

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

by

Deborah Lizanne Claire Schulze

Submitted in partial fulfillment of the requirements for the degree

*Philosophiae Doctor*

Department of Biochemistry

School of Biological Sciences

Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria

South Africa

April 2005

## **Acknowledgements**

I would like to thank the following people and institutions for their contribution to this thesis:

Prof. A.I. Louw, Department of Biochemistry, University of Pretoria, my supervisor, for his guidance, moral support and endless patience through numerous difficult project and thesis phases.

Prof. A.W.H. Neitz, Department of Biochemistry, University of Pretoria, my co-supervisor, for his guidance and support in the completion of this thesis.

Prof. J.A. Verschoor, Head, Department of Biochemistry, University of Pretoria, for supporting an extension of the final thesis completion date.

Prof. M. Meyer, Head, Department of Botany, University of Pretoria, for making the department's HPLC available for my use to complete vital experiments.

Dr. Erwin Prozesky, Department of Botany, University of Pretoria, for help in setting up the HPLC program as well as for his willingness to share the obligatory weekend and holiday parasite culturing shifts.

Dr. Fourie Joubert, Department of Biochemistry, University of Pretoria, for his help in the installation of the computer software used to perform RNA secondary structure analyses.

Jaco de Ridder, Department of Biochemistry, University of Pretoria, for his assistance in the  $\alpha$ -I-tubulin mRNA roll-off and ribosome breathing analyses as well as the retrospective BLAST searches. His invaluable assistance with later evaluation of these results as well as numerous computer-related problems is also much appreciated.

Prof. L. Visser for introducing me to the field of antisense technology.

The NRF and the University of Pretoria for financial assistance in the form of bursaries and assistantships respectively, as well as the assistance received from the FRD in the financing of this project.

Friends and family- in- law for their encouragement and support throughout this study.

My late mother, who passed away during the final stages of thesis completion, after a long illness bravely borne, and my father for their never-ending support, help, encouragement and love over all the years.

Last but not least my husband, Hanco and our darling son James for love, patience and always being there to put thesis-related problems back into perspective.

**Chapter 1: Literature Overview**

1.1 History and overview of malaria.....	1
1.2 Reasons for the current malaria problem.....	6
1.3 Anti-malaria drugs and parasite resistance .....	12
1.4 Malaria vaccine development .....	17
1.5 Additional malaria-combative strategies under investigation.....	20
1.5.1 Alternative Medicine (Traditional Medicine).....	21
1.5.2 Genetically modified mosquitoes .....	22
1.5.3 Inhibitor design .....	23
1.5.4 Small interfering RNAs (siRNAs).....	24
1.6 Background and overview of antisense technology.....	25
1.6.1 Antisense target protein .....	28
1.6.2 Phosphodiester oligodeoxynucleotide stability.....	29
1.6.2.1 Backbone modifications.....	29
1.6.2.2 Nitrogenous base modifications.....	34
1.6.2.3 Sugar modifications .....	34
1.6.2.4 Self-stabilizing loops .....	36
1.7 Cellular ODN uptake .....	36
1.8 Aims.....	39

**Chapter 2: The identification of target proteins and the design of antisense oligodeoxynucleotides**

2.1 Introduction .....	41
2.1.1 General.....	41
2.1.2 Target protein selection.....	44
2.1.3 Target sequence selection .....	47
2.1.3.1 Random shotgun approach or gene walk.....	48
2.1.3.2 Predictions of mRNA secondary structure .....	48
2.1.3.3 Oligonucleotide scanning arrays.....	49
2.1.3.4 Oligomer library/ribonuclease-H-digestion .....	50
2.1.4 Oligonucleotide modification selection .....	51
2.2 Materials and Methods.....	53
2.2.1 Materials .....	53
2.2.2 Methods.....	53
2.2.2.1 $\alpha$ -I-tubulin sequences.....	53
2.2.2.2 The RNA folding program, <i>Mfold</i> .....	53
2.2.2.2.1 Drawing and evaluation of mRNA structures.....	53
i RNA Structure 3.5 .....	53
ii RNAdraw .....	54
2.3 Results.....	55
2.3.1 Selection of target mRNA sequences .....	55

2.3.1.1	Secondary structure prediction of <i>P. falciparum</i> mature $\alpha$ -I-tubulin mRNA.....	55
2.3.1.2	Identification of accessible hybridization sites and design of antisense ODN.....	58
2.3.1.3	Design of an antisense ODN targeted against the translation initiation codon.....	66
2.4	Discussion.....	69

### **Chapter 3: Stability of modified antisense phosphodiester ODN under culture conditions**

3.1	Introduction.....	76
3.1.1	Capillary electrophoresis.....	76
3.1.2	High Performance Liquid Chromatography.....	78
3.1.2.1	Anion exchange HPLC.....	78
3.1.2.2	Reversed Phase HPLC.....	79
3.2	Materials and Methods.....	80
3.2.1	Materials.....	80
3.2.2	Methods.....	80
3.2.2.1	Optimization of separation and elution conditions.....	80
3.2.2.2	HPLC analysis of ODN samples.....	80
3.2.2.3	Method used for extraction of ODNs from parasite culture medium.....	81
3.2.2.3.1	Fluid phase extraction.....	81
3.3	Results.....	84
3.3.1	Extraction of ASL-1 and IS from serum containing medium and subsequent elution conditions from C <sub>18</sub> reversed phase HPLC column.....	84
3.3.2	Determination of ODN stability.....	85
3.4	Discussion.....	88

### **Chapter 4: Uptake and efficacy of modified phosphodiester ODNs to inhibit *in vitro* erythrocytic cultures of *P. falciparum***

4.1	Introduction.....	92
4.2	Materials and Methods.....	99
4.2.1	Materials.....	99
4.2.2	Methods.....	99
4.2.2.1	<i>In vitro</i> culturing of malaria parasites.....	99
4.2.2.2	Giemsa-stained blood smear preparation.....	100
4.2.2.3	<i>In vitro</i> synchronization of malaria parasites.....	101
4.2.2.4	Determination of antisense ODN efficacy to inhibit parasite proliferation.....	101
4.2.2.5	Flow cytometric (FC) measurement of parasitaemia in fixed parasite cultures.....	102
4.2.2.6	Uptake of FITC-labeled ODNs.....	103

4.2.2.7 Determination of hybridization site availability .....	104
4.3 Results.....	106
4.3.1 <i>In vitro</i> ODN inhibitory efficacy .....	106
4.3.2 Cellular ODN uptake .....	111
4.3.3 Retrospective analysis.....	114
4.4 Discussion .....	121
<b>Chapter 5: Concluding discussion.....</b>	<b>130</b>
<b>Summary.....</b>	<b>144</b>
<b>References.....</b>	<b>147</b>

## LIST OF TABLES

Table 1.1	Classification of the most commonly used anti-malarial drugs.....	12
Table 1.2	Principle polymorphisms in the <i>Plasmodium falciparum dhfr</i> and <i>dhps</i> genes with the resulting amino acid changes. ....	15
Table 2.1	Inhibition of proliferation of <i>P. falciparum</i> in cultured erythrocytes and mammalian cells by microtubule inhibitors.....	46
Table 2.2	The percentage prevalence of the first base in each of the mature mRNA structures predicted by the RNA Structure 3.5 program to be the most accessible hybridization site for a 27-mer antisense ODN. ....	60
Table 2.3	Three <i>P. falciparum</i> $\alpha$ -I-tubulin mature mRNA sequences predicted to be accessible for hybridization of a 27-mer antisense ODN. ....	60
Table 2.4	Sequences and Tm values of the antisense ODN (AS-1), targeted against <i>P. falciparum</i> alpha-I-tubulin initiation codon region as well as the sense and mismatch control sequences.....	66
Table 2.5	Sequences and Tm values of the structurally modified antisense ODNs AS-1, AS-2 and AS-3 targeted against <i>P. falciparum</i> $\alpha$ -I-tubulin initiation codon (ODN: ASL-1, together with the sense and mismatch control sequences) and <i>P. falciparum</i> $\alpha$ -I-tubulin internal sequences (ASL-2 and ASL-3, together with mismatch control sequences).....	68
Table 3.1	The gradient conditions used to establish successful separation between ASL-1 and internal standard (IS).....	82
Table 3.2	The recoveries of both ASL-1 and IS ODNs from culture medium after the specified incubation periods of ASL-1, followed by fluid phase extraction of both ASL-1 and IS.....	87
Table 4.1	Oligonucleotides with FDA approval or undergoing clinical trials.....	92
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. ....	114

## LIST OF FIGURES

Figure 1.1:	The complete life-cycle of the human malaria parasite, <i>P. falciparum</i> .	2
Figure 1.2:	Schematic representation of merozoite invasion of a host erythrocyte and an ultrastructural view of the parasite components involved in invasion.	4
Figure 1.3:	World map indicating the regions (red) in which indigenous malaria is prevalent.	5
Figure 1.4:	Schematic representation of the interaction at the cytoadhesive interface between a <i>P. falciparum</i> infected erythrocyte and host vascular endothelium.	9
Figure 1.5:	Structures of some of the most commonly used anti-malaria drugs.	13
Figure 1.6:	Illustration of an antisense ODN binding to a complementary segment of mRNA, inhibiting translation of the target protein.	27
Figure 1.7:	Illustration of the most popular first generation modifications of the ODN phosphodiester bond which protect against nuclease degradation.	30
Figure 1.8:	Illustration of a morpholino ODN where the entire sugar phosphate backbone was replaced by morpholino diamidates.	32
Figure 1.9:	Illustration of the peptide nucleic acid (PNA) analogue hybridized to target RNA.	33
Figure 1.10:	Illustration of sugar modifications used in attempts to enhance hybridization between antisense ODN and RNA and to protect the ODN against nuclease degradation.	35
Figure 2.1:	Graphic portrayal of the most common secondary and tertiary structure formations found in RNA.	43
Figure 2.2:	Illustration showing the spatial arrangement of $\alpha$ - and $\beta$ -tubulin in the heterodimers that are assembled into microtubules.	45
Figure 2.3:	Complete gene sequence of <i>P. falciparum</i> $\alpha$ -I-tubulin. The translation initiation codon is underlined and the exons are indicated in bold letters.	52
Figure 2.4A:	The predicted optimal structure of <i>P. falciparum</i> mature $\alpha$ -I-tubulin mRNA.	56
Figure 2.4B:	One example of the 17 sub-optimal structures of <i>P. falciparum</i> $\alpha$ -I-tubulin mRNA.	57
Figure 2.5:	Histogram depicting the first base of the mature mRNA sequence in each of the predicted secondary structures of <i>P. falciparum</i> mature $\alpha$ -I-tubulin mRNA predicted by the program RNA Structure 3.5, to be optimal for hybridization to a 27-mer antisense ODN.	59
Figure 2.6:	The mRNA secondary structure of nucleotides 92-118 and -9 to 18.	61
Figure 2.7A:	The optimal ODN hybridization sites in the predicted secondary mRNA structures of Tub 1,3,5,7,11, and 14: Nucleotides 118-144 are shown.	62



Figure 2.7B:	The optimal ODN hybridization sites in the predicted secondary mRNA structures of Tub 1, 3, 5, 7, 11 and 14: Nucleotides –9 to 18 are shown. ....	63
Figure 2.8A:	The most accessible hybridization sites in the predicted Secondary structures of Tub 4 and 16: nucleotides 164-190 are shown. ....	64
Figure 2.8B:	The most accessible hybridization sites in the predicted Secondary structures of Tub 4 and 16: Nucleotides –9 to 18 are shown. ....	65
Figure 3.1:	A typical reversed phase HPLC chromatogram (Varian 9012 HPLC) obtained using a Phenomenex LUNA C <sub>18</sub> column. ....	85
Figure 3.2:	Calibration curve ( $r^2 = 0.9985$ ) showing the relationship between sample ODN and peak height, obtained using HPLC analysis data, and ODN quantity ranging from zero to 0.48 $\mu$ M. ....	86
Figure 3.3:	Stability of a modified phosphodiester ODN, targeted against <i>P. falciparum</i> $\alpha$ -I-tubulin, after incubation in serum-containing culture medium for time periods ranging from zero to six hours. ....	86
Figure 4.1:	The nucleotide and amino acid sequences of <i>P. falciparum</i> $\alpha$ -I-tubulin (Genbank accession number: X15979) and <i>H. Sapiens</i> (Genbank accession number: X01703). ....	95
Figure 4.2:	Molecular structure of 1,3-di-oleoyloxy-2-(6-carboxy-spermyl)-propyl amide (DOSPER) cationic liposomal transfection reagent. ....	96
Figure 4.3:	Inhibition of parasite growth measured by flow cytometry after a 48 hour incubation period of non-synchronous PfUP1 cultures with antisense ODN ASL-1 at ODN concentrations ranging from 10 nM to 0.5 $\mu$ M. ....	106
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. The ODN concentrations ranged from 0.02 $\mu$ M to 0.5 $\mu$ M with an ODN: liposome ratio of 1:2. ....	107
Figure 4.5:	Inhibition of parasite growth inhibition by flow cytometry after a 48 hour incubation period of ring and trophozoite phase synchronous PfUP1 with 0.5 $\mu$ M antisense ODN, ASL-1, and mismatch ODN MSL-1. ....	108
Figure 4.6:	Inhibition of parasite growth measured by flow cytometry after a 48 hour incubation period of ring-phase synchronous PfUP1 with 0.5 $\mu$ M ASL-1, MSL-1 and sense sequence ODN (SL-1) as an additional control. ....	109
Figure 4.7:	a: Inhibition of parasite growth measured by flow cytometry after a 48 hour incubation period of ring-phase synchronous PfUP1 with 0.5 $\mu$ M ASL-1, ASL-2 and ASL-3, MSL-1, MSL-2 and MSL-3, respectively b: Inhibition of parasite growth measured by flow cytometry after a 48 hour incubation period of trophozoite-phase synchronized PfUP1 with 0.5 $\mu$ M ASL-1, ASL-2, ASL-3, MSL-1, MSL-2 and MSL-3 ODNs, respectively. ....	110

Figure 4.8:	The cellular uptake of 5' labeled FITC antisense ODN ASL-1 into non-synchronous parasitized and control erythrocyte cultures after incubation at 37 °C for 1, 2, 4 and 6 hours. ....	111
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.....	112
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. ....	113
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 accession number X15979) during both the transcription (mRNA rolloff) and translation (ribosome breathing) processes.....	120

## LIST OF ABBREVIATIONS

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