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).

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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             *  *  *  *  *   *  *  *      *           *      *  *  *   *  *  *   *  *  *  *   *  *  *      *      *  *
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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               *  *   *  *  *   *  *  *  *   *  *        *  *   *     *  *   *     *   *     *        *  *
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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.

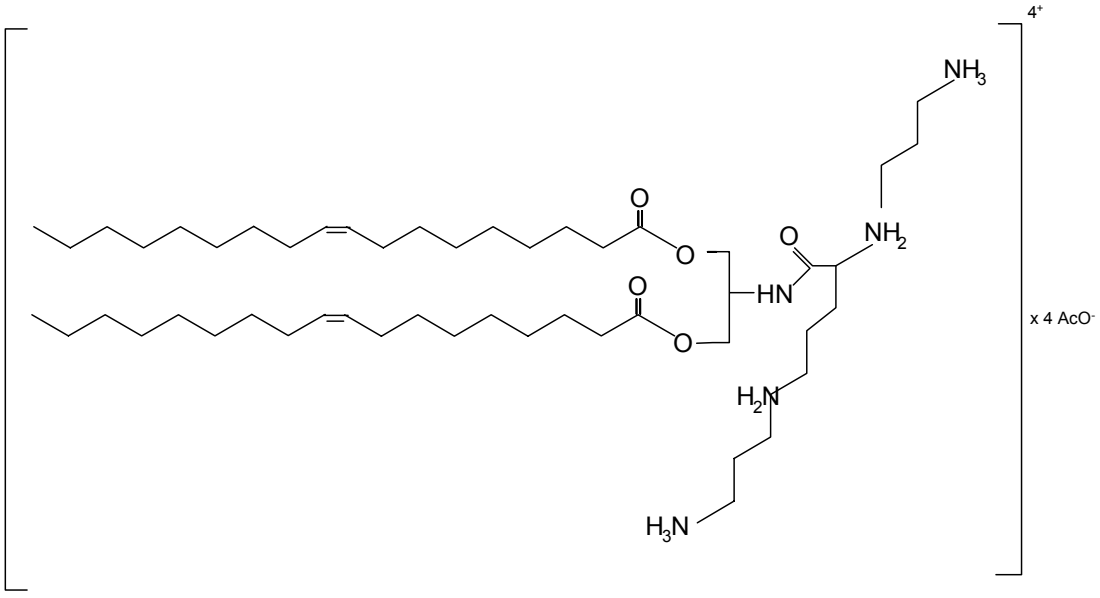

Figure 4.2: Molecular structure of 1,3-di-oleoyloxy-2-(6-carboxy-spermyl)-propyl amide (DOSPER) cationic lipsosomal transfection reagent (Boehringer Mannheim product information, 1996).

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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).

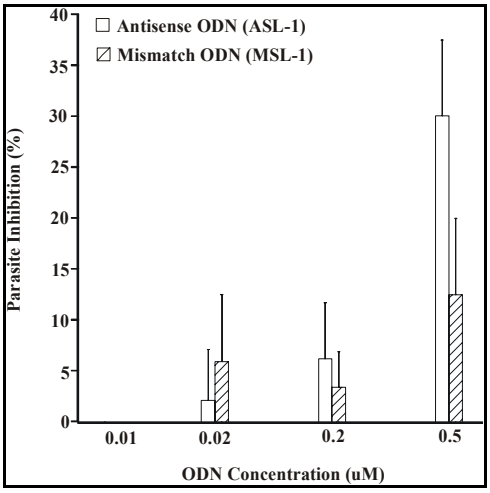

Figure 4.3: Inhibition of parasite growth, measured by flow cytometry after a 48 hour incubation period of non-synchronous PfUP1 cultures (52% ring phase; 48% trophozoite phase) with antisense ODN, ASL-1, targeted against *P. falciparum* α-I-tubulin, at concentrations ranging from 10 nM to 0.5 µM. The 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).

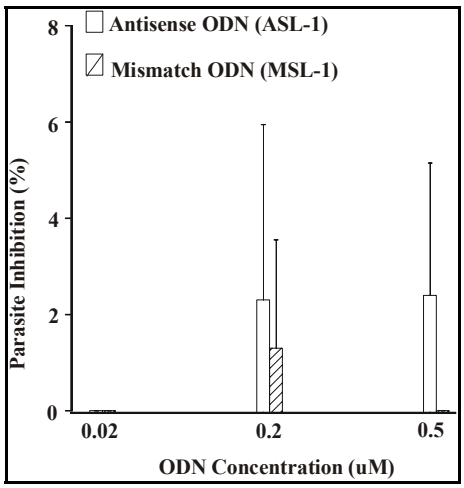

Figure 4.4: Inhibition of parasite growth measured by flow cytometry after a 48 hour incubation period of non-synchronous PfUP1 cultures with antisense ODN ASL-1, targeted against *P. falciparum* α-I-tubulin. The ODN concentrations ranged from 0.02 µM to 0.5 µM with a ODN:liposome ratio of 1:2. The results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

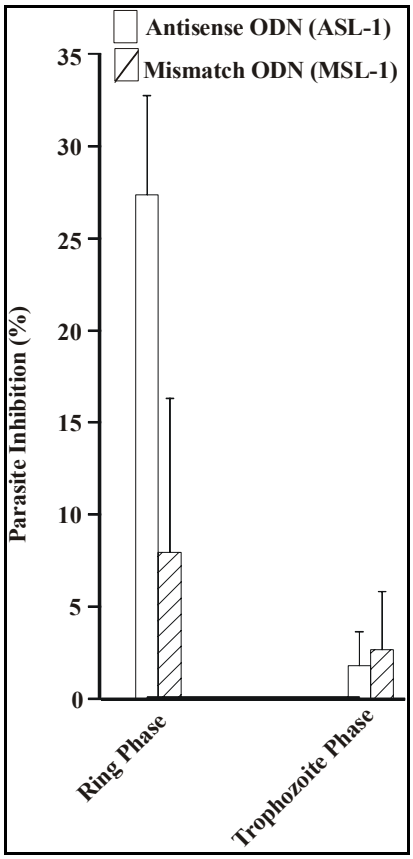

Figure 4.5: Inhibition of parasite growth, measured by flow cytometry after a 48 hour incubation period of ring (4-8 hours post invasion) and trophozoite phase (24-30 hours post invasion) synchronous PfUP1 cultures with 0.5 µM antisense ODN ASL-1, and mismatch ODN MSL-1. The results are given as the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

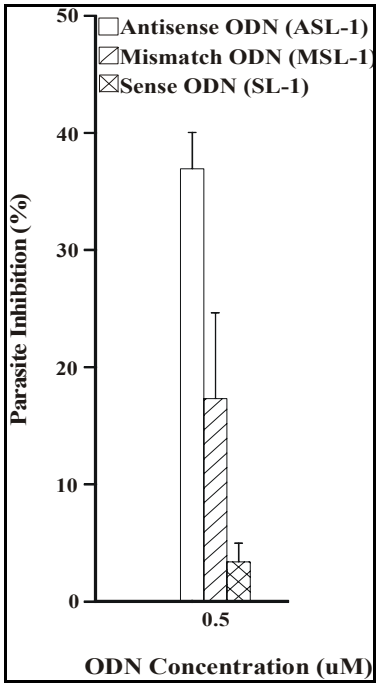

Figure 4.6: Inhibition of parasite growth measured by flow cytometry after a 48 hour incubation period of ring-phase (4-8 hours post invasion) synchronous PfUP1 with 0.5 µM ASL-1, MSL-1 and the sense sequence ODN (SL-1) as an additional control. The results are given as the mean of three samples ± SEM.

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.

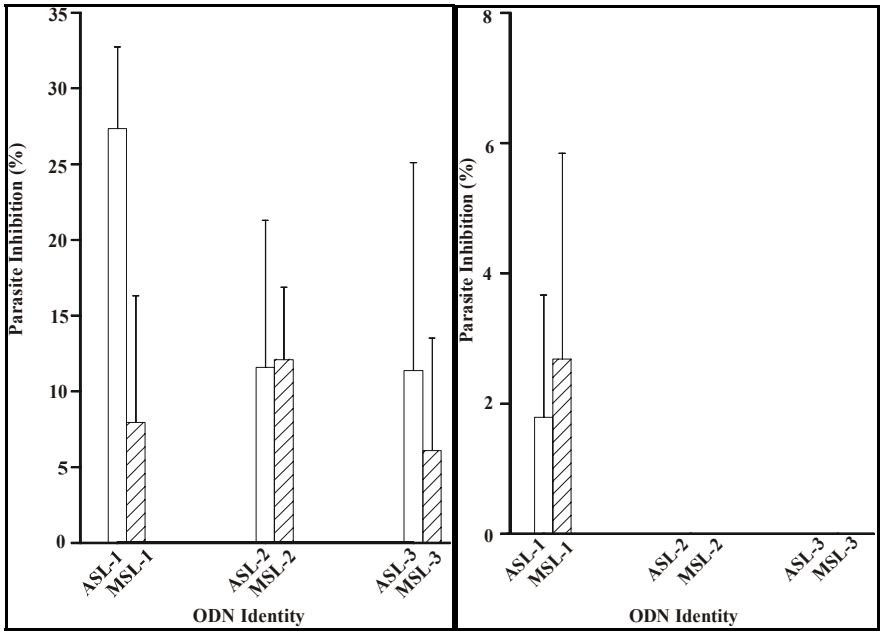

Figure 4.7: A: Inhibition of parasite growth measured by flow cytometry after a 48 hour incubation period of ring-phase (4-8 hours post invasion) synchronous PfUP1 with 0.5 µM ASL-1, ASL-2 and ASL-3 with 0.5 µM MSL-1, MSL-2 and MSL-3 mismatch ODNs, respectively. The results are given as the mean values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

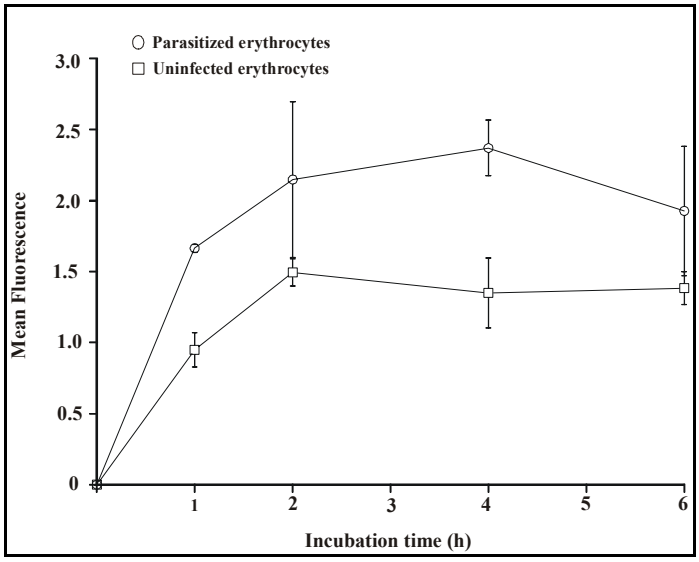

Figure 4.8: The cellular uptake of 5' -labeled FITC antisense ODN ASL-1 into non-synchronous parasitized and control uninfected erythrocyte cultures after incubation at 37°C for 1, 2, 4 or 6 hours. Fluorescence associated with the cell populations was determined by flow cytometry. Results are the mean 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.

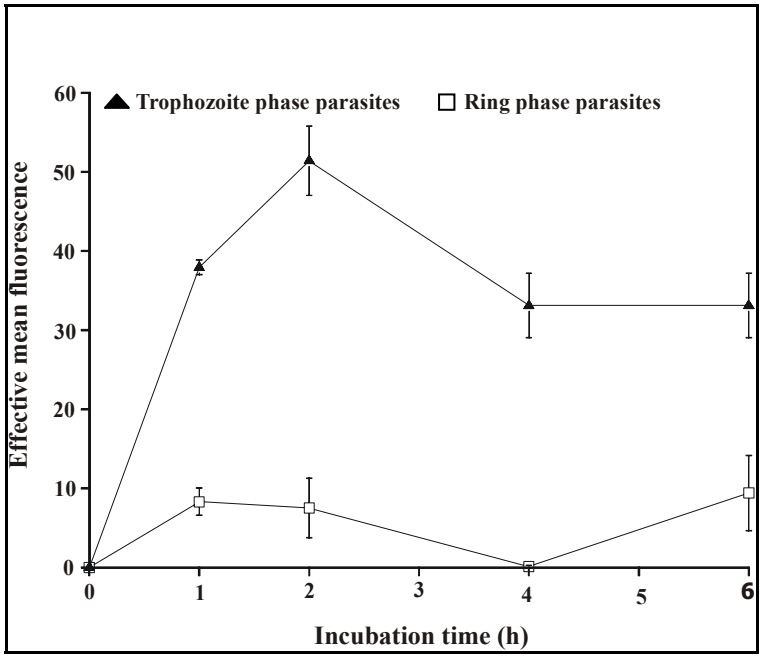

Figure 4.9: The cellular uptake of 5'FITC labeled antisense ODN ASL-1, by synchronous ring and trophozoite phase parasite cultures after incubation periods of 1, 2, 4 and 6 hours. Uninfected erythrocyte cultures treated in the same way were used to determine background fluorescence and were subtracted 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.

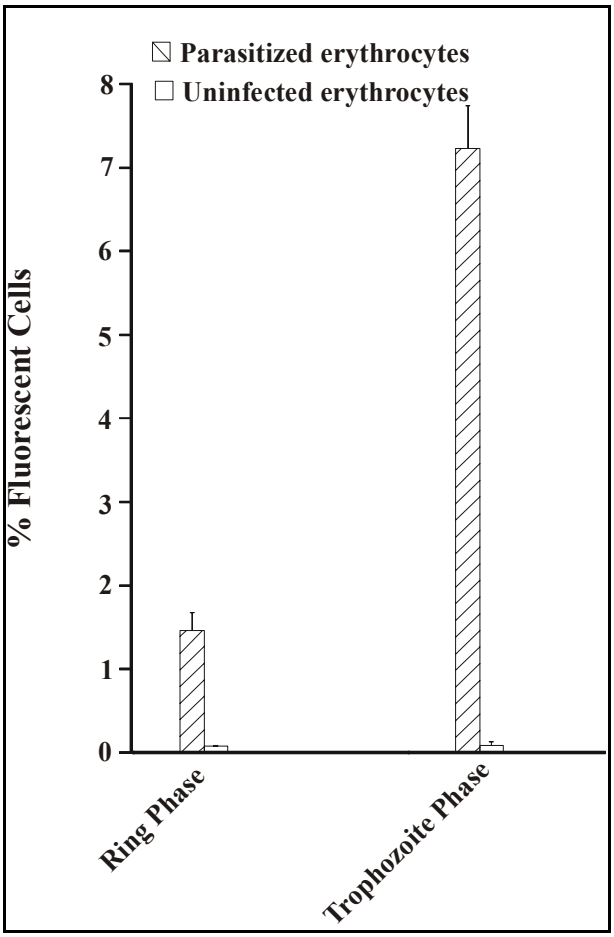

Figure 4.10: The percentage fluorescent cells present in trophozoite and ring infected cultures, respectively, after two hours incubation with 5'FITC conjugated antisense ODN, ASL-1. All values were normalised to a parasitaemia of 10%. Results are the mean of three samples ± SEM.

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).

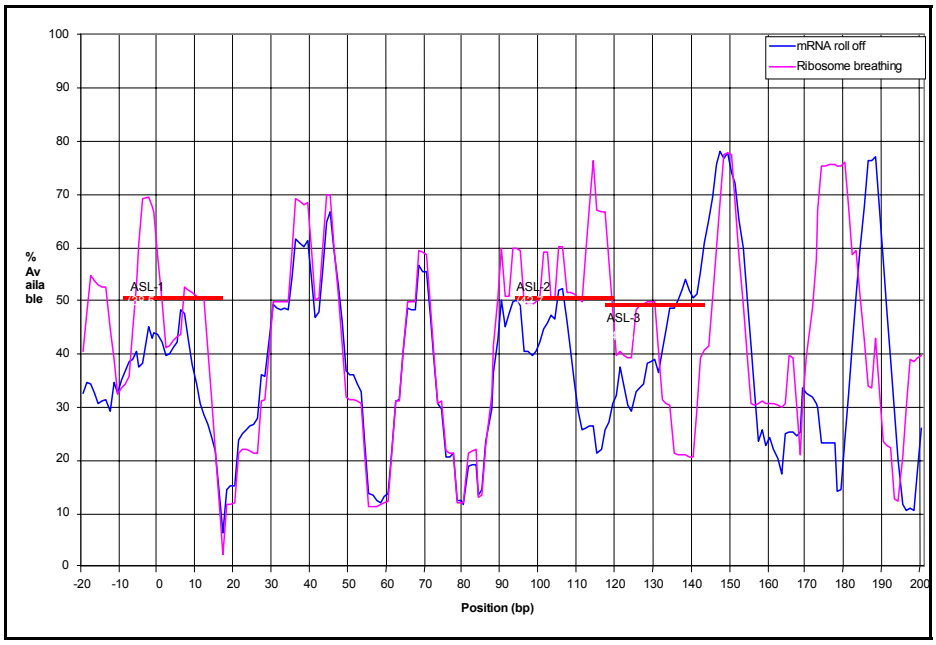

Figure 4.11: The percentage availability of the antisense–binding sites of ASL-1, ASL-2 and ASL-3 in the alpha-I- tubulin gene (Genbank acc No X15979) during both the transcription (mRNA roll off) and translation (ribosome breathing) processes.

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.