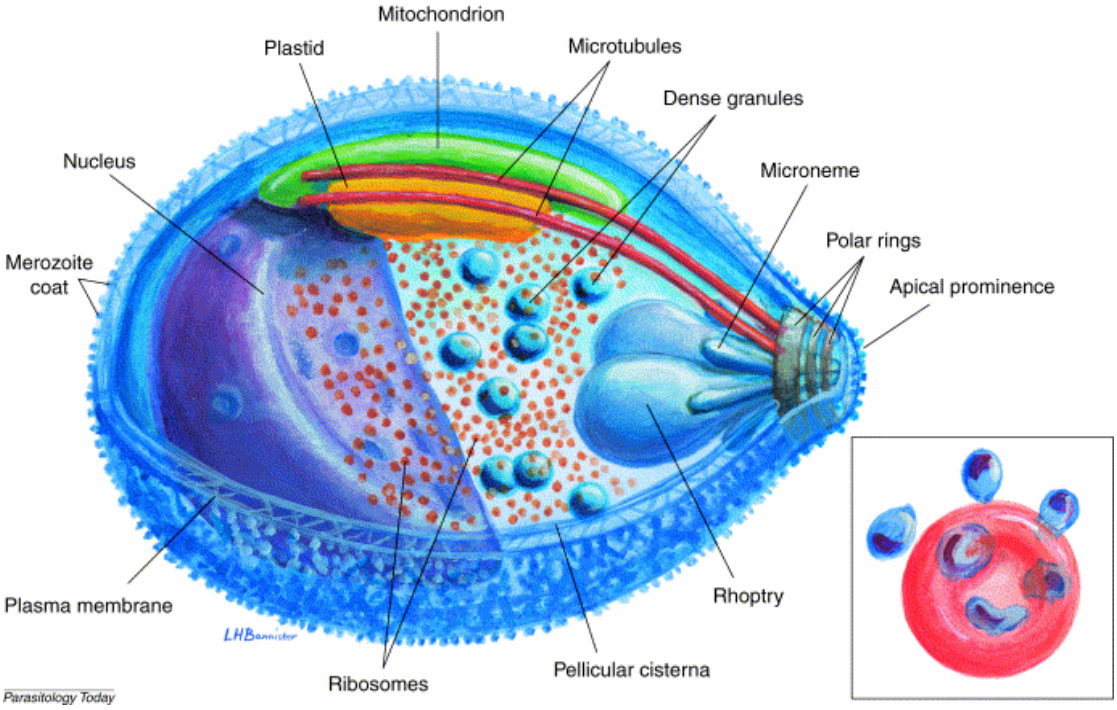


Figure 1.2: Schematic representation of merozoite invasion of a host erythrocyte (http://sites.huji.ac.il/malaria/) and an ultrastructural view of the parasite components involved in invasion (Bannister *et al*, 2000)

membrane. This is evidenced by the release of proteins such as RESA (ring-infected erythrocyte surface antigen), which is located in the dense granules in merozoites and is transported to the erythrocyte membrane shortly after parasite invasion.

The upward trend of reported cases and magnitude of the malaria problem highlights the fragility of the current control programmes. Malaria is a public health problem in more than 101 countries and afflicts ca. 40% of the world's population (WHO, 1998). This is illustrated in Figure 1.3.

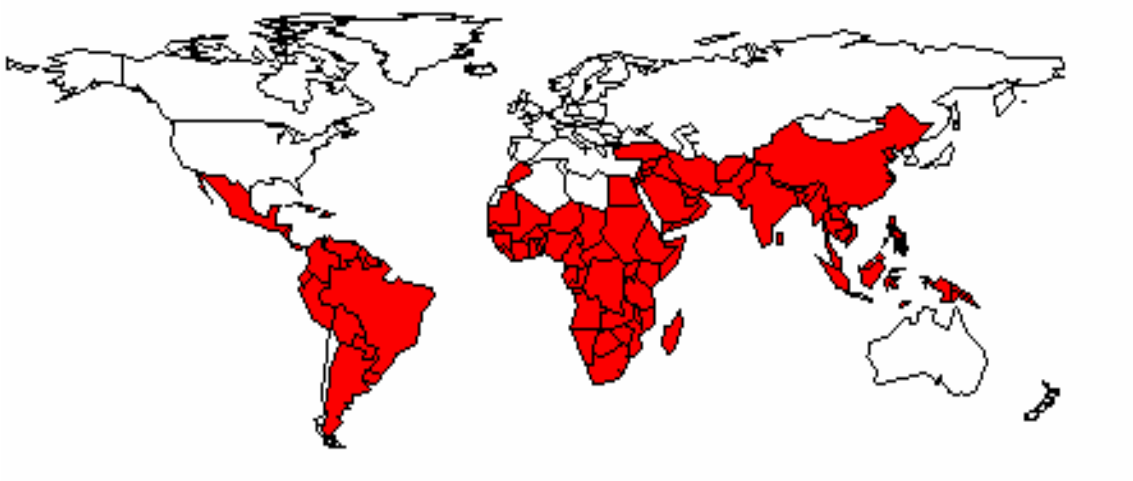


Figure 1.3: World map indicating the regions (red) in which indigenous malaria is prevalent. The red areas include countries such as Brazil, India, Afghanistan, Sri-Lanka, Thailand, Cambodia, Vietnam, Indonesia and China as well as large portions of the African continent (WHO, 1998).

The affected countries can be summarized as follows: 45 in Africa, 21 in the Americas, 4 in Europe, 14 in the Eastern Mediterranean, 8 in South East Asia and 9 in the Western Pacific (WHO, 1998). The magnitude of the problem is evidenced by the between 300 and 500 million clinical cases of malaria reported worldwide each year, with about 90% of these coming from sub-Saharan Africa. In real terms these figures translate to the

death of 3000 children under five years of age, each day. The direct and indirect costs associated with malaria, which include prevention, treatment and loss of income, amount to ca. US $2 billion annually (WHO, 1998).

1.2 The current malaria problem

The reasons for this grave problem are numerous and include inefficient control of the mosquito vectors and resistance of the latter to insecticides, the lack of an effective vaccine and increasing resistance of the parasites to the dwindling supply of effectual anti-malaria drugs (Nacher, 2001). Furthermore, failure to diagnose malaria in the early stages often leads to mortality that could have been avoided by timely treatment (Fischer and Bilek, 2002). Poverty and hence malnutrition increases the susceptibility of individuals in endemic areas to malaria and further increases the mortality rate (Kasis *et. al*., 2001).

One of the first systematic efforts to eradicate malaria began in the 1940s with the introduction of dichlorodiphenyltrichloroethane, more commonly known as DDT. The aim was to introduce a time-limited project to eradicate the mosquito vector populations. The widespread use of DDT however led to a number of health problems, including reproductive abnormalities in higher animals (Wooster, 2001). It was therefore decided to terminate the use of DDT in all industrialized and most third world countries (Wooster, 2001). In South Africa, DDT was replaced by synthetic pyrethroids in 1996. The ban on DDT and replacement thereof with other insecticides, resulted in a dramatic increase in

the incidence of malaria in countries such as South Africa, Sri Lanka, Madagascar and Swaziland, to name a few. In the Northern KwaZulu-Natal region of South Africa, for example, the incidence of malaria cases increased from the low base of ca. 5000 per year to between 60 000 and 70 000 in 1999 and 2000. This increase can be ascribed to two factors: firstly, the reappearance of the mosquito, *Anopheles funestus*, due to aquired resistance to pyrethroids. The second factor was parasite resistance to anti-malarial drugs, in particular sulfadoxine-pyrimethamine (SP) (Roper *et. al*., 2003). The resistance of the malaria parasite to SP is not limited to Africa but is also evident in Southeast Asia and South America (Hopkins-Sibley *et. al*., 2001). To illustrate the extent of the problem, epidemiological studies conducted in northern KwaZulu-Natal showed parasite resistance to SP in excess of 62% of cases in the 2001 malaria season (www.mrc.co.za).

The malaria problem is being addressed on two levels, namely vector and parasite control. KwaZulu-Natal reverted back to the controlled use of DDT for intra-domiciliary spraying in the winter of 2000 in an attempt to eradicate *Anopheles funestus* from the target regions and thereby reduce transmission of malaria (www.malaria.org). Combination drug therapy was also introduced in the region in February 2001 in order to limit the spread of parasite drug resistance. Malaria treatment currently entails a combination of artemesinin and lumafantrene (co-artemether). The vector control strategy together with combination drug therapy has yielded a 75% decrease in malaria cases in northern KwaZulu-Natal for 2001, compared with the previous year (www.malaria.org).

Antigenic variation and the polymorphism of key antigenic parasite proteins targeted by the host immune system are partly responsible for the inability of humans to develop long lasting immunity to malaria (Allred, 2001). Nucleotide sequence mutations that affect changes in the amino acid sequence of a protein (polymorphisms) can lead in turn to either positive or negative selection over a period of time (Forsdyke, 2002). Mutations affecting the antigenic determinants on the surface of pathogens such as malaria, that enable them to evade the host immune response lead to positive selection. MSP-1 and CSP are two *P. falciparum* proteins known to be highly polymorphic (Hartl *et. al*., 2002). *Msp-1*, the gene encoding the MSP-1 protein found at the surface of merozoite stage parasites for example, includes two divergent allele classes (known as *MAD* and *K1*) differing in their amino acid sequence by as much as 60% in some regions (Hartl *et. al*., 2002). CSP is found on the surface of malaria sporozoites and contains multiple tandem and thus polymorphic repeats (Hartl *et al*., 2002; Zambrano-Villa *et al*., 2002). Since both of these proteins are situated on the outer surface of the parasite they are ideal vaccine candidates. However, the polymorphic nature of these proteins serves as a successful protective mechanism for the parasite to evade the immune response by the production of new, vaccine-induced antigenic variants (Escalante *et. al*., 2002).

Rapidly dividing intra-erythrocytic parasites alter the host cell membranes, which would enable the reticuloendothelial system of the spleen to recognize parasitized erythrocytes and destroy them (Borst *et. al*., 1995). To prevent this the parasite expresses proteins on the infected RBC membrane that allows it to escape splenic destruction.

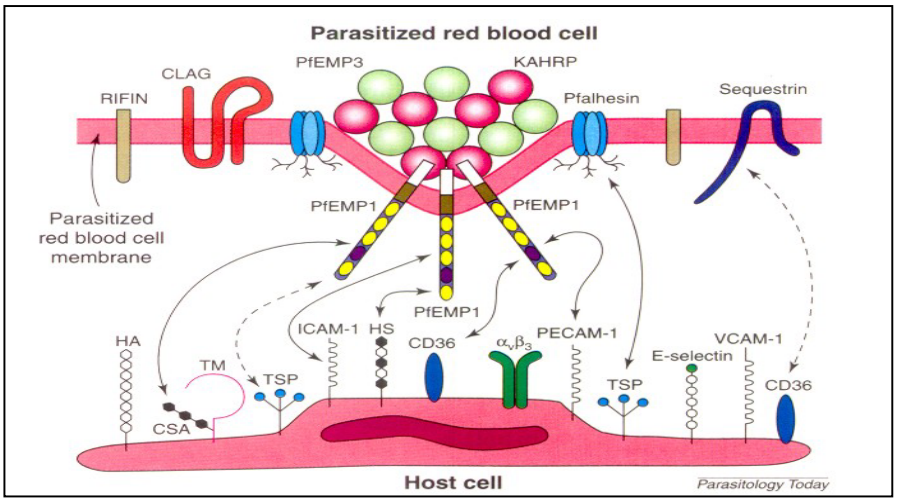


Figure 1.4: Schematic representation of the interaction at the cytoadhesive interface between a *P. falciparum* infected erythrocyte and the host vascular endothelium**.** CLAG: cytoadherence-linked asexual gene; CR1: complement receptor I; CSA: chondroitin sulphate A; HA: hyaluronic acid; HS: heparan sulphate; ICAM: intercellular adhesion molecule I; KAHRP: knob-associated His-rich protein; PECAM-1: platelet-endothelial cell adhesion molecule I; Pfalhesin: modified form of band 3; TM: thrombomodulin; TSP: thrombospondin; VCAM-1: vascular cell adhesion molecule 1. Adapted from Cooke *et.al.,* 2000.

P. falciparum-induced changes of the host erythrocyte cell membrane include the insertion of parasite proteins that are associated with cerebral malaria. The latter can be fatal within 24 – 72 hours after the appearance of symptoms (25 to 50% of cases) and has been ascribed to rosetting (sticking together of infected and uninfected erythrocytes) as illustrated in Figure 1.4 (Newton and Krishna, 1998; Wickham *et. al*., 2001). These parasite-specific proteins in addition, mediate the adhesion of infected erythrocytes to post-capillary microvascular endothelial cells (a process termed sequestration), other infected erythrocytes (auto-agglutination or clumping) and to platelets, monocytes and lymphocytes (Kyes *et. al*. 1999, Berendt*, et al.*, 1994, Miller*, et al.*, 2002, White, 1998). One such example is the parasite protein PfEMP1 (*P. falciparum* erythrocyte membrane

protein 1), which attaches to blood vessel endothelial cell ligands such as CD 36, ICAM-1 (intracellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule) causing reduced blood flow and fatal hypoxia (Gamain *et. al*., 2002; Smith *et. al*., 2000). As a consequence only the immature ring stage parasites are visible in peripheral blood.

The parasite specific proteins on the red blood cell surface are however exposed to the immune system and antigenic variation is utilized to evade the host immune response. Antigenic variation refers to the ability of an organism to alter the structural and antigenic characteristics of a specific component over a short period of time (Allred, 2001). By 1995 it was evident that malaria parasites contain large families of genes coding for variant antigens and that differential expression of these genes aid the parasite to escape detection by the host immune system and in particular recognition by the reticulo-endothelial system of the spleen (Borst *et al*., 1995). The *var* and *rif* multigene families of *P. falciparum* encode PfEMP-1 (*P. falciparum* erythrocyte membrane protein -1) and the Rifin protein families, respectively. Both these parasite protein families are expressed on the surface of infected erythrocytes and are thus under strong immune pressure to undergo antigenic variation. PfEMP1, expressed on the surface of erythrocyte membranes infected with trophozoite or early gametocyte parasites, has at least 3 distinct but inter-related functions, namely: i) continuous variation of antigen from a repertoire of 50-150 genes to evade immune recognition, ii) aiding infected erythrocytes to adhere to endothelial cells through binding to the host receptors, CD36 and chondroitin sulphate A to avoid splenic filtration and destruction, and iii) mediating the binding of infected

erythrocytes to dendritic cells, a process that prevents the ability of the latter to process antigen or to be stimulated (Allred, 2001; Gamain *et. al*., 2002; Piper *et. al*., 1999).

The Rifin proteins are transcribed for only a short period of time between the late ring and early trophozoite phases (Kyes *et. al*., 1999). It is estimated that there are more than 200 copies of the *rif* gene per haploid genome. This makes the *rif* genes at least four times more abundant than the *var* genes (Kyes *et. al*., 1999). The precise function(s) of Rifin proteins except to assist immune evasion mechanisms, is however not known at present and requires further elucidation.

Antigenic variation is achieved by different mechanisms, which include: i) *in situ* gene switching, ii) segmental gene conversion and iii) duplicative replacement. *In situ* gene switching is defined as alternative transcription by mechanisms that do not include movement, duplication or deletion of the controlled gene (Allred, 2001). Often the antigenic variant components are those to which there is a strong host immune response e.g. PfEMP1 and Rifin proteins. Segmental gene conversion entails the duplication of a gene segment and insertion thereof into an actively transcribed gene, thereby creating a mosaic gene. There is no exact copy of such a gene in the rest of the genome. Lastly, duplicative replacement or duplicative transposition is a process whereby an entire gene is duplicated and inserted into an active site of transcription (Allred, 2001).

1.3 Anti-malaria drugs and parasite resistance

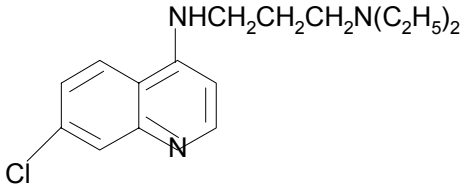
The most frequently used anti-malarial drugs are listed in Table 1.1 along with their class and biological activity.

Table 1.1: Classification of the most commonly used anti-malaria drugs (http://www.icp.ucl.ac.be/~opperd/parasites/chemo1.html).

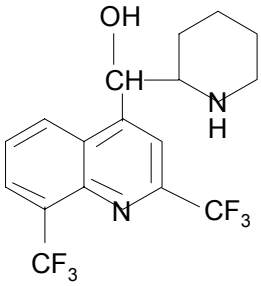
Class	Drug	Biological activity	
		Blood Schizontocide	**Tissue Schizontocide**
4-Aminoquinolines	Chloroquine	++	0
Arylaminoalcohols	Quinine	++	0
	Quinidine	++	0
	Mefloquine	++	0
Phenanthrene methanol	Halofantrine	++	0
Artemisinine and derivatives	Artemisinine	++	0
	Artemether	++	0
	Artesunate	++	0
Antimetabolites	Proguanil	+	+
	Pyrimethamine	+	0
	Sulfadoxine	+	0
	Sulfalene	+	0
	Dapsone	+	0
Antibiotics	Tetracyclin	+	+
	Doxycyclin	+	+
	Minocyclin	+	+
8-Aminoquinoline	Primaquine	0	+

++: effective; +: moderately effective; 0: not effective

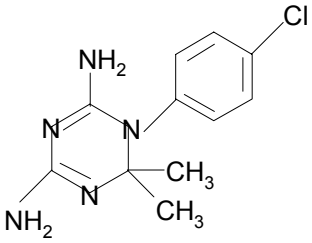
From the above list, existing or emerging parasite resistance to the following drugs will be discussed in more detail in this section: chloroquine, mefloquine, proguanil (active form: cycloguanil), pyrimethamine, sulfadoxine and dapsone. The structures of these compounds are shown in Fig. 1.5.



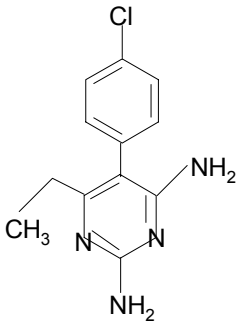
Chloroquine



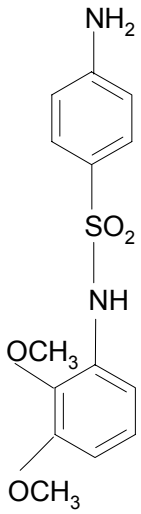
Mefloquine



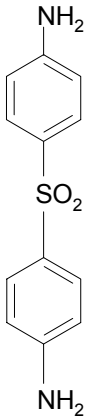
Cycloguanil



Pyrimethamine



Sulfadoxine



Dapsone

Figure 1.5: Structures of some of the most commonly used anti-malaria drugs (http://www.icp.ucl.ac.be/~opperd/parasites/chemo1.htm).

Parasite resistance against anti-malaria drugs is not a new phenomenon (Phillipson and Wright, 1991; Povoa *et. al*., 1998; Hopkins-Sibley *et. al*., 2001). The widespread resistance of *P. falciparum* to the first line and most cost-effective drug, chloroquine, has prompted the introduction of different prophylactic as well as therapeutic drugs for people entering areas where drug resistance is prevalent. For prophylaxis, mefloquine is often recommended and for treatment, either quinine with doxycycline or Fansidar (a combination of sulfadoxine and pyrimethamine). Resistance to SP has however already been reported in East Africa, where this has been the first-line drug for less than 5 years (Hopkins-Sibley *et. al*., 2001; Roper *et. al*., 2003). This is an alarming phenomenon. The target of pyrimethamine, dihydrofolate reductase (DHFR), is present as a bifunctional enzyme with thymidylate synthase (DHFR-TS) in *P. falciparum* but occurs as two separate enzymes in mammals (Hopkins-Sibley *et al*., 2001). The difference between the mammalian and parasite enzymes makes this an ideal anti-malaria target, which is further supported by the function of DHFR as a key enzyme in the production of tetrahydrofolate. The latter, in turn is a cofactor required in nucleotide and amino acid biosynthesis, specifically that of (d)TMP and methionine (Hopkins-Sibley *et. al*., 2001). Pyrimethamine is always administered together with sulfadoxine, an inhibitor of dihydropteroate synthase (DHPS) with which it acts synergistically to inhibit tetrahydrofolate synthesis. The reported resistance to SP is ascribed to point mutations in the *P. falciparum dhfr* and *dhps* genes (Hopkins-Sibley *et. al*, 2001). These point mutations have been confirmed by wide-ranging epidemiological investigations to be responsible for all cases of SP resistance observed in the field. These mutations are shown in Table 1.2.

Table 1.2: Principal polymorphisms in the *Plasmodium falciparum dhfr* and *dhps* genes. Shown are the amino acid changes resulting from the polymorphisms[a] (Hopkins Sibley *et. al.*, 2001).

DHFR codon	**16**		**51**		**59**		**108**		**164**	
	Codon	**AA**	**Codon**	**AA**	**Codon**	**AA**	**Codon**	**AA**	**Codon**	**AA**
Wild Type	GCA	A	AAT AAC	N N	TGT	C	AGC	S	ATA	I
Variants	G<u>T</u>A	V	A<u>T</u>T	I	<u>C</u>GT	R	A<u>A</u>C A<u>C</u>C	N T	<u>T</u>TA	L
DHPS codon	**436**		**437**		**540**		**581**		**613**	
	Codon	**AA**	**Codon**	**AA**	**Codon**	**AA**	**Codon**	**AA**	**Codon**	**AA**
Wild Type	TCT	S	GCT	A	AAA	K	GCG	A	GCC	A
Variants	<u>G</u>CT T<u>T</u>T	A F	G<u>G</u>T	G	<u>G</u>AA	E	G<u>G</u>G	G	<u>T</u>CC	S

a Nucleotides that are changed are underlined. Abbreviations: AA, amino acid; DHFR, dihydrofolate reductase; DHPS, dihydropteroate synthase. Other abbreviations are standard one-letter nucleotide and amino acid codes.

The A16V and S108T phenotype confers resistance to the activated form of the pro drug proguanil, namely cycloguanil. This phenotype does not however confer resistance to pyrimethamine. The other genotypes listed in the table as variants of *dhfr* confer resistance to pyrimethamine while the variants of *dhps* listed confer resistance to sulfadoxine. It is interesting to note that the S108N phenotype of DHFR increases the resistance to pyrimethamine by about 100 fold while the triple and quadruple mutants are about an order of magnitude more resistant to pyrimethamine. Selection for mutations in *dhfr* seem to occur first with selection for *dhps* mutations occurring only when parasites in the population carry at least a double or even a triple mutant allele of *dhfr (*Hopkins-Sibley *et. al.,* 2001).

The SP combination is considered to be a vulnerable anti-malarial, i.e. an anti-malarial susceptible to the onset of parasite resistance. This is due in part to the fact that only a few single point mutations in the targeted enzymes are needed by the parasite to evolve resistance to this drug combination, which is further aggravated by the long biological half-lives of sulfadoxine (116 hours) and pyrimethamine (81 hours) (Hopkins-Sibley *et. al*., 2001).

Some more inexpensive antifolate drugs such as chlorproguanil and dapsone are being investigated as alternatives to SP therapy. Chlorproguanil, which is metabolized to its active form, chlorcycloguanil, inhibits DHFR while dapsone acts on DHPS. The advantage that these two drugs have over SP is their higher affinity for the respective targets. The disadvantage of these drugs on the other hand is that sulfadoxine and pyrimethamine select mutations in the *dhfr* and *dhps* genes that in turn decrease the affinity of chlorproguanil and dapsone for the respective enzyme targets. The implication is therefore that as resistance to SP increases with prolonged use of the drug the likelihood exists that resistance to chlorproguanil – dapsone will emerge (Hopkins-Sibley *et. al*., 2001).

Artemisinin is a blood schizonticide and has been used successfully in Thailand to treat chloroquine and quinine resistant *P. falciparum* infections. Artemisinin and it's derivatives are the most potent of the anti-malaria agents, an attribute that may be ascribed to their 10 000 fold reduction of the parasite biomass and the 90% reduction in malaria transmission. This is brought about by a reduction in gametocyte carriage from

the infected individual to the mosquito vector (White *et. al*., 1999). Trials involving 4500 patients in sub-Saharan Africa are being conducted to evaluate the efficacy and safety of artesunate combined with SP. A study in the Gambia, investigated SP monotherapy and 3 days of artesunate treatment with a single dose of SP. A gametocyte carriage of 68% was found with SP mono-therapy versus 21% with the combination therapy (www.malaria.org). The key to successful artemesinin derivative treatment is that the onset of resistance is delayed due to the rapid decrease of asexual parasite load as well as decreased gametocyte carriage (Price *et. al*., 1996; White, 1997).

It is critical that the useful therapeutic life (UTL) of SP be prolonged with the use of combination anti-malarial therapy. If it becomes necessary to deploy the next line of drugs (mefloquine or halofantrine) it could spell disaster for sub-Saharan Africa as most countries in the region will not be able to afford this therapy, since it is more expensive than SP treatment. As is evidenced from the above the development of parasite resistance against new drugs is occurring at an alarming rate and is a cause for great concern.

1.4 Malaria vaccine development

Several vaccines have been investigated with varying degrees of success. Six of these will be discussed briefly. The proteins targeted by the vaccines include circumsporozoite protein (CSP), sporozoite surface antigen-2 (SSP-2), liver stage antigen-1 (LSA-1), merozoite surface protein (MSP-1), serine rich antigen (SERA), apical merozoite antigen (AMA), Pfs25 and schizont export antigen 5.1.

The vaccine that received the most attention worldwide was SPf66 developed by the Columbian scientist Manuel Patarroyo (Patarroyo *et. al,* 1992, Trager, 1995). This vaccine was aimed at the asexual stages of the parasite and consisted of three merozoite-derived proteins joined by the repeating domain of *P. falciparum* circumsporozoite protein (CSP). The claimed protection was controversial due to the design of the clinical trial protocols. In particular, the ability of the vaccine to reduce the incidence of the initial *P. falciparum* infection differed to a large degree between trials (Graves and Gelband, 2000). Subcatagorizing the trials by location revealed that SPf66 was ineffective in reducing the incidence of *P. falciparum* in children under five years of age in four African trials. These results led to the conclusion that there was no evidence supporting the vaccine potential of SPf66 against *P. falciparum* in Africa (Graves and Gelband, 2000).

Another potential vaccine was based on the parasite circumsporozoite protein (CSP) but also met with no success (Thomas *et. al*., 2001). A vaccine called NYVAC-Pf7 was also tested and consisted of a single pox-virus genome containing genes encoding 7 *P. falciparum* antigens (Ockenhouse *et. al*., 1998; Stanley, 1998). Two antigens were derived from the sporozoite stage, namely CSP and sporozoite surface protein 2 (SSP-2), one from the liver stage antigen (LSA-1), three blood stage antigens (merozoite surface protein (MSP-1), serine rich antigen (SERA) and apical merozoite antigen (AMA-1)) and one sexual stage antigen, Pfs25. The vaccine elicited specific immune responses to 4 out of the 7 antigens. These were CSP, SSP-2, MSP-1 and Pfs25. A cellular immune response but only poor antibody responses were observed. Of the thirty five volunteers

challenged with the bite of five *Anopheles* mosquitoes infected with *P. falciparum*, only one exhibited complete protection while the other volunteers showed a delay in the onset of symptoms compared to control groups (Ockenhaus *et. al*. 1998). Clinical trials are ongoing.

Another vaccine named [NANP]19 – 5.1 contained 19 repeats of the sporozoite surface protein (SSP) and the schizont export antigen 5.1 (Reber-Liske *et. al*. 1995). This vaccine was tested on 194 school children between the ages of 6 and 12 years, who already possessed a degree of immunity to malaria. During the twelve-week trial none of the children showed clinical signs of malaria. Serum was collected from the volunteers before and after immunization and revealed that in all but eight children the levels of antibodies to both antigens were increased (Reber-Liske *et. al*., 1995). Further work, using volunteers not previously exposed to malaria, was under investigation, with no further information available to date.

Still another vaccine named Pfs25 was targeted to the 25 kDa sexual-stage *P. falciparum* antigen (Lobo *et. al*., 1999; Stowers and Miller, 2001). Antibodies were elicited that blocked the infectivity of gametocytes to mosquitoes. This strategy is being investigated further.

The vaccine RTS,S/AS02A was developed by the Walter Reed Army Institute of Research and was designed to protect troops sent into malaria endemic areas (Bojang *et. al*., 2001; Kester *et. al*., 2001). This is a recombinant vaccine consisting of CSP fused

with a hepatitis B surface antigen. This fusion was undertaken due to the fact that CSP alone is poorly immunogenic. The adjuvants used in this vaccine have also proved crucial to its success. RTS,S/AS02A protects a vaccinated individual for a period of up to 6 months. This vaccine has been found to be safe, effective and was well tolerated in clinical trials (Alonso *et. al*., 2004). To date Pfs25 and RTS,S/AS02A are the most successful vaccine attempts to offer significant protection against malaria.

All of the above vaccines have been either protein- or peptide-based but it is important to note that as a result of the malaria genome-sequencing project vaccine development also focuses on DNA vaccines (Jones *et. al*. 2002). Such a vaccine, offering 100% protection against malaria infection in mice, has been developed (Bruna-Romero *et. al*., 2001). In this approach mice were inoculated with a replication deficient adenovirus expressing the circumsporozoite protein (CSP) of *P. yoelii* (AdPyCS). A booster of vaccinia virus also expressing CSP (VacPyCS) 8 weeks later yielded complete and long lasting protection against malaria infection. This has become known as the prime-boost strategy. Investigations are currently underway to develop and test a vaccine based on a similar prime-boost strategy in humans (Carey, 2001).

1.5. Additional malaria-combative strategies under investigation

Various additional strategies to the above are currently being investigated in an effort to eradicate or at least contain the malaria problem. The most important of these are discussed below:

1.5.1. Alternative Medicine (Traditional Medicine)

Several studies are underway to test plant extracts for anti-malaria activity. For example, in one such study, 14 Ghanian plant extracts were tested of which three were found to have significant anti-plasmodial activity (Addae-Kyereme *et. al*., 2001). Some 600 plants from 126 countries have been tested for antimalarial activity since 1947 but progress in this area was hindered by the lack of suitable screening methods (Phillipson and Wright, 1991). In South Africa a DACST (Department of Arts Culture Science and Technology) Innovation Fund Consortium was established recently to investigate several hundred local plants for anti-malaria activity. Project leader Professor Peter Folb of the Medical Research Council's (MRC) Traditional Medicines Research Unit and Professor of Pharmacology at the University of Cape Town (UCT) says that in this study the validity of traditional medicines will be scientifically determined (**http://www.sahealthinfo.org/noveldrug/index.htm**). Work currently underway aims to find active compounds from plants traditionally used for the treatment of fever-causing diseases such as malaria and to then discover the lead molecule for further development into an anti-malarial. Successful anti-malarials are expected to fall into one of three categories namely: i) Prophylactic agents, ii) Cures for malaria, and iii) Insecticides against *Anopheles* mosquitoes.

The use of plant extracts for the treatment or prevention of malaria has enormous potential since the plants investigated mainly stem from malaria endemic areas. Hence, plant-based medications would be more readily available and affordable to the communities most afflicted by malaria outbreaks.

1.5.2. Genetically modified mosquitoes

The above technology together with efforts to create genetically modified mosquitoes, which are unable to spread malaria parasites, offers an alternative approach to fight this killer disease (Catteruccia *et. al*., 2000; Coates, 2000). The life cycle of the parasite in the mosquito is completed by the production of sporozoites, which migrate from the midgut to the salivary glands (see Fig 1.1). Ito *et al*. (2002) used a rodent malaria model and transformed *Anopheles stephensi*, with a DNA cassette containing a gene coding for a peptide resembling a receptor in the mosquito's gut and salivary glands. This receptor is believed to facilitate the passage of the parasites between the gut and salivary glands. Thus the expressed peptide competitively inhibits parasite binding to the receptor and hence also progression of the latter within the mosquito. The results showed that oocyst formation was inhibited by ca. 80% in transgenic compared to wildtype mosquitoes containing control plasmids. There was thus a marked reduction in the development as well as the transmission of the parasite from the mosquito to the host. Since this experiment was performed using the rodent model, further investigations will follow to study the effect of this strategy in the human model. In other investigations use is also being made of plasmids containing transposable elements. Following injection of mosquito eggs with the plasmid the transposable elements move from the plasmid and are inserted into the mosquito chromosomes. In so doing it is hoped that genes, preventing the development and transmission of parasites, will be incorporated into the genome of the main vector of malaria in sub-Saharan Africa, *A. gambiae.* Once inserted into the germline cells the offspring will be genetically transformed and these genes will be

passed on from one generation to the next. Many more hurdles however need to be overcome before this strategy can be realized (Coates, 2000; Catteruccia *et al*., 2000).

Furthermore, it has been found that the innate immune system of the mosquito offers a set of targets that can be utilized in the blocking of parasite development (Osta *et. al*., 2004). Briefly, four proteins have been identified that regulate parasite development, two of them positively and two negatively. Two C-type lectins called CTL4 and CTLMA2 protect the parasite development within the mosquito while a thioester-containing protein (TEP1) and a leucine-rich immune protein (LRIM) destroy the parasite. Subsequent regulation of the production of these four proteins could inhibit the parasite life cycle within the vector and prevent its transmission to the human host.

1.5.3 Inhibitor design

Another approach in drug development is structure-based inhibitor design (Yuthavong, 2002). This approach requires the identification of feasible target proteins and knowledge of its three dimensional structure. Crystallization of the protein and determination of the structure by X-ray crystallography or solving the structure by NMR-based methods can achieve this. The successful crystallization of the *P. falciparum* enzyme DHFR-TS offered significant progress in better understanding the selective inhibition of present inhibitors of this enzyme as well as mutational changes leading to drug resistance (Rathold and Phillips, 2003; Yuvaniyama *et. al*., 2003). This research has made the visualization of novel targets and the rational design of inhibitors possible.

Alternatively, if the amino acid sequence of the target is sufficiently similar to a protein of known three-dimensional structure the latter can be used to predict a possible structure for the target protein using comparative homology modelling methods (Birkholtz *et. al*., 2003; Joubert *et. al*., 2001). The next stage of the development process involves finding suitable ligands for the target protein. In many cases ligands (or enzyme inhibitors in this case) are computationally designed. Hereafter the potential inhibitor, which is not a drug at this point, is required to undergo extensive testing and refinement. The typical problem areas that need to be addressed are chemical stability, solubility, toxicity and delivery to name a few. It therefore becomes evident that time and cost are notable factors to be borne in mind when embarking on this approach. This technology has potential since the *P. falciparum* genome sequence now offers a wealth of information that can be exploited for identification of parasite-specific features, determination and validation of a data base of therapeutic targets and vaccine and drug design (Gardner *et. al*., 2002). This then further allows the opportunity to elucidate the complex metabolic pathways of the parasite, amongst others, and to not only design a store of novel anti-malaria drugs but also gain a deeper understanding of the biochemical intricacies of the parasite. These can then be utilized in other approaches to combat the malaria problem (De Koning-Ward *et. al*., 2000).

1.5.4 Small interfering RNAs (siRNAs)

This approach is based on the addition of double stranded RNA (dsRNA) to the relevant cells or organism. The dsRNA is then cleaved into smaller RNA fragments of between 21 and 25 nucleotides in length by an RNase III-like enzyme called Dicer. These smaller

RNA fragments, known as small interfering RNAs (siRNAs), are assembled into complexes, with endoribonucleases, known as RNA induced silencing complexes (RISC). The ATP-dependant unwinding of the siRNA components of RISC serve to activate the latter, which are then able to hybridize to complementary mRNA molecules. The mRNA molecule is then degraded by the endoribonuclease components of RISC (Thompson, 2002).

Whether RNA interference (RNAi) is functioning in *Plasmodium* species is still a debateable issue (Ulla *et. al*., 2004). dsRNA against *P. falciparum* dihydro-orotate dehydrogenase (DHODH), essential to pyrimidine biosynthesis, inhibited parasite growth but no specific dsRNA effect could be shown (McRobert and McConkey, 2002). Next the down-regulation of *P. falciparum* cysteine proteases, falcipain-1 and falcipain-2, by homologous dsRNA was investigated. However no evidence for siRNA could be found (Malhotra *et. al*., 2002). To date no RNAi gene candidates have been identified in *Plasmodium* species (Ulla *et. al*., 2004).

Other novel approaches also include antisense technology. Since the antisense technique is the focus of this thesis it is discussed in more detail below.

1.6 Background and overview of antisense technology

In 1969 Bovre and Szybylski first observed the transcription of both complementary strands in the same region of phage lambda DNA (Uhlmann and Peyman, 1990). At that

time the possible regulatory role of such so-called antisense RNA could only be speculated upon (Barker *et. al*., 1998). It was already shown in 1983 that antisense RNA targeting the ribosome-binding site and initiation codon of a particular gene, could have an inhibitory effect (Crooke, 2000). The assumption was made that if antisense molecules occurred naturally in prokaryotes then the same should be true for eukaryotes. Several such antisense molecules have been identified since then (Kimelman, 1992). The first successful result using synthetic antisense oligodeoxynucelotides (ODNs) reported the inhibition of replication of the Rous Sarcoma Virus (RSV) by a synthetic 13-mer antisense ODN (Zamecnik and Stephenson, 1978).

Antisense ODNs have been shown to be clinically effective against diseases such as cancer, Crohn's disease and hepatitis C (Braasch and Corey, 2002). All of these antisense ODNs are undergoing clinical trials, while the antisense drug fomivirsen (ISIS Pharmaceuticals), designed to combat cytomegalovirus (CMV) retinitis, already has Federal Drug Administration (FDA) approval. Fomivirsen is a 21-mer phosphorothioate oligonucleotide complementary to human CMV. The drug is administered by intravenous injection to AIDS patients suffering from CMV-induced retinitis and is effective at nanomolar concentrations ().

Antisense differs from knock-out technology in so far that it blocks the expression of a gene against a physiologically normal background (Wanidworanum *et. al*., 1999). The effect of the antisense ODN can be rapidly detected in the experimental system. If the organism or system under investigation is merely affected by the antisense ODN but still

viable, removal of the antisense ODN may result in a return to normal functioning. A knock-out strategy does not offer this possibility. The implementation of antisense technology requires knowledge of the inferred nucleotide sequence of the target protein together with other information such as its function and expression patterns (Barker *et. al*., 1998). Antisense technology is based on the hybridization of an oligodeoxynucleotide (ODN) or RNA molecule to a complementary mRNA sequence, by means of Watson-Crick base pairing. This is illustrated in Figure 1.6.

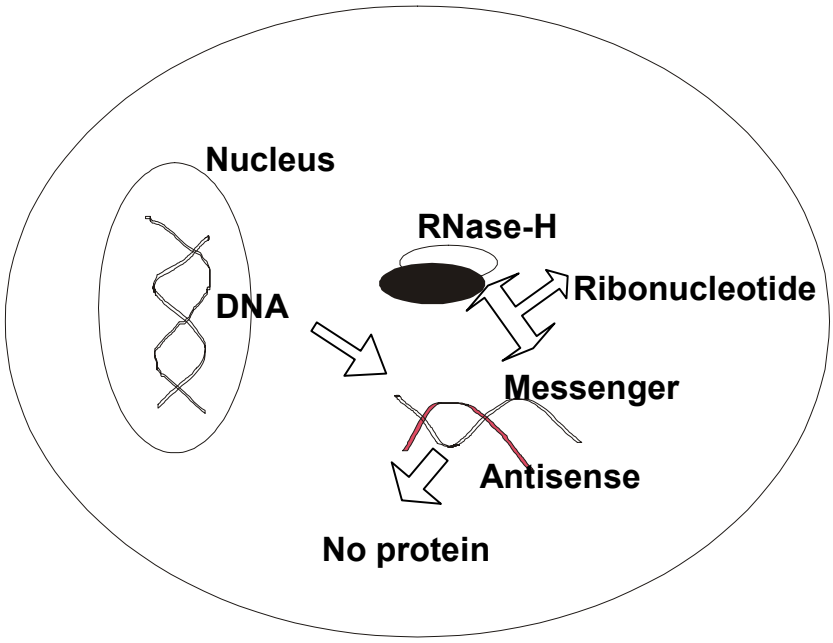


Figure 1.6: Illustration of an antisense ODN binding to a complementary segment of mRNA, inhibiting translation of the target protein.

There are two main mechanisms for antisense ODN action. The first entails the physical blocking of the complementary mRNA sequence, thereby preventing the ribosome complexes from translating the target protein (Agrawal, 1996; Crooke, 2000). The second entails the activation of RNase-H (see Figure 1.6) by the ODN/RNA duplex followed by

the degradation of the RNA component of the duplex (Crooke, 2000). The added advantage of this approach is that the released ODN is left intact and free to hybridize to other mRNA molecules (Phillips and Zhang, 2000).

However, several parameters require optimization in order to successfully implement antisense techniques.

1.6.1. Antisense target protein

The inferred nucleotide sequence of the target protein must be known in order to design a complementary antisense ODN. Knowledge of higher order protein structure is however not necessary, a distinct advantage of antisense technology over many other drug design approaches (Agrawal, 1996; Askari and McDonnell, 1996; Lonnberg and Vuorio, 1996; Stein and Cheng, 1993; Wanidworanum *et. al*., 1999). It is further important that the target protein be vital to the continued proliferation of the organism in question, if drug design is the aim of the study.

The choice of RNA target site is an important aspect in the design of any antisense experiment (Crooke, 2000; Phillips and Zhang, 2000). For a successful antisense mediated effect the mRNA target site must be accessible to ODN hybridization. This pre-requisite is complicated by the fact that RNA molecules have regions of complex 3-dimensional structure. There are a variety of secondary structures present in RNA molecules, all of which have been observed to have specific functions involved mainly in translation regulation (Riesner, 1997). These secondary structures can then also associate with each other to form tertiary structures such as pseudoknots and triple strand

complexes. Various computer-aided as well as laboratory methods have been developed to predict possible secondary structures but the prediction of the tertiary structures is still problematic (Riesner, 1997). The characteristics of the ideal ODN hybridisation site and methods used for their identification are detailed in Chapter 2.

1.6.2. Phosphodiester oligodeoxynucleotide stability

Phosphodiester ODNs are susceptible to nuclease degradation resulting in shortened sequences that in turn are less sequence specific. Strategies have been developed to inhibit their enzymatic degradation. A nucleotide dimer can be modified in up to 26 different positions, without negatively influencing ODN hybridization to target mRNA (Cook, 1998). Modifications may be of a chemical or structural nature. Some of the most common chemical modifications are described below.

1.6.2.1. Backbone modifications:

The most commonly used analogues in this category are the phosphorothioate (PS) and methylphosphonate (MP) modifications where a non-bridging oxygen atom is replaced by sulphur (PS ODNs) or a methyl group (MP ODNs) (Figure 1.7). Both PS and MP analogues have been successful at protecting the ODNs against nuclease degradation. Phosphorothioate ODNs have a half-life of between 12 and 24 hours in tissue culture medium compared to the half-life of unmodified phosphodiester ODNs, which is in the range of minutes (Crooke, 2000). The ability of these modifications to allow degradation of DNA:RNA heteroduplexes by RNase-H differs. PS ODNs allow degradation of the heteroduplexes by RNase-H whereas MP ODNs do not (Crooke, 2000). Degradation of

the mRNA component of the DNA: RNA heteroduplex is a valuable additional mechanism of antisense ODN action (Crooke, 2000).

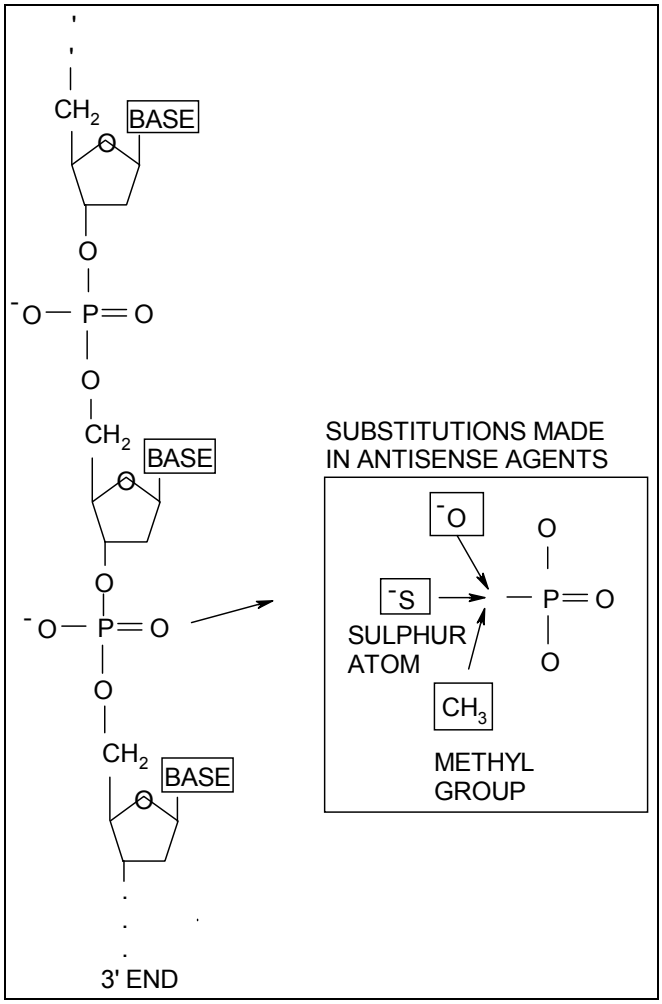


Figure 1.7: Illustration of the most popular first generation modifications of the ODN phosphodiester bond which protect against nuclease degradation (Adapted from Uhlmann and Peyman, 1990).

PS ODNs have, however, been reported to be responsible for unwanted non-sequence specific effects (Clark *et al*, 1994; Crooke, 2000; Dagle and Weeks, 2000; Uhlmann *et al*, 2000). These have been largely attributed to the ODN binding to proteins such as serum

albumin (Crooke, 2000; Geary *et. al*., 2001). The PS ODNs also interact with nucleic acid binding proteins including transcription factors, nucleases and polymerases.

To circumvent these problems use is being made of chimeric ODNs (Mixed backbone ODN - MBO), containing a limited number of PS or MP internucleotide linkages at the termini, or at the termini as well as a few internal ones (Furdon *et. al.,* 1989; Giles *et. al*., 2000). These modifications have met with a degree of success. For example one study investigated the *in vitro* nuclease sensitivity in tissue culture and RNase-H sensitivity of the mRNA/ODN heteroduplexes of ODNs with different arrangements of methylphosphonate linkages (Quartin *et. al*., 1989). Results indicated firstly that a span of 3 or fewer contiguous internal, unmodified phosphodiester linkages yielded the greatest resistance to nuclease degradation (regardless of ODN length) and secondly, that a span of 3 internal unmodified phosphodiester linkages were necessary to enable RNase-H cleavage of the mRNA component of the mRNA/ODN heteroduplex. However, such a large number of methylphosphonate internucleotide linkages have been shown to reduce the hybridization affinity of the MBO to the target mRNA (Giles and Tidd, 1992).

Studies have also been performed using MBOs containing phosphorothioate linkages at the 3' ends as well as at internal pyrimidine nucleotides since the latter are particularly sensitive to endonuclease degradation (Uhlmann *et. al*., 2000). The application of this modification to several systems has shown that the introduction of between 40 and 60% phosphorothioate linkages confers protection of the ODN to nuclease degradation but that the hybridization affinity to RNA decreased by 0.3 – 0.5 °C per phosphorothioate-

modification. The consequence of this is that the melting temperature of a given ODN, with a given sequence, decreases as the number of PS linkages increases. The cellular uptake into cells was also decreased with increasing percentage of phosphorothioate linkages. These MBOs were however, found to diminish ODN binding to proteins (Uhlamnn *et. al*., 2000).

There are other backbone modifications worth mentioning where the entire sugar-phosphate backbone has been replaced. One of these are the morpholino analogues, shown in Figure 1.8.

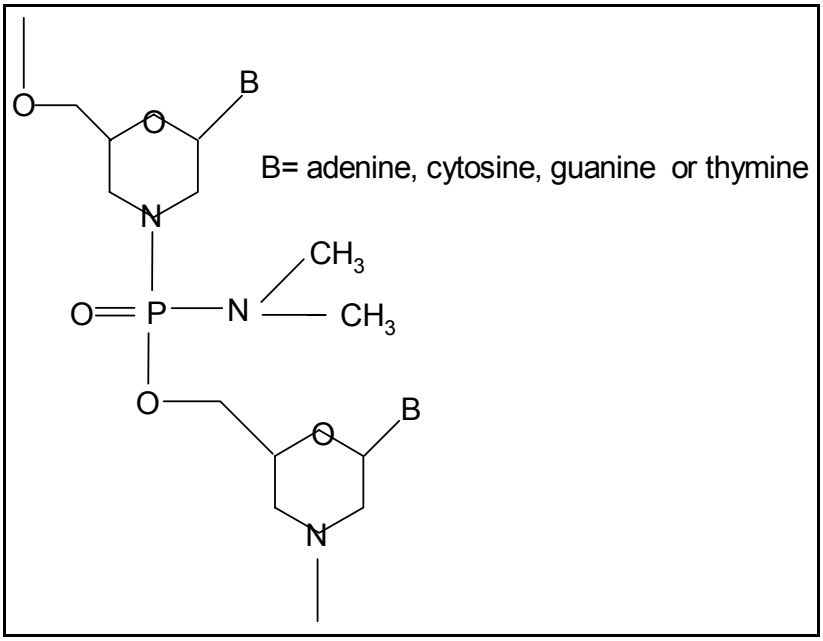


Figure 1.8: Illustration of a morpholino ODN where the entire sugar phosphate backbone is replaced by morpholino diamidates (Summerton and Weller, 1997).

Morpholino ODNs have been shown to be stable toward a wide variety of nucleases but do not support RNase-H cleavage of mRNA. It has, however, been shown that morpholino ODNs inhibit target RNA more efficiently than the corresponding PS ODNs, but both PS and morpholino analogues show satisfactory efficacy at 10 nM and above (Summerton and Weller, 1997).

Another analogue is where the sugar phosphate backbone is replaced by the peptide nucleic acid (PNA) with repeating units of N-(2-aminoethyl)glycine (Cook, 1998; Nielsen *et. al*., 1991). This modification is shown in Figure 1.9.

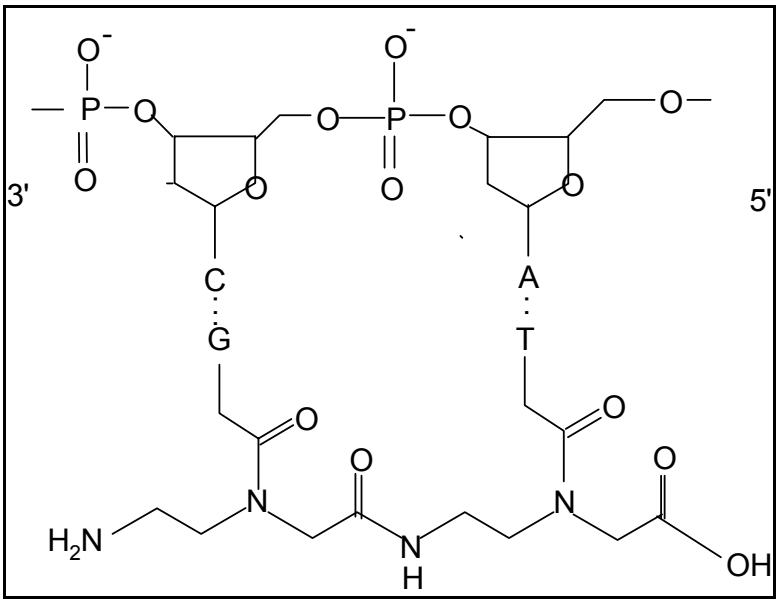


Figure 1.9: Illustration of the peptide nucleic acid (PNA) analogue (lower strand) hybridized to target RNA (upper strand).

PNAs have been shown to act as both transcription (anti-gene) as well as translation (antisense) inhibitors of gene expression and to be stable against both nuclease and peptidase attack (Cook, 1998, Crooke, 2000; Hyrup and Nielsen, 1996).

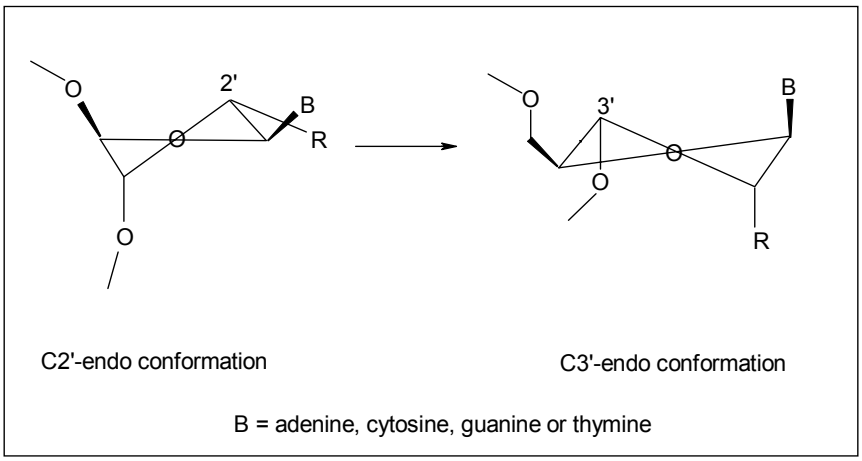
1.6.2.2. Nitrogenous base modifications

Nitrogenous base modifications serve the primary purpose of stabilizing and/ or enhancing ODN hybridization to its target sequence. Several modifications to the C-4 and C-5 positions of pyrimidines have been employed with success. These include 4-thio-pyrimidines and 5-propynyl-deoxycytidine (Cook, 1998; Crooke, 2000; Foehler *et. al*., 1992; Nikiforov and Connolly, 1991; Wagner, 1994). With the exception of 2,6 diamino purines and 3-deaza-adenosine, most purine analogues have however not met with the same degree of success as the pyrimidine analogues (Crooke, 2000).

1.6.2.3. Sugar modifications

Modifications to the sugar subunit have been introduced to enhance hybridization between ODN and RNA as well as to protect against degradation. These modifications convert the ribofuranosyl groups to a 3'-endo as opposed to a 2'-endo conformation since the former is the ideal ribose conformation for hybridization to RNA (Figure 1.10i) (Altman *et. al*., 1996). Further modifications included replacing the 4'-oxygen atom with methylene or sulphur but this proved not to be successful in protecting against nuclease degradation or supporting RNase-H cleavage (Figure 1.10ii). The 2'-position modifications proved to be the most useful. In this group the 2'-O- alkyls, particularly the 2'-O-(methoxyethyl) (MOE), have provided the best results (Figure 1.10iii) (Altman *et. al*., 1996; Cook, 1998). The stability of 20-mer oligonucleotides targeted against human intra-cellular adhesion molecule-1 (ICAM-1) mRNA was monitored in rats following intraduodenal administration. The results showed that 50% more of the 2'-O-MOE modified oligonucleotides were intact compared with the PS ODN of the same length and

sequence, 8 hours post administration (Braasch and Corey, 2002). This modification also does not support RNase-H cleavage unless a central window containing at least six phosphodiester linkages is available (Braasch and Corey, 2002). The 2'-O-MOE modified oligonucelotides are currently undergoing human trials.



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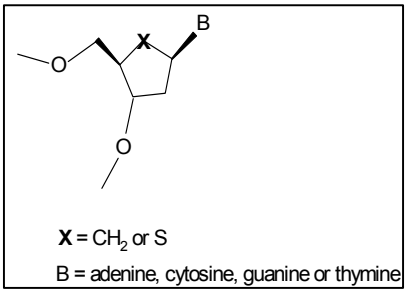

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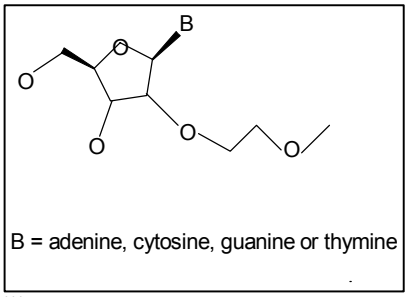

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Figure 1.10: Illustration of sugar modifications used in attempts to enhance hybridization between antisense ODN and RNA and to protect the ODN against nuclease degradation. i: Conversion of the ribofuranosyl groups from a 2' endo to a 3' endo conformation, ii: Replacement of the 4' oxygen with methylene or sulphur and iii: 2'-O-(methoxyethyl) (MOE) modification (Braasch and Corey, 2002; Cook, 1998).

1.6.2.4. Self stabilizing loops

Certain other ODN structural modifications have also been investigated for their efficacy to protect the ODN against nuclease degradation. These are alterations of the sequence so as to favour 3' terminal self-stabilizing loops (Barker *et al*., 1998). This type of modification has been tested using phosphorothioate modified ODNs and found to improve the inhibitory efficacy marginally (by 20-25%) (Barker *et al*, 1998).

1.7 Cellular ODN uptake

The efficient and adequate uptake of antisense ODNs is crucial to ODN efficacy. Therefore the proper delivery of antisense ODNs to the intra-cellular environment of the target cell must be taken into consideration when planning antisense experiments. Due to the polyanionic nature of the ODNs, passage through the cell membrane does not occur readily (Cooke *et. al*., 1998; Crooke, 2000; Wu-Pong *et. al*., 1994). The uptake mechanisms of ODNs into cells are still uncertain although many reports have indicated the possible involvement of receptor-mediated endocytosis (Cooke *et. al*., 1998; Loke *et. al*., 1989; Nakai *et. al*., 1996; Stein and Cheng, 1993).

A variety of physical methods have been used to increase cell permeation. These include the use of streptolysin-O (SLO) to permeabilize cells, microinjection and electroporation to name a few (Giles *et. al*., 2000; Lledo *et. al*., 2000). SLO has been used to introduce ODNs into cultured cells as well as cells resulting from bone marrow and peripheral blood stem cell harvests from patients diagnosed with chronic myeloid leukemia (Giles *et*

al., 2000). In the region of 5 x10^6 cells can be permeabilized per treatment and the permeability can then be reversed after ODN addition using a standard culture medium such as RPMI 1640, also used for the *in vitro* culturing of malaria parasites. In excess of 90% of the permeabilized cells could be resealed and it was shown that translation of the target protein was inhibited by ca. 60% relative to control levels. The amount of ODN that can be taken up by this method was not stated. Electroporation has been used for the identical application as described for SLO membrane permeabilization and no difference in the results could be detected. Microinjection has been also used for the loading of antisense ODNs into single cells maintained in cell culture (Lledo *et al*., 2000). Here antisense and control ODNs (10 µg/ml) were injected and 70% of all the cells were shown to have survived the procedure after a period of nine days. This was significant since it was then possible to induce the specific protein, the translation of which had been inhibited by the antisense ODNs, and observe the restoration of the original cell phenotype (Lledo, *et al*., 2000).

The application of several lipid-based systems to improve intra-cellular ODN delivery has also been investigated. Lipid conjugates can also increase lipophilicity, nuclease resistance and binding affinity (Cooke *et. al*., 1998). Three main approaches have been used to deliver antisense ODNs with the aid of lipids. Several *in vivo* studies have been performed using cholesterol conjugated to PS-ODNs (Cooke *et. al*., 1998, Desjardins *et. al*., 1995). These studies have shown the analogues to exhibit altered pharmacokinetic properties compared to the parent compound and to have a 10-fold increased antisense inhibitory efficacy. These altered properties have been largely attributed to the improved

ODN uptake mediated by lipoprotein receptors (Cooke *et. al*., 1998). The second approach involves complex formation between antisense ODNs and cationic lipids often referred to as lipoplexes (Semple *et. al*., 2000). Polyanionic ODNs are mixed with preformed cationic liposomes, at various charge ratios to generate complexes for delivery of ODNs to cultured cells (Felgner *et. al*., 1994; Semple *et. al*., 2000). These ODN-cationic lipid complexes are formed due to electrostatic interaction between the negative charges, contributed by the phosphate groups of the ODN internucleotide phosphodiester bonds and the positive charges (tertiary amine groups) present on the outside surface of the preformed cationic liposomes. The third approach involves encapsulating antisense ODNs in liposomes. Here the ODNs are enclosed in the inner aqueous space of the liposome. For this approach to be successful, several factors need to be considered namely, encapsulation efficiency and drug/lipid ratio, which is usually kept high so as to minimize the amount of lipid administered with the drug. This is due to the fact that these lipid formulations are often cytotoxic (Semple *et. al*., 2000). Lastly, vesicle size is important for the pharmacokinetic impact (typically ODN tissue and organ distribution, elimination, and half-life determinations) of the delivery system. Typically, particles larger than 200 nm are eliminated from the circulation more rapidly than particles having diameters between 80 - 100 nm (Senior, 1987).

From the above it is clear that the stability of the particular ODN, accessibility of the target site and cellular uptake of the ODN all have a significant bearing on the ultimate *in vitro* as well as *in vivo* antisense efficacy of ODNs.

One application of antisense technology is to determine the function of malaria parasite proteins especially now that the sequence of the *P. falciparum* genome has been completed (Gardner *et. al*., 2002). About 60% of the identified open reading frames have no described function. Reversible translation inhibition of specific parasite proteins may reveal their possible roles in metabolic pathways, cell processes and parasite survival. ODNs may even aid the preliminary validation of specific proteins as therapeutic targets by revealing the effects of their inhibition on parasite survival. A further advantage of antisense technology is that it targets nucleotide sequences rather than protein structural motifs that may be similar and shared between related or even unrelated proteins. This is particularly relevant in the application of antisense technology to malaria since the A + T richness of the malaria genome (80%) relative to that of the human host (60% G + C rich) allows for sequence specific targeting on gene and mRNA levels by antisense ODNs (Gardner *et al*., 1999).

1.8 Aims

The first objective of this thesis was to design an antisense ODN, aimed at a relevant target protein of *P. falciparum,* and to test the protective effect of a structural modification against nuclease degradation of the ODN. The second objective was to determine the *in vitro* efficacy of the designed antisense ODN. This included the determination of inhibitory effects on parasite cultures, uptake into infected and uninfected erythrocytes as well as the parasite phase on ODN uptake and inhibitory efficacy.

In Chapter 2 the design of the antisense ODNs using two different strategies is described. Chapter 3 provides experimental results of studies to determine the stability of structurally modified ODNs in parasite culture medium. Chapter 4 deals with the *in vitro* efficacy as well as cellular uptake of the ODNs and the effect of parasite phase on ODN uptake and efficacy. The thesis is finally concluded by Chapter 5, which provides an evaluation of the major results obtained, unresolved issues and future perspectives.