University of Pretoria etd – Schulze, D L C (2006)

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.

University of Pretoria etd – Schulze, D L C (2006)

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,

University of Pretoria etd – Schulze, D L C (2006)

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

University of Pretoria etd – Schulze, D L C (2006)

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.