Biochemical characterisation of putrescine and spermidine uptake as a potential therapeutic target against the human malaria parasite, *Plasmodium falciparum*

by

Jandeli Niemand

Submitted in partial fulfilment of the requirements for the degree

*Philosophiae Doctor* Biochemistry

in the Faculty of Natural & Agricultural Science

University of Pretoria

Pretoria

August 2011
Submission declaration:

I declare that the thesis/dissertation which is herewith submitted to for the degree Philosophiae Doctor Biochemistry at the University of Pretoria is my own work has and has not previously been submitted by them for a degree at this or any other tertiary institution.

Signed: Jandeli Niemand

Date: 24 August 2011
Full name: Jandeli Niemand  Student number: 21001953
Title of work: Biochemical characterisation of putrescine and spermidine uptake as a potential therapeutic target against the human malaria parasite, *Plasmodium falciparum*

Declaration

1. I understand what plagiarism entails and am aware of the University’s policy in this regard.
2. I declare that this thesis is my own, original work. Where someone else’s work was used (whether from a printed source, the internet or any other source) due acknowledgement was given and reference was made according to departmental requirements.
3. I did not make use of another student’s previous work and submit it as my own.
4. I did not allow and will not allow anyone to copy my work with the intention of presenting it as his or her own work.

Signature: Niemand  Date: 24 August 2011
Acknowledgements

I wish to thank my supervisor Prof. Lyn-Marie Birkholtz (Department of Biochemistry, University of Pretoria), for her innovative ideas, encouragement and leadership, and for allowing me to pursue my own interests. I am immensely grateful for the help Lyn-Marie has given me, ranging from PhD supervision and discussions of my future plans as a scientist to providing an additional pair of hands when the work required it. I am indebted to my co-supervisor, Prof. Braam Louw (Department of Biochemistry, University of Pretoria) for teaching me to think outside of the box, as well as for being always available for brainstorming sessions. I wish to also thank my co-supervisor Prof. Kiaran Kirk (Research School of Biology, The Australian National University) for his astute analysis of experimental findings, his willingness to explain scientific principles and his patience in teaching me scientific writing skills.

I thank Prof. Annie Joubert (Department of Physiology, University of Pretoria) for allowing a part of the work to be performed in her laboratory, and for helpful discussions regarding the FACS data. I wish to acknowledge Dr Rowena Martin (Research School of Biology, The Australian National University) for allowing me access to the *Xenopus laevis* expression system and Rosa Marchetti and Megan Nash for assistance with the expression studies. I am also grateful for Dr Heinrich Hoppe and Dr Musa Mhlanga (CSIR Biosciences, Pretoria) for allowing me access to their instrumentation, and to Caron Griffiths for assistance with the deconvolution microscopy. In addition I wish to thank Annette Exley (Department of Biochemistry, University of Pretoria) for help with HPLC analyses. You are sorely missed.

I wish to thank my past and present colleagues in the Molecular Parasitology laboratory (Department of Biochemistry, University of Pretoria) and the Saliba and Kirk laboratories (Research School of Biology, The Australian National University) all of whom have made this experience thoroughly enjoyable. In particular, I’d like to thank Dr Kevin Saliba, Dr Donelly van Schalkwyk, Dr Christina Spry, Dr Adele Lehane, Dr Richard Allen, Dr Gordon Wells, Dr Christine Maritz-Olivier, Dr Bridgette Cumming, Dr Thariena van Brummelen, Dr Salome Smit, Esmaré Human, Simon Cobbold, Marni Williams, Marli Botha, Rosa Marchetti, Bianca Verlinden and Natalie Spillman for their support, friendship, and many helpful discussions. I am also grateful for the support of the general staff at both the Department of Biochemistry and the Research School of Biology, with particular thanks to Sandra van Wyngaard.
Furthermore, I am grateful to my parents, Dewald and Hannelie Niemand, and my sister Andri Palk, as well as other family and friends for their continuing support throughout this study, without which I would not have been able to persevere to the end. Additionally, I would like to thank God, for giving me the strength and wisdom to see this through.

I would like to take the opportunity to thank the following funding agencies, namely the University of Pretoria’s mentoring scheme, the Carl and Emily Fuchs Foundation, as well as the Ernst and Ethel Ericksen Trust, all of which provided me with financial support whilst undertaking this degree.
Summary

*Plasmodium falciparum* causes the most severe form of human malaria, and the continual development of resistance of this parasite to current anti-malarial drugs underpins a pressing need for the discovery of novel chemotherapeutic approaches. Polyamines and their biosynthetic enzymes are present at high levels in rapidly proliferating cells, including cancer cells and protozoan parasites. Inhibition of the malaria parasite’s polyamine biosynthesis pathway causes cytostatic arrest in the trophozoite stage, but does not cure infections *in vivo*. This may be due to the salvage of exogenous polyamines from the host, replenishing the intracellular polyamine pool; however the mechanism(s) of polyamine uptake by the intra-erythrocytic parasite are not well understood. In this study the uptake of the polyamines putrescine and spermidine into *P. falciparum*-infected erythrocytes (iRBC) as well as into *P. falciparum* parasites functionally isolated from their host cell by saponin-permeabilisation of the erythrocyte membrane was investigated using radioisotope flux techniques. While the characteristics of transport of putrescine into infected erythrocytes were similar to those of transport into uninfected erythrocytes, spermidine entered iRBC in part via the ‘new permeation pathways’ induced by the parasite in the erythrocyte membrane. Both putrescine and spermidine were taken up across the plasma membrane of isolated parasites via a saturable, temperature-dependent process that showed competition between different polyamines as well as the polyamine precursor ornithine and basic amino acids. Inhibition of polyamine biosynthesis led to increased total uptake of both putrescine and spermidine. The influx of putrescine and spermidine into isolated parasites was independent of Na$^+$ but increased with increasing pH and showed a marked dependence on the membrane potential, decreasing with membrane depolarisation and increasing with membrane hyperpolarisation.

Both anthracene and polyamine derivatives have been shown to have anti-malarial activity. Anthracene-polyamine conjugates have been developed with the aim of utilising the polyamine uptake mechanisms of cancer cells to deliver the cytotoxic anthracene moieties to these cells. Here, several anthracene-polyamine conjugates showed promising anti-malarial activity. These compounds inhibited parasite proliferation with IC$_{50}$ values in the nM range, and caused an arrest in the cell cycle, as well as a decrease in the mitochondrial membrane potential. Cytotoxicity could not be reversed by the addition of exogenous polyamines, nor did the conjugates have an effect on intracellular polyamine levels.
This doctoral study showed that *P. falciparum* parasites not only synthesise polyamines, but can also acquire putrescine and spermidine from the extracellular environment and paves the way for interfering with polyamine metabolism as an anti-parasitic strategy.
# Contents

Submission declaration ........................................................................................................ ii
Plagiarism declaration ........................................................................................................ iii
Acknowledgements ........................................................................................................... iv
Summary ........................................................................................................................... vi
Table of contents ................................................................................................................ vii
I. List of Tables .................................................................................................................... xi
II. List of Figures .................................................................................................................. xii
III. List of Abbreviations ..................................................................................................... xv

## Table of Contents

1  Chapter 1: Literature review ............................................................................................... 1
   1.1  Malaria ......................................................................................................................... 1
       1.1.1  General .................................................................................................................. 1
       1.1.2  Lifecycle of *P. falciparum* .................................................................................. 2
       1.1.3  The control of malaria ......................................................................................... 4
   1.2  Polyamine metabolism ................................................................................................. 8
       1.2.1  Polyamine biosynthesis in mammals .................................................................... 9
       1.2.2  *P. falciparum* parasites’ polyamine metabolism ................................................. 11
   1.3  Membrane transport ..................................................................................................... 15
   1.4  Polyamine transport ..................................................................................................... 17
       1.4.1  General .................................................................................................................. 17
       1.4.2  Polyamine transport in multi-cellular organisms .................................................. 17
       1.4.3  Polyamine transport in bacterial cells ................................................................... 21
       1.4.4  Polyamine transport in yeast cells ....................................................................... 24
       1.4.5  Polyamine transport in parasitic protozoa ............................................................. 27
   1.5  Transport of solutes in intra-erythrocytic *P. falciparum* parasites ............................ 29
       1.5.1  Transport across the EPM ..................................................................................... 31
       1.5.2  Transport across the PVM .................................................................................... 31
       1.5.3  Transport across the PPM .................................................................................... 32
   1.6  Objective ..................................................................................................................... 33
2 Chapter 2: Polyamine uptake by the intra-erythrocytic malaria parasite, 

*P. falciparum.* ........................................................................................................ 35

2.1 Introduction........................................................................................................... 35

2.2 Materials and methods...................................................................................... 38

2.2.1 HEPES buffered solutions ........................................................................... 38

2.2.2 Cell culture and preparation ........................................................................ 38

2.2.3 Radioisotope uptake measurements ............................................................ 40

2.2.4 Creating RBCs with modified haemoglobin content ................................. 42

2.2.5 Cytosolic pH measurements of isolated *P. falciparum* parasites ............. 42

2.2.6 Data analysis ................................................................................................. 43

2.3 Results ............................................................................................................... 45

2.3.1[^3H]putrescine or[^3H]spermidine uptake into *P. falciparum*-infected RBCs .... 46

2.3.2[^3H]putrescine or[^3H]spermidine uptake into isolated *P. falciparum* parasites ... 51

2.4 Discussion ......................................................................................................... 66

3 Chapter 3: The effect of anthracene-polyamine conjugates on intra- 

erythrocytic *P. falciparum* parasites. ................................................................ 73

3.1 Introduction........................................................................................................... 73

3.2 Materials and methods...................................................................................... 76

3.2.1 *In vitro* cultivation of intra-erythrocytic *P. falciparum* parasites .......... 76

3.2.2 Parasite proliferation assays ........................................................................ 76

3.2.3 Determination of anthracene-polyamine uptake into iRBCs ....................... 76

3.2.4 Determination of the effects of polyamine conjugates on putrescine uptake into isolated *P. falciparum* trophozoites ............................................................................................................. 77

3.2.5 Determination of the effect of polyamines on Ant-4 uptake into intra-erythrocytic *P. falciparum* trophozoites ............................................................................................................. 78

3.2.6 Measurement of intracellular polyamine levels in intra-erythrocytic *P. falciparum* trophozoites ............................................................................................................. 78

3.2.7 Cytotoxicity and cell viability measurements .............................................. 79

3.2.8 Determination of oxidative stress ................................................................. 80

3.2.9 Investigation of DNA levels and DNA replication ..................................... 80

3.2.10 Determination of mitochondrial membrane potential ................................ 81

3.2.11 Statistical analysis ...................................................................................... 82

3.3 Results ............................................................................................................... 83
3.3.1 Effect of anthracene-polyamine conjugates on iRBC proliferation ..................... 83
3.3.2 Cytostatic vs. cytotoxic effects of Ant-4.......................................................... 85
3.3.3 Uptake of anthracene-polyamine conjugates by iRBCs.................................... 88
3.3.4 Effect of Ant-4 treatment on intracellular polyamine levels in iRBCs................. 92
3.3.5 Effect of Ant-4 on the reducing environment of iRBC.................................... 93
3.3.6 Effect of Ant-4 on iRBC mitochondrial membrane potential (Δψm) .................. 94
3.3.7 Ant-4 treatment affects DNA replication in iRBC.......................................... 95
3.4 Discussion ........................................................................................................ 97

4 Chapter 4: Concluding Discussion................................................................. 104

5 References...................................................................................................... 113
I: List of tables

Table 1.1: Currently used anti-malarial drugs. ................................................................. 6
Table 1.2: Reversal of in vitro intra-erythrocytic P. falciparum parasites’ growth inhibition with exogenous polyamines. ................................................................. 14

Table 2.1: Effect of various polyamines, amino acids and metabolic inhibitors on putrescine uptake in A) P. knowlesi-infected RBCs (Singh et al., 1997) and B) P. falciparum-infected RBCs (Ramya et al., 2006). ................................................................. 36
Table 2.2: Effect of pH on the protonation status of putrescine. ........................................ 62

Table 3.1: IC$_{50}$ values of anthracene-polyamine conjugates against the iRBCs, CHO cells and human (HL-60) and murine (L1210) leukaemia cell lines. ........................................ 85
Table 3.2: Measurement of H$_2$O$_2$ (DCFDA signal) and reduced glutathione levels as indicators for the reducing environment in Ant-4 treated intra-erythrocytic P. falciparum trophozoites. ................................................................. 93
Table 3.3: Flow cytometric analysis of nuclear division of intra-erythrocytic P. falciparum parasites. ......................................................................................................................... 96

Table 4.1: Polyamine transporter proteins identified in various organisms .................... 107
II: List of figures

Figure 1.1: World-wide occurrence of malaria. ................................................................. 2
Figure 1.2: The lifecycle of the *Plasmodium* parasite.................................................... 3
Figure 1.3: Current MMV portfolio (4th quarter 2010, www.mmv.org). ....................... 5
Figure 1.4: The structures of putrescine, spermidine and spermine. ........................... 9
Figure 1.5: Polyamine metabolism in humans. ............................................................... 10
Figure 1.6: Composite of polyamine levels and polyamine biosynthetic enzyme levels (PfAdoMetDC/ODC and PfSpdSyn), during the intra-erythrocytic developmental cycle of *P. falciparum*. ................................................................. 11
Figure 1.7: Schematic representation of polyamine metabolism in intra-erythrocytic *P. falciparum* parasites. .................................................................................. 12
Figure 1.8: Schematic representation of membrane transport proteins. ....................... 16
Figure 1.9: Schematic representation of the polyamine uptake and export systems in *E. coli*.. 21
Figure 1.10: Schematic representation of the mechanism of polyamine recognition of PotD and PotF, taken from (Igarashi and Kashiwagi, 1999). ................................................ 22
Figure 1.11: Polyamine import and export in *S. cerevisiae*. ........................................ 25
Figure 1.12: Polyamine transporters in parasitic protozoa. .......................................... 27
Figure 1.13: Schematic representation of transport processes in iRBCs. ....................... 30

Figure 2.1: Typical time course for the uptake of a radioactive polyamine (in this case [*3*H]spermidine) by isolated parasites. ...................................................................................... 45
Figure 2.2: Uptake of [*3*H]putrescine and [*3*H]spermidine by RBCs and iRBCs. .......... 47
Figure 2.3: Uptake of [*3*H]putrescine and [*3*H]spermidine by RBCs and iRBCs. ........ 48
Figure 2.4: Uptake of [*3*H]putrescine by RBCs with different haemoglobin concentrations. ..... 49
Figure 2.5: Effect of furosemide on uptake of [*3*H]putrescine or [*3*H]spermidine into iRBCs. ... 50
Figure 2.6: Time courses for the uptake of [*3*H]putrescine (■) and [*3*H]spermidine (□) by isolated *P. falciparum* trophozoites at 37°C. ........................................................................... 51
Figure 2.7: Temperature dependence of [*3*H]putrescine or [*3*H]spermidine uptake by isolated *P. falciparum* trophozoites. .............................................................................. 52
Figure 2.8: Glucose dependence of [*3*H]putrescine and [*3*H]spermidine uptake by isolated *P. falciparum* trophozoites. .............................................................................. 53
Figure 2.9: Kinetics of [*3*H]putrescine and [*3*H]spermidine uptake into isolated *P. falciparum* parasites at 37°C. ................................................................................... 55
Figure 2.10: Characterisation of kinetics of [*3*H]putrescine or [*3*H]spermidine transport into isolated *P. falciparum* parasites at 37°C. ........................................................ 56
Figure 2.11: Inhibition of \[^{3}\text{H}\]putrescine or \[^{3}\text{H}\]spermidine uptake by *P. falciparum* trophozoites by various metabolites (5 mM) at 37°C. ................................................................. 57
Figure 2.12: Effect of *in vitro* polyamine depletion on the uptake of \[^{3}\text{H}\]putrescine and \[^{3}\text{H}\]spermidine by *P. falciparum* trophozoites at 37°C. ................................................................. 59
Figure 2.13: \(\text{Na}^+\) dependence of \[^{3}\text{H}\]putrescine and \[^{3}\text{H}\]spermidine uptake by *P. falciparum* trophozoites at 37°C. ........................................................................ 60
Figure 2.14: p\(\text{H}\) Dependence of \[^{3}\text{H}\]putrescine and \[^{3}\text{H}\]spermidine uptake by isolated *P. falciparum* trophozoites at 37°C. ........................................................................ 61
Figure 2.15: p\(\text{H}_{\text{i}}\) response of isolated *P. falciparum* trophozoites following extracellular exposure to 10 mM NH\(_4\)Cl or 10 mM putrescine dihydrochloride at 37°C. ......................... 63
Figure 2.16: Effect of membrane potential perturbations on \[^{3}\text{H}\]putrescine and \[^{3}\text{H}\]spermidine uptake by *P. falciparum* trophozoites. ................................................................. 65

Figure 3.1: Structures of anthracene-polyamine conjugates. ........................................ 75
Figure 3.2: Dose-response curves showing the inhibitory effect of anthracene-polyamine conjugates on the proliferation of intra-erythrocytic *P. falciparum* parasites *in vitro* over 96 hrs (initiated with ring-stage parasites) at 37°C in the absence (filled symbols) or presence (empty symbols) of 0.5 mM aminoguanidine. ......................................................... 84
Figure 3.3: Effect of Ant-4 on the viability of intra-erythrocytic *P. falciparum* trophozoites. .... 86
Figure 3.4: Cytotoxicity of Ant-4 against intra-erythrocytic *P. falciparum* parasites in the absence and presence of putrescine. ................................................................. 87
Figure 3.5: Deconvolution fluorescence microscopy of trophozoite-stage iRBCs incubated with Ant-4 (100 µM) at 37°C. ...................................................................................... 88
Figure 3.6: Flow cytometric scatter plots of Ant-4 signal (DAPI channel) in (A) RBCs, (B) iRBCs and (C) isolated *P. falciparum* trophozoites. ................................................................. 89
Figure 3.7: Effect of DFMO pre-treatment on the cytotoxicity of anthracene-polyamine conjugates against iRBCs, using Ant-4. ................................................................. 90
Figure 3.8: Inhibition of \[^{3}\text{H}\]putrescine (black bars) and \[^{3}\text{H}\]spermidine uptake (grey bars) into isolated *P. falciparum* trophozoites by anthracene-polyamine conjugates (500 µM) at 37°C during a 30 min incubation. ........................................................................ 91
Figure 3.9: Inhibition of Ant-4 uptake (400 µM) into trophozoite-stage iRBCs by polyamines (500 µM) at 37°C following 1 hr incubation. ................................................................. 91
Figure 3.10: Polyamine levels of trophozoites-stage iRBCs following Ant-4 treatment. ........ 92
Figure 3.11: Flow cytometric profiles of JC-1 signal as indicator of changes in mitochondrial membrane potential due to Ant-4 treatment of iRBCs. ............................................. 94
Figure 3.12: Intra-erythrocytic *P. falciparum* DNA levels following treatment with Ant-4. ....... 95
### III: List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta \psi$</td>
<td>Membrane potential</td>
</tr>
<tr>
<td>$\Delta \psi_m$</td>
<td>Mitochondrial membrane potential</td>
</tr>
<tr>
<td>AdoMet</td>
<td>$S$-adenosylmethionine</td>
</tr>
<tr>
<td>AdoMetDC</td>
<td>$S$-adenosylmethionine decarboxylase</td>
</tr>
<tr>
<td>AMEL-3</td>
<td>Hamster melanoma cell line</td>
</tr>
<tr>
<td>APA</td>
<td>3-aminoxy-1-aminopropane</td>
</tr>
<tr>
<td>APAO</td>
<td>$N^\alpha$ acetylpolyamine oxidase</td>
</tr>
<tr>
<td>APC</td>
<td>Amino acid/Polyamine/Organocation</td>
</tr>
<tr>
<td>BCBD</td>
<td>$N^\alpha, N^\beta$-bis-(7-chloroquinoline-4-yl)butane-1,4-diamine</td>
</tr>
<tr>
<td>BCECF</td>
<td>2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein</td>
</tr>
<tr>
<td>BP</td>
<td>Bandpass</td>
</tr>
<tr>
<td>CCC</td>
<td>Cation-Cl$^-$ cotransporter</td>
</tr>
<tr>
<td>CCCP</td>
<td>Carbonyl cyanide-$m$-chlorophenylhydrazone</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>DAX</td>
<td>Diamine exporter</td>
</tr>
<tr>
<td>dcAdoMet</td>
<td>Decarboxylated $S$-adenosylmethionine</td>
</tr>
<tr>
<td>DCFDA</td>
<td>2'-7'-Dichlorodihydrofluorescein diacetate</td>
</tr>
<tr>
<td>DFMO</td>
<td>DL-$\alpha$-difluoromethylornithine</td>
</tr>
<tr>
<td>DHFR</td>
<td>Dihydrofolate reductase</td>
</tr>
<tr>
<td>eIF5A</td>
<td>Eukaryotic initiation factor 5A</td>
</tr>
<tr>
<td>EPM</td>
<td>RBC plasma membrane</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>Gpc-1</td>
<td>Glypican-1</td>
</tr>
<tr>
<td>HEK-293</td>
<td>Human embryonic kidney 293 cells</td>
</tr>
<tr>
<td>HEPES</td>
<td>$N$-(2-hydroxyethyl)piperazine-$N'$-(2-ethanesulphonic acid)</td>
</tr>
<tr>
<td>HL-60</td>
<td>Human leukaemia cell line</td>
</tr>
<tr>
<td>iRBC</td>
<td><em>P. falciparum</em> (strain 3D7)-infected red blood cell</td>
</tr>
<tr>
<td>iRBCs</td>
<td><em>P. falciparum</em> (strain 3D7)-infected red blood cells</td>
</tr>
<tr>
<td>JC-1</td>
<td>5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide</td>
</tr>
<tr>
<td>L1210</td>
<td>Murine leukaemia cells</td>
</tr>
<tr>
<td>MDL27695</td>
<td>$N,N'$-bis(3-(phenylmethyl)aminolpropyl)-1,7-diaminoheptane</td>
</tr>
<tr>
<td>MES</td>
<td>2-morpholinoethanesulfonic acid</td>
</tr>
</tbody>
</table>
MGBG  Methylglyoxal bis(guanylhydrazone)
MMV  Medicines for Malaria Venture
MR  Methionine recycling pathway
MTA  5’-methylthioadenosine
NMDG  N-methyl-D-glucamine
NO  Nitric oxide
NOS2  Nitric oxide synthase
NPP  New Permeation Pathways
ODC  Ornithine decarboxylase
PAH  Polycyclic aromatic hydrocarbons
PAO  Polyamine oxidase
PBS  Phosphate-buffered saline
PfAdoMetDC/ODC  *P. falciparum* S-adenosylmethionine decarboxylase/ornithine decarboxylase
PfATP4  *P. falciparum* Ca\(^{2+}\)ATPase
PfCHA  Putative *P. falciparum* Ca\(^{2+}\)/H\(^+\) anti-porter
PfCRT  *P. falciparum* chloroquine-resistance transporter
PfENT1  *P. falciparum* Equilibrative Nucleoside/nucleobase Transporter 1
pHi  Intracellular pH
pH\(_e\)  Extracellular pH
PPM  Parasite plasma membrane
PSAC  Plasmodial surface anion channel
PVM  Parasitophorous vacuolar membrane
RBC  Uninfected human red blood cell
RBCs  Uninfected human red blood cells
ROS  Reactive oxygen species
rpm  Revolutions per minute
S.E.  Standard error of the mean
SAM3  *S*-Adenosylmethionine transporter
SAM1  South African Malaria Initiative
-SH  Sulphydryl
SI  Selectivity index
SMO  Spermine oxidase
SMR  Small multi-drug resistance
SpdSyn  Spermidine synthase  
SpmSyn  Spermine synthase  
Spp  Species  
SSAT:  Spermidine/spermine N\textsuperscript{-}-acyltransferase  
TPO  Transporter for polyamine  
TS  Thymidylate synthethase  
TUNEL  Terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end-labelling  
WHO  World health organisation