The design and efficacy of an antisense oligodeoxynucleotide (ODN) targeted against alpha-I-tubulin of *Plasmodium falciparum*

by

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AMA:	Apical Merozoite Antigen
BLAST:	Basic Local Alignment Search Tool
CE:	Capillary electrophoresis
CMV:	Cytomegalovirus
CSP:	Circumsporozoite Protein
DEAE:	Diethylaminoethyl
DHFR:	Dihydrofolate reductase
DHPS:	Dihydropteroate synthase
DOSPER:	1,3-di-oleoyloxy-2(6-carboxy-spermyl)-propyl amide
FC:	Flow cytometry
FDA:	Federal drug administration
FITC:	Fluorescein isothiocyanate
HEPES:	N-2-hydroxyethylpiperazine-N-2'-ethane sulfonate
HPLC:	High performance liquid chromatography
ICAM:	Intracellular adhesion molecule
LSA:	Liver Stage Antigen
MBO:	Mixed Backbone ODN
MOE:	2'-O-(methoxyethyl)
MSP:	Merozoite Surface Protein
MTOs:	Microtubule organising centres
NPP:	New permeation pathway
ODN:	Oligodeoxynucleotide
PfEMP-1:	*Plasmodium falciparum* erythrocyte membrane protein 1
PO:	Phosphodiester
PS:	Phosphorothioate
RP HPLC:	Reversed phase high performance liquid chromatography
SAGE:	Serial Analysis of Gene Expression
SAX:	Strong anion exchange
SERA:	Serine Rich Antigen
SLO:	Streptolysine-O
SP:	Sulphadoxine-Pyrimethamine
SPP:	Sporozoite Surface Protein
TEAA:	Triethylammonium acetate
TRIS:	N-tris(hydroxymethyl)aminomethane
UTL:	Useful Therapeutic Life
WAX:	Weak anion exchange

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.

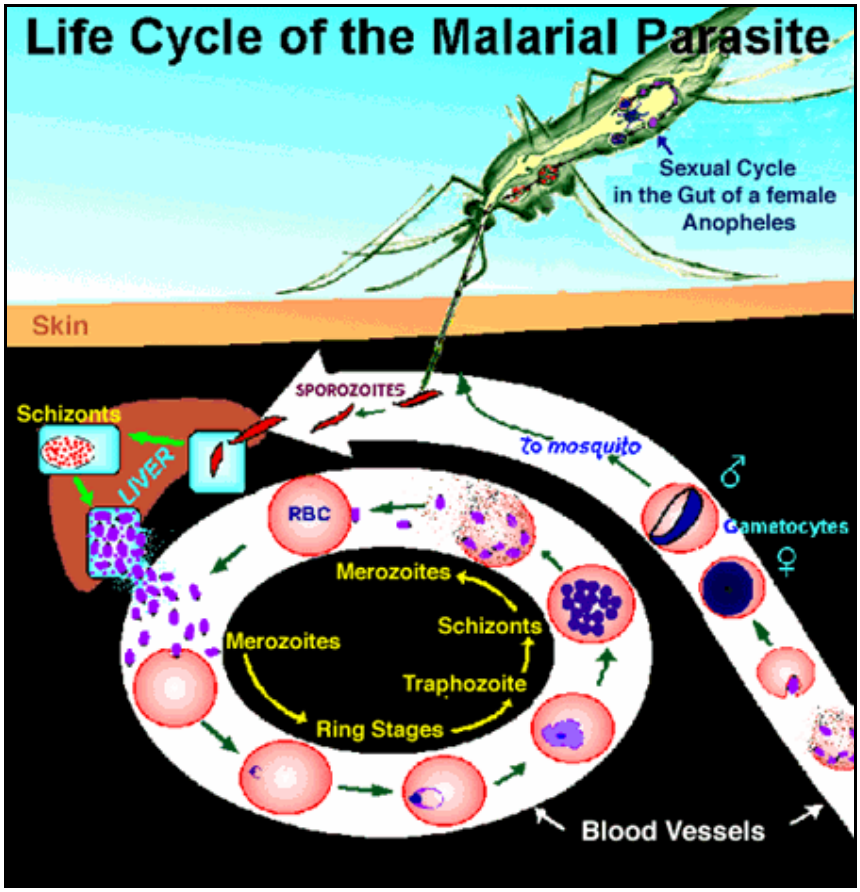


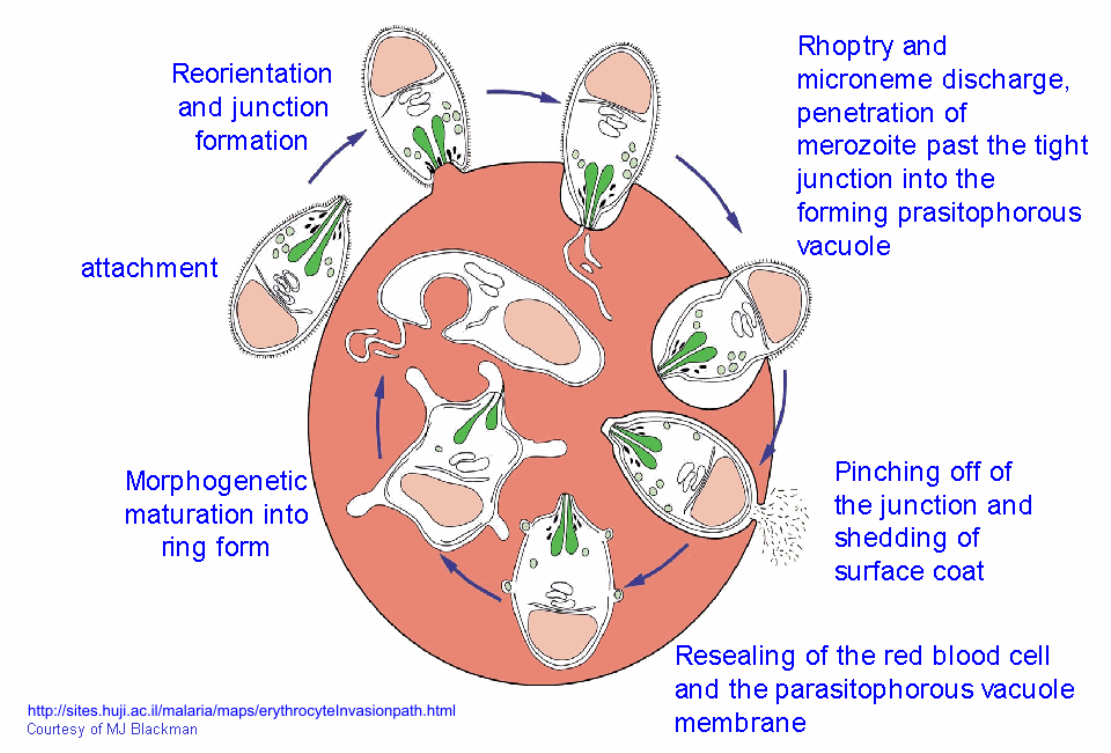
Figure 1.1: The complete life cycle of the human malaria parasite, *P.falciparum*. (Malaria Foundation International (www.malaria.org/lifecycle.html).

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



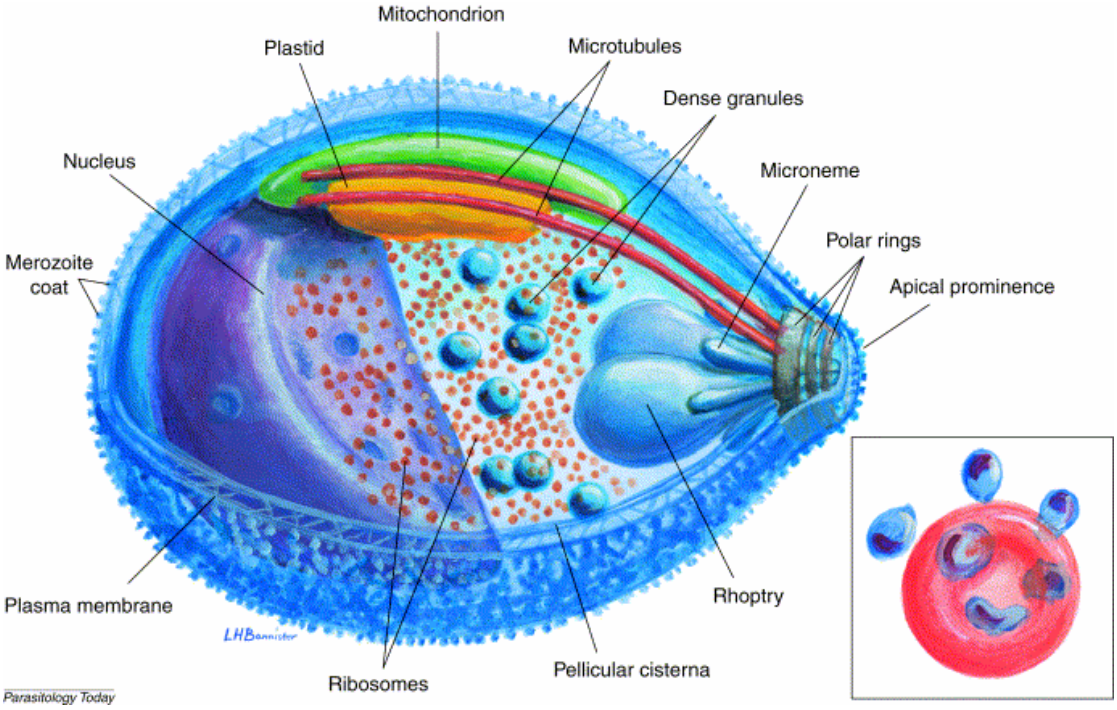


Figure 1.2: Schematic representation of merozoite invasion of a host erythrocyte (http://sites.huji.ac.il/malaria/) and an ultrastructural view of the parasite components involved in invasion (Bannister *et al*, 2000)

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.

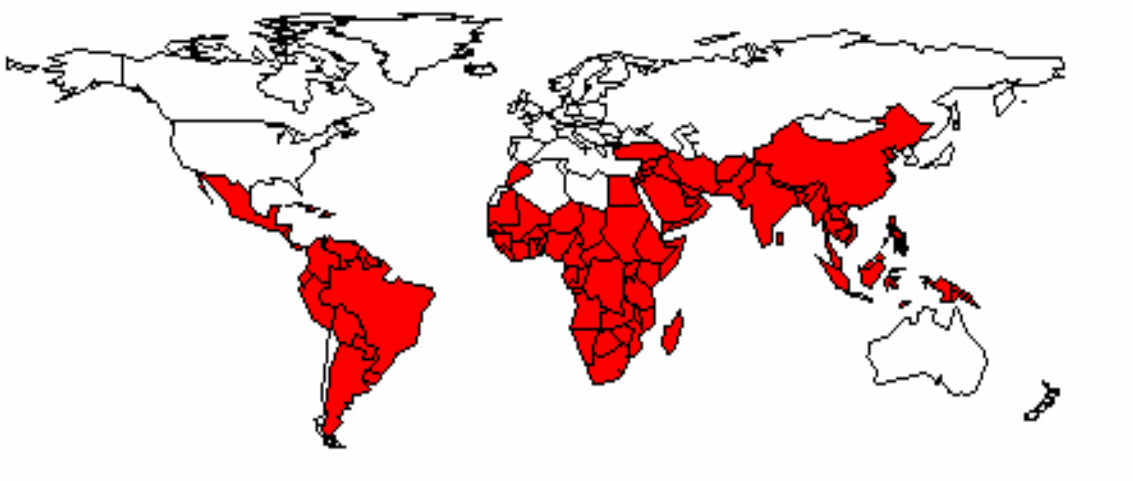


Figure 1.3: World map indicating the regions (red) in which indigenous malaria is prevalent. The red areas include countries such as Brazil, India, Afghanistan, Sri-Lanka, Thailand, Cambodia, Vietnam, Indonesia and China as well as large portions of the African continent (WHO, 1998).

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.

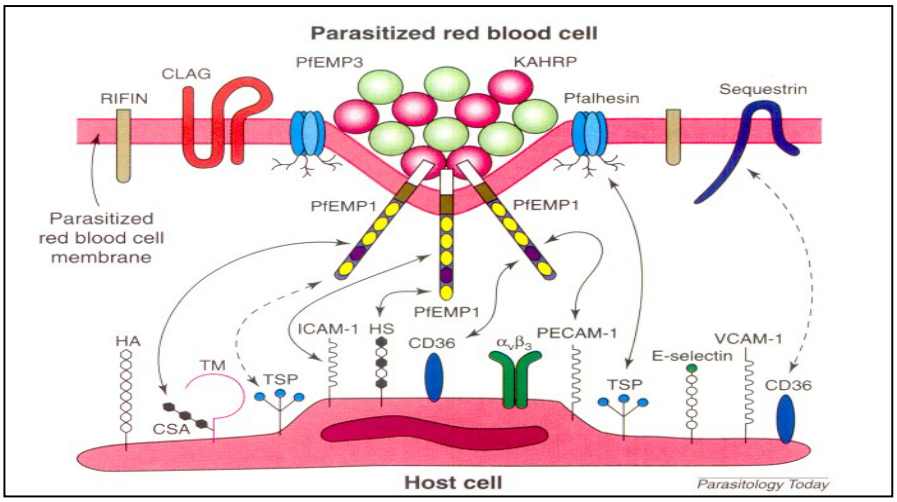


Figure 1.4: Schematic representation of the interaction at the cytoadhesive interface between a *P. falciparum* infected erythrocyte and the host vascular endothelium**.** CLAG: cytoadherence-linked asexual gene; CR1: complement receptor I; CSA: chondroitin sulphate A; HA: hyaluronic acid; HS: heparan 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

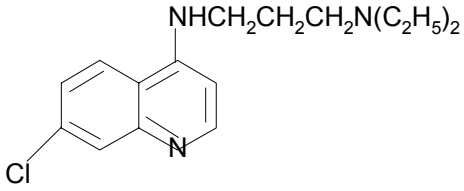
The most frequently used anti-malarial drugs are listed in Table 1.1 along with their class and biological activity.

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

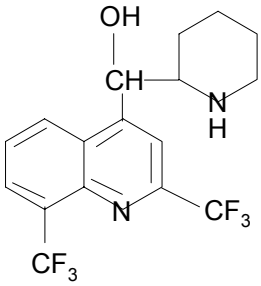
Class	Drug	Biological activity	
		Blood Schizontocide	**Tissue Schizontocide**
4-Aminoquinolines	Chloroquine	++	0
Arylaminoalcohols	Quinine	++	0
	Quinidine	++	0
	Mefloquine	++	0
Phenanthrene methanol	Halofantrine	++	0
Artemisinine and derivatives	Artemisinine	++	0
	Artemether	++	0
	Artesunate	++	0
Antimetabolites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

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

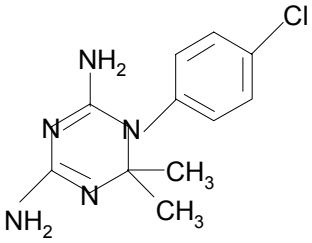
From the above list, existing or emerging parasite resistance to the following drugs will be discussed in more detail in this section: chloroquine, mefloquine, proguanil (active form: cycloguanil), pyrimethamine, sulfadoxine and dapsone. The structures of these compounds are shown in Fig. 1.5.



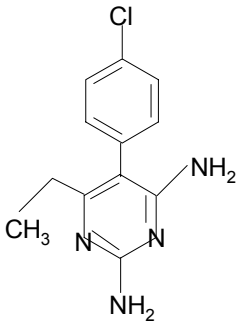
Chloroquine



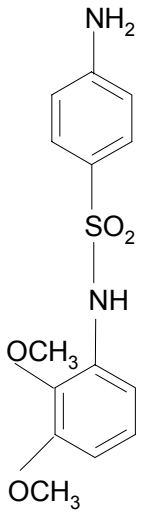
Mefloquine



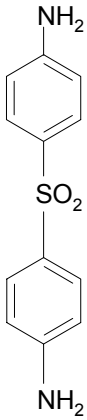
Cycloguanil



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.

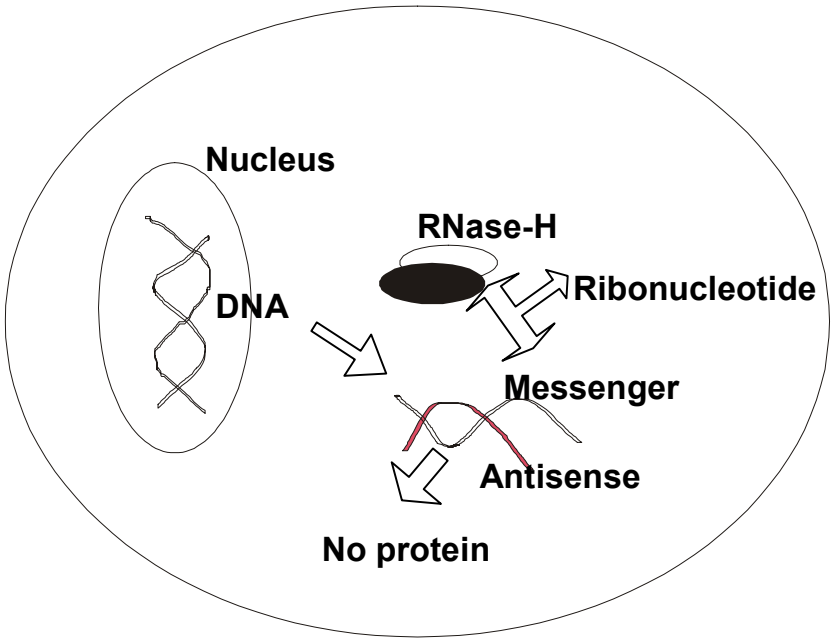


Figure 1.6: Illustration of an antisense ODN binding to a complementary segment of mRNA, inhibiting translation of the target protein.

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).

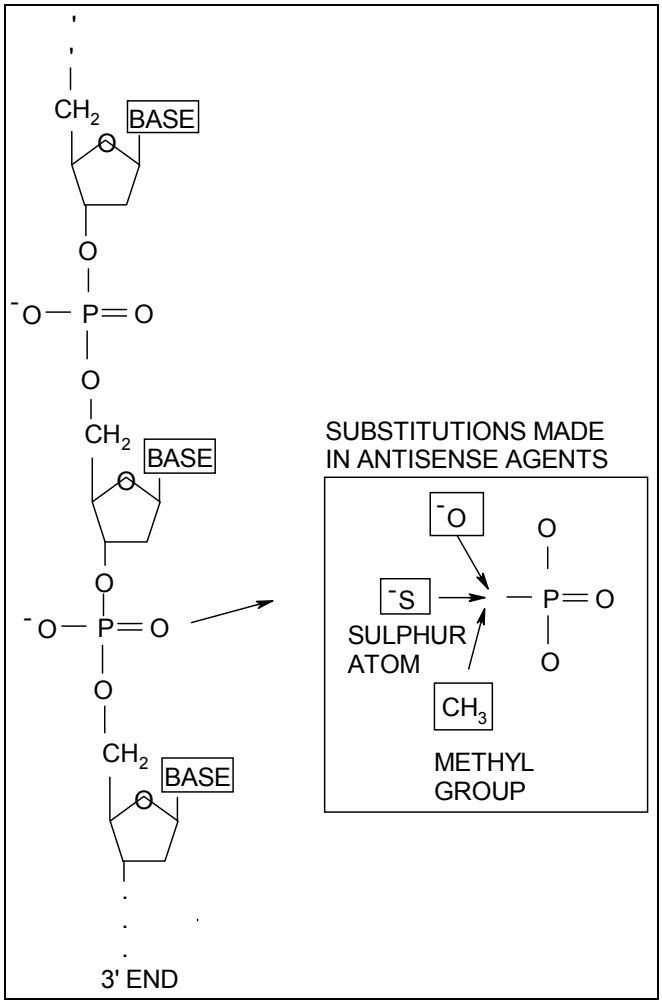


Figure 1.7: Illustration of the most popular first generation modifications of the ODN phosphodiester bond which protect against nuclease degradation (Adapted from Uhlmann and Peyman, 1990).

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.

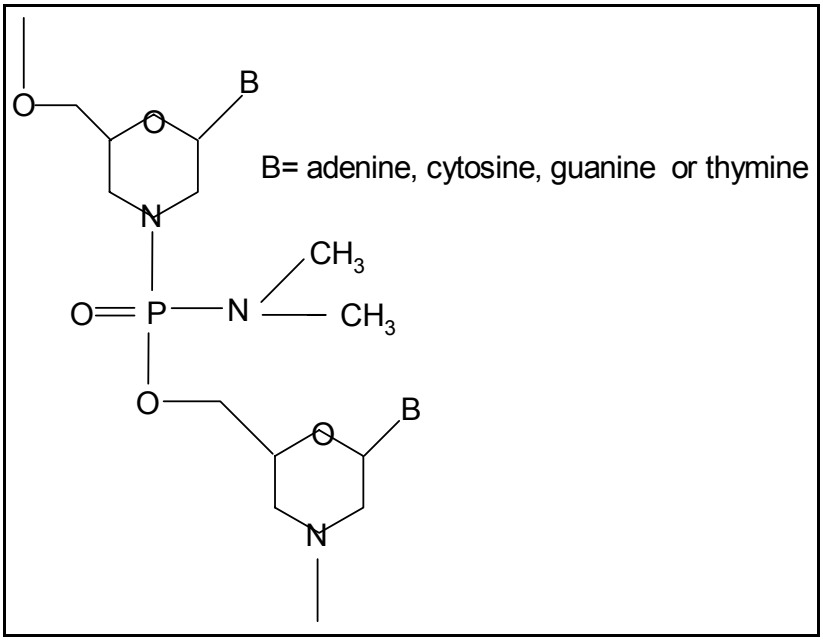


Figure 1.8: Illustration of a morpholino ODN where the entire sugar phosphate backbone is replaced by morpholino diamidates (Summerton and Weller, 1997).

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.

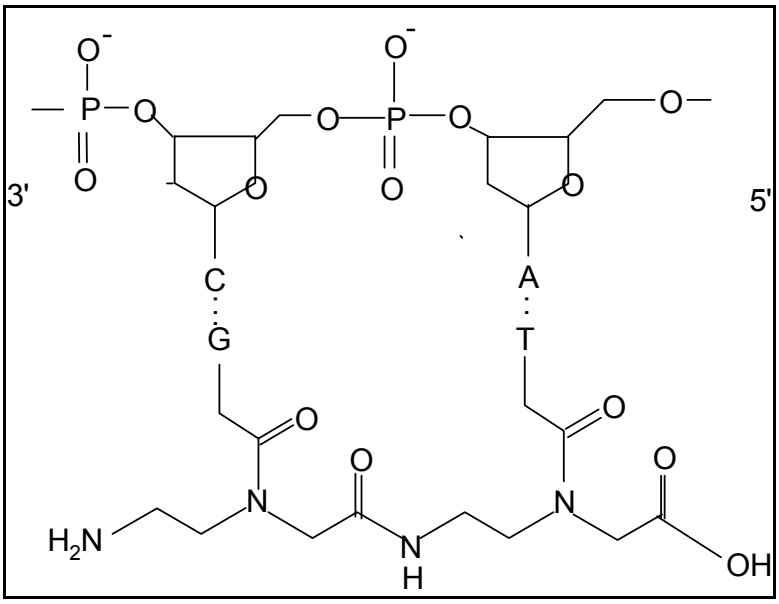


Figure 1.9: Illustration of the peptide nucleic acid (PNA) analogue (lower strand) hybridized to target RNA (upper strand).

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).

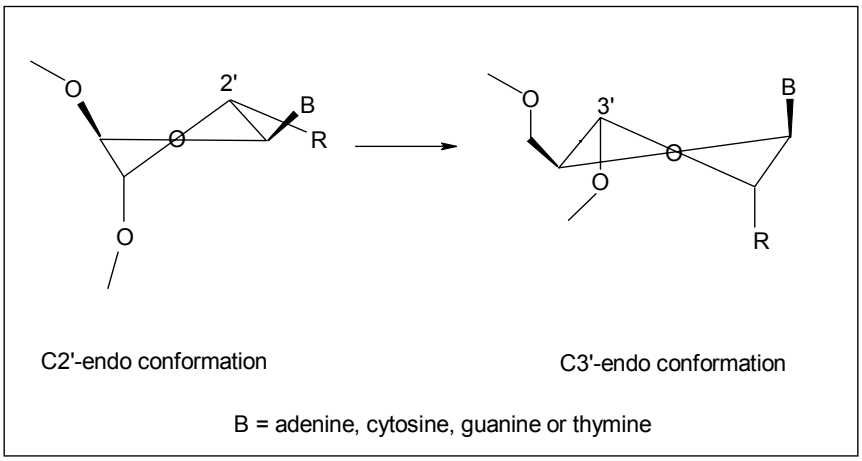
1.6.2.2. Nitrogenous base modifications

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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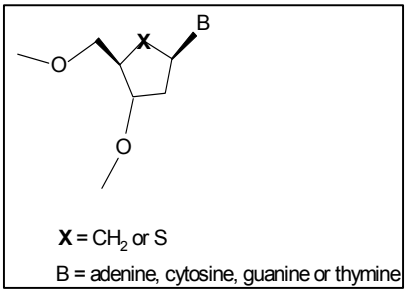

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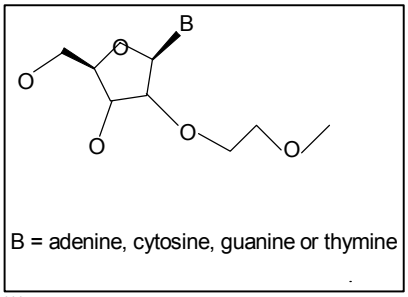

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Figure 1.10: Illustration of sugar modifications used in attempts to enhance hybridization between antisense ODN and RNA and to protect the ODN against nuclease degradation. i: Conversion of the ribofuranosyl groups from a 2' endo to a 3' endo conformation, 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.

CHAPTER 2

Identification of target proteins and the design of antisense oligodeoxynucleotides

2.1. Introduction

2.1.1 General

The aim of the work described in this chapter was to select a target protein for the inhibition of *P. falciparum,* with an antisense ODN targeted to the area surrounding the translation initiation codon as well as ODNs based on computational predictions of the mRNA secondary structure.

The design of antisense ODNs can be divided into several steps, each of which is important to the successful application of the end product. These steps include choosing the target protein, the targeted nucleotide sequence and type of ODN modification. Since the efficacy of antisense ODNs is largely dependent on their successful hybridization to mRNA, accessibility of the target sequence is of paramount importance (Lima and Crooke, 1997; Phillips and Zhang, 2000).

The aim of the study largely influences the choice of the target protein. If the antisense ODN is to be applied for therapeutic use, it is important that the target protein play a vital role in the organism. In many cases however, antisense studies are performed to validate the protein as a potential drug target. It is also possible that the protein has already been validated but that its specific inhibition, using conventional methods is problematic.

Another common aim of antisense studies is to determine the function of a particular protein or enzyme in a cell or metabolic pathway. For application in the current projects in our laboratory the most applicable aims would be to utilize the antisense technique in the validation of a protein as a potential drug candidate and/ or to determine the function of a particular protein or enzyme in a cell or metabolic pathway, akin to knock down applications.

Once the target protein has been identified, the secondary or higher order structures of the mRNA target sequence(s) must be determined since it is not a linear molecule (Smith *et al*, 2000; Sohail and Southern, 2000). There are several complex secondary and tertiary structures along the length of mRNA, which can have profound effects on antisense ODN hybridization to the target. Thermal denaturation studies performed in the 1950's, showed that between 40 and 60% of the bases in an RNA molecule are stacked and paired. The most common secondary structures include hairpin loops; internal loops; bulges and bifurcations (Figure 2.1). These secondary structures may associate with each other to form tertiary structures. An example of such tertiary structure formation is that of a pseudoknot. The latter forms when the single bases of two hairpin loops interact with each other (Riesner, 1997). Triple-stranded structures on the other hand, result from the interaction between the stems of two hairpin loops.

These different structures are distributed along the length of the mRNA molecule and influence the accessibility of these regions to antisense ODN hybridization. The secondary structures illustrated in Figure 2.1 represent certain functional signals (Riesner,

1997; Toulme, *et. al*., 1996).

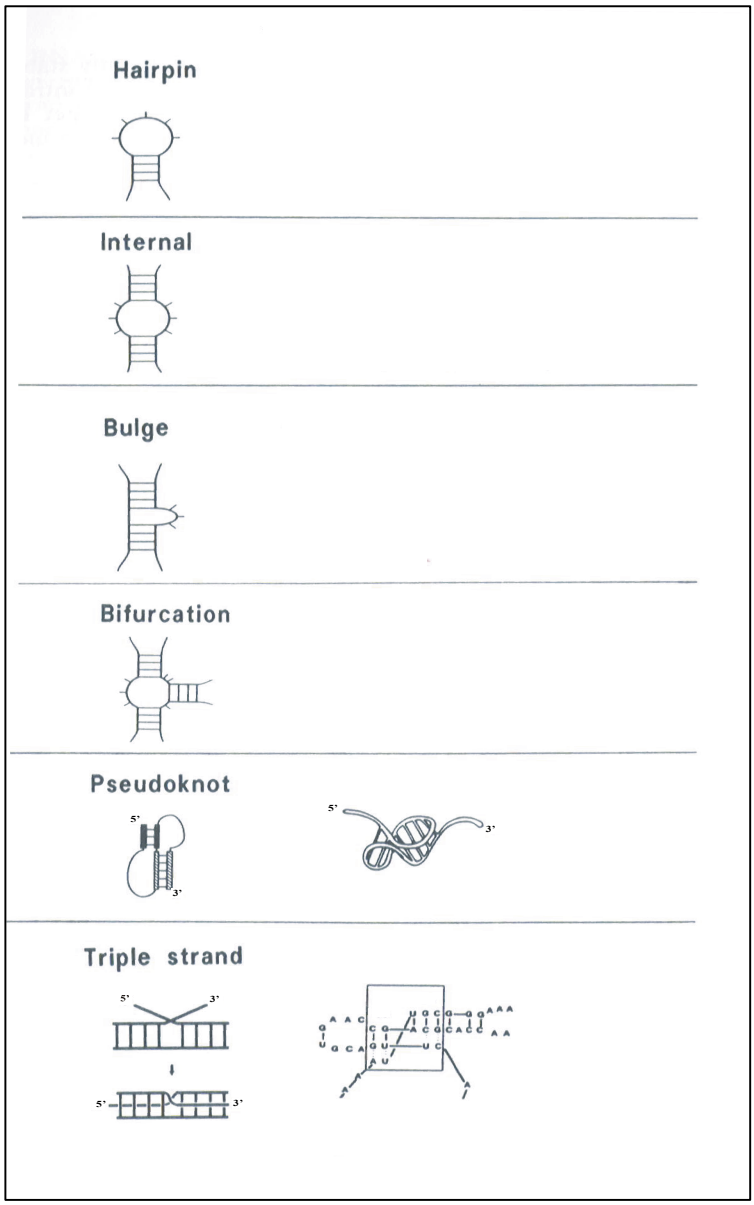


Figure 2.1: Graphic portrayal of the most common secondary and tertiary structure formations found in RNA. The hairpin loop, internal bulge, bulge and bifurcated loop are all examples of secondary structure formations, while the pseudoknot and triple strands are examples of tertiary structures. (Adapted from Riesner, 1997).

Hairpin loops are involved in the regulation of mRNA, which includes aspects such as stability, frame shifting and binding of specific proteins. Bulged loops serve as binding sites for proteins and are able to change their conformation after such binding has taken place. The tertiary structures too have specific functions such as pausing translation and frame shifting. From the nature of the tertiary structure formation it becomes clear that structures such as pseudoknots and triple strands are needed to define the relative orientation of distant helical regions (Riesner, 1997; Toulme *et. al*., 1996).

2.1.2. Target protein selection

Since the aim of the designed antisense ODN is to inhibit the *in vitro* proliferation of the malaria parasite, the target protein must fulfill certain criteria. Firstly, the protein must be vital for the continued survival of the parasite and secondly, the nucleotide sequence must be known.

Tubulin is a globular protein and ubiquitous to eukaryotic cells (Downing and Nogales, 1998). It is known to play a central role in cell division, cell movement and intra-cellular transport as a major component of microtubules (Bell, 1998). Inhibition of tubulin production and/or function therefore, has been shown to result in inhibition of cell proliferation and cell death (Downing and Nogales, 1998). Several anticancer drugs target tubulin proteins and thereby lead to the apoptosis of these cancer cells. Since cancer cells are rapidly dividing it is equally relevant and important to investigate the effect of tubulin inhibitors on the rapidly dividing malaria parasites and in so doing consider tubulin as a suitable target to inhibit parasite proliferation

The tubulin protein family can be divided into three subclasses, namely alpha (α), beta (β) and gamma (γ). The γ-tubulins can be found primarily at the microtubule organising centres (MTOCs), which act as the points of self-assembly in the cell, for example the spindle poles. The α- and β-tubulins, each with a molecular mass of 55 kDa, form heterodimers. The resultant 110 kDa proteins are the functional units that form the polar protofilaments. Staggered arrangement of thirteen protofilaments yields a helical arrangement of tubulin heterodimers to form the microtubule (Jordan and Wilson, 2004). Numerous ligands have been shown to bind to the tubulin component of microtubules in particular, of which several have proven anticancer properties (Downing and Nogales, 1998).

Most anticancer drugs such as colchicine, vincristine, vinblastine, taxotere and taxol interact with the β-tubulin subunit (Figure 2.2).

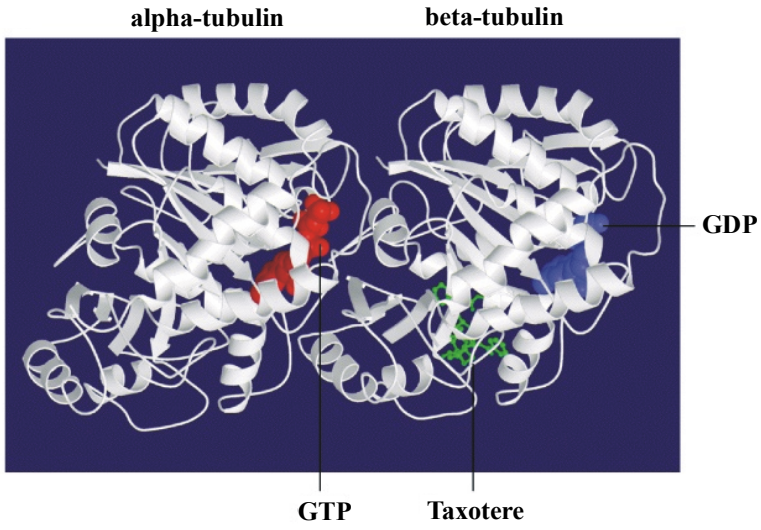


Figure 2.2: Illustration showing the spatial arrangement of α- and β-tubulin in the heterodimers that are assembled into microtubules. The binding site of taxotere, a microtubule inhibitor is indicated on the β-tubulin subunit (Downing and Nogales , 1998).

Jan *et al*. (2000) however discovered a new anticancer drug (COBRA-1) that exercises its action by binding to a cavity in α-tubulin. This drug has led to the destruction of microtubule organization and eventually apoptosis.

The effects of numerous known microtubule inhibitors on *P. falciparum* proliferation were investigated and the results are shown in Table 2.1.

Table 2.1: Inhibition of proliferation of *P.falciparum* in cultured erythrocytes and mammalian cells by microtubule inhibitors (Bell, 1998).

Compound	**IC_{50} (*P. falciparum*, µM)**	**IC_{50} (mammalian cell, µM)**
Colchicine	13	$3.2x10^{-3}$
Vinblastine	$2.8x10^{-2}$	$6x10^{-4}$
Vincristine	$6x10^{-3}$	$2.2x10^{-3}$
Taxol	$7.1x10^{-2}$	$2.2x10^{-3}$
Taxotere	$3.1x10^{-3}$	$3.9x10^{-2}$

All the above compounds bind to β-tubulin but have different modes of action. Colchicine binds to a single site of an unassembled β-tubulin and thereby blocks or inhibits tubulin polymerization. Vinblastine and vincristine cause depolymeriztion of the already formed microtubule while taxol and taxotere stabilize the assembly of tubulin into microtubules to the extent that mitosis is disrupted and eventually leads to apoptotic cell death (Bell, 1998; www.dentistry.leeds.ac.uk/biochem). It is important to note that all

the above drugs affect the proliferation of mammalian cells as is evident from the fact that these agents are also employed as anticancer agents. From the information provided it becomes evident that tubulin proteins are suitable targets for the inhibition of *P. falciparum* proliferation, if this can be achieved in such a way as to specifically target the parasitic tubulin. This is problematic since Table 2.1 shows that all the compounds tested, with the exception of taxotere, are more effective inhibitors of mammalian cell proliferation than of *P. falciparum* proliferation.

Since the malaria parasite has five different known types of tubulin, namely α-I, α-II, β, γ and a putative δ tubulin it is necessary to consider which of the above would be the most suitable target. α-II tubulin is expressed predominantly in the sexual stages of the parasite lifecycle, whereas α-I and β tubulin are expressed in both the sexual and asexual stages (Delves *et. al*., 1990). It was decided to target α-I-tubulin since it is proposed as the binding and regulatory site for the key glycolytic enzymes: phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase and aldolase in the parasite (Itin *et. al*., 1993). This is particularly important due to the high glycolytic activity of infected erythrocytes as reflected by the 50 to 100-fold increase in the uptake of glucose (Hess *et. al*., 1995).

2.1.3. Target sequence selection

Several methods have been described to determine accessible regions of the mRNA molecule in order to improve the targeting of antisense ODNs. A brief description of the most commonly used methods to select mRNA target sequences is given below.

2.1.3.1. The random shotgun approach or gene walking: In this method, several ODNs (usually containing between 50 and 100 nucleotides) are targeted to various regions of a mRNA molecule (Smith *et al.*, 2000; Sohail and Southern, 2000). Typically only between 2 and 5% of the ODNs are successful (Sohail and Southern, 2000). Stull *et al.* (1996) measured the apparent dissociation constants of 32 phosphodiester and 5 phosphorothioate antisense ODNs, targeted against different regions of murine TNF-mRNA. These studies showed that a slight change in the target site of the mRNA has a profound influence on the ODN affinity for the site. Results such as these emphasize the complexity of successful target site selection. This is obviously an expensive and time-consuming method to identify possible target sites (Akhatar, 1998; Sohail and Southern, 2000).

2.1.3.2. Predictions of mRNA secondary structures: There are two ways in which mRNA secondary structure can be predicted to aid the identification of antisense target sequences. The first one utilizes computer-based predictions of the mRNA secondary structure in order to identify areas free of intramolecular hydrogen bonds. Three programs are commonly used for the prediction of RNA secondary structure namely, *Mfold*, *LinAll* and RNA*fold*. *Mfold* is used for the prediction of optimal and sub-optimal structures of linear or circular sequences at 37 °C. *LinAll* predicts optimal and sub-optimal secondary structures of linear or circular sequences at different temperatures. Finally RNA*fold* predicts only the optimal secondary structure at a given temperature (Riesner, 1997). The optimal structure refers to the structure having the minimum ΔG

(free energy) value. The sub-optimal structures are those that are more than 20% higher than the ΔG value of the optimal structure (Matthews *et. al*., 1999).

One of the drawbacks of the computer prediction techniques is that higher order interactions such as those with cellular proteins as well as possible tertiary structures that can influence ODN hybridization are unknown and therefore cannot be taken into account (Smith *et al.*, 2000; Sohail and Southern, 2000). The predicted secondary RNA structures therefore offer the researcher only a first approximation and not a comprehensive picture of all possible RNA structures.

The second method utilizes a phylogenic approach that is based on the principle that sequences with similar function acquire homologous secondary structures (Michel and Westhof, 1990, Sohail and Southern, 2000). This approach has been particularly successful in predicting the folding of large mRNA molecules. The drawback of this method lies in the fact that it is of limited use when only a small number of comparative nucleotide sequences are available or if the functional significance of the folded structure is unknown.

2.1.3.3. Oligonucleotide scanning arrays: A set of oligonucleotides, with each one being complementary to a section of the target sequence, is synthesized directly on the solid surface (glass or polypropylene) on which hybridization studies are conducted (Sohail and Southern, 2000). Radiolabelled transcripts are then hybridized to the arrays of complementary ODNs at physiological temperature. Hybridization patterns are obtained

by exposure of the arrays to a phosphor storage screen and scanning in a phosphorimager. This method is costly but offers the accuracy vital to the identification of the optimal ODN hybridization site.

2.1.3.4. Oligomer library / Ribonuclease-H-digestion: RNase-H, which specifically cleaves RNA in a DNA:RNA heteroduplex, is used in combination with a random or semi-random library. A random library consists of a complete set of all possible ODNs of a defined length (usually 10-12 mers). Semi-random libraries consist of ODNs (typically 11-mers) that have one constrained (i.e. non-variable) nucleotide at an internal position (Ho *et al*., 1996). To obtain accurate results, four libraries are synthesized each with a different constrained nucleotide. The size of each library is dependent on the length of the transcript under investigation. The target mRNA is usually transcribed *in vitro*, end-labeled and mixed with a random or semi-random library. Those library components complementary to accessible mRNA regions are identified by electrophoresis after cleavage of the DNA oligomer:RNA heteroduplexes by RNase-H. The problem with this particular method is that it is difficult to accurately determine the precise cleavage site and hence identify the precise location of the ODN hybridization site. This is significant since the shift of a single base or a single base length difference can have significant effects on the antisense activity of the ODN (Sohail and Southern, 2000). This method is therefore not as accurate as the oligomer scanning arrays but can be applied as an initial screening for RNA accessible sites (Milner *et. al*., 1997).

From the above it is evident that there is no single, reliable or inexpensive method for accurately predicting the most successful antisense target sites on mRNA and that

selection is still largely based on trial and error. Fortuitously, the area surrounding the translation initiation codon can be often used as a starting point due to the number of successful results reported in the literature using this region as a target site (Phillips and Zhang, 2000).

2.1.4. Oligonucleotide modification selection

The next important decision to be taken when designing antisense ODNs is the choice of modification. These may serve several purposes, the most important being protection against nuclease degradation and improving cell membrane permeation. Phosphodiester bonds are particularly susceptible to 3' exonuclease degradation (Uhlmann *et al*, 2000) and the hydrophobic core of cell membranes does not readily facilitate the uptake of the polyanionic ODNs into cells. Several types of chemical modifications to protect the ODN against enzymatic degradation have already being discussed in Chapter 1. The addition of a stable hairpin loop to either the 3' or 5' terminus of ODNs have to date only been applied and tested in conjunction with phosphorothioate modifications (Barker *et. al*., 1998). No other information is available except that this modification was shown to improve the efficacy by about 20% compared to the corresponding unmodified phosphorothiate ODN. Since this structural modification is the most simple and cost-effective, hairpin loops were added to all the ODNs used in this study to investigate their stability and uptake into parasitized erythrocytes.

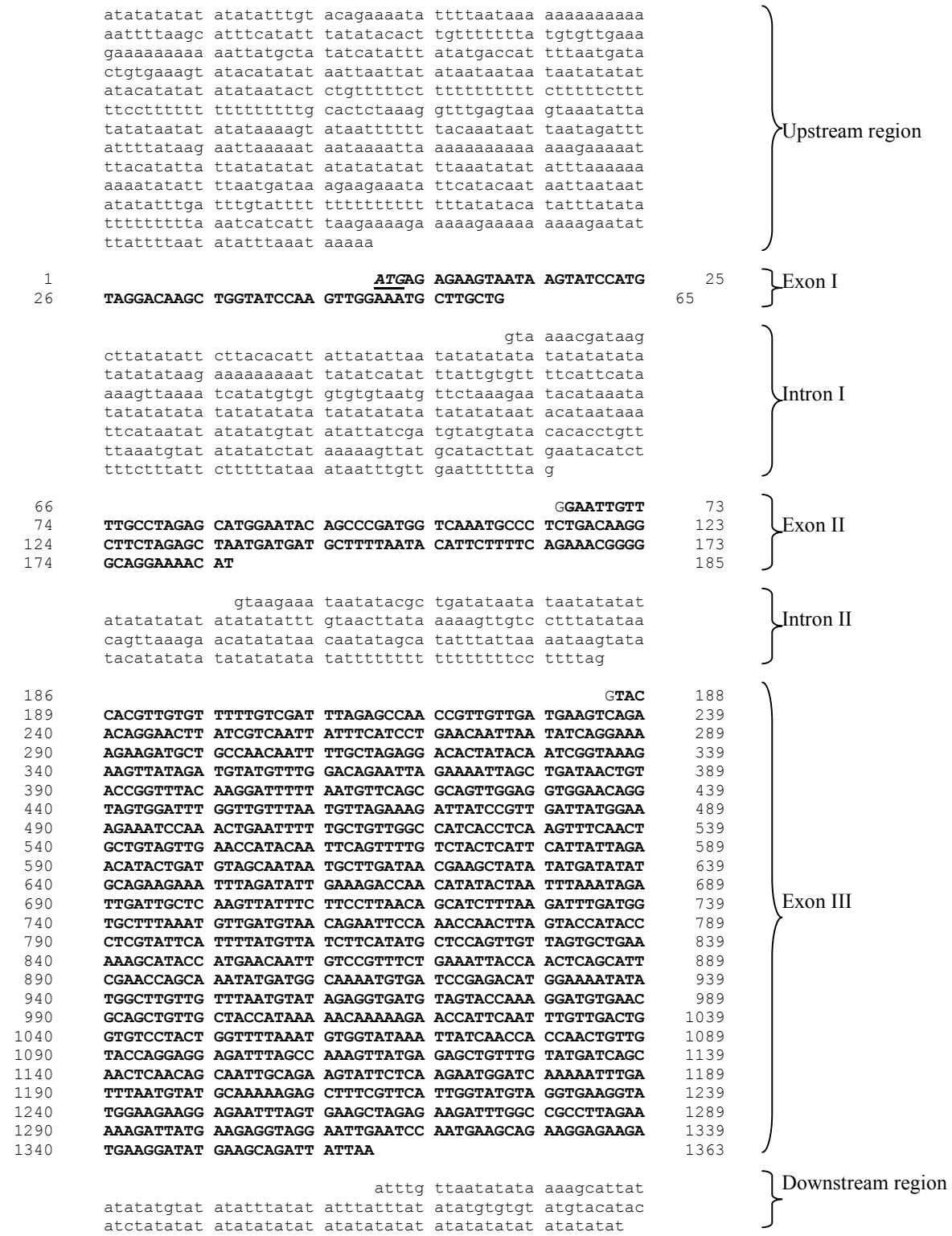


Figure 2.3: Complete gene sequence of *P. falciparum* α-I-tubulin. The translation initiation codon is underlined and the exons are indicated in bold letters (GenBank accession number: X15979).

2.2. Materials and Methods

2.2.1. **Materials**

The ODNs were purchased from Roche Diagnostics.

2.2.2. **Methods**

2.2.2.1. α-I-tubulin sequences

The complete sequence of *Plasmodium falciparum* α-I-tubulin gene (Genbank accession number: X15979; Holloway, 1989) is given in Figure 2.3.

The α-I-tubulin DNA sequence contains two introns, which were removed prior to analysis of the secondary structure of the mRNA by *Mfold* (Matthews *et. al.*, 1999 and Zuker, 1989).

2.2.2.2. The RNA folding program, *Mfold*

Mfold was used to predict RNA secondary structure by energy minimization. Michael Zuker and co-workers at the Institute for Biomedical Computing, Washington University, USA wrote this program, and it was downloaded (version 2.3) from the Zuker homepage at http://bioinfo.math.rpi.edu/~zukerm/home.html on a Silicon Graphics Workstation.

2.2.2.2.1. Drawing and evaluation of secondary mRNA structures

i) RNA Structure 3.5

This program utilizes the ct (connect) file format generated by the *Mfold* program to produce visual representations of the mRNA structures. The program was downloaded from the homepage of Doug Turner (sub-heading: The Turner Group) at http://www.chem.rochester.edu/Faculty/Turner.html.

RNA Structure 3.5 offers several features that allows a thorough investigation of the folded mRNA molecules. The two features used in these investigations, found under the *File* option, were *Draw* and *Oligo Walk*. The *Draw* function allows for the entry of an *Mfold* ct file and utilizes this information to generate a visual representation of the required RNA molecules. These can be printed as a hard copy or exported to Corel Draw or any word processing program.

The *Oligo Walk* function was extremely important in these investigations as it identified optimal ODN hybridization sites (a length of 27-mer at a concentration of 0.5 µM was specified) in each of the RNA structures predicted by *Mfold*. Each RNA structure is "walked" one base at time, and the overall ΔG of hybridization for each hybridization site is calculated. After conclusion of the *Oligo Walk* the energetically most favourable hybridization site (minimum ΔG) is identified. It is important to note that the overall hybridization energy took the energy required to break any local secondary structures into account.

ii) RNAdraw

The visual representations of the RNA molecules produced by this program were compared to those obtained with RNA Structure 3.5. This program, written by Ole Matzura, was downloaded from the RNAdraw homepage at: http://rnadraw.base8.sel. This program also utilizes the ct files generated by the *Mfold* program to produce visual representations of the folded RNA molecules.

All mentioned web addresses were last visited and confirmed in April, 2005.

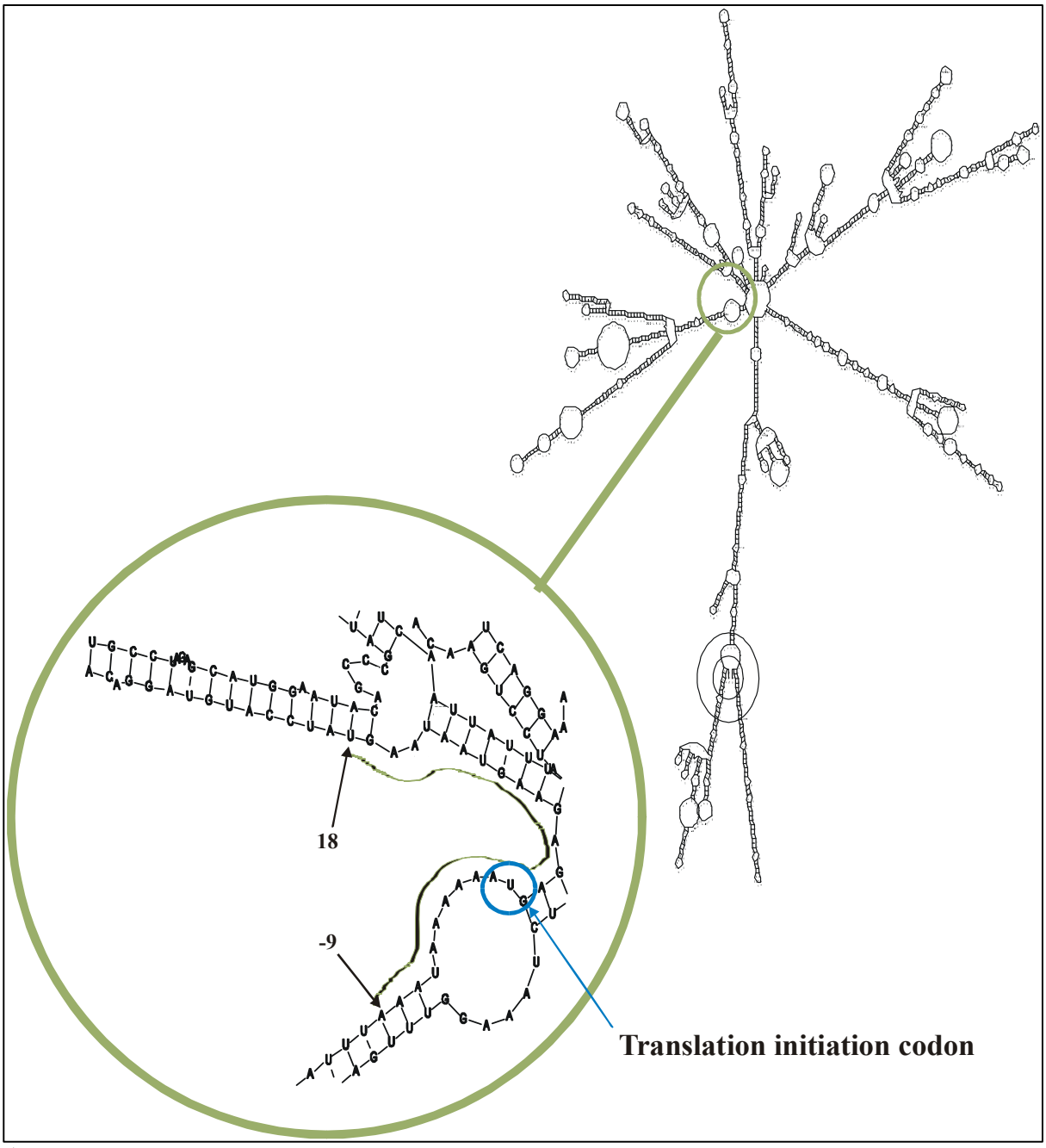
2.3 Results

2.3.1. Selection of target mRNA sequences

Three antisense ODNs, targeted against different regions of the mature α-I-tubulin mRNA sequence of *P. falciparum*, were designed as well as the appropriate controls. One targeted the region around the translation initiation codon, while the selection of the other two was based on mRNA secondary structure information (Refer to Tables 2.4 and 2.5 in section 2.3.1.3 for sequences).

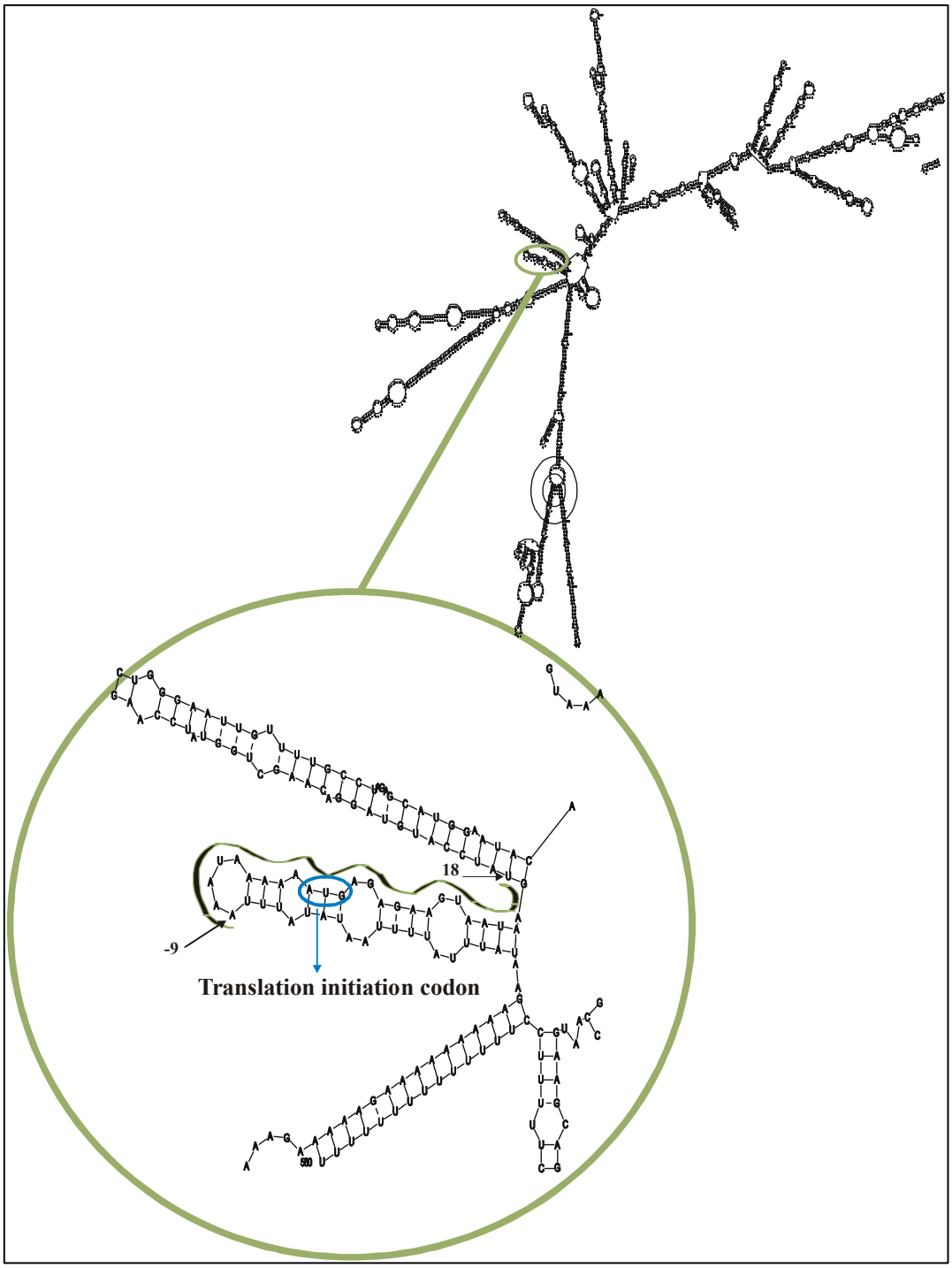
2.3.1.1. Secondary structure prediction of *P. falciparum* mature α-I-tubulin mRNA

The optimal and sub-optimal foldings of *P. falciparum* α-I-tubulin mature mRNA were computed with the aid of the program, *Mfold* (Zuker, 1989). The optimal structure, i.e. the structure having the most favourable (minimum) ΔG value (–417.1 Kcal/mol), is shown in Figure 2.4A. The 17 sub-optimal structures, predicted to have ΔG values within 20% of that of the optimal structure, were generated in the same way. An example of such a structure is shown in Figure 2.4.B.



A

Figure 2.4: A: The predicted optimal structure of *P. falciparum* mature α-I-tubulin mRNA. The location of AS-1 is shown in green. Refer to Table 2.5 for all ODN sequences. These structures were generated by the program RNA Structure 3.5.



B

Figure 2.4 B: One example of the 17 sub-optimal structures of *P. falciparum* mature α-I-tubulin mRNA. The location of AS-1 is in green. Refer to Table 2.5 for all ODN sequences. These structures were generated by the program RNA Structure 3.5.

Significant deviations in the secondary structures were evident after comparison of all the predicted mRNA secondary structures. Literature reports have indicated that the most successful antisense ODNs are those that target single-stranded hairpin loop regions i.e. mRNA regions involved in minimal local structure formation (Bruice and Lima 1997; Lima *et. al*., 1997; Vickers *et. al*., 2000; Francois *et. al*., 1994). It was therefore important to analyse the optimal as well as the 17 predicted sub-optimal structures in order to identify the best possible antisense ODN hybridization site. This entailed inspection of all single stranded regions in an attempt to identify common or shared sequences predicted to be available for hybridization to an antisense ODN.

Possible ODN hybridization sites fitting the stated criteria were identified from the optimal and 17 predicted sub-optimal mRNA secondary structures as described below.

2.3.1.2 Identification of accessible hybridization sites and design of antisense ODNs.

The software program RNA Structure 3.5 was used to identify accessible binding sites of a 27-mer ODN on the mature mRNA secondary structures. The first base of the hybridization site for each of the 18 predicted mRNA secondary structures (one optimal and 17 sub-optimal structures), is given in Figure 2.5.

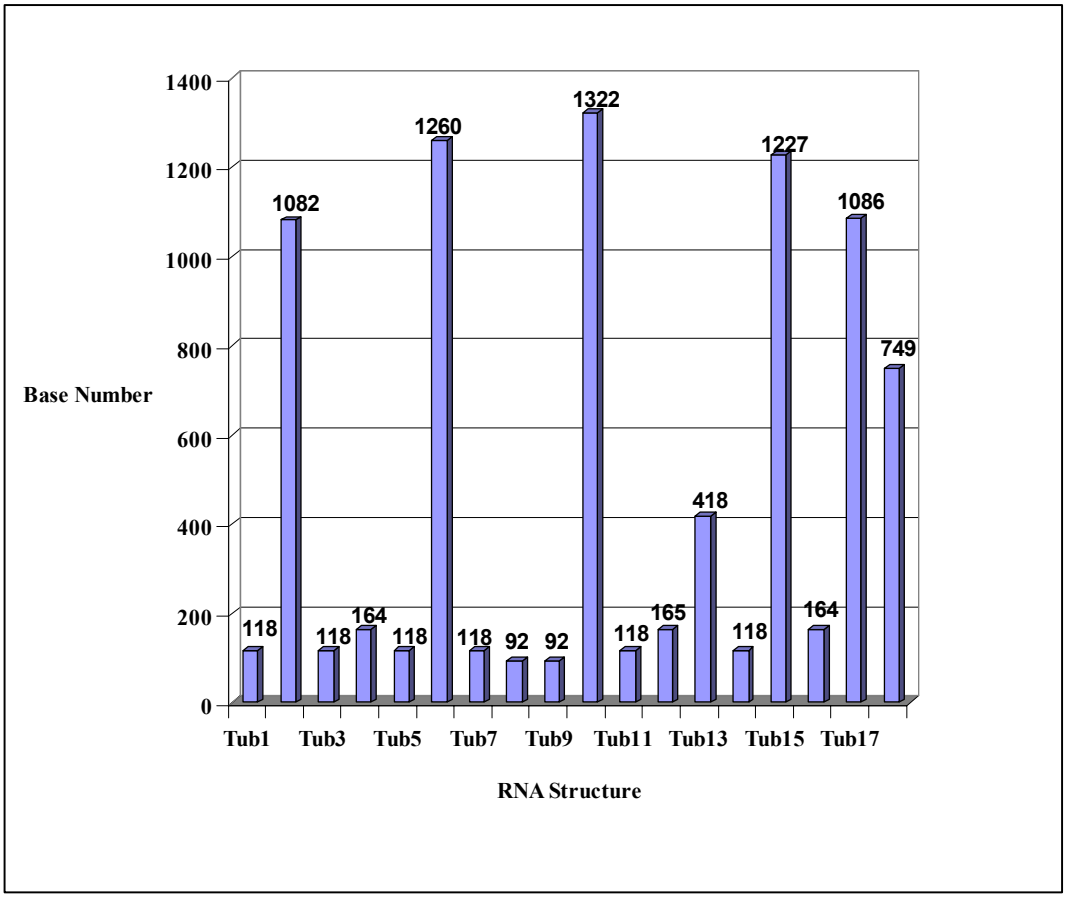


Figure 2.5 : Histogram depicting the first base of the mature mRNA sequence in each of the secondary structures of *P. falciparum* mature α-I-tubulin mRNA predicted by the program RNA Structure 3.5 to be optimal for hybridization to a 27-mer antisense ODN AS-1. Tub1 being the optimal structure.

The mRNA sequence starting at nucleotide 92 was predicted to be the most accessible hybridization site for a 27 mer antisense ODN in structures Tub 8 and 9. The sequence starting at nucleotide 118 was predicted to be the most accessible hybridization site for structures Tub 1, 3, 5, 7, 11 and 14 while the sequence commencing from nucleotide 164 was predicted to be the most accessible for the predicted structures of Tub 4 and 16, respectively.

The number of times a particular hybridization site is predicted to be accessible to the ODN in each of the different mRNA secondary structures, is given as % prevalence in Table 2.2.

Table 2.2: The percentage prevalence of the first base in each of the mature mRNA structures predicted by the RNA Structure 3.5 program to be the most accessible hybridization site for a 27-mer antisense ODN.

First Base Number	% Prevelance
92	11.10%
118	33%
164	11.10%
165	5.60%
418	5.60%
749	5.60%
1082	5.60%
1086	5.60%
1227	5.60%
1260	5.60%
1322	5.60%

Three 27-mer antisense ODN binding sites starting at nucleotides 92, 118 and 164 of α-I-tubulin mRNA, are suggested as the most accessible in all the predicted mature mRNA secondary structures. Their sequences are shown in Table 2.3.

Table 2.3: Three *P. falciparum* α-I-tubulin mature mRNA sequences predicted to be accessible for hybridization of a 27-mer antisense ODN.

mRNA Base Numbers	Sequence
92 – 118	5’ AGC CCG AUG GUC AAA UGC CCU CUG ACA 3’
118 – 144	5’ AAG GCU UCU AGA GCU AAU GAU GAU GCU 3’
164 – 190	5’ AAA CGG GGG CAG GAA AAC AUG UAC CAC 3’

The secondary structure motifs of the three mRNA sequences displayed in Table 2.3 are shown in Figures 2.7 – 2.9. The position of the antisense ODN (AS-1) targeted against the region surrounding the translation initiation codon is also indicated.

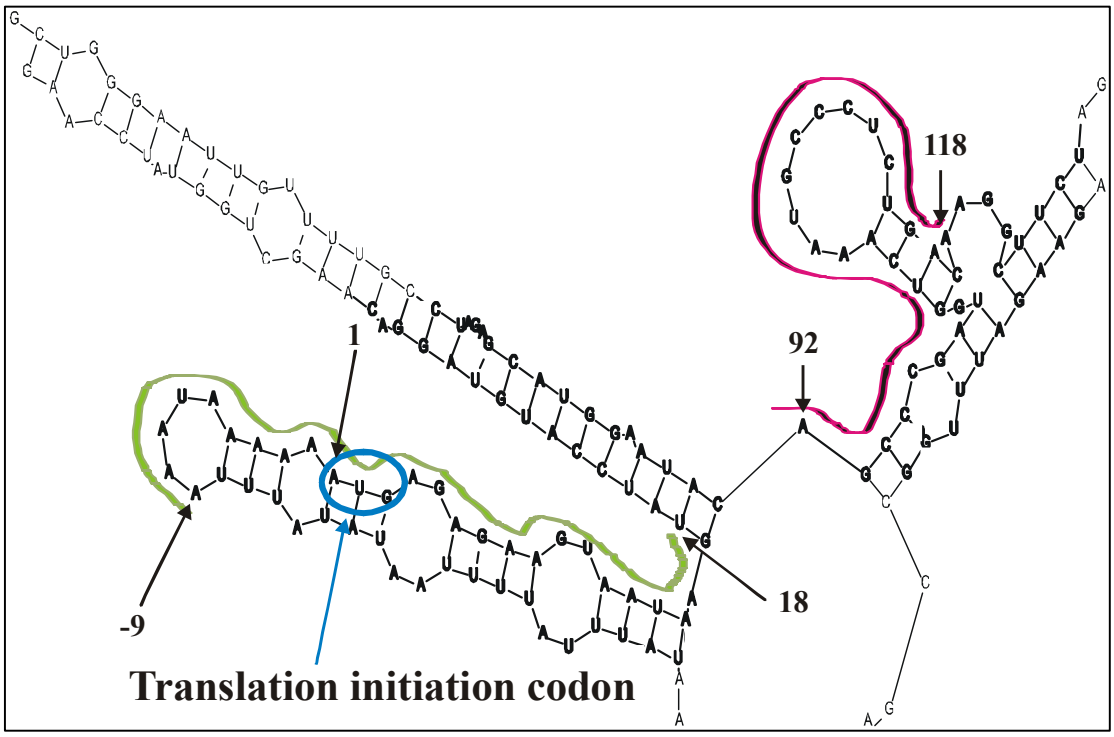
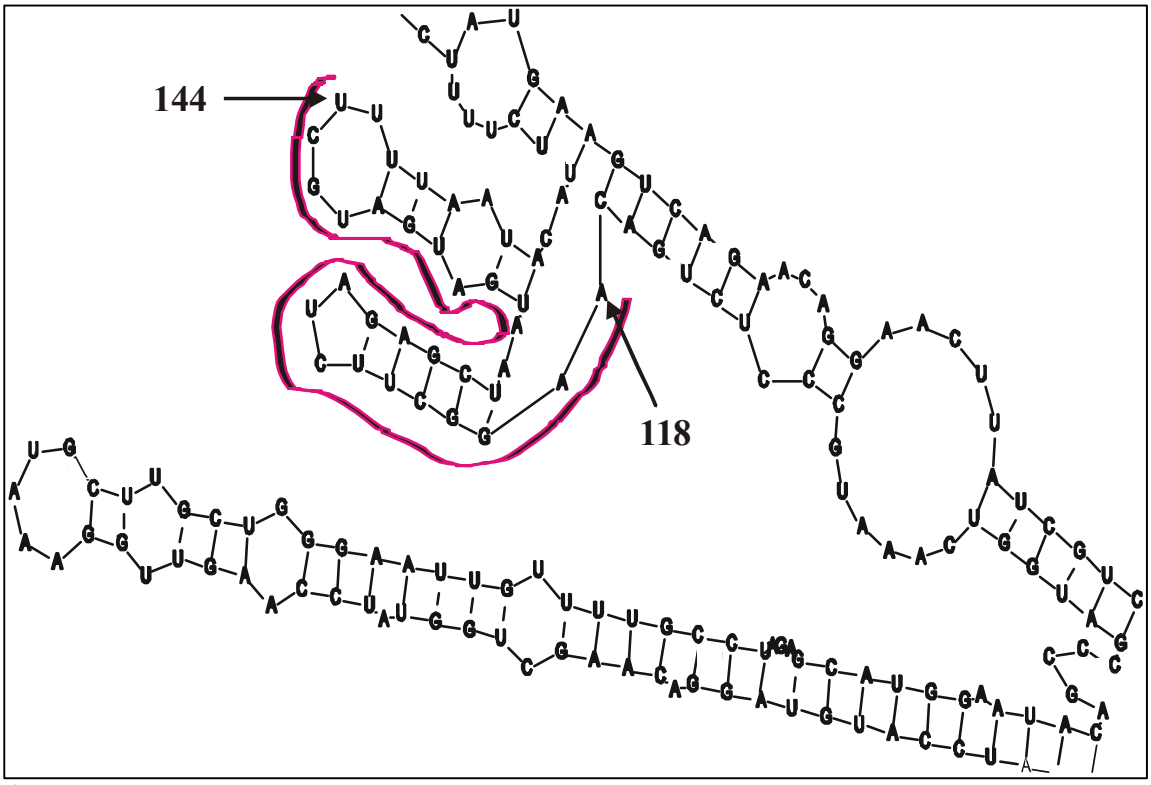


Figure 2.6: The mRNA secondary structure of nucleotides 92 to 118 (magenta area) and –9 to 18 (green area). The sequence 92 to 118 was predicted to be the optimal hybridization site for a 27-mer ODN in the predicted mRNA structures, Tub 8 and 9 (same secondary structure in the indicated regions). The sequence –9 to 18 is the region to which antisense ODN AS-1 is targeted and includes the initiation codon.

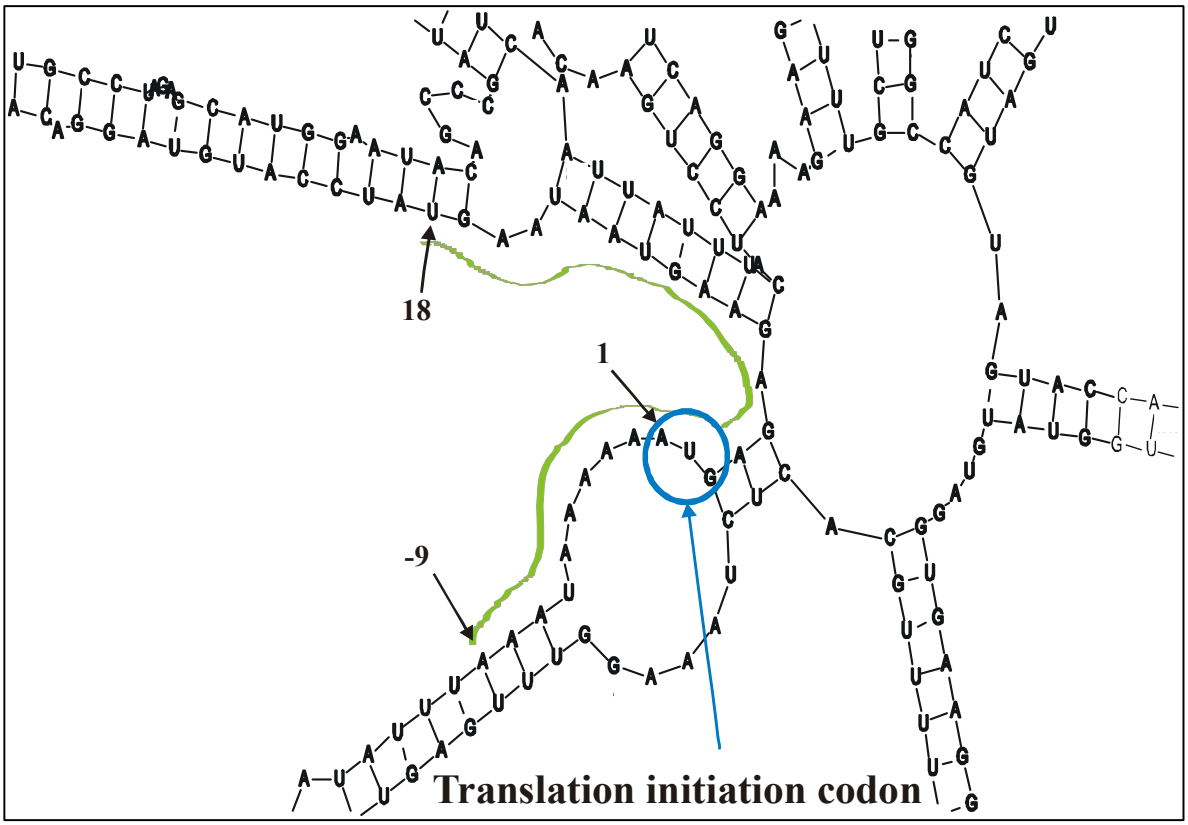
Part of an internal and a hairpin loop structure are common to both the predicted secondary mRNA structures Tub 8 and 9 in the region of bases 92 – 118. The bases –9 to 18 span three internal loops as well as part of a hairpin loop (Figure 2.6).



A

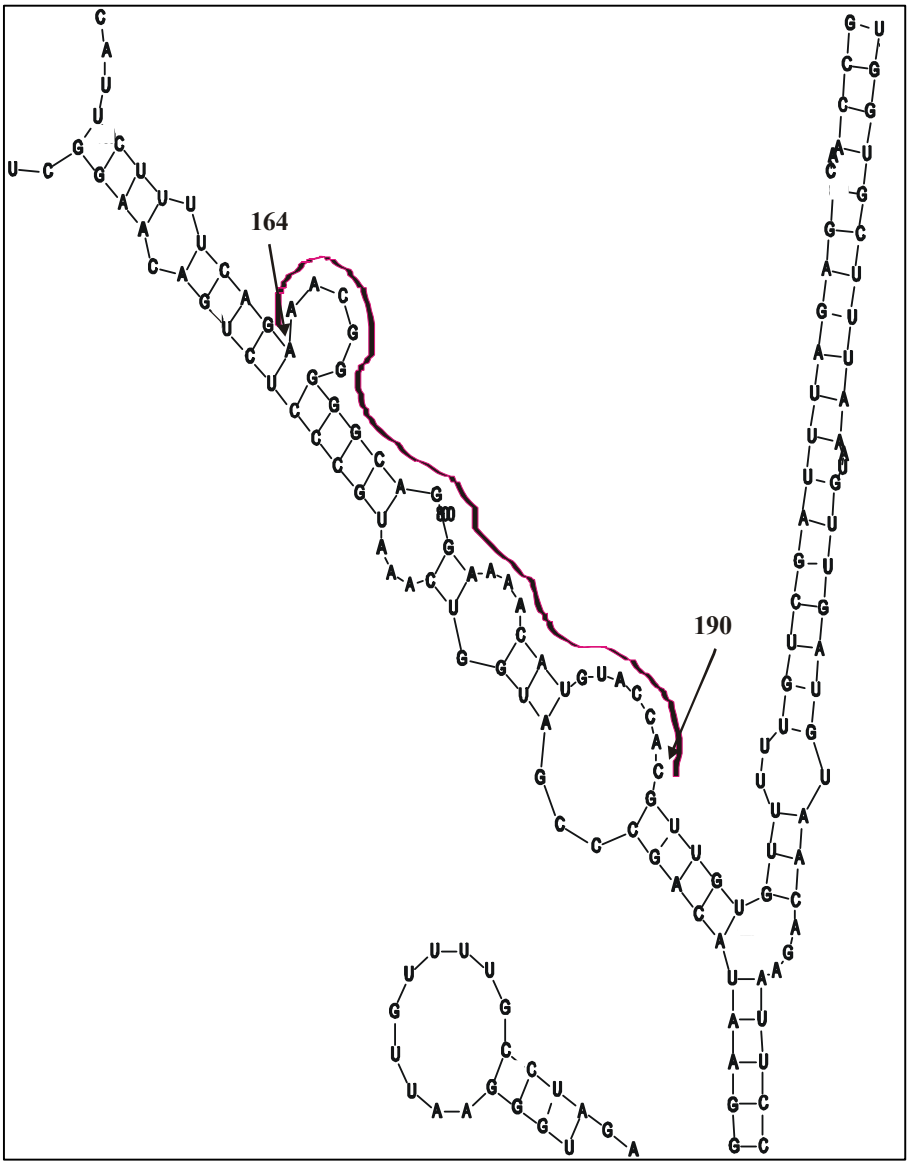
Figure 2.7 A: The optimal ODN hybridization sites in the predicted secondary mRNA structures of Tub 1, 3, 5, 7, 11 and 14: Nucleotides 118-144 (magenta area)(same secondary structure observed in the indicated region).

The nucleotide sequence 118 – 144 in the relevant mature mRNA structures where this sequence was predicted to be the optimal hybridization site, spans two hairpin loops and part of an internal loop (Fig 2.7 A). The bases –9 to 18 that were targeted by AS-1 include the initiation codon and span a bifurcation, an internal loop and part of a second bifurcation (Fig 2.7 B).



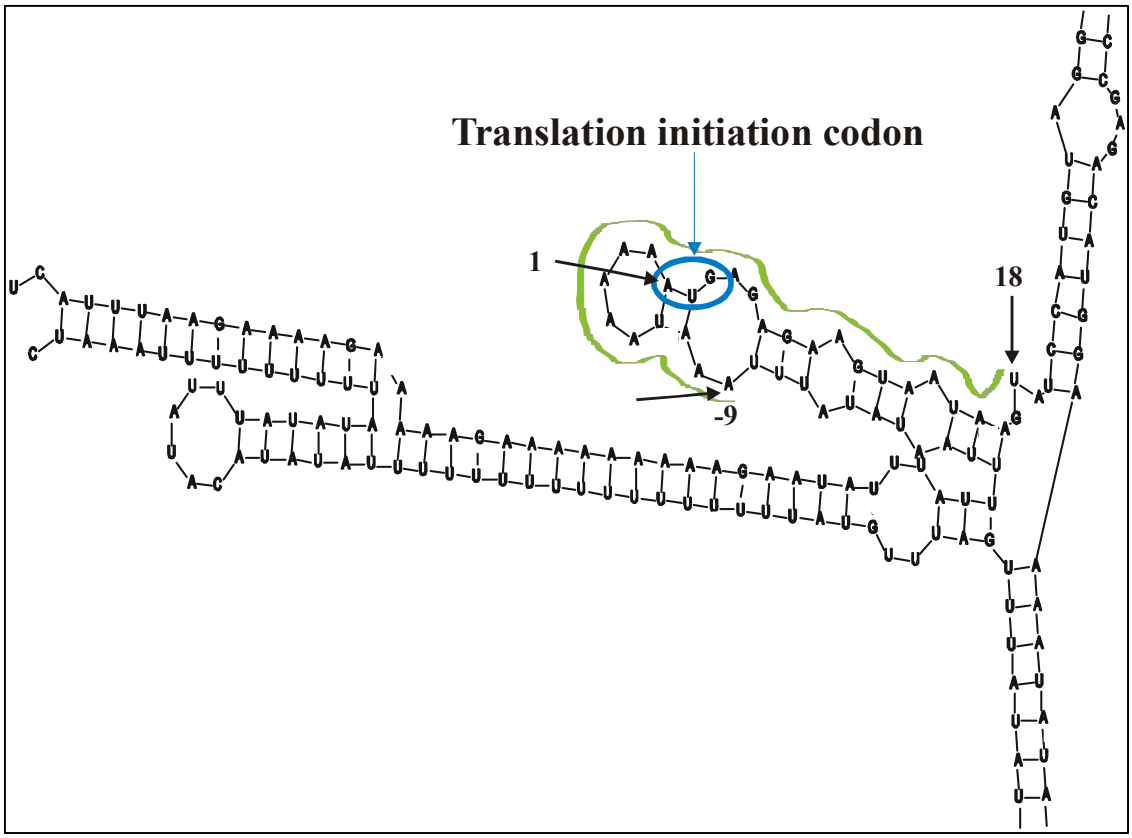
B

Figure 2.7: B: The optimal ODN hybridization sites in the predicted secondary mRNA structures of Tub 1, 3, 5, 7, 11 and 14: Nucleotides –9 to 18 (green area) is the region to which antisense ODN AS-1 is targeted and includes the initiation codon.



A

Figure 2.8: A : The most accessible ODN hybridization sites in the predicted secondary mRNA structures of Tub 4 and 16: Nucleotides 164-190 (magenta area)(same secondary structure observed in the indicated region).



B

Figure 2.8: B: The most accessible ODN hybridization sites in the predicted secondary mRNA structures of Tub 4 and 16: Nucleotides –9 to 18 (green area) is the region to which antisense ODN AS-1 is targeted and includes the initiation codon.

The predicted accessible nucleotide sequence 164 – 190 is shown to span one bulge and three internal loops (Fig 2.8 A). The sequence –9 to 18 targeted by AS-1 spans a hairpin loop, three internal loops and part of a bifurcation (Fig 2.8B).

Based on the criteria stated earlier on the properties of a ODN hybridization site and the results obtained from secondary structure predictions of α-1-tubulin mRNA, the most promising accessible binding areas for a 27-mer antisense ODN appear to include the

nucleotides 92 - 118 and 118 –144 (Figures 2.6 and 2.7A and Table 2.3). Both of these sequences span hairpin loop structures in the relevant predicted mRNA secondary structures. The nucleotides 164 – 190 do not span a hairpin loop structure in the relevant predicted mRNA structures and this sequence was therefore not included as a suitable antisense ODN binding site.

2.3.1.3. Design of an antisense ODN targeted against the translation initiation codon

The antisense ODN targeted against the translation initiation codon region, along with the sense and mismatch control sequences are shown in Table 2.4.

Table 2.4: Sequences and Tm values of the antisense ODN (AS-1), targeted against *P. falciparum* alpha-I-tubulin initiation codon region as well as the sense and mismatch control sequences. The translation initiation codon is underlined.

ODN Name Nt	**SEQUENCE**	**Tm[1]**
Antisense ODN (AS-1)	5' ACT TAT TAC TTC TCT CAT TTT TTA TTT 3’	64.1 °C
Mismatch ODN (MS-1)	5' TTT ATT TTT TAC TCT CTT CAT TAT TCA 3’	64.1 °C
Sense ODN (S-1)	5’ AAA TAA AAA ATG AGA GAA GTA ATA AGT 3’	64.1 °C

[1]81.5 + 16.6log1 + 0.41x(%GC) – (675/L), L=27 (%GC method) (Howley, 1979).

Fifteen bases downstream, nine bases upstream from and including the initiation codon were targeted by the antisense ODN. The mismatch control is the inverse of the antisense

sequence to ensure a similar base composition between the antisense and mismatch control ODNs (Francois *et. al*., 2000).

The 3' terminal of the antisense ODN, as well as the sense and mismatch control sequences were structurally modified by the addition of a 15 base self-complementary 3'-loop in an attempt to protect the phosphodiester ODNs against 3'exonuclease degradation (Barker *et. al*, 1998). The modified ODNs i.e. the ODNs containing this loop, are referred to as ASL-1, MSL-1, SL-1, ASL-2, MSL-2, ASL-3 and MSL-3, respectively. All ODNs were investigated for sequence specificity against the *P. falciparum* and human sequences available at the time of ODN design (1997/1998) as well as retrospectively (April 2005) and are shown and discussed in Chapter 4.

The full sequences of the ODNs including the chosen loop modification, are shown in Table 2.5.

Table 2.5: Sequences and Tm values of the structurally modified antisense ODN, ASL-1 targeted against *P. falciparum* α-I-tubulin initiation codon (ODN: ASL-1, together with the sense and mismatch control sequences) and *P. falciparum* α-I-tubulin internal sequences (ASL-2 and ASL-3, together with mismatch control sequences). The 3'-loop region is shown in bold and the translation initiation codon is underlined.

ODN Name	**SEQUENCE**	**Tm**[1]
Antisense ODN (ASL-1)	5' ACT TAT TAC TTC TCT CAT TTT TTA TTT **GCG CGC TTT GCG CGC** 3'	64.1 °C
Mismatch ODN (MSL-1)	5' TTT ATT TTT TAC TCT CTT CAT TAT TCA **GCG CGC TTT GCG CGC** 3'	64.1 °C
Sense ODN nt -9 – 18 (SL-1)	5' AAA TAA AAA ATG AGA GAA GTA ATA AGT **GCG CGC TTT GCG CGC 3'**	64.1 °C
Antisense ODN (nt 92 – 118) (ASL – 2)	5' TGT CAG AGG GCA TTT GAC CAT CGG GCT **GCG CGC TTT GCG CGC** 3'	79.2 °C
Mismatch ODN (MSL-2)	5' TCG GGC TAC CAG TTT ACG GGA GAC TGT **GCG CGC TTT GCG CGC** 3'	79.2 °C
Antisense ODN (nt 118 – 144) (ASL – 3)	5' AGC ATC ATC ATT AGC TCT AGA AGC CTT **GCG CGC TTT GCG CGC** 3'	73.2 °C
Mismatch ODN (MSL-3)	5' TTC CGA AGA TCT CGA TTA CTA CTA CGA **GCG CGC TTT GCG CGC** 3'	73.2 °C

[1]81.5 + 16.6log1 + 0.41x (%GC) – (675/L), L= 27 (% GC method) **(**Howley, 1979**).** The Tm of the hairpin loop alone (GCG CGC TTT GCG CGC) was calculated to be 69.3 °C with a ΔG value of –40.3 kcal/mol.

Analysis with the Oligo 4.1 Primer Analysis Software (National Biosciences Inc.) indicated no dimer or loop formations, other than the 15-base self-complementary 3' terminal loop.

2.4 Discussion

The choice of *P. falciparum* α-I-tubulin mRNA as the target for antisense ODNs in these studies was based on the central role that microtubules, of which tubulin is the major component, play in vital cellular functions such as cell division, to name but one (Jordan and Wilson, 2004). The α-I-tubulin protein, in particular is known to have regulatory functions in parasite glycolysis (Itin *et. al*., 1993). The choice of tubulin as target protein was further supported by the fact that numerous anticancer agents, inhibiting microtubule functioning were also effective inhibitors of *P. falciparum* proliferation (Bell, 1998). The problem with this approach however is the specific inhibition of the parasite tubulin without adversely affecting the mammalian protein. This aim is complicated by the fact that there is an 83% amino acid identity between the mammalian and *P. falciparum* α-tubulin proteins (Holloway *et al*., 1989). Antisense ODNs, that target the mRNA and thus the translation of the protein target and not the protein itself, are advantageous in this particular instance due to the 80% A + T rich *P. falciparum* genome as opposed to the 60% G + C rich human genome (Gardner *et al*., 2002). This difference in nucleotide composition enables the specific targeting of the *P. falciparum* α-I-tubulin mRNA.

The design of three phosphodiester antisense ODNs targeting different regions of *P. falciparum* α-I-tubulin mature mRNA is described in this chapter. RNA secondary

structures have been shown to affect the hybridization ability and hence the efficacy of antisense ODNs (Crooke, 2000; Smith *et al*, 2000; Sohail and Southern, 2000; Vickers *et al*, 2000). Two different approaches were used in the design of the ODNs. In the first approach the ODN was targeted to regions adjacent to and including the translation initiation codon. This strategy was chosen due to the results of the majority of studies reporting successful antisense-mediated inhibition by ODNs targeted to this region (Crooke, 2000; Francois *et al*., 1994; Sohail and Southern, 2000). In the second approach the predicted secondary structures of the mature mRNA were used in the design of two further ODNs targeted to the predicted accessible sites for ODN hybridization. Inclusion of the latter strategy was important since mRNA is known to have secondary and tertiary structures rendering certain sites inaccessible to ODN hybridization (Ho *et al*., 1996).

Several groups have reported the utility of computer-generated secondary structures to predict ODN hybridization sites (Cirino *et al*, 1997; Lima *et al*, 1992; Thierry *et. al*., 1993) whilst others have criticized this approach (Smith *et al*, 2000; Sohail and Southern, 2000). The points of criticism were aimed at two aspects of computer predictions. The first one being that tertiary structures are not taken into account when determining accessible antisense ODN target sites with computer algorithms used to predict the secondary mRNA structures. The second criticism is that large RNA molecules probably do not reach one global minimum energy structure, i.e. one optimal structure (Smith *et al*, 2000; Sohail and Southern, 2000). A structural hierarchy exists in the unfolding and folding process of RNA molecules, with secondary structures forming first (Leontis and Santa-Lucia, 1997). Tertiary structures then ensue from interactions between these

secondary structures and the reverse process takes place when the RNA molecules unfold. This structural hierarchy therefore refers to the preferential unfolding of the less stable tertiary structures compared to the more stable secondary structures. Earlier observations along these same lines led Tinoco and co-workers (1973) to postulate that the use of computer algorithms to predict secondary mRNA structure could indeed be useful in the identification of hybridization sites. Furthermore, most of the computer algorithms used to predict RNA secondary structures can generate optimal and sub-optimal structures, thereby fully acknowledging the fact that no single optimal RNA structure is continually present (Leontis and Santa-Lucia, 1997).

Computer generated RNA structures therefore appear to be useful due to the saving in time and cost, which is considerable compared with the random shot-gun or gene walking approach to identify successful RNA target sites for antisense ODNs. The number of possible antisense ODNs to be tested is narrowed down significantly. For example, calculations showed that 1981 oligonucleotides should be tested individually in order to identify the best 20-mer antisense ODN to target a 2-kilobase mRNA molecule when using the random shot gun approach (Sohail and Southern, 2000).

Of the three possible programs available for secondary structure predictions, the *Mfold* program allows the determination of optimal and sub-optimal secondary structures at a physiological temperature of 37 °C. The optimal as well as sub-optimal secondary structures of α-I-tubulin mature mRNA were determined. This was deemed necessary due firstly, to the fact that RNA molecules are dynamic structures and secondly due to the

fact that slight differences in the free energy parameters may have profound effects on the secondary structure of the RNA molecule. It is therefore important to utilize methods that allow an approximation of these parameters. *Mfold* utilizes three methods to achieve these goals.

Firstly an empirical method is used, which is based on the free energies of optical melting experiments for representative sequences. A second method bases the assignment of stability (or free energy) to unpaired regions on the frequency with which sequences occur in a specific motif in a database of phylogenetic structures. The third method varies the free energy parameters so as to optimize the accuracy of the computer-generated sequences against their phylogenetically determined structures (Riesner, 1997). The *Mfold* program is therefore capable of predicting optimal and sub-optimal structures for comparison in order to determine which regions remain the same upon slight changes of these free energy parameters (Riesner, 1997).

Eighteen secondary structures of the mature mRNA sequence of α-I-tubulin were predicted by *Mfold*, one optimal structure and 17 sub-optimal structures, the latter having ΔG values within 20% of the ΔG value of the optimal structure. Three potential (accessible) target sequences were identified (Figs 2.6 – 2.8 and Table 2.3) that incorporated nucleotides 92-118; 118-144 and 164-190). The identification of these three sequences was primarily based on the percentage prevalence, i.e. the number of structures in which the relevant sequence was predicted to be the most accessible hybridization site (Smith *et al*, 2000).

The sequence 118-144 was the most prevalent (33%) with sequences 92 -118 and 164-190 being accessible in 11% of the structures (Table 2.2). All the other sequences were found to be accessible in only 5.6% of the predicted structures and therefore not included in the list of potential target sites. The sequence 118-144 included 2 hairpin loops and part of an internal loop while the sequence 92-118 contained a hairpin loop and an internal loop. Sequence 164-190 on the other hand included 1 internal bulge and 3 internal loops. Sequences 92-118 and 118-144 were chosen as targets for antisense ODNs due to the presence of hairpin loop structures, which were shown to be the most effective targets for antisense ODNs and needed to initiate heteroduplex formation (Eguchi *et. al*., 1991; Frauendorf and Engels, 1996; Southern *et. al*., 1994; Southern *et. al*., 1999; Toulme *et. al*., 1996; Lima *et al.,* 1992; Thierry *et al.,* 1993). Studies using yeast tRNA which is structurally well characterized in solution, revealed that stable secondary structures prevent heteroduplex formation (Sohail and Southern, 2000).

The choice of sequence –9 to 18 targeted by AS-1 was based on the premise that this region is less obstructed by secondary structure formation due to the presence of amongst others, the ribosome binding site and the translation initiation codon (Sohail and Southern, 2000). Four of the predicted structures (Tub 4, 8, 9 and 16) contained a hairpin loop along with internal loops in this translation initiation region. This means that 22% of the predicted *P. falciparum* α-I-tubulin mRNA secondary structures contained a hairpin loop structure in this region. This compares favourably with sequences 92-118 and 118-144 in so far as each of these also contained at least 1 hairpin loop structure.

All ODNs were 27 nucleotides long, an acceptable length for specificity since a 17-mer ODN should already find a unique target within any genome (Francois *et al*, 1994). There is no fixed maximum limit for antisense ODN length, but generally the hybridization regions of the ODNs are in the range of 15-25 nucleotides (Phillips and Zhang, 2000; Riesner, 1997). However, since efficient cellular uptake is a prerequisite for all antisense ODNs and decreases with increasing ODN length, the latter should be kept as short as is possible while still allowing the achievement of sequence specific inhibition of target protein translation (Crooke, 2000). Furthermore, since ODN modifications and cell lines also have an influence on cellular ODN uptake, the decision of the length of ODN is largely an empirical one. In this particular study the choice of ODN length was also influenced by the high A + T content of the *P. falciparum* genome, a characteristic that decreases the Tm of any ODN targeted against the parasite DNA or RNA (Gardner, 2001). The lower Tm could in turn negatively influence the hybridization efficacy of the ODN. For this reason a longer 27-mer ODN with a correspondingly higher Tm was decided upon rather than a shorter, 15-mer ODN for example.

The inclusion of the correct control sequences is also extremely important in the design of antisense experiments. The selected mismatch ODN sequences were the inverse of their respective antisense sequences. This ensured a difference only in the base sequence and not the composition, which is regarded as a standard requirement for evaluation of antisense ODN results (Francois *et al*, 1994).

The effect of the self-stabilizing loop structural modification on the stability of phosphodiester ODNs in culture medium, targeted against *P. falciparum* mRNA has not yet been investigated. The stability of the three ODNs designed in this chapter was therefore investigated in culture medium supplemented with serum and the results are reported and discussed in the next chapter.

CHAPTER 3

Stability of modified antisense ODN under culture conditions

3.1. Introduction

Nuclease degradation may affect the specificity and /or efficiency of ODNs and their stability under physiological conditions is therefore particularly important. Fifteen self-complementary bases were added at the 3'-end of the antisense phosphodiester ODNs as described in the previous chapter in an effort to prevent exonuclease degradation. There are three commonly used and accepted techniques to investigate the stability of ODNs under standard culture conditions. These include capillary electrophoresis (CE), anion exchange HPLC or reversed phase HPLC analyses of culture extracts after pre-incubation with ODN (Giles *et. al*., 2000; Kemp, 1998; Deshmukh *et. al*., 2000). The most appropriate method for analyses was selected after consideration of their principles of operation and general applicability for such studies. The costs associated with the methods and availability of equipment were additional factors taken into consideration when deciding on the most appropriate method to be used for this application.

3.1.1. Capillary Electrophoresis

This method is based on the migration of charged species dissolved or suspended in an electrolyte under the influence of an electric current. Detection is typically performed by UV absorbance. Sample injection occurs by placing one end of the capillary tube into a sample vial followed by the application of an electric field or gas pressure (Kemp, 1998).

There are different types of CE of which capillary zone electrophoresis (CZE) is the most commonly used method to separate different sized ODNs as well as DNA based on the migration of particles determined by the charge/size ratio (Kemp, 1998). Most of these capillaries are typically 30 – 50 µm in diameter and may have a hydrophobic coating that serves to mask the surface charge intrinsic to the capillary tube and thus prevent undesired electro osmotic flow (EOF) (Kemp, 1998). The Hewlett Packard Oligonucleotide Analysis kit (Hewlett Packard) makes use of a PVA (polyvinyl alcohol)-coated capillary tube, which eliminates EOF and suppresses interactions between particles and the inner capillary wall. Alternatively, use is also made of a polymer sieving solution or low-percentage polyacrylamide gel-filled capillaries (Hewlett Packard Oligonucleotide Analysis Kit Information Brochure)

Although capillary electrophoresis has been successfully used for quantification of ODNs, the gel columns utilized in CE are subject to interference by biological media (Bansal *et al*, 2001). Extensive treatment (clean-up) of ODNs incubated in biological samples, including precipitation or anion exchange HPLC followed by reversed phase HPLC, is required prior to CE analysis. The reason for this is that protein and salt interfere with the amount of sample that can be applied to gel-filled capillaries by electrokinetic injection (Leeds *et. al*., 1996). The implication of the above is that determination of ODNs from biological samples by CE is a costly and time-consuming procedure (Bansal *et. al*., 2001; Leeds *et. al*., 1996).

3.1.2. High Performance Liquid Chromatography (HPLC)

Anion exchange or reversed phased HPLC are routinely employed for both the purification and analysis of oligonucleotides.

3.1.2.1. Anion Exchange HPLC (AE HPLC)

Separation by AE HPLC is based on the electrostatic interaction between the negatively charged phosphate groups of the phosphodiester ODN or DNA backbone and the positively charged anion exchange matrix. The ODNs are desorbed from the column by a gradual increase in the ionic strength of the eluting buffer. Due to the relationship between the length and net charge, shorter ODNs elute earlier from these columns (Borque and Cohen, 1993, 1993; Deshmukh *et. al*., 2000).

Weak anion exchange (WAX) columns are packed with a porous resin containing diethylaminoethyl (DEAE). The strong anion exchange (SAX) columns on the other hand are packed with resins containing quaternary alkylamines. The different properties of these column types are especially relevant for the resolution of phosphorothioate (PS) ODN analogues (non-bridging oxygen atoms replaced by sulphur atoms). The SAX column is generally employed if the PS ODN contains 10 nucleotides or less while longer ODNs are resolved with WAX columns. The strong association between the longer PS-ODNs (>10 nucleotides) and the SAX resin necessitates the use of strong halide displacers (LiBr or LiCl) in the mobile phase instead of the ordinary phosphate, sulphate or perchlorate containing mobile phases (Metlev and Agrawal, 1992; Borque and Cohen, 1993).

3.1.2.2. Reversed phase HPLC (RP HPLC)

In RP HPLC the elution order of the molecules to be separated is determined by their hydrophobicity. Usually the hydrophobicity of the mobile phases used to elute the sample from the column, is controlled by the addition of water miscible organic solvents such as alcohols or acetonitrile.

Phosphorothioate (PS) ODNs longer than 10 bases are not easily purified on reversed phase columns due to their increased hydrophobicity compared to phosphorodiester (PO) ODNs (Agrawal *et. al*., 1990; Thayer *et. al*., 1996). As a result, PS ODNs are usually separated by anion exchange HPLC (Agrawal *et. al*., 1990). Reversed phase HPLC has been shown to be very useful for the purification and analysis of PO ODNs provided that the negative charge of the phosphate groups in the phosphodiester backbone is first neutralized by a counter-ion such as triethylammonium (ion-pair reversed phase chromatography; Kwiatkowski *et al.*, 1984).

The latter method was utilized to determine the stability of the modified PO ODN, ASL-1, in culture medium. It was assumed that all the ODNs would exhibit similar properties to ASL-1 due to the equivalent length, similar base composition and the same 3'-self-complementary loop modification. The separation and stability of only ASL-1 (designed in chapter 2) is therefore described below.

3.2. Materials and Methods

3.2.1. **Materials**

Reversed phase C_{18} columns were purchased from Phenomenex (Torrance, California, USA). Triethylammonium acetate (TEAA) was purchased from Fluka as a 1 M stock solution (pH 7.0). Acetonitrile, chloroform, diethylether, ethanol, isoamyl alcohol, methanol (all analytical grade), D-glucose, EDTA, hypoxanthine, sodium chloride, sodium dodecyl sulphate (SDS), sodium hydrogen carbonate and TRIS were all purchased from Merck. Glycogen, proteinase K, LiCl and oligonucleotides were obtained from Roche Diagnostics. *Sec*-butanol, RPMI-1640, L-glutamine, gentamycin and HEPES buffer were purchased from Sigma.

3.2.2. **Methods**

3.2.2.1. Optimization of separation and elution conditions of ODN ASL-1 on reversed phase HPLC

The C_{18} column was conditioned and equilibrated with 0.1M triethylammonium acetate (TEAA) in 80% acetonitrile according to the manufacturer's instructions. TEAA is used as counter-ion in ion pair chromatography and was applied in these studies.

3.2.2.2. HPLC analysis of ODN samples

A Varian 9012 HPLC system (Waters) was used in these investigations. A Phenomenex LUNA C_{18} column (dimensions: 150 x 4.60 mm) was employed. Elution buffer A consisted of 0.1M TEAA and buffer B of 0.1M TEAA supplemented with varying

percentages of acetonitrile. Various gradients were investigated to optimize the elution conditions (Table 3.1).

For these investigations ODNs were injected onto the C_{18} column directly. Elution was conducted at a flow speed of 1 ml/min and monitored at a wavelength of either 260 nm or 270 nm (Borque and Cohen, 1983; Deshmukh *et. al*., 2000).

3.2.2.3. Method used for extraction of ODNs from parasite culture medium

3.2.2.3.1. Fluid phase extraction

For this extraction 0.25 µg ODN (dissolved in 0.5 µl double distilled, sterile water) was incubated at 37 °C for time periods specified for each experiment in 250 µl serum-containing culture medium. For extraction 250 µl DNA extraction buffer (50 mM SDS, 10 mM NaCl, 20 mM Tris, 10 mM EDTA, pH 9.0 and 20 µl of 10mg/ml proteinase K dissolved in 10 mM Tris (pH 7.5) for the digestion of serum proteins (Borque and Cohen, 1993) was added. Following incubation at 60°C for 90 minutes, 600µl chloroform/phenol/isoamyl alcohol (25:24:1) was added next, followed by vigorous mixing for 15 seconds. After centrifugation at 14 000g for 2 minutes the upper aqueous phase was removed and successively washed twice with 1 ml *sec*-butanol and once with 500 µl diethylether interspaced with a vortexing and centrifugation step as above. After removal of the upper phase, the ODN containing aqueous phase was allowed to air dry and the residue was suspended in 1 ml of 0.1 M TEAA buffer immediately prior to HPLC (Borque and Cohen, 1989).

An internal standard (IS), containing the first 15 nucleotides of the ASL-1 ODN designed in chapter 2, was included in all extractions but not incubated in culture medium. To determine the stability of ASL-1 in culture medium, the peak height of the ASL-1 (sample) ODN, incubated in culture medium for various periods of time, was expressed as a ratio of the peak height of the IS ODN, added after incubation. A decrease in this ratio would be indicative of ODN breakdown after a specified incubation time. The RP HPLC gradient conditions used in these investigations are given below in Table 3.1.

Table 3.1: The gradient conditions used to establish successful separation between ASL-1 and internal standard (IS). Buffer B: 0.1 M TEAA, 80% acetonitrile.

Mobile Phase and gradient composition	**Time period for each step (min)**	**Time at onset of each step (min)**
0% Buffer B	5.00	0.00
0% - 50% Buffer B	90.00	5.00
50% Buffer B	2.00	95.00
0% Buffer B	1.00	97.00
END		98.00
Data collection stopped after 90 minutes		

Depending on the separation distances of IS and ASL-1 it is possible to observe the appearance of degradation products only between the respective lengths of these two ODNs. This in turn would enable comparison of the results obtained with the half lives of unmodified PO ODNs as reported in the literature. ODN recoveries given in the results

were calculated by substituting the peak heights, obtained after each appropriate HPLC analysis into the equation for the standard curve (y = 85829.78x + 907.1053) and solving for the ODN concentration. This resulted in the following equation to be solved: x = (y-907.1053)/85829.78. This was done for both ASL-1 and SL-1.

3.3. Results

3.3.1. Extraction of ASL-1 and IS from serum containing medium and subsequent elution conditions from the C_{18} reversed phase HPLC column

The fluid-phase extraction method was used since standard extraction methods incorporating ethanol precipitation steps were not successful (Borque and Cohen, 1993). The method was used successfully by the latter authors for the extraction of 25-mer PS ODNs from human serum and urine. In the experiments reported here the phosphodiester ODNs were extracted from parasite culture medium and quantitated by ion pair reversed phase HPLC. ODNs were extracted after incubation at 37^{o}C for time periods ranging from 0-6 hours.

The detector wavelength was set to 270 nm so as to prevent possible interference from traces of *sec*-butanol used during the extraction process (Borque and Cohen, 1993, Niederwieser and Brenner, 1965). Several gradient conditions were investigated and the gradient settings that attained the most satisfactory separation are given in Table 3.1 (Methods). The separation achieved under these conditions is shown in Figure 3.1.

The separation time between the two ODNs is 2.29 min, with the IS eluting at 36.30 minutes and ASL-1 at 38.59 minutes. No peaks were observed after extraction of incubation medium without prior addition of ODN (results not shown).

To determine the extent of ODN degradation under incubation conditions, the above extraction and HPLC conditions were applied in the next series of experiments with ASL-1.

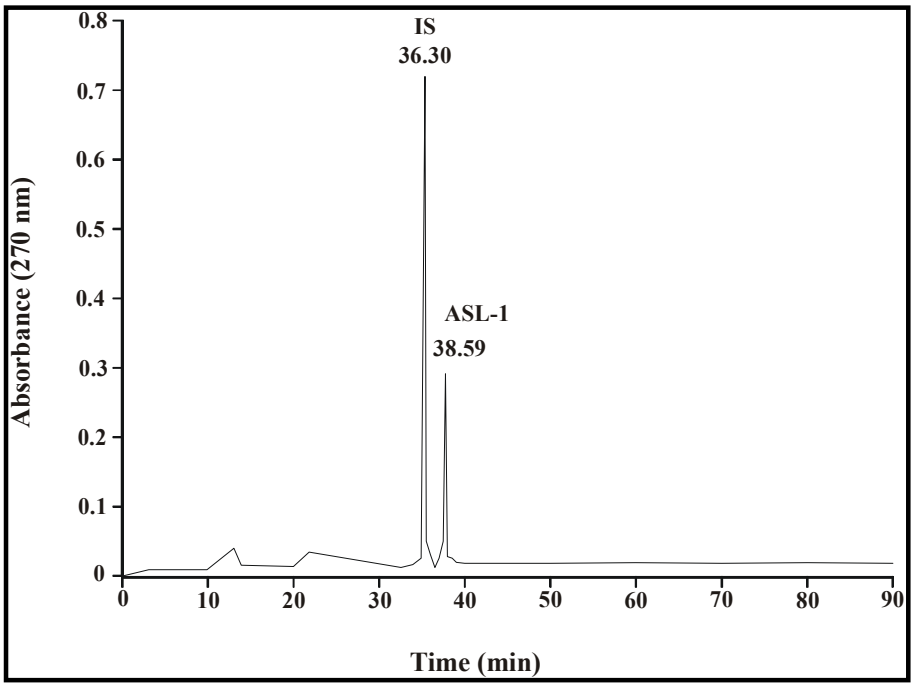


Figure 3.1: A typical reversed phase HPLC chromatogram (Varian 9012 HPLC) obtained using a Phenomenex LUNA C_{18} column. The antisense (0.3 µM) ODN ASL-1 was incubated at 37 °C for four hours followed by extraction of ASL-1 and IS (0.9 µM) ODNs using the fluid phase extraction procedure. Gradient conditions used are given in Table 3.1.

3.3.2. Determination of ODN stability

A calibration curve of peak height versus ASL-1 concentration was set up and is shown in Figure 3.2.

The calibration curve had a correlation coefficient (r^2) of 0.9985, which was indicative of linear ODN detection. The ODN, ASL-1 was incubated at 37°C in serum-containing culture medium from one to six hours, extracted along with the internal standard added at the end of the incubation period, and separated by reversed phased C_{18} column chromatography. The results are shown as the ratio of sample to standard peak height, plotted against incubation time (Figure 3.3).

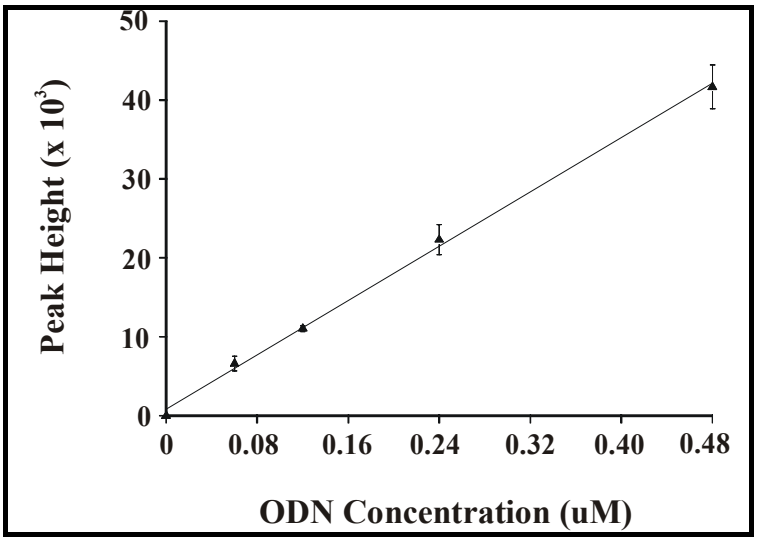


Figure 3.2: Calibration curve (r^2 = 0.9985) showing the relationship between sample ODN peak height obtained after HPLC and ODN concentrations ranging from zero to 0.48 µM. Each data point is expressed as the average ± SEM of three repeats. Antisense ODN ASL-1 was directly injected at the given concentrations. The straight line equation was y = 85829.78x + 907.1053.

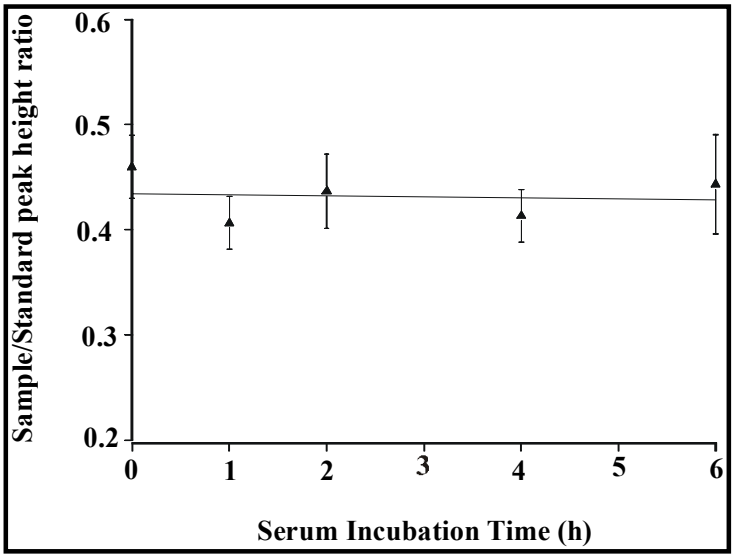


Figure 3.3: Stability of a modified phosphodiester antisense ODN, targeted against *P. falciparum* α-I-tubulin, after incubation in serum-containing medium for time periods ranging from zero to six hours. Prior to extraction and subsequent HPLC analysis, internal standard ODN (0.9 µM) was added to the incubated sample ODN (ASL-1) (0.3 µM). The sample/standard ODN peak height ratio was determined with increasing incubation time to assess ASL-1 stability. Each data point is expressed as the mean ± SEM of three repeats.

The average ratio of sample peak height to internal standard peak height over the 6 hour incubation period was 0.43 ± 0.024. This result is similar to a ratio of 0.44 ± 0.078 obtained for non-incubated, non-extracted ODNs (results not shown), suggesting that the modified antisense phosphodiester ODN, ASL-1 is stable up to 6 hours after incubation in serum containing medium.

The recoveries of both ASL-1 and IS after HPLC analysis were determined after each incubation and are shown in Table 3.2.

Table 3.2: The recoveries of both ASL-1 and IS ODNs from culture medium after the specified incubation periods of ASL-1, followed by fluid phase extraction of both ASL-1 and IS.

Incubation time of ASL-1(h)	**ASL-1 recovery (%) ± S.E.M**	**IS recovery (%) ± S.E.M**
1	12 ± 1.41	12 ± 1.89
2	11.2 ± 1.84	9.9 ± 1.50
4	9.7 ± 3.60	9.6 ± 4.20
6	11 ± 0.80	9.9 ± 0.78

From the table above it is noteworthy that the recoveries of both ASL-1 and IS after fluid phase extraction and HPLC analysis, differ from each other by no more than 1,3% for each specific incubation time.

3.4. Discussion

Nuclease degradation of unmodified phosphodiester ODNs is a problem in antisense studies since both sequence specificity and efficacy are compromised (Crooke, 2000). This is due to the fact that shorter ODN segments are more likely to be complementary to parasite mRNA sequences other than the target sequence as well as to host sequences. In order to prevent or limit degradation of ODNs several modifications are employed as were described in Chapter 1. The structurally modified ODN, ASL-1 targeted the upstream and downstream regions including the translation initiation codon of *P. falciparum* α-I-tubulin. The other two ODNs targeted downstream regions of α-I-tubulin mRNA and were designed using computer-based predictions of the most favourable hybridisation sites. All three ODNs were of the same length and included a 3'-hairpin loop for protection against 3' exonuclease degradation. After ASL-1 incubation in culture medium it was necessary to extract the ODN prior to HPLC analysis in order to remove the unwanted protein and lipid components of the medium.

A fluid-based extraction method was used since the standard precipitation methods using ethanol are designed to eliminate ODNs having the length of ASL-1 (Sambrook *et. al*., 1983). The method followed made use of a protein digestion step prior to a standard extraction with phenol/chloroform/isoamyl alcohol. This was followed by two successive *sec*-butanol washes, the purpose being to remove traces of phenol, lipid-soluble components as well as water. An extraction with diethylether was included to remove most of the *sec*-butanol and phenol from the concentrated ODN extract. This method was

considered applicable since it was first used to extract PS modified ODNs from human urine and serum (Borque and Cohen, 1993). Figure 3.2 showed that extracted ASL-1 was detected when using the HPLC method described.

The HPLC method was chosen in preference to capillary electrophoresis (CE) due to the additional purification steps required for samples extracted from biological media, prior to CE analysis (Bansal *et. al*., 2001; Leeds *et. al*., 1996). Reversed phase rather than anion exchange HPLC was the preferred method since the former was shown to be more suited for the purification and analysis of phosphodiester ODNs (Agrawal *et. al*., 1990; Bansal *et. al*., 2001; Bourque and Cohen, 1993; Toulme *et. al*., 1996). Elution of ASL-1 from the C_{18} column was achieved using 0.1 M TEAA, a standard ion-pairing reagent as mobile phase A and mobile phase B being supplemented with 80 % acetonitrile (Figure 3.1). These were standard elution conditions for PO-ODNs from this type of column (Apffel *et al*., 1997; Toulme *et al*., 1997). However, sample to sample variation made the use of an internal standard imperative. It was decided to use a 15-mer ODN (IS) for this purpose and to adjust the HPLC gradient such that ASL-1 and IS were completely separated (Figure 3.1).

The sample (ASL-1)/standard (IS) peak height ratios, obtained after the various ASL-1 incubation times, were calculated and plotted against ASL-1 incubation time. A decrease in this ratio over the 6 hour incubation period would be indicative of ASL-1 degradation. In this regard it is important to note that it is advantageous to make use of peak height rather than peak area. ODN degradation could give rise to a decrease in the peak height

accompanied by a simultaneous peak broadening. In this instance no change in the peak area would be measured and quantification of the intact would be problematic.

This chosen method is therefore capable of indicating the stability of a modified ODN over an unmodified counterpart. Different methods would have to be employed to accurately determine the degradation of a modified ODN under specified conditions over time. These methods would include capillary electrophoresis, which allows for the separation and detection of ODNs differing in length by only a single nucleotide.

After ASL-1 incubation in culture medium for 1, 2, 4 and 6 hours, respectively, ASL-1/IS peak height ratios were plotted against time of ASL-1 culture medium incubation (Figure 3.5). The results indicated that ASL-1 was not degraded after 6 hours of incubation in culture medium. This result is further supported by the similar recovery values calculated for both ASL-1 and IS over the 6 hour ASL-1 incubation period (Table 3.3). The observed stability of the modified PO-ODN is significant since the half-life of an unmodified PO-ODN in culture medium is in the range of minutes (Gilar *et. al*., 1997; Phillips and Zhang, 2000).

The observed recovery of ODNs from culture medium is consistently low and thereby highlight the difficulties associated with retrieving short ODNs from a variety of biological media (Borque and Cohen, 1993; Sambrook *et. al*., 1983). The extraction procedure performed in these experiments allowed for HPLC analysis of ODNs (ASL-1 and IS) after incubation in culture medium to determine the stability of the structurally

modified ASL-1. It must however be noted that the studies performed give no indication of ASL-1 half-life in culture medium or possible cleavage of smaller numbers of nucleotides (2-3 nucleotides for example) from the full length ODN. To obtain this information would require improvements in the efficiency of the extraction method and a method such as capillary electrophoresis to separate starting material from degraded products. A detailed analysis of each step of the extraction process could be undertaken in order to determine where most of the ODN is being lost. If the problem should prove to be in the actual extraction process it may be necessary to investigate the effect of changes in the composition of the extraction buffer (SDS, NaCl and proteinase-K) on the extraction efficacy of the method. It is also possible that extracted ODNs are being lost during the wash steps with *sec*-butanol and/or diethylether.

The inclusion of an unmodified PO-ODN, having the same sequence as ASL-1 but lacking the structural modification, would furthermore enable a direct comparison between the stabilities of the respective ODNs in culture medium as well as differences in inhibitory efficacy between the two ODNs.

The results obtained have however, shown that further studies on ASL-1, including inhibitory efficacy, are warranted. The results of investigations on the uptake of the modified antisense ODNs in relation to their inhibition of parasite propagation in erythrocyte cultures are presented in the next chapter.

CHAPTER 4

Uptake and efficacy of modified phosphodiester ODNs to inhibit *in vitro* erythrocytic cultures of *P. falciparum*

4.1. Introduction

Antisense technology has been successfully applied in several studies and Table 4.1 provides a list of the antisense ODNs that have been approved by the Federal Drug Administration (FDA) or ODNs that are currently undergoing clinical trials.

Table 4.1: Oligonucleotides with FDA approval, or undergoing clinical trials (Braasch and Corey, 2002).

Oligo/Target/Company	Disease	Status
Fomivirsen/CMV[1] IE2/ISIS	CMV retinitis	Approved
Genasense/Bcl2/Genta	Cancer	Clinical Trials
ISIS-3521/PKC-a/ISIS	Cancer	Clinical Trials
ISIS 2302	Psoriasis/Crohn's disease	Clinical Trials
ISIS 14803	Hepatitis C	Clinical Trials
ISIS-5132/c-raf/ISIS	Cancer	Clinical Trials
ISIS-2503/Ha-ras/ISIS	Cancer	Clinical Trials
ISIS-104838	Crohn's disease	Clinical Trials
Gem 230/PKA/Hybridon	Solid tumors	Clinical Trials
Gem 132/CMVUL36/Hybridon	CMV retinitis	Clinical Trials
GEM 92/HIV/Hybridon	AIDS	Clinical Trials
INX 3280/Myc/INEX	Restenosis	Clinical Trials
MG 98/DNA methyl transferase/MethylGene	Solid tumors	Clinical Trials

1: Cytomegalovirus

It is noteworthy that Formivirsen, a phosphorothioate DNA oligomer targeted against cytomegaloviral-induced retinitis, has received FDA approval. It is administered by injection into the eye and was regarded as a breakthrough in the field.

It has recently been discovered that antisense RNA transcription occurs more frequently in the malaria parasite than initially believed. When analyzing both sense and antisense transcript profiles from mixed asexual stage parasite populations it was found that approximately 12% of the expressed Serial Analysis of Gene Expression (SAGE) tags were in the antisense orientation (Gunasekera *et. al*., 2004). It is therefore possible that the malaria parasite uses natural antisense RNAs to modulate its own gene expression but this hypothesis requires further experimental evidence.

For studies reported here, *P. falciparum* α-I-tubulin was chosen as target. The reasons for this choice were discussed in chapter 2 and at this point it is important to consider some further aspects surrounding the amino acid and nucleotide sequences of the target. The high amino acid identity between the parasite and human protein makes specific targeting and inhibition of the parasite protein by any means problematic. However, the parasite and human codon preferences differ substantially from each other due to the remarkable A+T rich nature of the *P. falciparum* genome (Gardner *et al*, 1999; Gardner, 2001; Munasinghe *et al*, 2001; Rojas *et. al*., 1996; Withers-Martinez *et al*, 1999). Thus, even though the proteins differ very little at the amino acid sequence level, the nucleotide sequences differ enough to make antisense technology an attractive alternative therapeutic for malaria. This point is illustrated when the respective amino acid and nucleotide sequences of *P. falciparum* and *H. sapiens* are compared (Figure 4.1).

```
P. falciparum NUC   -625  atata tatatatata tttgtacaga aaatatttta ataaaaaaaa aaaaaaattt taagcatttc atatttatat acacttgttt ttttatgtgt tgaaa  -525
H. sapiens    NUC   -518  ----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------ga attca  -525

P. falciparum NUC   -526  gaaaa aaaaaaatta tgctatatca tatttatatg accattttaa tgatactgtg aaagtataca tatataatta attatataat aataataata tatat  -425
H. sapiens    NUC   -526  tgccg ttgggtggag tcagcgcccc caggctctac ttggaaaacc tttaagctct tttctttcgt aagctctctg ggcgagggtg gtggtatgtt ttgtg  -425

P. falciparum NUC   -426  ataca tatatatata atactctgtt tttctttttt tttttctttt tctttttcct ttttttttt ttttgcactc taaaggtttg agtaagtaaa tatta  -325
H. sapiens    NUC   -426  aggtt tagcttagcc ccaaatcctc aagccccgcc gccgccgcta gtgcggtgca ggaaccgggc cagtactgcg cccagggaca gagcgctggg gagga  -325

P. falciparum NUC   -326  tatat aatatatata aaagtataat ttttttacaa ataattaata gatttatttt ataagaatta aaaataataa aattaaaaaa aaaaaaaaga aaaat  -225
H. sapiens    NUC   -326  acaaa ggcggcgcta ggctgtgtta tccgagagat ctttcggggg ccgcgggcag cccgtcctgc cgcgaccgag ggtctgggcg tcccggctgg gcccc  -225

P. falciparum NUC   -226  ttaca tattattata tatatatata tatatttaaa tatatattta aaaaaaaaat atattttaat gataaagaag aaatattcat acaataatta ataat  -125
H. sapiens    NUC   -226  gtgtc tgtgcgcacg gtttcgctga tgctgagggg ccactttctg tctcgcgttg ttctctgggg accgggagag gaggaggcac ccaaaaagag cgggg  -125

P. falciparum NUC   -126  atata tttgatttgt attttttttt ttttttttat atacatattt atatattttt ttttaaatca tcatttaaga aaagaaaaag aaaaaaaaag aatat  -25
H. sapiens    NUC   -126  gcgtt gggcgagctc gggggacgtg ggaggggggaa cgggaacaaa gcgcagccta gggttagcgt gggaagaccc tccgcggtct ttggcgtttt ggaaa  -25

                                    ASL-1
                                    TTTATTTTT TACTCTCTT CATTATTCA
P. falciparum NUC    -26  ttatt ttaatatatt taaataaaaa atgagagaag taataagtat ccatgtagga caagctggta tccaagttgg aaatgcttgc tgggaattgt tttgc   75
H. sapiens    NUC    -26  gatac ccacacattc ccgggaaaac atgcgtgagt gcatctccat ccacgttggc caggctggtg tccagattgg caatgcctgc tgggagctct actgc   75
P. falciparum AA                                      M  R  E  V   I  S  I   H  V  G   Q  A  G  I   Q  V  G   N  A  C   W  E  L  F   C
H. sapiens    AA                                      M  R  E  C   I  S  I   H  V  G   Q  A  G  V   Q  I  G   N  A  C   W  E  L  Y   C
AA identity                                           *  *  *      *  *  *   *  *  *   *  *  *      *     *   *  *  *   *  *  *  *   *

                                    ASL-2
                                    TCGGGCTAC CAGTTTACG GGAGACTGT                                  ASL-3
                                                                    TTC CGAAGATCT CGATTACTAC TACGA
P. falciparum NUC     76  ctaga gcatggaata cagcccgatg gtcaaatgcc ctctgacaag gcttctagag ctaatgatga tgcttttaat acattctttt cagaaacggg ggcag  175
H. sapiens    NUC     76  ctgga acacggcatc cagcccgatg gccagatgcc aagtgacaag accattgggg gaggagatga ttccttcaac accttcttca gtgagacggg ggctg  175
P. falciparum AA        L  E   H  G  I   Q  P  D  G   Q  M  P   S  D  K   A  S  R  A   N  D  D   A  F  N   T  F  F  S   E  T  G   A  G
H. sapiens    AA        L  E   H  G  I   Q  P  D  G   Q  M  P   S  D  K   T  I  G  G   G  D  D   S  F  N   T  F  F  S   E  T  G   A  G
AA identity             *  *   *  *  *   *  *  *  *   *  *  *   *  *  *                  *  *      *  *   *  *  *  *   *  *  *   *  *

P. falciparum NUC    176  gaaaa catgtaccac gttgtgtttt tgtcgattta gagccaaccg ttgttgatga agtcagaaca ggaacttatc gtcaattatt tcatcctgaa caatt  275
H. sapiens    NUC    176  gcaag catgtgcccc gggcagtgtt tgtagacttg gaacccacag tcattgatga agttcgcact ggcacctacc gccagctctt ccaccctgag caact  275
P. falciparum AA        K   H  V  P  R   C  V  F   V  D  L   E  P  T  V   V  D  E   V  R  T   G  T  Y  R   Q  L  F   H  P  E   Q  L
H. sapiens    AA        K   H  V  P  R   A  V  F   V  D  L   E  P  T  V   I  D  E   V  R  T   G  T  Y  R   Q  L  F   H  P  E   Q  L
AA identity             *   *  *  *  *      *  *   *  *  *   *  *  *  *      *  *   *  *  *   *  *  *  *   *  *  *   *  *  *   *  *

P. falciparum NUC    276  aatat caggaaaaga agatgctgcc aacaattttg ctagaggaca ctatacaatc ggtaaagaag ttatagatgt atgtttggac agaattagaa aatta  375
H. sapiens    NUC    276  catca caggcaaaga agatgctgcc aataactatg cccgagggca ctacaccatt ggcaaggaga tcattgacct cgtgttggac cgaattcgca agctg  375
P. falciparum AA        I  S   G  K  E   D  A  A   N  N  F  A   R  G  H   Y  T  I   G  K  E  V   I  D  V   C  L  D   R  I  R  K   L
H. sapiens    AA        I  T   G  K  E   D  A  A   N  N  Y  A   R  G  H   Y  T  I   G  K  E  I   I  D  L   V  L  D   R  I  R  K   L
AA identity             *      *  *  *   *  *  *   *  *     *   *  *  *   *  *  *   *  *  *      *  *         *  *   *  *  *  *   *

P. falciparum NUC    376  gctga taactgtacc ggtttacaag gatttttaat gttcagcgca gttggaggtg gaacaggtag tggatttggt tgtttaatgt tagaaagatt atccg  475
H. sapiens    NUC    376  gccga ccagtgcacg cgtctccagg gcttcttggt tttccacagc tttggtgggg gaactggttc tgggttcacc tcgctgctca tggaacgtct ctcag  475
P. falciparum AA        A  D   N  C  T   G  L  Q  G   F  L  M   F  S  A   V  G  G  G   T  G  S   G  F  G   C  L  M  L   E  R  L   S  V
H. sapiens    AA        A  D   Q  C  T   R  L  Q  G   F  L  V   F  H  S   F  G  G  G   T  G  S   G  F  T   S  L  L  M   E  R  L   S  V
AA identity             *  *      *  *      *  *  *   *  *     *            *  *  *  *   *  *  *   *           *        *  *  *   *  *

P. falciparum NUC    476  ttgat tatggaaaga aatccaaact gaatttttgc tgttggccat cacctcaagt ttcaactgct gtagttgaac catacaattc agttttgtct actca  575
H. sapiens    NUC    476  ttgat tatggcaaga agtccaagct ggagttctct atttacccgg cgccccaggt ttccacagct gtagttgagc cctacaactc catcctcacc accca  575
P. falciparum AA        D  Y  G  K  K   S  K  L   N  F  C   C  W  P  S   P  Q  V   S  T  A   V  V  E  P   Y  N  S   V  L  S   T  H
H. sapiens    AA        D  Y  G  K  K   S  K  L   E  F  S   I  Y  P  A   P  Q  V   S  T  A   V  V  E  P   Y  N  S   I  L  T   T  H
AA identity             *  *  *  *  *   *  *  *      *           *      *  *  *   *  *  *   *  *  *  *   *  *  *      *      *  *
```

```
P. falciparum NUC    576  ttcat tattagaaca tactgatgta gcaataatgc ttgataacga agctatatat gatatatgca gaagaaattt agatattgaa agaccaacat atact  675
H. sapiens    NUC    576  cacca ccctggagca ctctgattgt gccttcatgg tagacaatga ggccatctat gacatctgtc gtagaaacct cgatattgag cgtccaacct atact  675
P. falciparum AA          S  L   L  E  H   T  D  V   A  I  M  L   D  N  E   A  I  Y   D  I  C  R   R  N  L   D  I  E   R  P  T  Y   T
H. sapiens    AA          T  T   L  E  H   S  D  C   A  F  M  V   D  N  E   A  I  Y   D  I  C  R   R  N  L   D  I  E   R  P  T  Y   T
AA identity                  *   *  *  *      *      *     *      *  *  *   *  *  *   *  *  *  *   *  *  *   *  *  *   *  *  *  *   *

P. falciparum NUC    676  aattt aaatagattg attgctcaag ttatttcttc cttaacagca tctttaagat ttgatggtgc tttaaatgtt gatgtaacag aattccaaac caact  775
H. sapiens    NUC    676  aacct gaataggtta ataggtcaaa ttgtgtcctc catcactgct tccctgagat ttgatggagc cctgaatgtt gacctgacag aattccagac caacc  775
P. falciparum AA          N  L   N  R  L   I  A  Q  V   I  S  S   L  T  A   S  L  R  F   D  G  A   L  N  V   D  V  T  E   F  Q  T   N  L
H. sapiens    AA          N  L   N  R  L   I  G  Q  I   V  S  S   I  T  A   S  L  R  F   D  G  A   L  N  V   D  L  T  E   F  Q  T   N  L
AA identity               *  *   *  *  *   *     *        *  *   *  *  *   *  *  *  *   *  *  *   *  *  *   *     *  *   *  *  *   *  *

P. falciparum NUC    776  tagta ccataccctc gtattcattt tatgttatct tcatatgctc cagttgttag tgctgaaaaa gcataccatg aacaattgtc cgtttctgaa attac  875
H. sapiens    NUC    776  tggtg ccctatcccc gcatccactt ccctctggcc acatatgccc ctgtcatctc tgctgagaaa gcctaccatg aacagctttc tgtagcagag atcac  875
P. falciparum AA            V   P  Y  P  R   I  H  F   M  L  S   S  Y  A  P   V  V  S   A  E  K   A  Y  H  E   Q  L  S   V  S  E   I  T
H. sapiens    AA            V   P  Y  P  R   I  H  F   P  L  A   T  Y  A  P   V  I  S   A  E  K   A  Y  H  E   Q  L  S   V  A  E   I  T
AA identity                 *   *  *  *  *   *  *  *      *        *  *  *   *     *  *   *  *  *   *  *  *  *   *  *  *   *     *   *  *

P. falciparum NUC    876  caact cagcattcga accagcaaat atgatggcaa aatgtgatcc gagacatgga aaatatatgg cttgttgttt aatgtataga ggtgatgtag tacca  975
H. sapiens    NUC    876  caatg cttgctttga gccagccaac cagatggtga aatgtgaccc tggccatggt aaatacatgg cttgctgcct gttgtaccgt ggtgacgtgg ttccc  975
P. falciparum AA          N  S   A  F  E   P  A  N   M  M  A  K   C  D  P   R  H  G   K  Y  M  A   C  C  L   M  Y  R   G  D  V  V   P
H. sapiens    AA          N  A   C  F  E   P  A  N   Q  M  V  K   C  D  P   G  H  G   K  Y  M  A   C  C  L   L  Y  R   G  D  V  V   P
AA identity               *         *  *   *  *  *      *     *   *  *  *      *  *   *  *  *  *   *  *  *      *  *   *  *  *  *   *

P. falciparum NUC    976  aagga tgtgaacgca gctgttgcta ccataaaaac aaaaagaacc attcaatttg ttgactggtg tcctactggt tttaaatgtg gtataaatta tcaac  1075
H. sapiens    NUC    976  aaaga tgtcaatgct gccattgcca ccatcaagac caagcgtacc atccagtttg tggattggtg ccccactggc ttcaaggttg gcatcaacta ccagc  1075
P. falciparum AA          K  D   V  N  A   A  V  A  T   I  K  T   K  R  T   I  Q  F  V   D  W  C   P  T  G   F  K  C  G   I  N  Y   Q  P
H. sapiens    AA          K  D   V  N  A   A  I  A  T   I  K  T   K  R  T   I  Q  F  V   D  W  C   P  T  G   F  K  V  G   I  N  Y   Q  P
AA identity               *  *   *  *  *   *     *  *   *  *  *   *  *  *   *  *  *  *      *  *   *  *  *   *  *     *   *  *  *   *  *

P. falciparum NUC   1076  cacca actgttgtac caggaggaga tttagccaaa gttatgagag ctgtttgtat gatcagcaac tcaacagcaa ttgcagaagt attctcaaga atgga  1175
H. sapiens    NUC   1076  ctccc actgtggtgc ctggtggaga cctggccaag gtacagagag ctgtgtgcat gctgagcaac accacagcca ttgctgaggc ctgggctcgc ctgga  1175
P. falciparum AA            P   T  V  V  P   G  G  D   L  A  K   V  M  R  A   V  C  M   I  S  N   S  T  A  I   A  E  V   F  S  R   M  D
H. sapiens    AA            P   T  V  V  P   G  G  D   L  A  K   V  Q  R  A   V  C  M   L  S  N   T  T  A  I   A  E  A   W  A  R   L  D
AA identity                 *   *  *  *  *   *  *  *   *  *  *   *     *  *   *  *  *   *  *  *      *  *  *   *  *           *      *

P. falciparum NUC   1176  tcaaa aatttgattt aatgtatgca aaaagagctt tcgttcattg gtatgtaggt gaaggtatgg aagaaggaga atttagtgaa gctagagaag atttg  1275
H. sapiens    NUC   1176  ccaca agtttgacct gatgtatgcc aaacgtgcct ttgttcactg gtacgttggg gaggggatgg aggaaggtga gttttcagag gcccgtgagg acatg  1275
P. falciparum AA          Q  K   F  D  L   M  Y  A   K  R  A  F   V  H  W   Y  V  G   E  G  M  E   E  G  E   F  S  E   A  R  E  D   L
H. sapiens    AA          H  K   F  D  L   M  Y  A   K  R  A  F   V  H  W   Y  V  G   E  G  M  E   E  G  E   F  S  E   A  R  E  D   M
AA identity                  *   *  *  *   *  *  *   *  *  *  *   *  *  *   *  *  *   *  *  *  *   *  *  *   *  *  *   *  *  *  *

P. falciparum NUC   1276  gccgc cttagaaaaa gattatgaag aggtaggaat tgaatccaat gaagcagaag gagaagatga aggatatgaa gcagattatt aa              1362
H. sapiens    NUC   1276  gctgc ccttgagaag gattatgagg aggttggtgt gcattctgtt gaaggagagg gtgaggaaga aggagaggaa ------tact aa              1356
P. falciparum AA          A  A   L  E  K   D  Y  E  E   V  G  I   E  S  N   E  A  E  G   E  D  E   G  Y  E   A  D  Y  *
H. sapiens    AA          A  A   L  E  K   D  Y  E  E   V  G  V   H  S  V   E  G  E  G   E  E  E   G  E  E   -  -  Y  *
AA identity               *  *   *  *  *   *  *  *  *   *  *        *  *   *     *  *   *     *   *     *        *  *
```

Figure 4.1: The nucleotide (NUC) and amino acid (AA) sequences of *P. falciparum* α-I-tubulin (Genbank accession number: X15979) and *H. Sapiens* (Genbank accession number: X01703) respectively. Amino acid identity is indicated. The hybridization sites for antisense ODNs, ASL-1, ASL-2 and ASL-3 are also shown.

From Fig 4.1 it can be calculated that an 83% amino acid identity exists between *P. falciparum* and *H. sapiens*, while the nucleotide identity between the two is only 68%. This data supports the application of antisense ODNs in an attempt to specifically inhibit parasite α-I-tubulin.

For the antisense ODNs to be successful however, sufficient concentrations of the latter must reach the intra-parasitic target mRNA. Cationic liposomal transfection reagents may be employed in an attempt to increase membrane permeation of polyanionic ODNs and thereby achieve the aforementioned objective (Braasch and Corey, 2002). One such liposomal reagent is 1,3-di-oleoyloxy-2-(6-carboxy-spermyl)-propyl amide (DOSPER), the structure of which is shown in Figure 4.2.

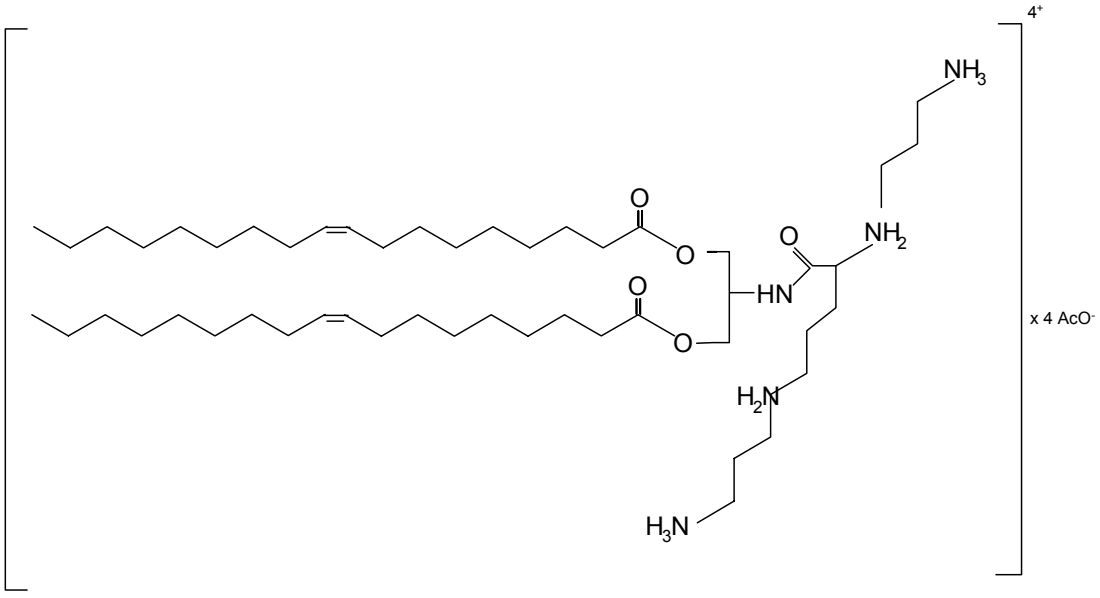


Figure 4.2: Molecular structure of 1,3-di-oleoyloxy-2-(6-carboxy-spermyl)-propyl amide (DOSPER) cationic lipsosomal transfection reagent (Boehringer Mannheim product information, 1996).

.

The four positively charged amine groups give DOSPER a polycationic nature, making it capable of binding to DNA or polyanionic ODNs. Furthermore the carboxy-spermine residue, that carries these four amine groups, stabilizes the DNA or ODN. It is also interesting to note that ester linkages are used to connect the two oleic acid chains to a propane backbone. These ester linkages are metabolized with greater ease than the ether linkages used in many other liposomal transfection reagents (Boehringer Mannheim product information, 1996).

The intra-erythrocytic parasite is involved in extremely intensive anabolic activity while it resides in the metabolically quiescent host. The fact that the parasite is separated from the serum by the erythrocyte, parasitophorous vacuolar and parasite plasma membranes makes the trafficking pathways complex. The necessary fast uptake of nutrients and discharge of waste products are facilitated by parasite-induced alterations of the constitutive transporters of the host cell and the production of new parallel pathways also known as new permeation pathways (NPPs) (Ginsburg, 1994; Thomas and Lew, 2004). Evidence suggesting that the NPPs are anion-selective channels, resulting from parasite control of endogenous channels, has mounted (Thomas and Lew, 2004). Merely six hours after parasite invasion, the permeability of the host cell membrane to anions and small nonelectrolytes starts to increase (Ginsburg and Stein, 1987).

Pouvelle and colleagues (1991) showed that macromolecules do not cross the erythrocyte and parasitophorous membranes but rather gain direct access to the aqueous space surrounding the parasite through a parasitophorous duct. The parasite can then internalize macromolecules from this aqueous compartment by endocytosis.

The Nucleotide Basic Local Alignment Search Tool (BLAST) program can be utilized to compare a given nucleotide sequence to the NCBI nucleotide databases. Matches found are then reported. These analyses are of particular importance and interest to antisense studies in order to determine the specificity of the chosen antisense ODN, DNA or RNA prior to further investigations such as inhibitory efficacy testing. These analyses are also extremely useful retrospectively in aiding the interpretation of results obtained by taking additions of new sequences in the databases into account. The program can also perform translated comparisons. One such translation entails converting a nucleotide query sequence to the protein sequences and then comparing the converted protein products against the NCBI protein databases. It is also possible to perform a pairwise BLAST in which two sequences are compared using the BLAST algorithm. The program considers a "sequence 1" to be the query sequence and "sequence 2" to be the subject. For this type of BLAST there are several program options one of which is the so-called *blastn*, which performs nucleotide – nucleotide comparisons. All the BLAST options can be accessed from the NCBI homepage (http://www.ncbi.nlm.nih.gov).

The aim of these investigations was to determine the efficacy of the modified antisense ODNs ASL-1, ASL-2 and ASL-3 to inhibit *in vitro* parasite proliferation. These studies included the determination of the intra-cellular ODN concentration as well as the effect of the parasite phase on cellular ODN uptake. The inclusion of sufficient controls is important for the correct interpretation of antisense results. These controls typically include a mismatch ODN sequence which is the inverse of the antisense sequence and the sense sequence which is identical to the mRNA target sequence (Crooke, 2000).

4.2 Materials and Methods

4.2.1. **Materials**

Materials used for the culture medium are listed in section 3.2.1. The thiazole orange was a kind gift from Molecular Probes Inc. Eugene, OR. The special gas mixture of 5% O_2, 5% CO_2 and 90% N_2 was purchased from Afrox (Germiston). Cationic liposomal transfection reagent DOSPER was purchased from Roche Diagnostics.

4.2.2. **Methods**

4.2.2.1. *In vitro* culturing of malaria parasites

Frozen stocks of a Southern African isolate of the malaria species *P. falciparum*, PfUP1, were used and cultured by a modified method as described (Trager and Jensen, 1976; Hoppe, 1993). Briefly, the medium consisted of 10.4 g RPMI 1640 - L glutamine, 5.94g HEPES, 4g glucose, 44 mg hypoxanthine, 5% sodium hydrogen carbonate and 4 mg of gentamycin, dissolved in 900 ml deionised, sterile water. The culture medium was supplemented with 10% human uninfected serum of blood group A^+ or O^+. Parasite cultures were suspended in 10 ml of culture medium in a 75 ml culture flask to which fresh, human erythrocytes (blood group O^+) to a hematocrit of 5% was added.

Erythrocytes were obtained from human whole blood collected in 4.5 ml blood collection tubes containing 15% K_4 EDTA to prevent clotting. The collected blood was centrifuged in a Hermle Z 320 bench centrifuge at 500g for 5 minutes. The supernatant was removed as well as the leukocyte buffy coat covering the erythrocyte top layer. The erythrocytes were then suspended in wash medium (culture medium

without serum) and centrifuged in the Hermle centrifuge at 500g for 5 minutes. The supernatant was removed and the procedure was repeated three times. Washed erythrocytes were stored in wash medium for two weeks at 4°C.

Culture flasks were gassed with a special gas mixture consisting of 5% O_2, 5% CO_2 and 90% N_2 prior to being incubated at 37°C. Parasitaemia was maintained at ca. 2% with daily replacement of culture medium and removal of and replacement of erythrocytes every second day or as needed.

4.2.2.2. Giemsa-stained blood smear preparation

A drop of parasite culture was placed at the end of a microscope slide nearest the frosted edge. The blood was then smeared across the length of the slide using a second slide and allowed to dry. The blood smear was fixed with methanol (analytical grade) and allowed to stand for ca. 5 minutes, after which the methanol was removed and the DNA stained with Giemsa solution. The Giemsa solution was prepared in glycerol as recommended by the supplier (Merck), diluted in a phosphate buffer (2 drops per 1 ml) containing 9.5 g/L NaH_2PO_4 and 9.2 g/L Na_2HPO_4 at a pH of between 6.8 and 7.2. The slide was covered with this solution for ca. 15 minutes and then allowed to air dry. A drop of microscope immersion oil was placed on the slide and it was viewed under the oil immersion 100x objective of a Nikon microscope.

Counting of the number of parasitized erythrocytes, in a given field, and expressing this as a percentage of the total number of erythrocytes counted in that field determined the percentage parasitaemia of a culture. Typically 6000 erythrocytes were counted over 10 fields for each slide.

4.2.2.3. *In vitro* synchronization of malaria parasites

For synchronization, cultures containing approximately 80% ring phase parasites were used (Lambros and Vanderberg, 1979; Vernes *et al*, 1984). Ten ml culture with 5 % hematocrit and ca. 2% parasitemia, was transferred from the culture flasks to 50 ml centrifuge tubes and centrifuged in a Hermle bench centrifuge at 500g for 5 minutes. The supernatant was removed and 4 ml of a 15% D-sorbitol solution was added for every 1 ml of parasite pellet. After careful mixing by tube inversion, the solution was incubated at 37°C for 5 minutes. After this period, 8 ml of 0.1% D-glucose was added to the tube and the solution mixed by gentle tube inversion. The parasite culture-sorbitol-glucose mixture was incubated at 37°C for 5 minutes. This method caused trophozoite- and later phase infected erythrocytes to lyse via osmotic shock, leaving only ring-phase and uninfected erythrocytes intact. The suspension was centrifuged at 500g for 5 minutes and the pelleted ring-phase-infected and uninfected erythrocytes were resuspended in 10 ml of culture medium. The hematocrit of the culture was returned to 5% by the addition of fresh erythrocytes whereafter the culture was returned to the culture flasks and filled with the special gas mixture before being returned to the incubator. This procedure was repeated every second day for three times in succession.

4.2.2.4. Determination of antisense ODN efficacy to inhibit parasite proliferation

Cultures of PfUP1 were grown in culture flasks and synchronised when necessary, as described above. To initiate an experiment, 5 ml of the parasite culture at the appropriate stage of development was removed and diluted to a 1% parasitaemia by the addition of culture medium and fresh erythrocytes to a hematocrit of 5%. Parasitaemia of ca. 1% was confirmed by microscope smears after which 90 µl of the

culture was placed into each well of a 96-well flat-bottomed microtiter plate (Corning). The lyophylised ODNs were redissolved in sterile, deionised water and stored as stock solutions (0.25 mM) at -20^{o}C. The ODNs were diluted as needed, using wash medium, to the required concentrations and 10 µl was added to the wells of the microtiter plate. Cultures to which sterile, distilled water had been added served as controls for uninhibited parasite growth. When required, ODNs were mixed with the cationic liposome, DOSPER (ODN:liposome ratio of either 1:2 or 1:5) prepared in HEPES-buffered saline, HBS (20 mM HEPES, 150 mM NaCl, pH=7.4), as per the supplier's instructions (Roche), prior to being added to the appropriate well. The ODN-liposome mixtures were gently mixed by tube inversion and allowed to stand at ambient temperature to allow the ODN-liposome interaction to take place.

The microtiter plates were then placed inside a humid box filled with the special gas mixture. The humid box was incubated at 37°C for 24 hours after which culture medium in all the wells was replaced by removing 50µl of medium from the settled cell suspension followed by replenishment with 40 µl fresh medium and 10 µl fresh ODN solutions. The gas-filled humid box was returned to the incubator for an additional 24 hours. After this incubation period the samples were prepared for flow cytometric analysis.

4.2.2.5. Flow cytometric (FC) measurement of parasitemia in fixed parasite cultures

The malaria cultures (100µl) were fixed in a 1:1 ratio with the fixing solution consisting of 10% formaldehyde and 4% D-glucose dissolved in a pH 7.3 Tris-saline buffer (10 mM Tris, 150 mM NaCl and 10 mM NaN_3) as described previously (Schulze *et al*, 1997). After incubation at 4°C for at least 18 hours, 50 µl fixed

parasite culture was added to 1 ml PBS containing 0.25µg thiazole orange. The parasite-PBS-thiazole orange mixture was gently mixed by 2-3 tube inversions and further incubated at ambient temperature in the dark, for 1 hour. The samples were then placed on ice to inhibit further staining of the parasite DNA and immediately analysed by flow cytometry.

A Coulter Epics XL MCL (Coulter) was programmed to have three electronic gates. All uninfected erythrocytes were counted in gate 1, which covered the region of zero fluorescence intensity. Gate 2 counted ring-infected erythrocytes that had a fluorescence intensity lower than that of the later-phase parasites, and gate 3 counted the later phase trophozoite and schizont phase infected erythrocytes. Two hundred µl samples in each case were analysed by the flow cytometer and at least 200 000 cells were counted.

4.2.2.6. Uptake of FITC-labeled ODN's

The ODN ASL-1 was purchased with a 5' FITC label attached (MWG-Biotech). The ODNs were incubated with synchronised parasite cultures in microtiter plates as described in section 4.2.2.4, with the following modifications. The ODNs were incubated for time periods of 1, 2, 4 and 6 hours, respectively, before removal from the wells and pelleting of the cells in a Hermle bench centrifuge at 1000g for 2 minutes. The supernatants were removed and the pellets were washed in 100 µl wash buffer twice before being resuspended in 1 ml wash buffer. The cell-associated fluorescence was measured by flow cytometry as described above. Live cells were gated with the aid of propidium iodide (0.3 µg/ml), added to the culture prior to flow cytometric measurement of internalized FITC ODNs (Zhao *et al*, 1993). Uninfected

erythrocyte cultures were included in all assays to determine background fluorescence. This background was subtracted from the fluorescence obtained with the parasitized erythrocytes.

In order to facilitate the comparison of fluorescence associated with ring and trophozoite cultures, respectively, the parasitaemias of the parasite cultures used in the investigations were determined using thiazole orange, as described in section 4.2.2.5. The parasitaemias of the different parasite cultures used were normalised to 10% and the fluorescence measured in the different experiments was adjusted accordingly. The difference in fluorescence allowed for direct comparison of the uptake of FITC ODNs into ring and trophozoite phase parasite cultures taking into consideration the effect of varying parasitaemia values.

4.2.2.7 Determination of hyridization site availability

The results in this chapter were analyzed by comparing the experimentally obtained efficacies of the ODN and mismatch ODNs to the calculated "availability" scores at their predicted binding sites on the target RNA. The average availability for all the RNA bases in the predicted target RNA's was calculated during mRNA roll off (original transcription of RNA from its DNA template) as well as during ribosome breathing events (when a ribosome bound to the target RNA moves along the length of the RNA during the translation progress).

A Perl script run on a Silicon Graphics O2 workstation was used to generate a set of template molecules simulating the RNA roll of process by the extension of the sequences by three RNA bases per template. A similar template set was generated to

simulate ribosome breathing by masking out the secondary structure interaction (during the downstream structure prediction calculation) of a sliding window of 12 bases (shifted 3bp for each sequence in the template set). The script pipelined each sequence in both template sets (for both RNA roll off and ribosomal breathing) to *Mfold* version 2.3. Using the default parameters, *Mfold* generated the energetically most favourable secondary structure for each sequence.

An availability score for each RNA base in the sequence was calculated by analysing the availability (defined as a base not involved in a predicted base-pair bond with another base) of that base in the optimal predicted secondary structure (*Mfold*) for each sequence in a template set generated and was expressed as a percentage for all sequences in the template set. The results are shown as the average availability for a sliding 20 bp window (step 1 bp).

4.3. Results

4.3.1. *In vitro* ODN inhibitory efficacy

The efficacy of the modified ODNs designed in Chapter 2 to inhibit parasite growth of *in vitro* erythrocyte cultures of *P. falciparum* (isolate PfUP1), was investigated. The first ODN tested (ASL-1), was targeted against the region surrounding the translation initiation codon of *P. falciparum* α-I-tubulin. The mismatch ODN MSL-1 was used as a negative control. Inhibition of parasite proliferation was measured by flow cytometry (Figure 4.3).

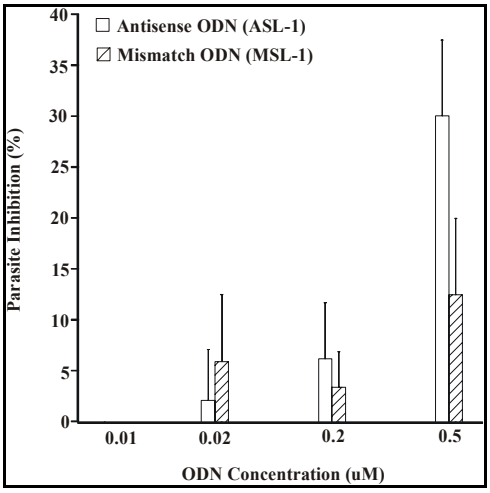


Figure 4.3: Inhibition of parasite growth, measured by flow cytometry after a 48 hour incubation period of non-synchronous PfUP1 cultures (52% ring phase; 48% trophozoite phase) with antisense ODN, ASL-1, targeted against *P. falciparum* α-I-tubulin, at concentrations ranging from 10 nM to 0.5 µM. The means of six repeats ± SEM are shown. No inhibiton observed for ASL-1 ans MSL-1 at 0.02 µM and MSL-1 at 0.5 µM.

These results indicate a 30% inhibition of parasite growth by antisense ODN ASL-1 and 12% by mismatch ODN, MSL-1, at a concentration of 0.5 µM. The inhibitory effect appears to be concentration dependent, with no specific inhibition being

observed at ODN concentrations below 0.2 µM. Furthermore, ODN concentrations of 1 and 2 µM showed no increase in the observed inhibition (results not shown).

The effect of inclusion of the cationic liposome, DOSPER, on ODN efficacy was determined in an attempt to improve on the observed inhibition of parasite growth. Non-synchronous cultures were used and inhibition of parasite growth was determined using flow cytometry (Figure 4.4).

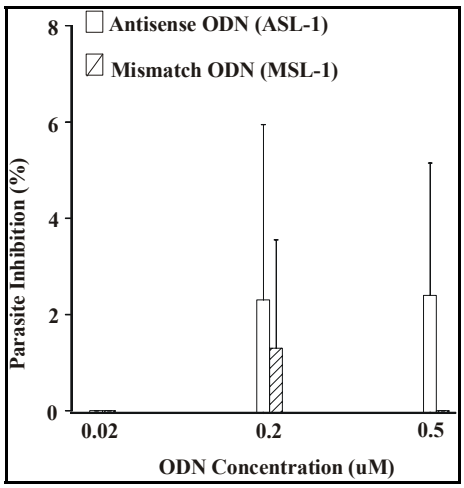


Figure 4.4: Inhibition of parasite growth measured by flow cytometry after a 48 hour incubation period of non-synchronous PfUP1 cultures with antisense ODN ASL-1, targeted against *P. falciparum* α-I-tubulin. The ODN concentrations ranged from 0.02 µM to 0.5 µM with a ODN:liposome ratio of 1:2. The results are given as the mean of three samples ± SEM.

The results obtained with an ODN:liposome ratio of 1:2 indicated insignificant inhibition of parasite growth at all the concentrations tested. Similar results were obtained using a higher ODN:liposome ratio of 1:5 (results not shown). No further experiments were undertaken with these liposomes.

The possible relationship between parasite development phase and antisense ODN efficacy was investigated by repeating the studies above on synchronous parasite cultures. Antisense ODN, ASL-1 and mismatch ODN, MSL-1 were added to ring and

trophozoite-phase synchronous cultures at a concentration of 0.5 µM. Parasitaemia was measured using flow cytometry. Results are shown in figure 4.5.

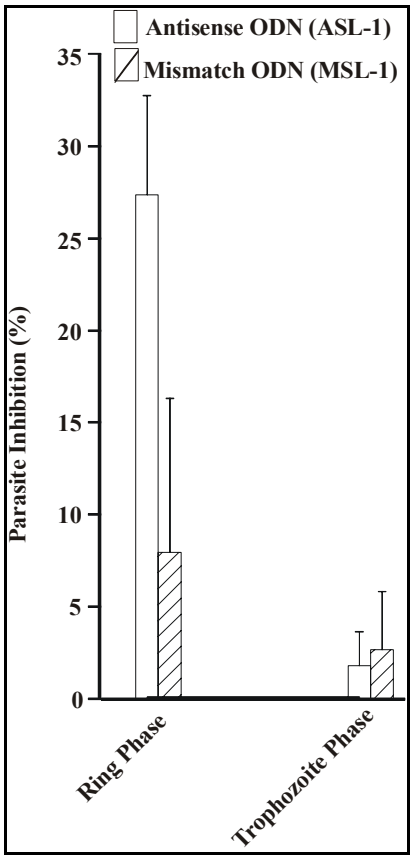


Figure 4.5: Inhibition of parasite growth, measured by flow cytometry after a 48 hour incubation period of ring (4-8 hours post invasion) and trophozoite phase (24-30 hours post invasion) synchronous PfUP1 cultures with 0.5 µM antisense ODN ASL-1, and mismatch ODN MSL-1. The results are given as the mean of six samples ± SEM.

The above results showed that the ring-phase parasites were inhibited by 27% at an ASL-1 concentration of 0.5 µM, while the corresponding inhibition by MSL-1 was 8%. No effective and specific inhibition was observed with ASL-1 on trophozoite-phase parasites when the inhibition by the mismatch control was taken into account.

The ring-phase parasite inhibition by ASL-1 was confirmed by inclusion of a sense sequence control, ODN (SL-1). The sequence of this ODN is given in chapter 2, Table 2.5. The results of an experiment with ring-infected parasite cultures are shown in Figure 4.6.

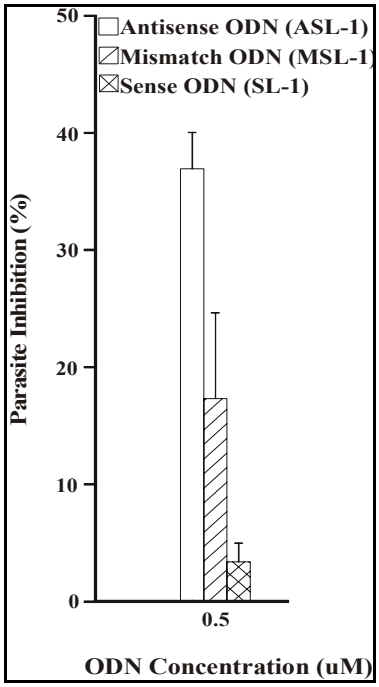


Figure 4.6: Inhibition of parasite growth measured by flow cytometry after a 48 hour incubation period of ring-phase (4-8 hours post invasion) synchronous PfUP1 with 0.5 µM ASL-1, MSL-1 and the sense sequence ODN (SL-1) as an additional control. The results are given as the mean of three samples ± SEM.

These results correlated with those obtained in Figure 4.5, since an inhibition of 37% was obtained after incubation with the antisense ODN, ASL-1, while 17% was obtained after incubation with the mismatch ODN, MSL-1. Inhibition observed after incubation of the culture with the sense ODN was less than 4%.

The efficacy of the antisense ODNs ASL-2 (targeted against nucleotides 92-118 of α-tubulin) and ASL-3 (targeted against nucleotides 118 -144 of α-tubulin) were tested using ring and trophozoite phase synchronised parasite cultures. The mismatch ODNs MSL-2 and MSL-3 were used as the respective negative controls. For purposes of comparison the results obtained previously for ODNs ASL-1 (targeted against nucleotides –9 to 18 of α-tubulin) and MSL-1 were included in these results. Parasite growth inhibition was measured by flow cytometry. The results are shown in Figures 4.7 A and B.

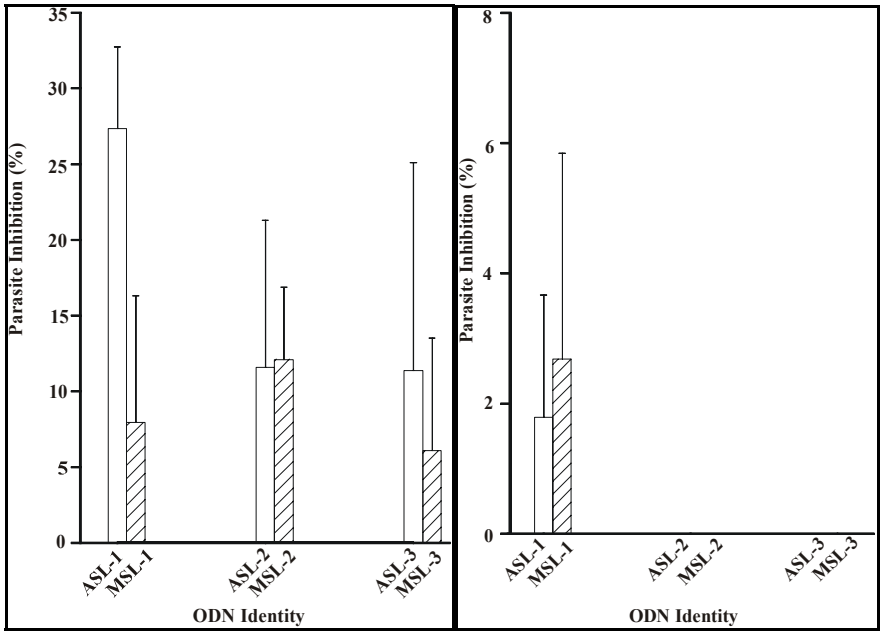


Figure 4.7: A: Inhibition of parasite growth measured by flow cytometry after a 48 hour incubation period of ring-phase (4-8 hours post invasion) synchronous PfUP1 with 0.5 µM ASL-1, ASL-2 and ASL-3 with 0.5 µM MSL-1, MSL-2 and MSL-3 mismatch ODNs, respectively. The results are given as the mean values of six samples ± SEM. B: Inhibition of parasite growth measured by flow cytometry after a 48 hour incubation period of trophozoite-phase synchronised PfUP1 with 0.5 µM ASL-1, ASL-2 and ASL-3 with 0.5 µM MSL-1, MSL-2 and MSL-3 mismatch ODNs, respectively. The results are given as the mean values of six samples ± SEM.

An inhibition of 27% was observed after incubation of ring phase synchronised cultures with ASL-1. Incubation of MSL-1 under the same conditions only yielded a 7.8% inhibition of parasite growth. The ODNs, ASL-2, ASL-3 along with MSL-2 and MSL-3 yielded inhibitions of 11.4%, 10.9%, 11.6% and 6.1%, respectively (Fig 4.7A). After incubation of trophozoite phase synchronised cultures with any of the ODNs, ASL-1, ASL-2, ASL-3, MSL-1, MSL-2 or MSL-3 no significant inhibition of parasite growth was observed (Figure 4.7B). Since no significant antisense effect was observed with the other ODNs (ASL-2 and ASL-3), further investigations were conducted using ASL-1 only.

4.3.2. Cellular ODN Uptake

It is apparent from the results obtained thus far that the ring-phase parasites are more prone to ASL-1 inhibition than the trophozoite-phase parasites. In the next series of experiments the erythrocytic uptake of 5' FITC labeled antisense ODNs was investigated using flow cytometry. In the first experiment the uptake of the modified antisense ASL-1 into non-synchronous parasitized and uninfected erythrocytes was determined at a constant ODN concentration of 0.5µM. The results are shown in Figure 4.8.

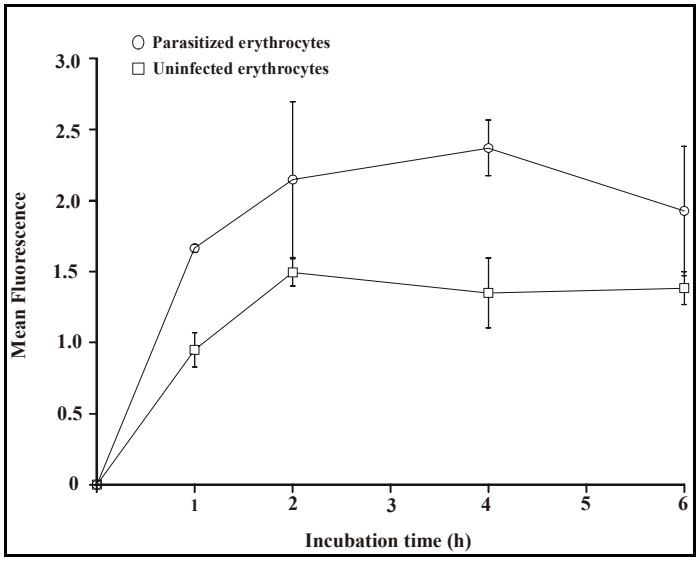


Figure 4.8: The cellular uptake of 5' -labeled FITC antisense ODN ASL-1 into non-synchronous parasitized and control uninfected erythrocyte cultures after incubation at 37°C for 1, 2, 4 or 6 hours. Fluorescence associated with the cell populations was determined by flow cytometry. Results are the mean of three samples ± SEM. Only viable cell populations, as evidenced by the absence of fluorescence after propidium iodide addition were measured.

Up to 50% more fluorescence was associated with parasitized compared to uninfected erythrocytes after an incubation period of 1 hour at 37^{o}C.

In the next set of experiments synchronised parasite cultures in different phases of development as well as uninfected erythrocytes as control, were also incubated with 5'-FITC conjugated antisense ODN for time periods of 1, 2, 4 and 6 hours. Fluorescence was determined by flow cytometry as described above.

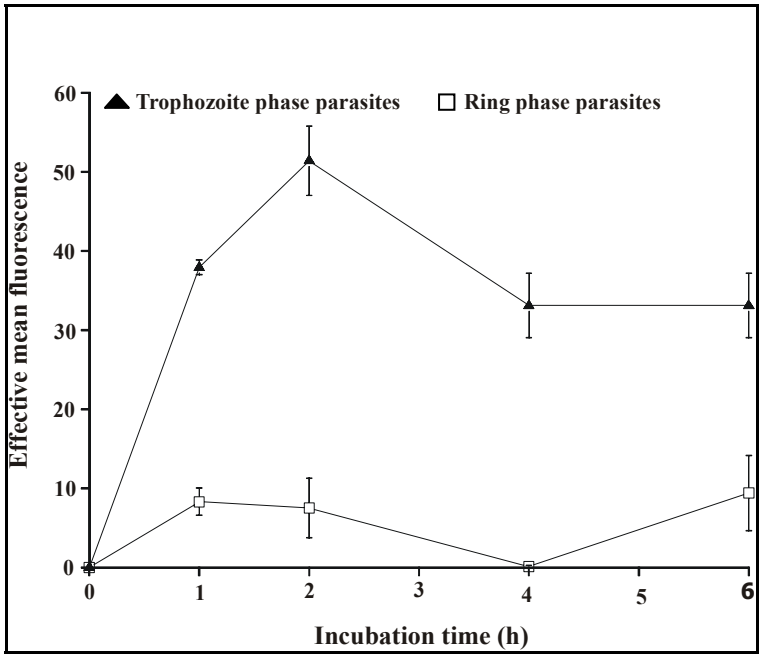


Figure 4.9: The cellular uptake of 5'FITC labeled antisense ODN ASL-1, by synchronous ring and trophozoite phase parasite cultures after incubation periods of 1, 2, 4 and 6 hours. Uninfected erythrocyte cultures treated in the same way were used to determine background fluorescence and were subtracted from the data obtained from the parasitized cultures. Parasitaemia measurements by flow cytometry were made for all cultures using thiazole orange as a DNA intercalator. Results are expressed as the mean values of six samples ± SEM.

The results in Figure 4.9 represent the mean fluorescence measured in ring and trophozoite infected cultures, respectively, after subtraction of the background fluorescence of the uninfected erythrocyte cultures. The parasitaemia of the various cultures used was measured and the fluorescence values obtained were normalized to 10% as described in section 4.2.2.6.

These results showed that about 10 times more fluorescence was associated with trophozoite- than ring-infected erythrocyte cultures after a two-hour incubation period. The percentage fluorescent cells present after two hours of incubation with 5'FITC antisense ODN, with trophozoite and ring-phase infected cultures, respectively, are shown in Figure 4.10. Uninfected erythrocytes served as a control to

determine background values. The parasitaemia was normalised to 10% as described in section 4.2.2.6.

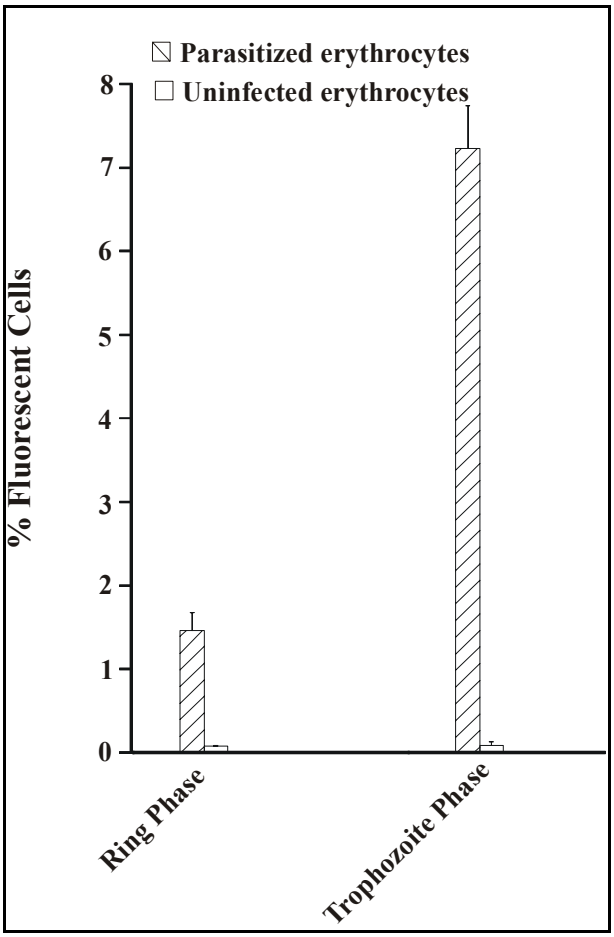


Figure 4.10: The percentage fluorescent cells present in trophozoite and ring infected cultures, respectively, after two hours incubation with 5'FITC conjugated antisense ODN, ASL-1. All values were normalised to a parasitaemia of 10%. Results are the mean of three samples ± SEM.

Figure 4.10 shows 7.2% of parasitized erythrocytes in the trophozoite-infected culture exhibited fluorescence as opposed to the 1.46% in the ring-infected erythrocytes. The percentage fluorescent cells in the uninfected erythrocyte control cultures for both experiments were comparable at 0.08% and 0.07%, respectively.

4.3.3. Retrospective analysis

The availability of the complete *P. falciparum* genome sequence in December 2002 (Gardner *et al.*, 2002) made possible a retrospective analysis of additional mRNA hybridization sites of the ODNs designed during 1997 and 1998. This was of particular importance since unexpected inhibition of parasite proliferation had been observed after incubation of parasite cultures with the ODNs MSL-1, MSL-2 and MSL-3. The results of a BLAST analysis performed in April 2005 are illustrated in Table 4.2.

Table 4.2: The five best matches, obtained from BLAST searches matching the ODN sequences of ASL-1, ASL-2, ASL-3, MSL-1, MSL-2 and MSL-3 are given. The binding orientation of the ODN to target site mRNA is indicated as Plus/Minus (indicating binding), Plus/Plus (Matching on Plus strands, no binding) and N/A if the target site does not encode a protein.

ODN	**Nucleotide Sequence Match** (gi Number | Database | Acession number Description)	**E-Value**	**S (Identities)**	**Binding orientation on target mRNA (Target Site Match)**
ASL-1	gi|9979|emb|X15979.1|PFTUBAI Plasmodium falciparum alpha-tubulin I gene	$4x10^{-8}$	54 bits (27/27 nt)	Strand: = Plus / Minus (1 - 27 on ODN matching 617 - 643 on nucleotide sequence match)
	gi|23504903|emb|AL929355.1|PFA929355 *Plasmodium falciparum* strain 3D7, chromosome 9; segment 1/5 Matching region codes for alpha-tubulin	$4x10^{-8}$	54 bits (27/27 nt)	Strand: = Plus / Minus (1 - 27 on ODN matching 175531 - 175557 on nucleotide sequence match)

ASL-1 (cont)	gi|23497422|gb|AE014822.1| *Plasmodium falciparum* 3D7 chromosome 14 section 7 of 13 of the complete sequence Matching region codes for hypothetical protein.	0.033	34.2 bits (17/17 nt)	Strand: = Plus / Minus (9 – 25 on ODN matching 90173 – 90189 on nucleotide sequence match)
	gi|23497188|gb|AE014818.1| *Plasmodium falciparum* 3D7 chromosome 14 section 3 of 13 of the complete sequence Matching region codes for hypothetical protein.	0.033	34.2 bits (17/17 nt)	Strand: = Plus / Minus (3 – 19 on ODN matching 238494 – 238510 on nucleotide sequence match)
	gi|23496321|gb|AE014842.1| *Plasmodium falciparum* 3D7 chromosome 11 section 7 of 8 of the complete sequence Matching region is in AT-rich tandem repeats region.	0.033	34.2 bits (17/17 nt)	Strand: = N/A (11 – 27 on ODN matching 8229 – 8245 on nucleotide sequence match)
ASL-2	gi|9979|emb|X15979.1|PFTUBAI *Plasmodium falciparum* alpha-tubulin I gene.	$9x10^{-9}$	56 bits (28/28 nt)	Strand: = Plus / Minus (1 – 28 on ODN matching 1070 - 1097 on nucleotide sequence nucleotide sequence match)
	gi|23504903|emb|AL929355.1|PFA929355 *Plasmodium falciparum* strain 3D7, chromosome 9; segment 1/5 Matching region codes for alpha-tubulin.	$9x10^{-9}$	56 bits (28/28 nt)	Strand: = Plus / Minus (1 – 28 on ODN matching 176001 - 175974on nucleotide sequence match)
	gi|23497066|gb|AE014816.1| *Plasmodium falciparum* 3D7 chromosome 14 section 1 of 13 of the complete sequence Matching region codes for hypothetical protein.	7.9	26.3 bits (13/13 nt)	Strand: = Plus / Plus (4 – 16 on ODN matching 116506– 116518 on nucleotide sequence match)
	gi|23496131|gb|AE014839.1| *Plasmodium falciparum* 3D7 chromosome 11 section 4 of 8 of the complete sequence Matching region codes for hypothetical protein.	7.9	26.3 bits (13/13 nt)	Strand: = Plus / Plus (10 – 22 on ODN matching 138308– 138320 on nucleotide sequence match)

ASL-2 (cont)	gi|23510587|emb|AL031745.8|PFMAL1P2 *Plasmodium falciparum* DNA from MAL1P2 Matching region codes for STEVOR.	1938	18.3 bits (9/9 nt)	Strand: = Plus / Minus (13 – 21 on ODN matching 28845–28853 on nucleotide sequence match)
ASL-3	gi|9979|emb|X15979.1|PFTUBAI *Plasmodium falciparum* alpha-tubulin I gene	$9x10^{-9}$	56.0 bits (28/28 nt)	Strand: = Plus / Minus (1 – 28 on ODN matching 1096 - 1123 on nucleotide sequence match)
	gi|23504903|emb|AL929355.1|PFA929355 *Plasmodium falciparum* strain 3D7, chromosome 9; segment 1/5 Matching region codes for alpha-tubulin	$9x10^{-9}$	56.0 bits (28/28 nt)	Strand: = Plus / Minus (1 – 28 on ODN matching 176000 - 176027 on nucleotide sequence match)
	gi|23497369|gb|AE014821.1| *Plasmodium falciparum* 3D7 chromosome 14 section 6 of 13 of the complete sequence Matching region codes for hypothetical protein	2.0	28.2 bits (14/14 nt)	Strand: = Plus / Minus (2 – 15 on ODN matching 220730 - 220743 on nucleotide sequence match)
	gi|6165410|emb|AJ133811.1|PFA133811 *Plasmodium falciparum* var gene, partial	2.0	28.2 bits (14/14 nt)	Strand: = Plus / Plus (1 – 14 on ODN matching 45 - 58 on nucleotide sequence match)
	gi|23497478|gb|AE014823.1| *Plasmodium falciparum* 3D7 chromosome 14 section 8 of 13 of the complete sequence Matching region codes for hypothetical protein.	7.9	26.3 bits (13/13 nt)	Strand: = Plus / Minus (1 – 13 on ODN matching 246779 - 246791 on nucleotide sequence match)
MSL-1	gi|23496531|gb|AE014844.1| *Plasmodium falciparum* 3D7 chromosome 12, section 1 of 9 of the complete sequence Matching region codes for hypothetical protein	0.002	38.2 bits (19/19 nt)	Strand: = Plus / Plus (9 – 27 on ODN matching 94500 – 94518 on nucleotide sequence match)
	gi|23498195|emb|AL035476.7|PFMAL4P3 *Plasmodium falciparum* MAL4P3 Matching region codes for hypothetical protein	0.008	36.2 bits (18/18 nt)	Strand: = Plus / Minus (11 – 28 on ODN matching 237903 – 237920 on nucleotide sequence match)

MSL-1 (cont)	gi|23496193|gb|AE014840.1| *Plasmodium falciparum* 3D7 chromosome 11 section 5 of 8 of the complete sequence Matching region codes for hypothetical protein	0.13	32.2 bits (22/24 nt)	Strand: = Plus / Minus (1 – 24 on ODN matching 166305 – 166328 on nucleotide sequence match)
	>gi|23495098|gb|AE014833.1| *Plasmodium falciparum* 3D7 chromosome 10 section 5 of 7 of the complete sequence Matching region is16 bp at 3' side of hypothetical protein	0.13	32.2 bits (19/20)	Strand: = N/A (3 – 224 on ODN matching 126325 – 126344 on nucleotide sequence match)
	gi|23497540|gb|AE014824.1| *Plasmodium falciparum* 3D7 chromosome 14 section 9 of 13 of the complete sequence Matching region codes for putative pyridine nucleotide transhydrogenase	0.51	30.2 bits (15/15 nt)	Strand: = Plus / Minus (13 – 27 on ODN matching 184386 – 184400 on nucleotide sequence match)
MSL-2	gi|26985284|gb|AF547122.1| *Plasmodium falciparum* Gb35 erythrocyte membrane protein 1 Gb35var6 gene	31	24.3 bits (12/12 nt)	Strand: = Plus / Minus (4 – 15 on ODN matching 338 – 349 on nucleotide sequence match)
	gi|23497369|gb|AE014821.1| *Plasmodium falciparum* 3D7 chromosome 14 section 6 of 13 of the complete sequence Matching region codes for hypothetical protein	124	22.3 bits (11/11 nt)	Strand: = Plus / Plus (13 – 23 on ODN matching 114150 – 114160 on nucleotide sequence match)
	gi|23497188|gb|AE014818.1| *Plasmodium falciparum* 3D7 chromosome 14 section 3 of 13 of the complete sequence Matching region codes for putative dimethyladenosine transferase.	124	22.3 bits (11/11 nt)	Strand: = Plus / Minus (11 – 21 on ODN matching 136931 – 136941 on nucleotide sequence match)
	gi|23495940|gb|AE014836.1| *Plasmodium falciparum* 3D7 chromosome 11 section 1 of 8 of the complete sequence Matching region is 644 bp to 5' side of hypothetical protein.	124	22.3 bits (11/11 nt)	Strand: = N/A (6 – 16 on ODN matching 207993 – 208003 on nucleotide sequence match)

MSL-2 (cont)	gi|21591791|gb|AY069958.1| *Plasmodium falciparum* mitochondrial processing peptidase alpha subunit mRNA, complete cds; nuclear gene for mitochondrial product	124	22.3 bits (11/11 nt)	Strand: = Plus / Plus (13 – 23 on ODN matching 1264 – 1274 on nucleotide sequence match)
MSL-3	gi|3647343|emb|Z97348.1|PFMAL3P1 *Plasmodium falciparum* MAL3P1, complete sequence Matching region is in a var pseudogene.	0.51	30.2 bits (15/15 nt)	Strand: = Plus / Minus (15 – 29 on ODN matching 16817 – 16831 on nucleotide sequence match) N/A
	gi|23504570|emb|AL929352.1|PFA929352 *Plasmodium falciparum* strain 3D7, chromosome 5, segment 2/4 Matching region codes for hypothetical protein.	2.0	28.2 bits (14/14 nt)	Strand: = Plus / Plus (11 – 21 on ODN matching 194937 – 194950 on nucleotide sequence match)
	gi|46019179|emb|CR382402.1| *Plasmodium falciparum* chromosome 6, complete sequence; segment 5/5 Matching region is in telomeric repeat region.	7.9	26.3 bits (13/13 nt)	Strand: N/A (15 – 27 on ODN matching 23388 – 23400 on nucleotide sequence match)
	gi|23495940|gb|AE014836.1| *Plasmodium falciparum* 3D7 chromosome 11 section 1 of 8 of the complete sequence Matching region is 13900 bp at 3' side: erythrocyte membrane protein 1 (PfEMP1).	7.9	26.3 bits (13/13 nt)	Strand: N/A (15 – 27 on ODN matching 10248 – 10260 on nucleotide sequence match)
	gi|23496259|gb|AE014841.1| *Plasmodium falciparum* 3D7 chromosome 11 section 6 of 8 of the complete sequence Matching region codes for hypothetical protein.	7.9	26.3 bits (13/13 nt)	Strand: Plus/Plus (17 – 29 on ODN matching 189898 – 189886 on nucleotide sequence match)

Comparison of E-values of BLAST hits gives a good indication of the significance of the results obtained. The bit score (S) of a BLAST hit represents the amount of similarity between the query sequence, in this case the respective ODNs, and the part

of the gene to which that similarity exists. The E value gives the number of alignments with scores greater than or equal to S that are expected to occur by chance in a database search. The lower the E value therefore, the more significant the match. E-values are inversely related to the S (bit) scores, i.e. high scores equal low E-values, indicating higher significance of a match. When querying a database with a short ODN sequence, the E-value becomes less relevant as a higher number of irrelevant matches are to be expected for a short sequence. A better indication of significance in this case will thus be a comparison of the number of matching nucleotides.

In this regard only matches longer than 15 nucleotides can be considered relevant for further consideration in this analysis since this is generally accepted as the minimum length for effective antisense ODN efficacy (Phillips and Zhang, 2000). The antisense ODNs, ASL-1, ASL-2 and ASL-3 are all complementary to the regions within *P. falciparum* alpha-tubulin sequences that they have been designed to target, having low E values and matching over their complete length. In addition, complementarity of these ODNs has also been found to several *P. falciparum* hypothetical proteins, although here the E values are markedly higher and fewer nucleotides are involved in the complementary region.

The mismatch control sequences, MSL-1, MSL-2 and MSL-3 were however also found to be complementary to regions within certain *P. falciparum* sequences. In addition to complementarity to regions coding for *P. falciparum* hypothetical proteins, the most profound of these included the match of MSL-1 to a sequence involved in coding for a putative pyridine nucleotide transhydrogenase (15/15 complementary nucleotides). MSL-2 was found to be complementary to a region coding for PfEMP-1

(12/12 complementary nucleotides) as well as for a region coding for a putative dimethyladenosine transferase (11/11 complementary nucleotides).

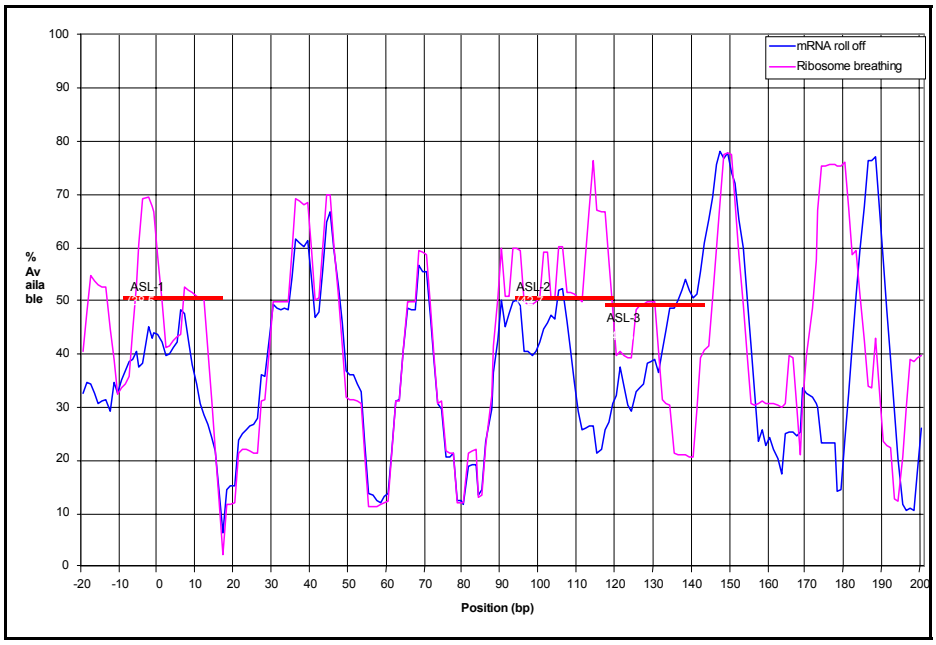


Figure 4.11: The percentage availability of the antisense–binding sites of ASL-1, ASL-2 and ASL-3 in the alpha-I- tubulin gene (Genbank acc No X15979) during both the transcription (mRNA roll off) and translation (ribosome breathing) processes.

As a further part of the retrospective analysis (January 2003), it was decided to investigate the percentage availability of the mRNA for the binding of ASL-1, ASL-2 and ASL-3 during mRNA roll off (transcription) as well as during ribosome breathing (translation). These results are depicted in Figure 4.11

It is important to note that in the *P. falciparum* α-I-tubulin mRNA the ASL-1 hybridization site is available on average 40 to 50% of the time during both mRNA roll off and during ribosome breathing (Figure 4.11). The same is true for ASL-2 while for ASL-3 between 30 and 40% of α-I-tubulin mRNA is available during mRNA roll off and between 40 and 50% during ribosome breathing.

4.4 Discussion

The inhibitory efficacy of phosphodiester antisense ODNs targeted against α-I-tubulin of the human malaria parasite, *P. falciparum*, was investigated. These studies included the determination of cellular uptake by the different phases of parasite - infected erythrocyte cultures.

Since the initiation of this research the Steering Committee on Drugs for Malaria (CHEMAL) of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) has identified tubulin as a possible malaria drug target (Bell, 1998). The tubulin inhibitors colchicine, vinblastine, vincristine, taxol and taxotere were shown to reduce malaria parasite growth both in culture and *in vivo*, with IC_{50} values of 13 μM, 28 nM, 6 nM, 71 nM and 3.1 nM respectively. The current tubulin-targeted drugs, however, inhibit both parasite and mammalian proliferation due to the 83% amino acid identity between the parasite α-I tubulin and human α-tubulin (Fig 4.1; Holloway *et. al*., 1989).

In the above situation the application of antisense technology can be useful. The mechanism of action of antisense ODNs differs from protein-based drugs since the biosynthesis of the targeted protein is interrupted by the inhibition of its translation. The difference between the codon preferences of the human host and parasite as reflected by the ~80% A+T-rich nature of the malaria genome, provides strong support for the development of antisense-based strategies for the specific inhibition of the malaria parasite (Gardner *et. al*., 2002).

Studies with the antisense ODN, ASL-1 using non-synchronous parasite cultures (Figure 4.3) showed a 30% inhibition of parasite proliferation. The mismatch control ODN, MSL-1, also inhibited parasite proliferation, but only by 12%. When the inhibition obtained with MSL-1 is subtracted from that obtained with ASL-1 a possible sequence specific antisense mediated inhibition of 18% is obtained. From these results it became evident that it was necessary to increase the observed parasite inhibition. One reason for the low inhibition observed could be insufficient ODN uptake by the parasitized erythrocytes (Crooke, 2000).

To investigate whether inhibition by antisense ODNs could be improved, the liposomal transfection reagent, DOSPER, was included in antisense efficacy assays (Bennett *et. al*., 1992; Felgner *et. al*., 1994; Lappalainen *et. al*., 1994; Semple *et. al*, 2000). The inclusion of DOSPER at two ODN:liposome ratios (1:2 and 1:5) in the culture medium of non-synchronous cultures abolished the inhibition observed in its absence by ASL-1 at a concentration of 0.5 µM (Figure 4.4). The above results could be explained by the fact that liposome, liposome-ODN and ODN uptake have been linked to endocytosis in several cell types (Kanagaratnam *et. al*., 1998; Wanidworanum *et. al.*, 1999). Parasite endocytosis of host cell cytoplasm is known to occur within the parasitized erythrocyte. Mature, parasite-infected or uninfected erythrocytes have however not been observed to endocytose nutrients from the extracellular medium (Hoppe *et. al*. 2004; Kanagaratnam *et. al*., 1998; Wanidworanum *et. al*., 1999). It is therefore probable that the ODN-liposome complexes were not taken up from the extracellular medium by the erythrocytes, as evidenced by the complete absence of inhibition (Kanagaratnam *et. al*., 1998).

In view of the above, the next set of investigations focused on the possible relationship between antisense ODN inhibitory efficacy and parasite phase. The phase was thought to have an important influence on ODN uptake due to the fact that the membrane of the parasitised erythrocyte is known to become more permeable to extracellular nutrients due to the induction of new permeation pathways (NPPs) as the parasite matures (Desai, 1999; Ginsburg, 1994; Goodyer *et. al*., 1997; Thomas and Lew, 2004). For these investigations the ODNs ASL-1 and MSL-1, were added to ring and trophozoite synchronous cultures, respectively, and inhibition of parasite proliferation measured (Figure 4.5). The results showed a 17% sequence-specific inhibition of parasite proliferation after treatment of ring synchronous cultures with ASL-1. The trophozoite synchronous cultures on the other hand showed insignificant inhibition after treatment with ASL-1 or MSL-1. Thus only proliferation of ring-phase parasites appears inhibited with little or no inhibition of trophozoite-phase parasite proliferation after treatment with ASL-1. The experiment was repeated on ring-phase parasite cultures with the inclusion of the sense ODN, SL-1, as additional control (Figure 4.6). These results correlated well with those obtained in Figure 4.5 since again only about 20% ASL-1 sequence-specific inhibition was observed. The SL-1 control ODN inhibited parasite proliferation by less than 4%.

The unexpected inhibition of ring-phase cultures by MSL-1 could be due to the presence of hybridization sites on *P. falciparum* mRNAs not known at the time of ODN design (1997 and 1998). With the availability of the partially annotated *P. falciparum* genome sequence (Gardner *et. al*., 2002) it was possible to perform a retrospective analysis in order to determine possible MSL-1 hybridization sites on *P. falciparum* mRNA (Table 4.2) that may explain the unexpected inhibition observed.

As became evident from this analysis MSL-1 was found to be complementary to a region coding for a putative pyridine nucleotide transhydrogenase (E.C. 1.6.1.1). This enzyme is involved in the formation of NADH and NADPH from NAD^+ and $NADP^+$, respectively (http:\\plasmodb.org/plasmodb). These cofactors are important in glycolysis and redox metabolism, respectively, making pyridine nucleotide transhydrogenase important to parasite vitality. It is possible that MSL-1 hybridizing to 15 nucleotides of mRNA coding for this enzyme was responsible for the observed inhibition of parasite proliferation after culture incubation with this ODN. The identity of the hypothetical proteins and the possible effect of MSL-1 hybridization to regions of mRNA encoding these proteins, remains to be determined.

The about 20% sequence-specific inhibition of ring-phase cultures by ASL-1 was however a value obtained after the inhibition observed with MSL-1 had been deducted since this inhibition was believed to be non-sequence specific. From the retrospective analysis however it has become evident that it is probable that MSL-1 inhibition of parasite proliferation could be sequence-specific due to complementarities discussed above. This would then imply that MSL-1 is a sequence-specific ODN and that inhibition observed with this ODN therefore cannot be subtracted from ASL-1 inhibition. From this it seems that sequence-specific inhibition by ASL-1 of parasite proliferation is about 33% (37%-4%), after deducting the apparent non-sequence specific of SL-1 of about 4%. In order to verify this finding, at least one further, carefully designed, negative control should however be tested in future experiments. The ODN SL-1 was complementary to ASL-1, and a plus/minus strand annotation would indicate a SL-1 match (Table 4.2). No such matches were however observed (data not shown).

The search for obstacles preventing even higher inhibition of parasite proliferation can be found when evaluating results discussed above in conjunction with the results in Figure 4.9. These results showed 10 times more ASL-1 associated with trophozoite- than ring-infected parasite cultures after 2 hours incubation, indicating that ODN uptake into parasitized erythrocytes is related to parasite phase and that maximal ODN uptake occurs during the trophozoite and later developmental phases. In fact 7.2% of all cells in trophozoite-phase cultures exhibited fluorescence while only 1.46% of the cells in the ring-phase cultures fluoresced. Since the parasitaemia was normalized to 10% these results imply that 72% of the trophozoite-phase parasitised erythrocytes contained ODNs while only 14.6% of the ring-phase parasitized erythrocytes contained ODNs.

Additionally, α-I-tubulin transcription in *P. falciparum*, has been shown to occur mainly in trophozoite-phase cultures (Bozdech et. al., 2003; http://plasmodb.org/). The limited inhibition of parasite proliferation by ASL-1 was therefore likely due to limited hybridization of the ODN to its target mRNA sequence. This in turn can be due to several factors. One of these was discovered during retrospective analyses of the predicted availability of the ASL-1 hybridization site during mRNA roll off as well as during ribosome breathing (Figure 4.11). From these results it appears that the mRNA hybridization site was only available for ASL-1 binding for about 40 to 50% of the time during mRNA roll-off and ribosome breathing. It was recently established that any gene involved in the intra-erythrocytic development cycle (IDC) of *P. falciparum*, is induced only once per cycle, with this induction occurring only at the time when the gene product is required (Bozdech *et. al.*, 2003). These findings

therefore imply that mRNA transcripts are available for antisense ODN hybridization for a limited time period only during the ring phase of development during which very little ODN is taken up. Once transcription and translation are in full swing during the development phase, very little inhibition of parasite growth is observed at a phase during which uptake of ODN reaches its peak. It is thus possible that the affinity of ASL-1 for the hybridization site is too low to compete with that of the initiation complex machinery during active translation.

A further two factors that could contribute to the reduced ASL-1 hybridization include the lack of knowledge of the exact α-I-tubulin mRNA sequence 5' of the initiation codon and the possible steric effect of the 3' loop modification. It has been shown that the first four nucleotides preceding the initiation codon in *P. falciparum* mRNA is the consensus sequence AAAA (Saul and Battistutta, 1990). The identity of the other five nucleotides upstream of this sequence and also targeted by ASL-1, are not known with certainty. It is therefore possible that these nucleotides are not identical to the genomic DNA sequence, thereby further reducing the hybridization efficiency of ASL-1 to its target sequence. It must also be borne in mind that little is known about the steric effects of 3' loop modifications on ODN hybridization. It is therefore advisable to investigate the effect of decreasing the loop size on both ODN serum stability as well as inhibitory efficacy.

The inhibitory efficacy of ODNs ASL-2, ASL-3 along with their negative controls, MSL-2 and MSL-3 was also investigated on ring- and trophozoite-phase parasite cultures (Figures 4.7A and B). From these results it is apparent that all of the above ODNs exhibited low inhibition of ring-phase cultures, while trophozoite-phase

cultures were once again not inhibited, a result that correlated well with the results obtained in Figures 4.5 and 4.6. The hybridization site availability of ASL-2 and ASL-3 was predicted to be similar to that of ASL-1. The possibility that neighbouring secondary or tertiary structures interfered with antisense ODN hybridization therefore cannot be excluded. The prediction of the latter structures is still problematic (Smith et. al., 2000). Retrospective analyses of the inhibition observed with the mismatch ODNs, MSL-2 and MSL-3 (Table 4.2) indicated that MSL-2 is complementary to a region coding for *P. falciparum* PfEMP-1 (12/12 complementary nucleotides) as well as to a region coding for a putative dimethyladenosine transferase (11/11 complementary nucleotides). The complementarity to the region coding for PfEMP-1 is however not regarded as relevant to these studies. PfEMP-1 is one of the essential parasite-specific proteins that mediates the adhesion of infected erythrocytes to post-capillary microvascular endothelial cells (a process termed sequestration), thereby preventing detection of the parasite by the host immune system (Gamain et. al., 2002) Inhibition of PfEMP-1 could therefore lead to inhibition of parasite proliferation, but would be relevant only under the *in vivo* scenario.

Uptake studies presented in figure 4.8, indicated that more than double of the FITC labeled ODNs were associated with parasitized than with uninfected erythrocytes. Barker *et. al*. (1998) and Upston and Gero, (1995) showed that ODNs were unable to enter mature human erythrocytes whereas most investigations have found that ODNs are able to enter virtually all eukaryotic cells (Crooke, 2000; Phillips and Zhang, 2000). Therefore, the fluorescence associated with the uninfected erythrocytes was considered to be due to non-specific association with red blood cell membranes and was treated as background fluorescence in all subsequent studies.

The higher uptake of ODNs by trophozoite- than ring-phase parasites could be due to modifications to the host membrane as described in section 4.1. Evidence suggests that macromolecules from the extra-cellular medium reach the parasitophorous vacuolar space through new permeation pathways (NPPs), formed during the trophozoite-phase of parasite development (Desai, 1999; Ginsburg, 1994; Goodyer *et. al*., 1997; Taraschi, 1999; Thomas and Lew, 2004). Importantly, macromolecules with a diameter of up to 50 – 70 nm have been shown to have direct access to the intra-erythrocytic parasites (Goodyer *et. al*., 1997; Taraschi, 1999). The predicted diameter of a single stranded ODN is in the range of 1 –2 nm, making its uptake into the parasitised erythrocytes possible. Once the ODN reaches the parasite plasma membrane its uptake into the parasite presumably occurs via endocytosis (Crooke, 2000; Hoppe *et. al*., 2004; Pouvelle *et. al*., 1991). The exact nature of these NPPs is still under investigation but evidence is mounting in support of the hypothesis that the NPPs are part of the anion channel family (Thomas and Lew, 2004). It has further been suggested that this anion exchange channel is large and poorly selective, letting through both organic and inorganic anions.

The results presented in this chapter showed lower than expected inhibition of parasite proliferation by antisense ODNs and further went on to offer explanations for this observation. Retrospective analyses of the results, using information that has recently become available, further shed light on and supported the results obtained. The main factors influencing inhibition of parasite proliferation included phase-dependant uptake of antisense ODNs by the parasitized erythrocytes coupled with the limited availability of and/or hybridization with the parasite α-I-tubulin mRNA target with the ODNs. The importance of accurate control ODN design was also highlighted by these

results as MSL-1 was found to be complementary to a region encoding for a protein vital to parasite proliferation and therefore is likely to act as antisense ODNs itself.

CHAPTER 5

Concluding Discussion

Malaria is a disease that has plagued mankind from the times of Hippocrates (4 BC) to the present day (Goodwin, 1992). The protozoal nature of the parasite causing malaria was reported in 1880, with the identification of the mosquito as the vector of the parasite following only in 1897 (Krettli and Miller, 2001; McKenzie, 2000). Later, the female mosquitoes of the genus *Anopheles* were specifically linked to the transmission of human malaria (Krettli and Miller, 2001). The malaria parasite belongs to the phylum Apicomplexa, which includes *Plasmodia* and *Toxoplasma* to name but two members. Of the four *Plasmodia* species infecting humans (*falciparum*, *ovale*, *vivax* and *malariae*), *P. falciparum* is the most virulent.

Of the 101 countries in which malaria is a public health problem, 45 are situated in Africa. The fact that 90% of about 500 million clinical malaria cases, reported annually, occur in sub-Saharan Africa, further highlights the magnitude of the problem on this continent (WHO, 1998). The reasons for the gravity of the malaria problem include inefficient control of the mosquito vectors and resistance of the latter to insecticides, the lack of an effective vaccine, and an increase in parasite resistance to currently available anti-malaria drugs. The malaria problem is being addressed on two levels, namely vector and parasite control. A vector control strategy, entailing intra-domiciliary spraying with DDT, and drug combination therapy (artemesinin and co-artemether) started in South

Africa (Kwa-Zulu Natal) in 2000 and yielded a 75% reduction in malaria cases in the region for 2001 (www.malaria.org.za).

The widespread resistance of *P. falciparum* against chloroquine is not a new phenomenon (Phillipson and Wright, 1991; Povoa *et. al*., 1998; Hopkins-Sibley *et. al*., 2001). A more alarming observation is the resistance to the drug combination sulphadoxine pyrimethamine (SP) reported from east Africa where it has been used as a first line drug for less than 5 years (Hopkins-Sibley *et. al*., 2001). Resistance to this drug has been ascribed to point mutations in the parasite *dhfr* and *dhps* genes, coding for DHFR and DHPS, respectively. These enzymes are involved in the production of tetrahydrofolate, a key cofactor in the biosynthesis of nucleotides and amino acids (Hopkins-Sibley *et. al*., 2001). To aid in the alleviation of this problem drugs such as chlorproguanil and dapsone, acting on the same targets as SP are being used as cheaper alternatives to SP therapy.

Antigenic variation and polymorphism of key antigenic parasite proteins, targeted by the human immune system, are largely responsible for the inability of humans to develop long lasting immunity to malaria (Allred, 2001). This is also an important obstacle in the development of a successful malaria vaccine. Of the numerous vaccines developed to date the most successful is known as RTS,S/AS02A, developed by the Walter Reed Army Institute of Research (Alonso *et. al*., 2004; Bojang *et. al*., 2001; Kester *et. al*., 2001). This is a recombinant vaccine consisting of *P. falciparum* CSP fused with a hepatitis B surface antigen due to the fact that CSP alone is poorly immunogenic.

Furthermore the adjuvants used have proved vital to the success of this particular vaccine, which has been shown to provide protection against malaria in children for a period of up to six months.

Various additional strategies are also being investigated in an effort to combat malaria. These include testing extracts from plants growing in malaria endemic areas, for anti-malaria activity (Addae-Kyereme *et. al*., 2001). Mosquitoes have been genetically modified so as to prevent the completion of the parasite phase in the vector, which in turn prevents the spread of malaria to the human host (Catteruccia *et. al*., 2000; Coates, 2000). Much attention is also being paid to structure-based inhibitor design, which involves identification of a feasible target protein and availability of the three dimensional structure of the latter (Birkholtz *et. al*., 2003; Chen *et. al*., 2002; Cody *et. al*., 2002, Davies *et. al*., 2002; Joubert *et. al*., 2001).

Antisense technology offers an attractive alternative to the use of chemotherapeutic drugs. Here use is made of short oligonucleotides (antisense ODNs) that are complementary to specific sequences of parasite mRNA (Agrawal, 1996; Crooke, 2000). Inhibition of mRNA translation occurs via two main mechanisms. These entail the physical blocking of the complementary mRNA sequence, thereby preventing ribosome complexes from translating the target mRNA (Crooke, 2000). The second mechanism is based on the ability of the ODN/mRNA heteroduplex to activate RNase-H, which then proceeds to degrade the mRNA component of the duplex (Crooke, 2000; Phillips and Zhang, 2000). There are several parameters that require careful optimization to ensure the

success of the above strategy. These include choice of the protein target, choice of target mRNA sequence, modifications needed to stabilize ODNs as well as mechanisms possibly required to increase ODN uptake into the target cell (Crooke, 2000).

The aims of this thesis included the design of a structurally modified antisense ODN targeting a relevant protein of *P. falciparum*, with the subsequent testing of the protective effect of the modification against nuclease degradation of the ODN. It was further aimed to determine the *in vitro* inhibitory efficacy of the designed antisense ODN, as well as investigation of the relationship between parasite phase and ODN cellular uptake.

For these studies α-I-tubulin was chosen as the parasite target protein for some very important reasons. Firstly α-tubulin is a vital component of microtubules, which in turn play a central role in cell division (Bell, 1998; Downing and Nogales, 1998). Since *P. falciparum* is a rapidly dividing parasite, inhibition of parasite α-I-tubulin translation by antisense ODNs should significantly affect parasite proliferation over the 48 hour life cycle. This hypothesis is supported by results obtained from studies on cancer cells in which tubulin inhibitors such as colchicine, taxol, vincristine and taxotere were found to inhibit these rapidly dividing cells (Jan *et. al*., 2000). Further investigations performed to ascertain the effect of the above-mentioned anti-cancer drugs on *P. falciparum* proliferation showed these to inhibit parasite proliferation extensively as is shown in Table 2.1 (Bell, 1998).

Since the parasite and human α-tubulin share an 83% amino acid identity the specific inhibition of parasite α-tubulin is problematic. Antisense technology is not hindered by this fact however since the parasite has an 80% A + T rich genome as compared to the 60% G + C rich human genome (Gardner *et. al*., 2002). These differences are clearly identifiable in Figure 4.1 and therefore allow for the targeting of the parasite α-tubulin mRNA sequence. Of the three available tubulin molecules (α-I, α-II and beta), α-I-tubulin was specifically chosen due to the fact that it is expressed in the asexual stage of parasite development, as opposed to α-II-tubulin, which is expressed in the sexual stages of development. α-I-tubulin plays a role in the regulation of the key glycolytic enzymes, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase and aldolase (Itin *et. al*., 1993). This is of particular importance due to the high glycolytic activity of the malaria parasite as evidenced by the 50-100 fold increase in glucose uptake by parasitised erythrocytes (Hess *et. al*., 1995).

Three phosphodiester ODNs (ASL-1, ASL-2 and ASL-3) were designed, each with a structural modification in the form of a stabilizing 3' self-complementary loop consisting of 15 nucleotides. This modification was chosen above the more popular phosphorothioate (PS) modifications since it has been reported that the latter modified ODNs bind to a variety of proteins, thereby causing non-sequence specific inhibition of parasite growth (Clark *et. al*., 1994; Crooke, 2000; Vollmer *et. al*., 2002). In previous unpublished work done in our laboratories using PS-ODNs, non-sequence specific inhibition was also observed (Schulze *et. al*., 1997). Antisense ODN, ASL-1 was designed complementary to the region upstream, downstream and including the initiation

codon, while ASL-2 and ASL-3 were designed complementary to regions predicted to be accessible to ODN hybridization on the grounds of computer predictions of the secondary structure α-I-tubulin mRNA. Interestingly, even those sequences predicted to be favourable for ODN hybridization were not completely free from secondary structure. Research has shown that ODN binding to its target sequence is not negatively affected if binding occurs so as to include the 5' side of the loop part of the hairpin loop structure (Lima *et. al*., 1992). The predominant secondary structure of the mRNA target sequence of ASL-2 (Figure 2.6) shows the inclusion of an internal loop as well as a hairpin loop. This sequence was therefore calculated as being a favourable ODN hybridization site due to the fact that minimal energy is required to disrupt local secondary structure in order to facilitate thermodynamically favourable ODN hybridization (Lima *et. al*., 1992; Toulme *et. al*., 1997). A similar situation is likely for the hybridization of ASL-3 to its target mRNA, where the local secondary structure included two hairpin loops and one internal loop (Figure 2.7).

Much controversy currently still exists around the accuracy and usefulness of RNA secondary structure predictions (Smith *et. al*., 2000; Sohail and Southern, 2000; Toulme *et. al*., 1997). The prediction of secondary structure by energy minimization used in the *Mfold* program is known to provide useful structural information, but no information of higher order interactions with protein components (Sohail and Southern, 2000). Computational prediction of RNA secondary structure has the added advantage of time and money saving when compared to the gene walking and oligonucleotide scanning array alternatives described in section 2.1.3.

A fluid-phase extraction method followed by C_{18} reversed phase HPLC were used to determine whether the 3' self-complementary loop conferred sufficient stability to the PO-ODN to warrant inhibitory efficacy studies. The chromatographic data showed no significant degradation of ASL-1 after six hours incubation in culture medium. This was an encouraging and significant result since the half-life of unmodified PO-ODNs in culture medium is in the range of minutes (Gilar *et. al*., 1997; Phillips and Zhang, 2000). The combination of reversed phase HPLC as well as the fluid-phase extraction methods, to determine ODN stability under culture conditions have not been described previously (Apffel *et. al*., 1997; Borque and Cohen, 1993; Toulme *et. al*., 1997).

The *in vitro* inhibitory efficacy assays showed a possible 17% sequence-specific inhibition of parasite growth after incubation of ASL-1 with non-synchronous parasite cultures containing 52% ring-phase parasites (Figure 4.3). One explanation for the low inhibition observed is that insufficient ODN is available for hybridization to target mRNA (Crooke, 2000). Attempts to improve the inhibitory efficacy of ASL-1 by the introduction of cationic liposomal transfection reagent, DOSPER, were not successful (Figure 4.4). The most probable explanation for these results is that ODN, ODN-liposome and liposome uptake is linked to endocytosis in most cell types (Kanagaratnam *et. al*., 2000; Wanidworanum *et. al*., 1999). While the parasite is known to endocytose large amounts of the host cell cytoplasm the mature erythrocytes themselves have not been shown to endocytose molecules from the extra-cellular medium (Hoppe *et. al*., 2004; Kanagaratnum *et. al*., 2000). It is therefore probable that the ODN-liposome

complexes were unable to enter the parasitized erythrocyte and were therefore unable to hybridize to the target mRNA sequence and affect inhibition of parasite growth.

The results obtained at this point seemed to indicate that insufficient ODNs were being taken up by the parasitized erythrocytes. It is known that with progression of the parasite phase the permeability of the erythrocyte membrane increases to facilitate the increasing nutritional requirements of the parasite (Desai, 1999; Ginsburg, 1994; Goodyer *et. al*., 1997). It was therefore considered possible that ODN uptake was related to the parasite phase, with ODN uptake expected to increase with parasite maturation. Experiments were performed to investigate the uptake of antisense ODNs in ring and trophozoite-phase synchronized cultures, respectively, as well as their inhibitory efficacy (Figures 4.5, 4.6, 4.8 and 4.9). The results showed that ODN uptake into trophozoite-phase cultures was 10-fold higher than into ring-phase cultures. However, about 20% sequence-specific inhibition of parasite growth was obtained after treatment of ring-phase cultures with ASL-1 with no apparent inhibition of trophozoite-phase cultures. These results were unexpected since the α-I-tubulin transcript is present mainly in the trophozoite-phase of parasite development (Bozdech *et. al*., 2003). Retrospective analyses performed in 2003 (ODNs were designed during 1997 and 1998) showed that the α-I-tubulin mRNA hybridization site for ASL-1 is only available between 40% and 50% of the time during RNA roll off and ribosome breathing (Figure 4.11). In addition, recent evidence shows that gene induction during the IDC occurs at the time when the gene product is needed. The time period during which the transcript is freely available prior to translation initiation could be limited (Bozdech *et. al*., 2003). Since translation principally occurs in

the trophozoite-phase, when uptake of the ODN is highest, it appears that the affinity of ASL-1 aimed at the initiation site and surrounding areas is too low to compete with the initiation machinery complex. The exact mRNA sequence of 5 of the 9 nucleotides targeted upstream of the initiation codon is also not certain. The consensus sequence of the four nucleotides directly upstream of the initiation codon is known to be AAAA, while the sequence of the other 5 upstream nucleotides is not known with certainty (Saul and Battistutta, 1990). If the sequences of the mRNA and genomic DNA differ in the region 9 bases upstream of the initiation codon, the hybridization efficiency of ASL-1 would be compromised.

Taken together it is probable that the low ODN concentrations in the ring-phase of parasite development combined with the availability of the hybridization site during RNA roll off and ribosome breathing, is responsible for the relatively low inhibition of α-I-tubulin translation in the late ring phase.

Inhibition studies performed on synchronous parasite cultures with ASL-2 and ASL-3 showed insignificant sequence-specific inhibition of ring-phase parasite cultures (Figure 4.7). Three possible explanations can be forwarded for this result. Firstly, the local secondary structure of the three hybridization sites differ, thereby presenting different energy requirements for ODN hybridization and affecting the inhibitory efficacy of the antisense ODNs (Crooke, 2000; Lima *et. al*., 1992; Toulme *et. al*., 1997). Secondly, it is possible that secondary structures adjacent to the hybridization sites of ASL-1, ASL-2 and ASL-3 may have different influences on the hybridization affinity of the ODNs,

thereby also affecting antisense ODN inhibitory efficacy (Riessner, 1997). Thirdly, it must be borne in mind that higher order interactions of the mRNA with proteins may also influence ODN hybridization (Smith *et. al*., 2000; Sohail and Southern, 2000). These effects are not amenable to prediction.

The unexpected inhibition observed with the mismatch ODNs, MSL-1, MSL-2 and MSL-3 could also be explained by retrospective analyses that were performed (April 2005), after the partially annotated sequence of the *P. falciparum* genome became available (Table 4.2). These results showed that MSL-1 was complementary to a region coding for a putative pyridine nucleotide transhydrogenase (15/15 complementary nucleotides). Since this is a parasite protein vital to the formation of NADH and NADPH, inhibition thereof could inhibit parasite proliferation. This ODN was also found to be complementary to a number of *P. falciparum* hypothetical proteins, the identity and thus function of which remains to be elucidated. The effective usefulness of MSL-1 as control in these experiments therefore requires revision. From the discussed retrospective analysis it would appear that MSL-1 is itself acting as an antisense ODN and the inhibition obtained with this ODN can thus not be subtracted from that obtained with ASL-1. The ODN SL-1, which inhibited parasite growth by less than 4% (Figure 4.6), then serves as the only control. This in turn implies that ASL-1 inhibited parasite growth by more than 33% instead of 17%.

MSL-2 and MSL-3 were also found to inhibit ring-phase parasite proliferation by 11.6% and 6.1%, respectively (Figure 4.7). Retrospective analyses showed that MSL-2 is

complementary to a region coding for PfEMP-1 (12/12 complementary nucleotides) as well as to region coding for a putative dimethyladenosine transferase (11/11 complementary nucleotides). These complementary regions, despite being under the minimum length of 15 complementary nucleotides for typical antisense ODNs, could be responsible for the slight but yet unexpected MSL-2 inhibition of parasite proliferation.

The increased ODN uptake by trophozoite-phase cultures appears to be related to the increased red blood cell permeability to a variety of compounds in the late ring-phase. The existence of a parasitophorous duct has been the point of much debate in past years and was described for the first time by Pouvelle *et. al*. (1991). Evidence indicated that macromolecules macromolecules with a diameter of up to 50-70 nm from the extra-cellular medium are able to reach the parasite via a parasitophorous duct (Pouvelle *et. al*., 1991; Taraschi *et. al*., 1999). Newer studies have provided convincing evidence that parasite uptake of anions occurs via a poorly selective anion channel (Thomas and Lew, 2004). Taking these results into account, it is therefore entirely possible for a single stranded ODN with a diameter of 1-2 nm, to gain access to the parasite.

For future studies utilizing antisense ODNs in malaria research it is worthwhile to take the following aspects into consideration. The stage of parasite development during which the RNA transcript is available for ODN hybridization and hence inhibition of protein translation, appears to be important. The mRNA transcript should preferably be available during the trophozoite-phase of parasite development. If this is not possible it must be considered to introduce sufficient ODN during the earlier phases by utilizing techniques

such as micro-injection or electroporation or alternatively by constitutively expressing an antisense ODN or RNA transcript. The mRNA secondary structures should be determined experimentally if possible, by using techniques such as RNA structure mapping with dimethyl sulphate (DMS) or by the analysis of structural transitions and complex formation of RNA by temperature-gradient gel electrophoresis (TGGE) (Riesner, 1997). Antisense ODNs should then be targeted to regions calculated to be thermodynamically favourable hybridization sites. Control ODNs should also be carefully designed and database searches should be performed to avoid any unwanted hybridization of the control ODNs to any *P. falciparum* sequences, and thereby prevent the latter from acting as antisense agents themselves.

It is interesting to note that 50% inhibition of parasite proliferation was obtained with phosphorothioate antisense ODNs (11 nM) targeting *P. falciparum* aldolase (Wanidworanum *et. al*., 1999). Here it is particularly interesting to note that aldolase is expressed during the schizont phase of the parasite life cycle suggesting inhibition of mRNA translation at the time of maximum ODN uptake by the parasitized erythrocytes, with presumed hybridization to target mRNA (Knapp *et. al*., 1990).

Once sufficient inhibition of parasite proliferation has been achieved the sequence specificity of the inhibition should be indicated on the RNA and protein levels, i.e. it should be shown that the antisense ODN hybridized to the correct mRNA transcript as well as that the translation of the targeted protein is affected.

It would further be necessary to determine the exact half-life of the structurally modified ODNs, designed and investigated in these studies, in culture medium as well as in the infected red blood cell. It is also imperative to investigate the effect of varying loop sizes on both ODN stability as well as inhibitory efficacy.

For future work investigating alternative ways of inhibiting malaria parasite proliferation, it would be interesting to determine the effect of small interfering RNAs (siRNAs) on parasite proliferation. The two main advantages of this technique over antisense technology is that lower concentrations are needed to attain the same inhibitory effect as with antisense ODNs and secondly, that siRNAs can be expressed intracellulary from RNA polymerase III promoters (Thompson, 2002). Antisense technology however also has distinct advantages, at this stage. These include the fact that more information is available on the modifications that offer ODNs resistance to nuclease degradation. This makes antisense technology easier to apply to mammalian systems (Thompson, 2002). Due to the fact that antisense technology is more mature than siRNAs, stricter and better controls are in place to aid evaluation of results obtained. Furthermore, it is known that more than half of the human genes produce more than one protein by using alternative splicing. In connection with the above it is also known that antisense ODNs can be targeted to inhibit specific splice site variants by targeting exon-exon junctions in mature mRNAs, while this still needs to be ascertained in siRNA technology (Thompson, 2002).

In conclusion it is interesting to note that no naturally occurring siRNAs have been observed in *P. falciparum* to date, while it has been found that the latter utilizes antisense

RNA extensively in the regulation of gene expression (Gunasekera *et. al*., 2004). It is can therefore be hypothesized that continued research, utilizing synthetic antisense ODNs, to modulate *Plasmodium falciparum* gene expression should yield profitable results. This should hold true particularly in enabling researchers to acquire an increased understanding of vital parasite proteins and their metabolic functions.

SUMMARY

The high incidence of malaria, coupled to the increasing occurrence of parasite resistance against commonly used anti-malaria drugs and the absence of an effective vaccine, makes the implementation of alternative strategies to combat this killer disease imperative. Antisense technology is one such alternative strategy, which makes use of complementary oligodeoxynucleotides designed to inhibit specific parasite mRNA sequences. These bind to their complementary mRNA target sequences and inhibit their translation. In this study α-I-tubulin was chosen as protein target due to its vital role in cell division.

Three antisense ODNs (ASL-1, ASL-2 and ASL-3) were designed, complementary to different regions of the parasite α-I-tubulin mRNA. ASL-1 was complementary to the region surrounding the translation initiation codon, with ASL-2 and ASL-3 being complementary to nucleotides 92-118 and 118-144, respectively. These regions were predicted to be accessible for ODN hybridization by RNA secondary structure determination and subsequent analysis. The above ODNs were modified to offer protection against nuclease degradation, by the inclusion of a 15 nucleotide, self-complementary 3' terminal loop. The stability of these modified ODNs was investigated in serum-containing culture medium, by means of reversed-phase HPLC. These studies showed that the ODN was not significantly degraded after 6 hours of incubation at 37°C in complete culture medium.

A sequence-specific inhibition of parasite proliferation of 20% was obtained after antisense ODN, ASL-1 was incubated with ring-phase parasitised cultures. The inclusion of cationic liposomal transfection reagent, DOSPER, abolished inhibition of parasite growth. Only minimal parasite inhibition was observed with ASL-2 and ASL-3. Studies on the effect of parasite phase on ODN uptake showed ODN uptake into trophozoite phase cultures to be 10x higher than in ring-phase cultures. No inhibition was obtained after ASL-1 ODN incubation with trophozoite-phase cultures. Higher uptake in the trophozoite-phase could be explained by modifications introduced into the erythrocyte membrane, by the maturing parasite (72% of trophozoite and only 14.6% of ring-phase parasitised cultures contained ODNs).

Retrospective analyses showed that alpha-I-tubulin mRNA was available for ASL-1 hybridization only 40%-50% of the time. The lack of inhibition of the trophozoite developmental stage, by any of the ODNs tested, suggests that the targeted site is no longer available for hybridization. Since ASL-1 was directed to the initiation site and the surrounding sequences it is likely that the ODN is unable to compete with the initiation complex during active translation. The low inhibition observed during the ring phase appears to be due to two factors, low uptake in this phase and availability of the target site prior to translation. Retrospective analysis further showed that the mismatch control ODNs, particularly MSL-1, are complementary to several parasite nucleotide sequences. Inhibition due to antisense ODN ASL-1 could therefore be increased to 33%, when sequence-specific MSL-1 inhibition was not deducted.

Inhibitory efficacy of ODNs targeted against the malaria parasite is therefore dependent on the choice of target protein, the local mRNA secondary structure of the target sequence and the transcription and translation stages of the target mRNA and protein, respectively, which include the parasite phases during which these take place.

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