Evaluation of the vaccine potential of malarial TCTP

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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December 2008
Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and ethics procedures and guidelines have been followed.

Kim Juliet Taylor
3rd December 2008
Acknowledgements

I would firstly like to thank my senior supervisor Dr. Peter Smooker for his support and encouragement, for providing me with ideas and assurance when I needed them, and for helping me stay on track with the thesis.

I would like to thank my second supervisor, Prof. Ian Macreadie, for his ideas and encouragement, and for providing me with support during protein purification.

I would like to thank Dr. Ross Fernley at CSIRO Health Sciences and Nutrition for all of his assistance during protein purification. I would like to thank many other people who assisted me at CSIRO Health Sciences and Nutrition, including Peter, Onisha, Janelle, Sonia, Helma and Jo.

I would like to thank Dr. Tania De Koning-Ward at WEHI for the immense amount of help she provided with the *P. berghei* transfections.

I would like to thank the people in the RMIT Biotechnology laboratory for all of their support and friendship, with a special thankyou to Emily Gan for her help with Southern blots. I would also like to thank the staff of the RMIT Animal Facility for their assistance.

Lastly, I would like to thank my family and my partner Alan for all of their support and patience.

Publications:

A scientific paper containing the major findings of the three results chapters is currently being prepared.
Table of Contents:

Declaration........................................................................................................... i
Acknowledgements................................................................................................. ii
Publications: ........................................................................................................... ii
Table of Contents:..................................................................................................... iii
List of Figures:.......................................................................................................... xii
List of Tables: ........................................................................................................... xiv
List of Abbreviations:............................................................................................. xv
Summary .................................................................................................................. 1

Chapter 1: Literature review...................................................................................... 3
  1.1 Introduction........................................................................................................ 3
  1.2 Malaria ............................................................................................................. 4
    1.2.1 Causative agents of malarial infection ....................................................... 4
    1.2.2 Global incidence of malaria ....................................................................... 4
    1.2.3 Mechanisms of malaria infection ............................................................... 5
    1.2.4 Malaria pathogenesis ............................................................................... 7
    1.2.5 Mouse models of malaria ......................................................................... 8
    1.2.6 Malaria prevention and treatment .............................................................. 9
    1.2.7 Malaria vaccines .....................................................................................10
    1.2.8 Malarial immunity and parasite immune evasion.......................................11
  1.3 Functions of TCTP ........................................................................................... 12
    1.3.1 Discovery of TCTP ..................................................................................12
    1.3.2 Translational regulation of TCTP ...............................................................12
    1.3.3 Identification of TCTP isoforms ...............................................................13
    1.3.4 TCTP involvement in cell growth and differentiation...............................14
    1.3.5 Induction of TCTP in response to stress ...................................................14
    1.3.6 Self-interaction of TCTP .........................................................................15
    1.3.7 Identification of TCTP pseudogenes ........................................................15
    1.3.8 Calcium binding by TCTP .......................................................................16
    1.3.9 Tubulin binding activity of TCTP ..............................................................17
    1.3.10 Chaperone activity of TCTP ..................................................................17
    1.3.11 Anti-apoptotic functions of TCTP ............................................................18
    1.3.12 TCTP and cancer ....................................................................................19
1.3.13 Studies of the conserved domains of TCTP ___________________19
1.3.14 Summary of intracellular functions of TCTP _________________20
1.4 Secretion of TCTP _____________________________________________21
1.5 Extracellular functions of TCTP _________________________________23
  1.5.1 Actions of TCTP in promoting histamine release from basophils __23
  1.5.2 Studies of the interaction of TCTP and IgE___________________24
  1.5.3 Actions of TCTP in promoting IL-8 release ___________________25
  1.5.4 Actions of TCTP in stimulation of IL-4 release from basophils___26
  1.5.5 TCTP secretion from macrophages ___________________________26
  1.5.6 TCTP involvement in B cell proliferation____________________26
  1.5.7 Inhibition of T-cell activation by TCTP______________________27
  1.5.8 Interaction of TCTP and SHIP_______________________________27
1.6 Parasite TCTP functions ________________________________________28
  1.6.1 Malarial TCTP induces histamine and IL-8 release___________28
  1.6.2 Malaria TCTP may competitively inhibit activation of B-cells by
     host TCTP ________________________________ _________________28
  1.6.3 Malarial TCTP may self-interact, and binds calcium ____________29
  1.6.4 Other parasite TCTPs induce histamine release from basophils
     and induce host immune responses_______________________________29
  1.6.5 The role of histamine and eosinophils in malaria________________31
1.7 Artemisinin and TCTP: _________________________________________33
  1.7.1 Antimalarial action of artemisinin __________________________33
  1.7.2 Artemisinin and TCTP ________________________________34
  1.7.3 Other malarial targets of artemisinin_________________________35
  1.7.4 Artemisinin acts as an anti-cancer agent ______________________36
1.8 TCTP gene knockout or expression inhibition experiments___________38
1.9 Malarial gene knockout experiments______________________________39
1.10 Research Aims_______________________________________________43

Chapter 2: Materials and Methods_______________________________ 44

  2.1 Materials and equipment suppliers _______________________________44
  2.2 Preparation of materials ________________________________________50
  2.3 General DNA Materials and Methods______________________________50
    2.3.1 DNA Materials __________________________________________50
    2.3.2 DNA Methods ___________________________________________52
      2.3.2.1 DNA extraction and purification methods_________________52
      2.3.2.2 PCR Methods ________________________________________53
2.3.2.3 Creation of cDNA _________________________________________56
2.3.2.4 Restriction endonuclease digestion of DNA _________________56
2.3.2.5 Agarose gel electrophoresis ________________________________56
2.3.2.6 Quantification of DNA samples ______________________________57
2.3.2.7 Cloning methods _________________________________________57
2.3.2.8 Ethanol/sodium acetate precipitation of DNA from solutions ______58

2.4 Bacteriological Materials and Methods ____________________________59
2.4.1 Bacteriological Materials ______________________________________59
2.4.2 Bacterial strains used ________________________________________60
2.4.3 Bacteriological methods ______________________________________60
2.4.3.1 Spectrophotometric quantification of bacterial cultures _________60
2.4.3.2 Electro-transformation of *E. coli* ____________________________60
2.4.3.3 Preparation of glycerol stocks of *E. coli* ______________________61

2.5 Yeast Materials and Methods ____________________________________62
2.5.1 Yeast Materials ____________________________________________62
2.5.2 Yeast methods _____________________________________________62
2.5.2.1 Transformation of *S. cerevisiae* with pYEULCBX vectors _________62
2.5.2.2 Preparation of glycerol stocks of *S. cerevisiae* ________________63

2.6 Protein Purification Materials and Methods ________________________64
2.6.1 Protein Purification Materials __________________________________64
2.6.2 Yeast protein expression and purification methods ________________65
2.6.2.1 Confirming expression of TCTP in *S. cerevisiae* by Western blot ____65
2.6.2.2 TCTP expression in *S. cerevisiae* _____________________________65
2.6.2.3 Protease-minimising protein expression method in *S. cerevisiae* __65
2.6.2.4 Lysis of yeast cultures _______________________________________66
2.6.2.5 Purification of His-tagged proteins expressed in *S. cerevisiae* using TALON™ resin _________________________________________67
2.6.2.6 Further purification of yeast-derived proteins using an anion-exchange column _____________________________________________68
2.6.2.7 N-terminal sequencing of PyTCTP _____________________________69
2.6.2.8 Protein lyophilisation and reconstitution ________________________69

2.6.3 *E. coli* protein expression and purification methods ___________69
2.6.3.1 PbTCTP and PyTCTP expression in *E. coli* ____________________69
2.6.3.2 Lysis of *E. coli* ___________________________________________69
2.6.3.3 Py and PbTCTP purification using Profinity™ metal affinity chromatography ____________________________________________70
2.6.3.4 Endotoxin removal _________________________________________70
CHAPTER 3: Evaluation of *P. yoelii* and *P. falciparum* TCTP as potential malaria vaccines

3.1 Introduction

3.2 Materials and Methods

3.3 Results

3.3.1 Amplification and cloning of *P. yoelii* TCTP

3.3.1.1 Primers used for amplification of *P. yoelii* TCTP

3.3.1.2 Transformation and purification of pYEULCBX vector

3.3.1.3 Amplification of PyTCTP from cDNA

3.3.1.4 TA cloning of PyTCTP into the pCR2.1 vector and ligation of PyTCTP into the pYEULCBX vector

3.3.2 Expression of PyTCTP and PfTCTP in *S. cerevisiae*

3.3.2.1 Growth of pYEULCBX.PyTCTP and pYEULCBX.PfTCTP transformants of *S. cerevisiae*

3.3.2.3 Western blot to test for protein expression

3.3.2.5 Large-scale expression and purification of PyTCTP and PfTCTP

3.3.2.6 Further purification of PyTCTP and PfTCTP

3.3.2.7 Analysis of protein storage methods

3.3.2.8 Sequence analysis of TCTP following Mono-Q chromatography

3.3.2.9 Analysis of PfTCTP obtained by an alternate expression method

3.3.3 Analysis of TCTP samples under reducing and non-reducing conditions

3.3.4 Evaluation of PfTCTP and PyTCTP as potential malarial vaccines

3.3.4.1 Mouse immunisation groups and schedule

3.3.4.2 Health of mice during immunisation period
3.3.4.3 Survival rates of groups challenged with *P. yoelii* XNL and YM malaria

3.3.4.4 Comparison of parasitemia

3.3.4.5 Analysis of antibody titres by ELISA

3.3.5 Recognition of *P. yoelii* TCTP by anti-TCTP antibodies present in immunised mouse sera

3.4 Discussion

Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as potential malaria vaccines using three malarial species

4.1 Introduction

4.2 Materials and methods

4.3 Results

4.3.1 Analysis of the modelled structure of PyTCTP and PbTCTP

4.3.2 Cloning of PyTCTP and PbTCTP into pRSETA vectors

4.3.2.1 Amplification of PbTCTP by PCR

4.3.2.2 Ligation of PyTCTP and PbTCTP into pCR2.1 and pRSETA vectors

4.3.2.3 Transformation and analysis of pRSETA.PyTCTP and pRSETA.PbTCTP

4.3.3 Py and PbTCTP expression in *E. coli* BL21

4.3.4 Immunisation of mice with Py or PbTCTP

4.3.4.1 Preparation of vaccines, mouse immunisation groups and schedule

4.3.4.2 Analysis of antibody titres

4.3.5 Challenge and monitoring of immunised mice

4.3.5.1 Assessment of parasite strains in pre-challenge trial

4.3.5.2 Malaria challenge of immunised mice

4.3.6 Trial results in PyTCTP-immunised mice challenged with *P. c. chabaudi* AS

4.3.6.1 Parasitemia and infection progression in control and immunised mice

4.3.5.3 Individual reciprocal antibody titres in mice immunised with PyTCTP and challenged with *P. c. chabaudi* AS

4.3.7 Trial results in PyTCTP-immunised mice challenged with *P. yoelii* YM
4.3.7.1 Parasitemia and infection progression in control and immunised mice ................................................................. 155
4.3.7.2 Survival of mice challenged with *P. yoelii* YM ____________________________________________________________ 158
4.3.7.3 Individual reciprocal antibody titres of mice immunised with PyTCTP and challenged with *P. yoelii* YM .......................... 158
4.3.8 Trial results in BALB/c mice immunised with PbTCTP and challenged with *P. berghei* ANKA ____________________________ 160
4.3.8.1 Infection progression in control and immunised mice ......................................................................................... 160
4.3.8.2 Individual reciprocal titres of BALB/c mice immunised with PbTCTP ................................................................. 160
4.3.9 Trial results in C57BL/6 mice immunised with PbTCTP and challenged with *P. berghei* ANKA ____________________________ 164
4.3.9.1 Infection progression and cerebral symptom development in control and immunised mice ................................ 164
4.3.9.2 Individual reciprocal antibody titres in C57BL/6 mice immunised with PbTCTP .................................................... 164
4.3.10 Recognition of *P. yoelii* and *P. berghei* TCTP in parasite lysates by anti-TCTP antibodies __________________________ 166
4.4 Discussion ................................................................................................................................................................. 171

Chapter 5: Development of TCTP-knockout strains of *P. berghei* ANKA ........................................................................... 176
5.1 Introduction ............................................................................................................................................................... 176
5.2 Materials and Methods ........................................................................................................................................ 180
5.3 Results ....................................................................................................................................................................... 180
5.3.1 Feasibility assessment of the TCTP knockout experiments by analysis of the *P. berghei* ANKA genome ................. 180
5.3.2 Cloning of the short integration construct ........................................................................................................... 181
5.3.2.1 Rationale for the creation of the short target b3Dint1int2 construct .................................................................... 181
5.3.2.2 PCR amplification of the short integration fragments and cloning into pCR2.1 vectors ........................................... 184
5.3.2.3 Cloning of the int1 and int2 products into the b3D vector .................................................................................. 186
5.3.3 First transfection of *P. berghei* ANKA parasites using the short target b3D.int1.int2 vector ........................................ 189
5.3.3.1 Preparation of target DNA prior to transfection ............................................................................................... 189
5.3.3.2 Growth of parasites and secondary infection of mice following first transfection ................................................. 191
5.3.3.3 Analysis of the genotype of the transfected parasites by PCR .................................................................. 192
5.3.3.4 Genetic analysis of parasite DNA from the first transfection experiment by Southern blot ................................................................. 196
5.3.3.4.1 PCR amplification of the TCTP probe ........................................... 196
5.3.3.4.2: PCR amplification of the TgDHFR/TS probe ............................... 196
5.3.3.4.3: Southern blot of transfected parasite and wt berghei DNA ________ 198
5.3.3.5 Secondary drug-selection of parasites obtained from the first transfection experiment .......................................................... 200
5.3.3.5.1 Growth of parasites during drug selection ______________________ 200
5.3.3.5.2 Analysis of the transfected parasite DNA from the secondary drug-selection by PCR ................................................................. 201
5.3.3.5.3 Analysis of the transfected parasite DNA from the secondary drug-selection by Southern blot ................................................... 203
5.3.3.5.4 Southern blot using HindIII-digested DNA ______________________ 205
5.3.3.6 Repeat of secondary drug selection of parasites from the first transfection experiment ........................................................ 206
5.3.3.6.1 Growth of parasites during drug selection ______________________ 206
5.3.3.6.2 Genetic analysis of drug-selected parasites by Southern blot ________ 206
5.3.4 Second transfection experiment ..................................................... 208
5.3.4.1 Design of the long target b3D.Lf1.Lf2 construct ____________________ 208
5.3.4.1.1 PCR amplification of the long integration fragments and cloning into pCR2.1 vectors ........................................................................ 210
5.3.4.1.2 Cloning of the Lf1 and Lf2 products into the b3D vector ___________ 211
5.3.4.2 Rationale for the second transfection experiment ............................ 213
5.3.4.2.1 Preparation of short and long target DNA prior to transfection ____ 213
5.3.4.2.2 Parasite transfection using the short and long target DNA ________ 215
5.3.4.3 Analysis of parasites obtained from second transfection experiments ............................................................................................... 215
5.3.4.3.1 Initial PCR analysis of transfected parasites ___________________________ 215
5.3.4.3.2 Further PCR analysis of the short and long target-transfected parasites ...................................................................................... 217
5.3.4.4 Secondary drug-selection of parasites obtained from second transfection experiment ............................................................. 218
5.3.4.5 Genetic analysis of initial and secondary drug-selected parasites from the second transfection experiment by Southern blot ________ 218
5.3.4.5.1 Preparation of the long TCTP probe ______________________________ 218
5.3.4.5.2 Southern blot ................................................................................ 220
5.3.5 Third transfection experiment .......................................................... 222
5.3.5.1 Rationale for final transfection experiment .................................. 222
5.3.5.2 Preparation of long target DNA prior to transfection .................. 222
5.3.5.3 Transfection of parasites with long target DNA ......................... 222
5.3.5.4 Initial genetic analysis of parasite DNA ..................................... 222
5.3.5.5 Further genetic analysis of parasite DNA obtained from the 1st, 2nd
and 3rd transfection experiments ..................................................... 224
5.3.5.6 Secondary drug selection of parasites from 3rd transfection
experiment .................................................................................... 227
5.3.5.6.1 Secondary drug selection of parasites from mouse 6.1 and 6.2 .... 227
5.3.5.6.2 Genetic analysis of parasites from 3rd transfection experiment
after secondary drug selection .......................................................... 227
5.3.5.7 Genetic analysis of parasites from 3rd transfection
experiment by Southern blot ........................................................... 229
5.3.5.7.1 Preparation of Lf2 and TgDHFR/TS probes ............................ 229
5.3.5.7.2 Preparation of λ/NdeI marker .............................................. 229
5.3.5.7.3 Southern blot of parasite DNA from 3rd transfection experiment .. 231
5.4 Discussion ..................................................................................... 235

Chapter 6: Final discussion and future directions ........................................ 242
References ....................................................................................... 249
Appendix 1: Markers used ................................................................. 261
Appendix 2: Quantifying Pain/Distress/Suffering ................................... 263
Appendix 3: Titration curves used to calculate reciprocal titres:.............. 264
List of Figures:

Figure 1.1: The global incidence of malaria ..................................................................................... 5
Figure 1.2: The life cycle of malaria within the human host and mosquito vector............................ 6
Figure 1.3: Structure of \textit{P. yoelii} TCTP, modelled using the Swiss-model server............... 20
Figure 3.1: Map of the pYEULCBX vector...................................................................................... 97
Figure 3.2: Cloning of PyTCTP into the pYEULCBX yeast expression vector:........................... 102
Figure 3.3: Sequence analysis of the His-tag region of the pYEULCBX.PyTCTP vector, and sequence alignment of PyTCTP and PfTCTP ................................................................. 103
Figure 3.4: Confirmation of PittCPT and PyTCTP expression in yeast ....................................... 106
Figure 3.5: Purification of PittCPT and PyTCTP by IMAC using Talon™ resin............................ 108
Figure 3.6: Elution profile of TCTP during Mono-Q purification ................................................... 111
Figure 3.7: TCTP following Mono-Q purification and PBS dialysis............................................... 112
Figure 3.8: Comparison of protein yield and quality following storage in PBS or in 50% glycerol ................................................................................................................................. 113
Figure 3.9: Purification of PittCPT following alternate expression method .................................... 115
Figure 3.10: PyTCTP and PittCPT separated by SDS-PAGE under reducing and non-reducing conditions ..................................................................................................................... 117
Figure 3.11: Survival of TCTP-immunised mice and controls following malaria challenge........ 121
Figure 3.12: Parasitemia in TCTP-immunised and control mice.................................................... 123
Figure 3.13: Reciprocal titres of vaccine trial mice......................................................................... 126
Figure 3.14 Recognition of TCTP in \textit{P. yoelii} YM lysates by PyTCTP anti-serum ...................... 128
Figure 4.1: Alignment of \textit{P. yoelii}, \textit{P. berghei}, \textit{P. chabaudi} and \textit{P. falciparum} TCTP amino acid sequences ............................................................................................................................. 137
Figure 4.2: pRSET vector diagram.................................................................................................. 137
Figure 4.3: Cloning of PyTCTP and PbTCTP into pRSETA vectors ............................................. 141
Figure 4.4: Purification on PyTCTP and PbTCTP from E. coli BL21 lysates using Profinity™ resin ........................................................................................................................................... 143
Figure 4.5: Western blot of Py and PbTCTP .................................................................................. 145
Figure 4.6: Malarial progression in BALB/c mice challenged with \textit{P. chabaudi} AS .................. 152
Figure 4.7: Cumulative survival of mice challenged with \textit{P. chabaudi} AS ................................. 154
Figure 4.8: Individual reciprocal antibody titres for PyTCTP-immunised mice prior to challenge with \textit{P. chabaudi} AS .......................................................... 154
Figure 4.9: Malarial progression in PBS controls and PyTCTP-immunised mice challenged with \textit{P. yoelii} YM. .......................................................... 157
Figure 4.10: Cumulative survival of PyTCTP-immunised and PBS control mice.......................... 159
Figure 4.11: Individual reciprocal antibody titres for PyTCTP-immunised mice prior to challenge with \textit{P. yoelii} YM .......................................................... 159
Figure 4.12: Malarial progression in BALB/c mice challenged with \textit{P. berghei} ANKA ............ 162
Figure 4.13: Cumulative survival of BALB/c mice challenged with \textit{P. berghei} ANKA .................. 163
Figure 4.14: Individual reciprocal antibody titres for mice in PbTCTP group #7 ...................... 163
Figure 4.15: Malarial progression in C57BL/6 mice challenged with *P. berghei* ANKA ............ 165
Figure 4.16: Survival of C57BL/6 mice challenged with *P. berghei* ANKA .......................... 165
Figure 4.17: Individual reciprocal antibody titres for mice in PbTCTP group #8 ..................... 165
Figure 4.18: Recognition of PyTCTP in malarial lysates, and cross-reactivity of Sc- and Ec-TCTP .......................................................................................................................... 168
Figure 4.19: Recognition of PbTCTP in *P. berghei* lysates ..................................................... 168
Figure 5.1: The b3D vector ........................................................................................................... 179
Figure 5.2 Creation of the b3D.int1.int2 vector, and disruption of the TCTP gene with the int1.int2 target .............................................................................................................................................. 183
Figure 5.3: Amplification and pCR2.1 cloning of the int1 and int2 fragments ........................... 185
Figure 5.4: Creation of the b3D.int1.int2 construct .................................................................... 187
Figure 5.5: Preparation of short integration target DNA prior to first transfection .................... 190
Figure 5.6: Amplification products of primers initially used to confirm disruption of TCTP with the DHFR gene ...................................................................................................................................................... 192
Figure 5.7: PCR analysis of parasite DNA obtained from the first transfection experiment ....... 195
Figure 5.8: Preparation of probes for Southern blot of parasite DNA from the first transfection experiment .................................................................................................................................................. 197
Figure 5.9: Southern blot of transfected and wild-type (wt) parasite DNA using TCTP and DHFR probes ................................................................................................................................................. 199
Figure 5.10: Tree diagram of parasite recipients from the first transfection experiment .............. 200
Figure 5.11: PCR analysis of parasite DNA from secondary drug selection .............................. 202
Figure 5.12: Genetic analysis by Southern blot of transfected parasites following secondary drug selection .................................................................................................................................................. 204
Figure 5.13: Southern blot on repeated secondary drug-selection of transfected parasites ...... 207
Figure 5.14: Disruption of TCTP on the *P. berghei* ANKA genome with the Lf1.Lf2 target DNA. 209
Figure 5.15: Creation of the b3D.Lf1.Lf2 construct ...................................................................... 212
Figure 5.16: Preparation of short and long integration target DNA prior to second transfection .......................................................................................................................................................... 214
Figure 5.17: Initial genetic analysis of parasites from second transfection experiment by PCR . 216
Figure 5.18: Separation of whole PCR reactions used to amplify long TCTP probe ............... 219
Figure 5.19: Southern blot of parasite DNA from second transfection experiment ................... 221
Figure 5.20: Preparation of long target DNA prior to third transfection experiment .................. 223
Figure 5.21: TCTP PCR analysis of parasite DNA from 3rd transfection experiment ............... 223
Figure 5.22: Analysis of target DNA integration in parasites from the 1st and 3rd transfection experiments .......................................................................................................................................................... 226
Figure 5.23: Genetic analysis of parasites from 3rd transfection experiment following secondary drug selection ................................................................................................................................................. 228
Figure 5.24: Preparation of Lf2 and DHFR probes ....................................................................... 230
Figure 5.25: Southern blot of parasite DNA obtained from 3rd transfection experiment .......... 233
List of Tables:

Table 2.1: Standard Taq PCR reaction parameters ................................................................... 53
Table 2.2: Standard Pfu PCR parameters ............................................................................... 54
Table 2.3: Standard Expand PCR parameters ....................................................................... 55
Table 2.4: PCR to incorporate dye terminators for DNA sequencing ..................................... 55
Table 3.1: Mouse immunisation groups ................................................................................. 118
Table 3.2: Average survival of mouse groups challenged with *P. yoelii* XNL ...................... 120
Table 3.3: Average survival of mouse groups challenged with *P. yoelii* XL ....................... 120
Table 4.1: Mouse immunisation groups .................................................................................. 146
Table 4.2 Reciprocal titres of pooled TCTP sera to homologous and heterologous antigen ..... 148
Table 4.3: Mean parasitemia for PyTCTP-immunised mice and PBS controls challenged
with *P.c. chabaudi* AS ............................................................................................................. 150
Table 4.4: Mean parasitemia for PyTCTP-immunised mice and PBS controls challenged
with *P. yoelii* YM .................................................................................................................... 155
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D-PAGE</td>
<td>One-dimensional polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>2D-PAGE</td>
<td>Two-dimensional polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>Abs</td>
<td>Absorbance</td>
</tr>
<tr>
<td>Ac</td>
<td>Acetate</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>CM</td>
<td>Cerebral malaria</td>
</tr>
<tr>
<td>CPD</td>
<td>Citrate phosphate dextrose</td>
</tr>
<tr>
<td>CV</td>
<td>Column volume</td>
</tr>
<tr>
<td>DHFR/TS</td>
<td>Dihydrofolate reductase/thymidylate synthase</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl formamide</td>
</tr>
<tr>
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<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>dpi</td>
<td>Day post-infection</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double-stranded ribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>ECM</td>
<td>Experimental cerebral malaria</td>
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<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>Genomic deoxyribonucleic acid</td>
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<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
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<tr>
<td>His</td>
<td>Histidine</td>
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<tr>
<td>HRF</td>
<td>Histamine releasing factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>∞</td>
<td>infinity</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>irbc</td>
<td>infected red blood cell</td>
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<tr>
<td>i.v.</td>
<td>intravenous</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>kV</td>
<td>kilovolt</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>mA</td>
<td>milliampere</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MFS</td>
<td>malaria freezing solution</td>
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<tr>
<td>µF</td>
<td>micro Faraday</td>
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<td>µg</td>
<td>microgram</td>
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<td>microlitre</td>
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<td>micrometre</td>
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<td>µM</td>
<td>micromolar</td>
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<td>mg</td>
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<td>millimetre</td>
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<td>millimolar</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MT-PBS</td>
<td>mouse tonicity phosphate-buffered saline</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>nm</td>
<td>nanometre</td>
</tr>
<tr>
<td>nM</td>
<td>nanomolar</td>
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<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>o/n</td>
<td>overnight</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>Pb</td>
<td>Plasmodium berghei</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>Pf</td>
<td><em>Plasmodium falciparum</em></td>
</tr>
<tr>
<td>pmol</td>
<td>picomoles</td>
</tr>
<tr>
<td>pRBC</td>
<td>parasitised red blood cell</td>
</tr>
<tr>
<td>Py</td>
<td><em>Plasmodium yoelii</em></td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RNAi</td>
<td>ribonucleic acid interference</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>rt</td>
<td>room temperature</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCTP</td>
<td>translationally-controlled tumour protein</td>
</tr>
<tr>
<td>TEMED</td>
<td><em>N</em>,<em>N</em>,<em>N</em>,<em>N</em>'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tg</td>
<td><em>Toxoplasma gondii</em></td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Tween-20</td>
<td>polyoxylethylene sorbitan monolaurate</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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<tr>
<td>V</td>
<td>volt</td>
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<tr>
<td>v/v</td>
<td>volume for volume</td>
</tr>
<tr>
<td>wt</td>
<td>wild-type</td>
</tr>
<tr>
<td>w/v</td>
<td>weight for volume</td>
</tr>
<tr>
<td>x g</td>
<td>times gravity</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside</td>
</tr>
</tbody>
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Summary

The translationally controlled tumour protein (TCTP) family are highly conserved eukaryotic proteins that have been assigned a variety of functions. Most studies of TCTP have focused on the intracellular functions of the protein, including its roles in microtubule binding (Gachet et al., 1999), in the inhibition of apoptosis (Li et al., 2001b; Zhang et al., 2002), and calcium binding (Arcuri et al., 2004; Sanchez et al., 1997). The human TCTP has also been reported to have a second extracellular function, in the induction of histamine and interleukin (IL)-8 release from immune cells of allergic donors in vitro (e.g. Bheekha-Escura et al., 2000; MacDonald et al., 1995). The TCTP of the malarial species *Plasmodium falciparum* has been demonstrated to induce histamine release from basophils and IL-8 release from eosinophils, and is present in the sera of malaria-infected individuals (MacDonald et al., 2001). The TCTP from the parasites *Schistosoma mansoni* and *Brugia malayi* can induce histamine release in vitro and can induce eosinophil recruitment in mice in vivo (Gnanasekar et al., 2002; Rao et al., 2002). Little other research has been conducted into the functions of malarial TCTP, however it is known to bind to the antimalarial drug artemisinin (Bhisutthibhan and Meshnick, 2001; Bhisutthibhan et al., 1998).

Malaria is a widespread parasitic disease, causing 300-500 million infections per year and resulting in over 1 million deaths. There is widespread resistance of the parasite to most of the antimalarial treatments available, indicating the need for a vaccine (http://www.rbm.who.int/wmr2005/). Several candidate malarial vaccines are currently in clinical trials, however due to the ability of the parasite to rapidly mutate to evade host immune responses the most effective malarial vaccine may consist of several subunits. Various malarial species exist that infect non-human hosts, allowing for candidate vaccine studies using animal models.

Previously, we studied the vaccine potential of *P. falciparum* TCTP in a malarial challenge model in mice infected with the lethal malaria *P. yoelii* YM. In that study, a significant delay in the establishment of infection was observed in immunised mice, as judged by reduced parasitemia and prolonged survival (Taylor, 2002). It was thought that the protective effect conferred by immunisation might have been due to the inhibition of the extracellular actions of malarial TCTP by the acquired host immune response.
This study was undertaken to confirm the modest protective effect conferred by *P. falciparum* TCTP immunisation, and to investigate if immunisation using homologous proteins would induce greater protection. *P. falciparum* and *P. yoelii* TCTP were initially expressed in *S. cerevisiae*, as in the previous study. The recombinant proteins were used to immunise mice, which were then challenged with two strains of *P. yoelii*. No protective effect was observed for either vaccine, and so the previous results using PfTCTP could not be confirmed.

Due to low yields and significant proteolysis being encountered using the yeast expression system, the TCTP of *P. yoelii* and *P. berghei* was expressed in *E. coli*. Proteins of greater than 95% purity were obtained from a single purification step using Profinity™ resin, and no significant proteolysis was observed. These recombinant proteins were used as vaccines in mice challenged with *P. yoelii* YM, *P. c. chabaudi* AS, or *P. berghei* ANKA. A significant delay in disease progression was observed in PyTCTP-immunised mice challenged with the non-lethal *P. c. chabaudi*, as determined by a significantly reduced parasitemia at each day post-infection leading up to a delayed peak parasitemia. A significant reduction in parasitemia was also observed in the early stages of *P. yoelii* YM infection in PyTCTP-immunised mice.

*P. berghei* ANKA was used to challenge C57BL/6 mice to evaluate the potential of PbTCTP immunisation to protect mice from cerebral malaria development. No significant differences were observed between immunised mice and controls. *P. berghei* ANKA was also used as a second lethal malaria challenge model in BALB/c mice, again no significant differences in disease progression were observed in immunised mice.

To further assess the functions of malarial TCTP, several attempts were made to create a TCTP-knockout strain of *P. berghei* ANKA. A TCTP-knockout malaria strain could be assessed for alterations in morphology, infectivity and artemisinin sensitivity compared with wild-type parasites. Initial genotype analysis of parasites resulting from several transfection experiments indicated that TCTP disruption had been successful, however TCTP-disrupted parasites were strongly selected against, and stable knockout strains could not be obtained. This indicates that TCTP performs an important role within the malaria parasite.
Chapter 1: Literature review

1.1 Introduction

Malaria is a parasitic disease caused by members of the *Plasmodium* genus, infecting 300-500 million people per year and resulting in 1-3 million deaths. Malarial species have high host specificity, and rodent malarial species are commonly used as models of human infection. As yet, there is no commercially available malaria vaccine, although several promising candidates are in clinical trials. In a small-scale vaccine trial in mice, immunisation with *P. falciparum* TCTP induced a significant delay in the progression of a *P. yoelii* YM infection (Taylor, 2002).

A member of the translationally controlled tumour protein (TCTP) family was first identified in mouse tumour cell lines (Chitpatima *et al*., 1988), with expression under translational control (Gross *et al*., 1989). Since then, TCTP expression has been found in normal tissues, as well as in tumours, in all eukaryotes examined. Members of the TCTP family are highly conserved and abundantly expressed (Thaw *et al*., 2001).

Several functions, mostly intracellular, have been ascribed to members of the TCTP family. Some of the intracellular functions attributed to TCTP include calcium binding (e.g. Arcuri *et al*., 2005; Feng *et al*., 2007; Gnanasekar *et al*., 2002; Kim *et al*., 2000; Sanchez *et al*., 1997) and microtubule stabilisation (Gachet *et al*., 1999). Several research groups have also identified secretion and extracellular functions of TCTP from several species, including humans, ticks, malaria and filarial worms. These extracellular functions include the induction of histamine release from basophils and IL-8 release from eosinophils (Gnanasekar *et al*., 2002; MacDonald *et al*., 2001; Rao *et al*., 2002).

The TCTP vaccine trial represented a different approach to the generation of protective responses to malaria, as other vaccines so far trialled have consisted of attenuated whole parasites (e.g. Renia *et al*., 2006a) or of proteins exposed on the surface of the parasite (e.g. Anders *et al*., 1998; Daly and Long, 1996). In contrast, an induced immune response to TCTP would recognise and inhibit the actions of an extracellular protein. This project was designed to further evaluate the potential of malarial TCTP as a vaccine, and to further investigate the functions of the protein.
Chapter 1: Literature review

1.2 Malaria

1.2.1 Causative agents of malarial infection

The name malaria is derived from the Italian mal’aria, meaning bad air. The disease is caused by unicellular eukaryotic parasites belonging to the genus *Plasmodium*. More than one hundred species of *Plasmodium* exist, with a broad range of hosts including reptiles, birds and mammals. Four *Plasmodium* species can infect humans under natural conditions, these being *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. The most widespread human malaria species is *P. vivax*, but this rarely causes fatal disease. *P. falciparum* is the major cause of human malaria deaths, mostly in children under five in sub-Saharan Africa (Tuteja, 2007).

1.2.2 Global incidence of malaria

According to the most recent figures (2005), 3.2 billion people live in areas at risk of malaria transmission. It is estimated that 350-500 million cases of clinical malaria occur annually. Malaria caused by *P. falciparum* results in more than one million deaths per year, and contributes indirectly to many more deaths. Around 60% of total malaria cases, 75% of falciparum malaria cases and 80% of malaria deaths occur in areas of Africa south of the Saharan desert. In these areas, *P. falciparum* infections are responsible for approximately 18% of total deaths in children under 5 years of age. In African countries in which malaria is endemic, malaria infections account for 20-45% of hospital admissions (http://www.rbm.who.int/wmr2005/).

Malaria has highest incidence in children; studies in areas of Liberia with continual malarial transmission show that blood density of *P. falciparum* peaks between six months and two years of age. Malaria morbidity also decreases with age, peak incidence of cerebral malaria and other malaria-related deaths in Gambia occurs at four years old (Ramasamy, 1998).

The global distribution of malaria transmission areas has decreased between 1946 and 1994 (Figure 1.1). Malarial transmission has been successfully halted in temperate climates, with the result that the disease is now confined to tropical and sub-tropical climates. This is due to a combination of factors. The reproduction rate of malaria is lower in colder areas (below 18°C), meaning that malarial prevention schemes such as insecticide treatment and the use of antimalarial drugs are much more likely to successfully eradicate the parasite. Also, the main species of vector mosquitoes (*Anopheles gambiae*) in sub-Saharan Africa has a strong tendency to feed on humans.
There is also a strong correlation between poverty and malarial incidence (Sachs and Malaney, 2002).

1.2.3 Mechanisms of malaria infection

Malarial parasites are transmitted between hosts by Anopheles mosquito vectors. Mosquitoes inject the sporozoite stage of malaria into subcutaneous tissue, or directly into the bloodstream. The sporozoites then travel to the liver, where they invade hepatocytes. Each sporozoite develops into thousands of merozoites, which invade erythrocytes following hepatocyte rupture. Disease symptoms occur following the multiplication of the asexual parasite stages within erythrocytes (Miller et al., 2002).

The time required for parasites to complete an asexual life cycle varies between malaria species. The following times relate to the *P. falciparum* cycle, which is completed in 48 hours. Parasites grow from merozoites to ring stages and then trophozoites within the host erythrocyte, finally forming schizonts that produce daughter merozoites capable of infecting new erythrocytes. The merozoites form rings, which develop to trophozoite stage in 15-18 hours. The mature trophozoite undergoes schizogony to produce around 16 merozoites, which are released by rupture of the erythrocyte membrane. These merozoites then invade new erythrocytes, leading to rapid expansion of the parasite population within the host. A small proportion of the merozoites develop into male or female gametocytes within the erythrocytes. These gametocytes are essential for transmission to new hosts via the Anopheles vector.
Gametocytogenesis occurs 10-12 days after the start of the erythrocytic cycle. The gametocytes are ingested by feeding mosquitoes, where they are fertilised in the midgut. The diploid zygote undergoes meiosis and differentiates into the motile ookinete. Ookinetes develop into oocysts and then into sporozoites, which migrate to the mosquito salivary glands where they are injected into new mammalian hosts (Figure 1.2) (Tuteja, 2007).

Figure 1.2: The life cycle of malaria within the human host and mosquito vector (Tuteja, 2007)
Chapter 1: Literature review

1.2.4 Malaria pathogenesis

Much research has been conducted into the mechanisms of malaria pathogenesis. In terms of symptoms observed in human patients, the principal malarial complication that is the most important determinant of survival is metabolic acidosis, which is observed in both cerebral malaria and severe malarial anaemia. Metabolic acidosis leads to a syndrome of respiratory distress, most likely caused by decreased oxygenation of tissues. The pathogenic processes that lead to severe disease include rapid expansion of infected erythrocytes, destruction of both infected and uninfected erythrocytes, obstruction of small blood vessels, and inflammatory processes (Miller et al., 2002). Other common clinical complications include liver failure, circulatory collapse, hypoglycaemia, anaemia, hyperpyrexia, pulmonary oedema, and renal failure, as well as maternal death, premature delivery, and low birth weight (Ramasamy, 1998).

A major factor in the pathogenesis of *P. falciparum* infections is the ability of the parasite-infected erythrocytes to sequester in various organs such as the brain, lung and placenta. Sequestration is the result of parasite proteins on the surface of infected erythrocytes binding to host adhesion molecules on the surface of uninfected erythrocytes and endothelial cells (Tuteja, 2007). After invasion, the major parasite protein expressed on the surface of the infected erythrocyte is known as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). This is encoded by a multigene family of between 50 and 150 var genes (Newbold 1999, Ramasamy, 1998). PfEMP1 proteins on the surface of red blood cells mediate binding to host adhesion molecules, including glycoprotein, thrombospondin, intercellular adhesion molecule-1 (ICAM-1), E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and chondroitin sulphate A (CSA), on endothelial cells. Cytoadherence by the parasite is believed to be a mechanism to avoid circulation through the spleen, where infected erythrocytes can be destroyed by T cell-derived cytokines, phagocytosis and physical trapping (Ramasamy, 1998).

Sequestration causes obstruction in small blood vessels, which contributes to cerebral malaria (CM), the most dangerous complication of malarial infection (Ramasamy, 1998). In humans, CM mostly occurs in people living in malaria-endemic areas with a ‘ naïve’ immune system, i.e. children under 5 and recent immigrants. Immunity to CM develops slowly. Recently, the proposed mechanisms of human cerebral malaria pathogenesis were reviewed (van der Heyde et al., 2006). It was hypothesised that cerebral malaria development is due the binding of parasitised erythrocytes to small blood vessels in the brain, partly via the upregulation of host endothelial adhesion molecules, and partly via mechanical plugging of vessels due to the
increased rigidity of parasitised erythrocytes. Host systemic inflammatory responses are also involved in cerebral malaria development, via the actions of malarial toxins such as glycoprophosphoinositol (GPI) inducing the release of proinflammatory cytokines such as IL-1 and -6, macrophage colony-stimulating factor (M-CSF) and tumour necrosis factor (TNF). These proinflammatory cytokines upregulate the expression of host adhesion molecules. In the mouse model of experimental cerebral malaria (ECM) (discussed in the next section) the involvement of the inflammatory response has been proven, however the model fitness of ECM to human cerebral malaria is debatable, and not all cerebral malaria pathology can be accounted for by inflammatory responses. It is hypothesised that platelets are also involved in cerebral malaria development via hemostasis, and via adhesion to microvasculature (van der Heyde et al., 2006).

1.2.5 Mouse models of malaria

Animal models of malaria are mostly used for studying immune mechanisms. Malaria has narrow host specificity, and species exist that infect rodents, primates, and birds. Four species of rodent malaria exist: *P. berghei*, *P. yoelii*, *P. chabaudi* and *P. vinkei*. No single mouse model completely mimics human infection (Taylor-Robinson, 1998). *P. yoelii* is commonly used in mouse immunisation and challenge models. Lethal strains (e.g. YM, 17XL) exist, as well as non-lethal strains (e.g. 17 XNL) (Kim et al., 1980; Li et al., 2001a; Ma et al., 2007; Taylor et al., 1986).

*P. chabaudi* species are used in both lethal and non-lethal malaria models, depending on the parasite and mouse species used. Using both susceptible and resistant mouse models, it was found that levels of pro-inflammatory cytokines such as interferon (IFN) -γ and TNF-α correlate with disease severity (Cross and Langhorne, 1998).

Several strains of *P. berghei*, most commonly *P. berghei* ANKA, are used as models of cerebral malaria. The susceptibility of mice to CM is strain-specific. In susceptible mouse strains, including CBA/Ca and C57BL/6, around 80-90% of mice develop CM following *P. berghei* ANKA infection. These mice develop neurological symptoms including ataxia, paralysis and convulsions at low parasitemia. In the mouse model of CM, sequestration of parasitised red blood cells (pRBC) is not seen, instead vessel haemorrhages are due to obstruction with leukocytes. Similar malarial antigens and similar immune responses exist in both diseases. Disease symptoms shared between human and murine CM include hypoglycaemia, hyper immunoglobulin (Ig), high
serum TNF levels and perivascular haemorrhages. Neutralising anti-TNFα antibodies prevented CM in mice, and CM-resistant strains of mice (BALB/c) that were treated daily with TNFα during *P. berghei* ANKA infection developed CM (Chen *et al.*, 2000). Non-CM-susceptible strains of mice, such as BALB/c, succumb to *P. berghei* ANKA 3-4 weeks after infection due to hyperparasitemia and anaemia. The remaining 10% of C57BL/6 mice that do not develop CM also follow this disease progression (Lou *et al.*, 2001).

### 1.2.6 Malaria prevention and treatment

The major methods used to prevent and treat malaria are control of the mosquito vector, through the use of insecticides and bed nets, and treatment using antimalarial drugs. Drugs commonly used to treat malaria include chloroquine, which inhibits haemoglobin digestion, and drugs such as sulfadoxine and pyrimethamine that inhibit enzymes in the parasite folate synthesis pathway. Alkylating agents, such as artemisinin, are a newer class of drug that are becoming more widely used as the parasite develops increasing resistance to chloroquine and the antifolate drugs (Winstanley *et al.*, 2004). The actions of artemisinin are discussed further in section 1.7.

The Roll Back Malaria (RBM) partnership was implemented in 2000 with the goal of halving the global malaria burden by 2010. Initiatives implemented by this partnership included distribution of insecticide-treated bed nets and the replacement of chloroquine treatment for malaria infections with artemisinin-based combination therapy (ACT). Other treatment and prevention programs recommended by the partnership included intermittent preventative treatment for pregnant women and indoor residual spraying (IRS) with insecticides. Early results indicate that usage rates of insecticide-treated bed nets and IRS have increased in many countries since 2000, and that 23 African countries had changed their national drug policy to ACT. In many Asian countries, rates of reported malaria cases and deaths have decreased since the introduction of these control measures ([http://www.rbm.who.int/wmr2005/](http://www.rbm.who.int/wmr2005/)).

It is estimated that the minimum cost of effective malarial control measures is around US $3.2 billion per year. This is partly due to the fact that ACT is 10 to 20 times more expensive than chloroquine. Financial commitment to malaria control has increased, with governments in malaria-affected countries providing most of the funds (between 71 and 96%). As poor countries tend to have the highest malaria burden, in many cases the required level of funding is not available. Several international funding
organizations exist, including the Global Fund to Fight AIDS, Tuberculosis and Malaria, and the Bill and Melinda Gates Foundation. These organizations have helped to fill the gap between the funds required and those available (http://www.rbm.who.int/wmr2005/).

1.2.7 Malaria vaccines

As yet, no vaccine to protect against malaria development is commercially available. Malaria sporozoites rendered uninfected by methods such as irradiation have previously been shown to confer immunity in humans, however a widespread vaccine using whole sporozoites is not economically viable in the developing countries in which malaria is concentrated. Recently, sporozoites with attenuated infectivity through newer methods such as gene disruption have shown some promise as vaccine candidates (Renia et al., 2006a).

Extensive research has been conducted into the subunit vaccine potential of several malarial proteins, generally using mice as the challenge model. Merozoite surface proteins (MSPs) are integral membrane proteins on the surface of free merozoites. They are involved in recognition and invasion of host erythrocytes. The carboxyl terminal region of MSP-1 is a promising malarial vaccine candidate and is currently in clinical trials (Shi et al., 2007). The processed 42-kDa form of this protein can induce protective immunity in mice and monkeys (e.g. Daly and Long, 1996; Hirunpetcharat et al., 1997). However, this vaccine candidate has narrow specificity, as immunisation with the P. yoelii MSP-1 could induce protection in mice challenged with P. yoelii but not P. chabaudi or P. berghei (Rotman et al., 1999). In natural human infections, MSP-1-specific antibody responses can be low, despite repeated infection (Hensmann et al., 2004).

Another promising vaccine candidate is apical membrane antigen 1 (AMA-1), present on the surface of merozoites during erythrocyte invasion. Production of the recombinant protein in yeast can produce an economically viable vaccine for use in clinical studies (Kennedy et al., 2002). Antibodies against AMA-1 prevent merozoite invasion of erythrocytes in mice and monkeys (Anders et al., 1998; Collins et al., 1994). Adoptive transfer of AMA-1 antibodies induced protection against P. yoelii infection in mice (Crewther et al., 1996). AMA-1 has been evaluated in clinical trials by several groups (Ballou et al., 2004).

Research into many other malarial vaccine candidates is continuing. The circumsporozoite (CS) protein present on the surface of sporozoites can confer
Chapter 1: Literature review

protection in mouse malaria models (Bergmann-Leitner et al., 2007). Another candidate vaccine is MSP-8, which could confer immunity in mice particularly when combined with MSP-1 (Shi et al., 2007). Used alone, MSP-8 immunisation could prevent malaria mortality but could not prevent patent infection, with mice infected with P. yoelii 17XL displaying a delayed parasitemia that was eventually resolved (Petritus and Burns, 2008). The MSP 4/5 proteins of P. yoelii have also shown promise in vaccine trials in mice, with complete prevention of patent infection (Alonso et al., 2004; Goschnick et al., 2004; Kedzierski et al., 2000).

The most clinically advanced malaria vaccine candidate currently available is the RTS,S/AS recombinant protein, which is a comprised of part of the P. falciparum CS protein fused with the hepatitis B surface antigen combined with a proprietary adjuvant system. This vaccine candidate was developed by GlaxoSmithKline (GSK) in collaboration with the Walter Reed Army Institute of Research, and in 2001 GSK and the PATH Malaria Vaccine Initiative partnered to develop RTS,S/AS as a vaccine for children in sub-Saharan Africa (http://www.malariavaccine.org/files/12052008__RTSSfactsheet.pdf). An RTS,S/AS02A vaccine trial in Mozambique in 2003 resulted in a 37% reduction in incidence of clinical P. falciparum malaria (Alonso et al., 2004) and a 49% reduction in the incidence of severe malaria for at least 18 months following vaccination (Alonso et al., 2005). A large-scale Phase III multi-center efficacy trial in both infants and young children is planned for 2009 (http://www.malariavaccine.org/files/12052008__RTSSfactsheet.pdf).

1.2.8 Malarial immunity and parasite immune evasion

Maintenance of malarial semi-immunity is dependent on persistent sub-clinical infection and is lost when people leave malaria-endemic areas (Taylor-Robinson, 1998). The malaria parasite is able to evade the host immune system via several mechanisms. One mechanism is antigen switching. In P. falciparum, only one PfEMP1 variant is expressed on the surface of the infected erythrocyte at a given time, but the parasite is able to alter expression of the multigene var family to evade host immune responses by expressing a different PfEMP1 variant (Newbold, 1999). There is some evidence that the tandem repeats in the circumsporozoite (CS) protein can participate in immune evasion. Antibodies are usually generated to these repeats, and it has been hypothesis that cross-reactions between the repeats can interfere with affinity maturation of the antibody response. The repeats may also act to mop up antibodies, preventing them from binding to functionally important parasite proteins (Ramasamy, 1998).
1.3 Functions of TCTP

Many intracellular and extracellular functions have been ascribed to TCTP, a comprehensive review of which was published in 2004 (Bommer and Thiele, 2004). Many of the functions that have been determined for TCTP are unrelated, and some seem to be contradictory. An overall function of TCTP has yet to be determined, and much is left to be learned.

1.3.1 Discovery of TCTP

The nucleotide sequence of the mouse gene later identified as TCTP was first published in 1988. The cDNA was derived from an abundant mRNA species in mouse tumour cell lines. This mRNA was found to exist mainly as messenger ribonucleoprotein (mRNP) particles that were unable to interact with translational apparatus within the cells (Chitpatima et al., 1988).

TCTP was originally named p21/p23 by several research groups. One group identified the mouse TCTP, which they named p23, as a growth-dependent protein in Ehrlich ascites tumour (EAT) cells. The synthesis of TCTP varied depending on the growth phase of the tumour, and was increased in the presence of serum (Benndorf et al., 1988). Further work by this research group provided evidence that the synthesis of TCTP was regulated at the post-transcriptional level. Several transcription inhibitors were added to EAT cell culture, no inhibition of TCTP synthesis was seen. Using a cell-free translation system, a similar amount of TCTP mRNA was found in early and late phase EAT cells, indicating that the increased protein seen in early phase cells was due to translational control (Bohm et al., 1989).

The name TCTP was first used to describe the human homolog of the mouse p23 gene. The human TCTP homolog was isolated from a mammary carcinoma cDNA library using probes derived from the mouse sequence, the two genes shared 86% nucleotide and 96% amino acid similarity. Three putative translation inhibitory elements were identified in the 3’ non-coding region (Gross et al., 1989).

1.3.2 Translational regulation of TCTP

The untranslated regions (UTRs) of TCTP have several features typical of translationally controlled proteins including a 5’-terminal polypyrimidine tract found mainly in mRNAs of ribosomal proteins, a high GC content in the 5’-UTR indicating a high degree of secondary structure, and two AUUUA motifs in the 3’-UTR, which are typical
recognition sequences for regulated degradation of mRNA (Bommer et al., 1994). Previous studies had reported that the 5’-UTR was important in the translation of TCTP mRNA (Bohm et al., 1989).

There is evidence that the translational control of TCTP involves the action of the cap-binding initiation factor complex eIF-4F, particularly its smallest subunit (eIF-4E/eIF-4Fα). The initiation factor mediates unwinding of mRNA secondary structure prior to translation. TCTP synthesis and eIF-4E phosphorylation were induced by the same mitogenic stimuli. Over-expression of eIF-4E resulted in a higher basal rate of TCTP synthesis and knockdown of eIF4E using anti-sense RNA reduced TCTP levels (Bommer et al., 1994).

The ability of TCTP mRNA to bind to the protein kinase PKR has been reported. PKR is activated by double-stranded RNA (dsRNA), and is hypothesised to be involved in signal transduction pathways that mediate cell growth and differentiation, stress responses and apoptosis. Full-length double-stranded TCTP mRNA, but not the coding region alone, could bind to PKR in vitro by both co-precipitation and retention experiments, and could activate PKR in vivo (Bommer et al., 2002).

Investigations of the TPT1 promoter of the TCTP gene revealed that expression was regulated by CREB transcription factors (Andree et al., 2006). In recent work by the same group, the transcriptional and translational regulation of TCTP by several different compounds was examined. Copper induced the greatest levels of TCTP expression, and a metal-responsive element was found in the TPT1 promoter (Schmidt et al., 2007).

1.3.3 Identification of TCTP isoforms

In one study, analysis of several tissue lysates by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) found three isoforms of human TCTP present in erythrocytes, liver cells, non-differentiated keratinocytes, melanoma, hepatoblastoma, and glioma cells. The TCTP isoforms had an isoelectric point (pI) of 4.8-4.9. TCTP was also expressed in platelets, lymphoma, erythroleukemia, astrocytes and macrophages. The proportion of each isoform differed between normal and tumour cells, however the relative abundance of TCTP was similar in all of the tissues analysed. TCTP was not found in normal kidney cells or in renal cell carcinoma (Sanchez et al., 1997). In another study, high levels of TCTP mRNA were detected in human and rabbit
kidney (Thiele et al., 2000). It could be that translation of TCTP is repressed in kidney tissue.

**1.3.4 TCTP involvement in cell growth and differentiation**

TCTP is generally accepted to have a role in cell growth and differentiation. TCTP is involved in the induction of flowering in violets (Sage-Ono et al., 1998), and high levels of TCTP mRNA and protein were found in regions of active cell proliferation, but not in regions that had undergone terminal differentiation, in the aquatic Hydrozoa *Hydra vulgaris* (Yan et al., 2000).

Differential mRNA analysis was performed on exponentially growing and ammonium-starved *Schizosaccharomyces pombe* cells. Most of the mRNA species down-regulated during nitrogen starvation encoded ribosomal proteins, so the authors conclude that the major transcriptional control induced by nitrogen starvation is the inhibition of ribosomal protein expression. The expression of TCTP was down-regulated in a similar manner to ribosomal proteins during nitrogen starvation, indicating shared expression control mechanisms (Bonnet et al., 2000).

**1.3.5 Induction of TCTP in response to stress**

Xu et al., (1999) studied altered protein synthesis in response to loss of calcium homeostasis in Cos7 cells. The endoplasmic reticulum (ER) is the major dynamic calcium storage site in non-muscle cells. Rapid calcium exchange is essential for correct protein folding and post-translational modifications, as well as for translation of mRNA and in maintaining the structural integrity of the ER. Sustained elevation of cytosolic calcium levels can induce apoptosis and necrotic cell death. TCTP was up-regulated in response to loss of calcium homeostasis, and the steady-state mRNA abundance increased. Depletion of ER levels of calcium up-regulated TCTP transcription, while an increase in cytosolic calcium up-regulated TCTP translation (Xu et al., 1999). A study on skeletal muscle proteins altered during prolonged potassium depletion revealed that TCTP was up-regulated (Thongboonkerd et al., 2006).

TCTP was found to be up-regulated in response to heavy metal stress in earthworms. In this study, TCTP expression was found to also be regulated at the transcriptional level (Sturzenbaum et al., 1998).
In *S. cerevisiae* yeast, induction of apoptosis by stress leads to translocation of TCTP from the cytoplasm to the outer surface of mitochondria. Deletion of the TCTP gene in yeast confers sensitivity to benomyl, a fungicide that destabilises microtubules (Rinnerthaler *et al.*, 2006). In other studies, TCTP has been shown to bind to and stabilise microtubules, as discussed in section 1.3.10.

The TCTP from the nematode species *Trichinella psuedospiralis* was reported to have a role in heat-stress adaptation. The TCTP expressed in response to heat stress was shown to be due to regulation at the translational level. It was suggested that TCTP might be up-regulated when *T. psuedospiralis* is transferred from a mammalian to an avian host, as an adaptive response (Mak *et al.*, 2007). The up-regulation of expression of the parasitic filarial worm TCTP was also observed upon transfer from a cold-blooded vector to a warm-blooded host (Gnanasekar *et al.*, 2002).

### 1.3.6 Self-interaction of TCTP

Rat TCTP was found to self-interact using a yeast two-hybrid screen. TCTP was found to form stable dimers and oligomers under non-denaturing conditions, whereas a single band at around 22 kDa was seen on SDS gels. The use of several truncated TCTP mutants determined that the C-terminal domain of TCTP, involving residues 126-172, was important in self-interaction (Yoon *et al.*, 2000). In another study, native mouse and human TCTPs were found to form at least one complex of 100-150 kDa, whereas recombinant mouse TCTP eluted at 40-60 kDa (Gachet *et al.*, 1999).

### 1.3.7 Identification of TCTP pseudogenes

In dot-blot experiments of a rabbit genomic DNA (gDNA) library 30 TCTP-positive plaques were found. A Southern blot on rabbit liver gDNA using a TCTP exon probe resulted in multiple bands whereas a re-screening of the blot with a TCTP intron probe found only one band, suggesting that several processed pseudogenes exist in the rabbit genome. It was suggested that these pseudogenes arose from retrotransposition of mRNA. All pseudogenes had complete 5'UTRs and a short 3' polyadenylation structures. There was some evidence that the TCTP pseudogenes were transcribed (Thiele *et al.*, 2000).

The mouse *Tpt1* gene encoding TCTP was examined. Seven putative *Tpt1* genes were identified in the mouse genome. Six of these had traits typical of pseudogenes that had arisen from retrotransposition of mRNA back into the genome.
These traits include flanking direct repeats in the 5’ and 3’ UTRs and residual poly(A) tails. Only one transcript was found to be expressed in all tissues. The mouse Tpt1 gene was found to be located on chromosome 14, and to contain six exons (Fiucci et al., 2003).

### 1.3.8 Calcium binding by TCTP

Several reports have identified calcium-binding activity of TCTP. TCTP was identified to migrate abnormally on SDS-PAGE, which is common for calcium-binding proteins. While TCTP has a hypothetical Mr of ~19.5 kDa, several reports have identified the protein as migrating above 25 kDa (e.g. Bhisutthibhan et al., 1998; Gnanasekar et al., 2002; Rao et al., 2002). The addition of 1 mM calcium caused TCTP to migrate faster on 1D-PAGE (Sanchez et al., 1997).

TCTP was found to be up-regulated in response to a loss of calcium homeostasis. While this could be part of a general stress response, this finding also lends support to the action of TCTP as a calcium-binding protein. TCTP could act as a calcium chelator in the cytoplasm (Xu et al., 1999).

Human prostate TCTP was shown to bind calcium in vitro, and had the highest expression levels of the calcium-binding proteins examined (Arcuri et al., 2004). Further work by this group examined the calcium-binding activity of human placental TCTP using small interfering RNA (siRNA) to knock-down protein expression. The concentration of calcium in foetal blood is much higher than the maternal circulation, indicating that an active transport mechanism exists within the placenta. Down-regulation of TCTP using siRNA resulted in a decreased calcium-uptake activity and an altered intracellular calcium profile in HTR-8/SV neo trophoblastic cells, suggesting that TCTP may have a direct role in placental calcium transport (Arcuri et al., 2005).

Deletion constructs of rat TCTP determined that that the calcium-binding region of TCTP is confined to residues 81-112 using a $^{45}\text{Ca}^{2+}$-overlay assay (Kim et al., 2000). However, in a recent report the calcium-binding site of human TCTP was determined by NMR, and was found to involve the residues N131, Q133, L149 and D150, with very low affinity (Feng et al., 2007).

TCTP was reported to repress activity of a Na⁺,K⁺-ATPase pump and induce systemic hypertension in mice. Increased calcium mobilisation from the ER to the cytoplasm was also observed (Kim et al., 2008).
1.3.9 Tubulin binding activity of TCTP

TCTP has been found to bind to microtubules. Around 10% of total TCTP could be immuno-precipitated from HeLa cell extracts using α-tubulin antibodies, indicating that either the interaction between TCTP and tubulin is weak, or that only a fraction of TCTP binds. TCTP and tubulin were localised in similar areas of Cos7 cells at different stages of the replication cycle. TCTP co-localised with microtubules during the G1, S, and G2 phases of the cell cycle. TCTP could also bind to Taxol-stabilised rat brain microtubules in vitro. Over-expression of mouse TCTP in bovine mammary epithelial cells (MACs) resulted in a much slower cell growth rate; cell morphology was also altered. A general increase in cell size was seen, and there were a higher percentage of cells in the G2/M phase of the cell cycle. Cells over-expressing TCTP had a 30-40% higher density of microtubules. It was thought that the reduced growth rate could be due to the over-expression of TCTP inhibiting mitosis (Gachet et al., 1999). In a recent study, TCTP the only protein found to be up-regulated in the metaphase II-arrested oocytes compared to the germinal vesicle stage (Vitale et al., 2007).

1.3.10 Chaperone activity of TCTP

Several reports have given evidence for the involvement of TCTP in guanine nucleotide exchange for several proteins. In an early report, the structure of the S. pombe TCTP was solved by NMR (Thaw et al., 2001). Structural similarity to mammalian suppressor of Sec4 (Mss4) was observed. Mss4 is a small GTPase accessory protein, binding to the GDP/GTP free form of Rab proteins and acting as a guanine nucleotide-free chaperone (GFC) or as a guanine nucleotide exchange factor (GEF). Rabs are part of the Ras superfamily of small G proteins involved in the secretory pathway (Takai et al., 2001). The Rab binding site of Mss4 coincided with the region of highest sequence conservation in TCTP, however, the H2 and H3 helices of TCTP were missing in Mss4, and the zinc-binding motif of Mss4 was not present in TCTP (Thaw et al., 2001). It has previously been stated that the Zn-motif of Mss4 is essential for Rab binding (Yu and Schreiber, 1995).

Therefore, the activity of TCTP as a chaperone is debatable. It was reported that in Drosophila TCTP associated with Rheb, a Ras superfamily GTPase. TCTP was immunoprecipitated together with Rheb in cell extracts, and bound preferentially to the nucleotide-free form. In vitro, TCTP stimulated GDP/GTP exchange, indicating that it acts as a GEF for Rheb. In vivo, cells with TCTP expression inhibited using RNAi had a
lower percentage of GTP-bound Rheb (Hsu et al., 2007). However, another study refuted the claim that TCTP acts as a GEF for Rheb. In this study, knockdown of TCTP expression by RNAi did not affect the ratio of GDP/GTP-bound Rheb, no interaction or exchange activity of TCTP with Rheb was detected, and TCTP depletion did not affect the direct downstream targets of Rheb in vivo. It was hypothesised that the additional structures present in TCTP compared with Mss4 would inhibit the binding of TCTP to Rheb (Rehmann et al., 2008).

In another study, TCTP was reported to interact with the translation elongation factor eEF1A, which is a GTPase. TCTP acted as a guanine nucleotide dissociation inhibitor (GDI), stabilising the GDP form of eEF1A and inhibiting the GDP exchange reaction performed by eEF1Bβ (Cans et al., 2003).

1.3.11 Anti-apoptotic functions of TCTP

Using a yeast two-hybrid screen, TCTP was found to interact with the Bcl-2 homolog myeloid leukaemia protein-1 (Mcl-1). Bcl-2 is a major negative regulator of apoptosis and binds to Bax, a pro-apoptotic protein, preventing the formation of cytotoxic pores in the mitochondrial membrane (Li et al., 2001b). A recent report stated that TCTP acts as an anti-apoptotic protein due to its function in inhibiting Bax dimerisation (Susini et al., 2008). Western blot showed greater TCTP abundance in cancer cell lines than in normal cell lines, especially in cancers of epithelial origin. Over-expression of TCTP in HeLa cells and in U2OS osteosarcoma cells prevented them from undergoing drug-induced apoptosis, and anti-sense depletion of TCTP from the breast cancer cell line MCF-7 led to increased cell death. Unlike the majority of reports that have found TCTP located in the cytoplasm or secreted, the authors reported that TCTP was localised in the nucleus, as determined by immunocytohistochemistry (Li et al., 2001b).

The interaction of TCTP with Mcl-1 was investigated further. Whereas other members of the Bcl-2 family are constitutively expressed, Mcl-1 is inducible in cells exposed to growth and differentiation stimuli. TCTP was found not to interact with other Bcl-2 proteins. Depletion of Mcl-1 by siRNA significantly reduced TCTP protein levels, and was associated with rapid degradation of TCTP. It was concluded that Mcl-1 binds to and stabilises TCTP in vivo (Zhang et al., 2002). Another report stated that over-expression of TCTP increased Mcl-1 stability, and inhibition of TCTP expression by siRNA destabilised Mcl-1 (Liu et al., 2005).
1.3.12 TCTP and cancer

Sinha et al. (2000) investigated proteins involved in the development of chemotherapeutic drug resistance in melanoma cells, which have a high level of intrinsic drug resistance. In chemoresistant cells a general down-regulation of protein expression was observed, however TCTP was over-expressed, along with 15 other proteins in the same pI range (2.8-5) (Sinha et al., 2000).

One model of tumour reversion uses human leukaemia and breast cancer cell lines transfected with SIAH-1, a gene involved in cell death and tumour suppression. In malignant cells that had reverted to a non-malignant phenotype, TCTP was the most strongly down-regulated protein following reversion. Additionally, inhibition of TCTP expression using siRNA resulted in a suppressed malignant phenotype and resulted in cellular reorganisation. Decreased TCTP expression led to a 15% increase in the rate of apoptosis (Tuynder et al., 2002). A more recent report by this group studied tumour revertants from colon, lung and melanoma cell lines. In most of the tumour revertant cell lines tested, TCTP expression was reduced compared to the parental tumour lines. Using antisense TCTP to inhibit protein expression, anchorage-dependent cell growth was re-established (Tuynder et al., 2004). Down-regulation of TCTP expression was also observed in a colorectal cell line that had lost the tumorigenic phenotype (Stierum et al., 2003).

Chemicals that could inhibit TCTP expression in cancer cells were evaluated for their potential cytopathic effects. As described in section 1.5, evidence exists for TCTP involvement in the induction of histamine release from basophils. Chemicals that could inhibit the histaminic pathway of the human leukaemia U937 cell line were examined. Two of the four drugs (hydroxyzine and promethazine) had a significant cytopathic effect. However, structurally related chemicals with no antihistaminic properties had a more pronounced effect. In drugs with a cytopathic effect, the expression of TCTP mRNA was increased, but the protein levels were decreased (Tuynder et al., 2004).

1.3.13 Studies of the conserved domains of TCTP

Thorough bioinformatics analysis was performed to investigate the sequence and predicted structure of the TCTPs from many species (Hinojosa-Moya et al., 2008). Extensive sequence analysis revealed that most species, including malaria parasites, have only a single TCTP gene. Mammals possess several copies of TCTP pseudogenes. Three residues are highly conserved in the region that likely corresponds
to the binding surface for G proteins, including Glu12 (Hinojosa-Moya et al., 2008). It was previously reported that glutamic acid at residue 12 was essential for interaction with the Rheb GTPase (Hsu et al., 2007).

TCTP is generally reported to have two conserved domains, named TCTP1 and TCTP2. The TCTP1 domain is predominantly hydrophilic, comprising of a mobile loop at around residues 45-55. The TCTP2 domain is also an irregular loop, together with the two flanking beta-sheets, at around residues 130 to 150 (Thaw et al., 2001). An additional conserved domain was suggested to be present in the first 16 amino acids following the N-terminus. The amino acid at position 15 is nearly always a serine, which may be a phosphorylation site (Venugopal, 2005). In a model of P. yoelii TCTP using the solved 3D structure of P. knowelsi as a template, an extra helical segment is present at the end of the TCTP1 loop, at residues 53-58 (Figure 1.3).

![Figure 1.3: Structure of P. yoelii TCTP, modelled using the Swiss-model server](http://swissmodel.expasy.org/SWISS-MODEL.html)

### 1.3.14 Summary of intracellular functions of TCTP

Several proposed activities of TCTP support a role for the protein in cell growth, including interaction with anti-apoptotic proteins, decreased expression in tumour cells that reverted to a non-malignant phenotype, and tubulin binding. However, how these discrete functions integrate is unknown. The upregulation of TCTP in response to stress could be related to its calcium binding activity, but this again is a separate function, as is the proposed role of TCTP as a chaperone in the secretory pathway. Therefore, much is yet to be determined regarding the true role of TCTP within the cell.
1.4 Secretion of TCTP

The majority of published reports on TCTP focus on the intracellular actions of the protein. However, an increasing number of reports have observed secretion and extracellular actions of TCTP. Most secretory and membrane proteins contain a hydrophobic N-terminal sequence which mediates translocation across the ER membrane, the sequence is then cleaved (Palade, 1975). However, more than 15 proteins with a defined extracellular function have been found to lack a secretory signal sequence. The proteins include the human IL-1β, thioredoxin, Annexin 1, the coagulation factor XIII a-chain, the platelet-derived endothelial cell growth factor and the fibroblast growth factor. These proteins share several biochemical features, including a lack of N-linked glycosylation at potential sites and a lack of disulphide bonds between cysteines, indicating that their route of secretion bypasses the ER. Many of the proteins secreted without a signal sequence have functions related to inflammation or tissue damage, and TCTP has been shown to have inflammatory functions (as discussed in section 1.5). It was hypothesised that these proteins are secreted via an alternative pathway as secretion via the classical pathway may destroy the biological function of the protein. Many of these proteins have free SH groups, and secretion via the ER might cause misfolding (Rubartelli and Sitia, 1991).

Research into the mechanisms of TCTP secretion concluded that the tumour suppressor activated pathway-6 (TSAP6) gene product can facilitate the secretion of TCTP (Amzallag et al., 2004). TSAP6 is a transmembrane protein, and was shown to interact with TCTP in a yeast two-hybrid screen. TCTP partially co-localised with TSAP6 in vesicular-like structures at the plasma and nuclear membranes. The TSAP6 gene product increases cell susceptibility to apoptosis, and is inducible by the p53 tumour suppressor protein. TSAP6 associates with the Bcl-2-related protein Nix, and the two proteins work together to promote apoptosis (Passer et al., 2003). The interaction of TCTP with the Bcl-1 homolog Mcl-1 has previously been reported (as described in section 1.3.11).

Only ~0.3% of the total intracellular TCTP was secreted after three hours incubation in cultured 293T cells. The active secretion of TCTP was confirmed by the absence of cytosolic proteins, such as tubulin and actin, in the culture supernatants. TCTP secretion was found to be insensitive to either brefeldin A or monesin, suggesting that it is not processed through the ER/Golgi complex, but instead is secreted through a non-classical pathway. TCTP was found in small secreted vesicles (exosomes), which
have been implicated in non-classical secretion. The implication of the interaction of the pro-apoptotic protein TSAP6 and TCTP, which has been widely reported to inhibit apoptosis, was not discussed (Amzallag et al., 2004). Although only a small amount of the total intracellular TCTP was observed to be secreted, it is probable that over longer time periods or under specific conditions a greater proportion of the intracellular TCTP would be exported out of the cell. The removal of the anti-apoptotic TCTP from the cell could result in favourable conditions for apoptosis.

In other studies, dioxin exposure increased secretion of TCTP from in vitro cultures of human B and T cells (Oikawa et al., 2002). In a review of TCTP structure, it was suggested that two pools of TCTP may exist, and that one isoform may be secreted while the other has intracellular activities (Hinojosa-Moya et al., 2008).
1.5 Extracellular functions of TCTP

1.5.1 Actions of TCTP in promoting histamine release from basophils

Although the majority of research has focused on the intracellular functions of TCTP, several groups have reported extracellular activities for the protein. As yet, no attempt has been made to integrate the extracellular functions of TCTP with the variety of intracellular functions ascribed to the protein family.

A protein later identified as TCTP was partially purified from nasal lavage fluids of allergic donors. The protein was initially named histamine-releasing factor (HRF), due to its ability to induce histamine release in allergic donor basophils in an IgE-dependent manner. The IgE dependency of this HRF was shown by removal of surface IgE from basophils using lactic acid. Additionally, HRF was dependent on a particular form of IgE, as only certain basophil donors would respond, and passive sensitisation of lactic acid-stripped donor basophils could be achieved using sera from donors responsive to HRF. The sensitising property of these donor sera was shown to be IgE, as heating of sera at 56°C, passage of sera over anti-IgE sepharose, or competitive inhibition using non-responsive donor IgE all reduced the subsequent response of basophils to HRF (MacDonald et al., 1987). IgE that could sensitise basophils to respond to HRF was designated as IgE⁺, IgE that did not induce this sensitivity was designated IgE⁻ (MacDonald et al., 1991).

The protein first named HRF was found as one of four major bands present in macrophage culture (U937) supernatants, and was identified as human TCTP by N-terminal amino acid sequencing. The genes for mouse and human TCTP were expressed in E. coli as GST fusion proteins; the GST was cleaved before activity trials. The recombinant proteins were tested in basophil histamine release assays alongside native HRF purified from nasal lavages and PMBC culture. Antibodies raised to mouse TCTP recognised recombinant mouse and human TCTP, and the HRF present in nasal lavages. Both of the recombinant proteins had identical histamine releasing activity, and were less active than the native HRF. The proteins were tested on donated basophils from IgE⁺ and IgE⁻ donors; only IgE⁺ basophils were responsive. The kinetics of histamine release from basophils induced with HRF were slow, with around 50% of maximum release between 5 and 10 minutes. The release kinetics were identical between the recombinant TCTPs and the native HRF (MacDonald et al., 1995). These results supported the hypothesis that the HRF purified from nasal lavages was TCTP.
Chapter 1: Literature review

TCTP has also been reported to prime IgE- basophils. In stripped basophils passively sensitised with IgE+, treatment with TCTP for 15 minutes enhanced the secretion of histamine, IL-13 and IL-4 after activation with anti-IgE, in a dose-dependent manner (Schroeder et al., 1997).

TCTP expression and secretion in basophils was identified. TCTP synthesis was increased after stimulation with rIL-3, and to a lesser extent with α-IgE. There was a dose-dependent increase in intracellular and secreted TCTP levels in response to rIL-3 up to 100 ng/ml. Only a small amount of the TCTP was found in the supernatants compared with the intracellular amounts, however there was evidence of active secretion of TCTP, including the fact that basophil viability counts were >95% and that other cellular proteins were not seen on culture supernatant gels. Basophil culture supernatants could induce histamine release from other basophils, and histamine release was decreased by an average of 28% when TCTP was partially precipitated out of the supernatants (Nielsen et al., 1998).

1.5.2 Studies of the interaction of TCTP and IgE

It was suggested that action of IgE as IgE+ and IgE- could be due to differential glycosylation. This hypothesis was investigated by testing the responsiveness of IgE+ and IgE-sensitised basophils to eight different oligosaccharide-specific lectins. A large variation in histamine release was observed from basophils of different donors to all of the lectins tested, and it was concluded that lectins could not distinguish between IgE+ and IgE- (Kleine-Tebbe et al., 1996). It has also been suggested that the IgE+/IgE- heterogeneity could be due to alternative mRNA splicing (Schroeder et al., 1996).

In several experiments, attempts to show TCTP interacting with IgE were unsuccessful. By analysis using ELISA or affinity chromatography, TCTP did not bind to soluble IgE (Schroeder et al., 1997). In a rat basophilic leukaemia cell line was transfected with a functional human FcεRI, cells were incubated with IgE+ or IgE- along with α-IgE, human rTCTP or mouse rTCTP. Neither human nor mouse TCTP induced any histamine release in cells sensitised with IgE+. It was concluded that TCTP doesn’t cause histamine release through IgE, and that its receptor is not functional on rat RBL-2H3 cells (Wantke et al., 1999).

The effect of different chemicals thought to inhibit protein kinase C was studied on the release of histamine by human basophils in response to rTCTP, α-IgE, or specific
antigen. The chemical rottlerin enhanced histamine release mediated by TCTP and had no effect on the histamine release induced by α-IgE or antigen. Rottlerin is a non-staurosporine-derived inhibitor isolated from Mallotus philippinensis; it targets calcium-dependent PKC isozymes. Therefore, it was suggested that TCTP works through a separate signalling pathway to IgE (Bheekha-Escura et al., 1999).

1.5.3 Actions of TCTP in promoting IL-8 release

TCTP was shown to act on eosinophils, promoting chemotaxis, calcium flux and IL-8 production (MacDonald, 1996). Eosinophils contribute to pathology in allergic diseases by secreting proinflammatory granule proteins, including major basic protein (MBP) and eosinophil cationic protein (ECP), which induce damage of bronchial epithelial cells. TCTP could induce IL-8 secretion from a proportion of eosinophil preparations primed with granulocyte-macrophage colony stimulating factor (GM-CSF), however a large variability in the amount of IL-8 secreted in response to TCTP was observed in different donors. Constant stimulation with TCTP was needed for IL-8 release, whereas this was not needed for TCTP-primed histamine release (Bheekha-Escura et al., 2000). TCTP signalling may be different between basophils and eosinophils, as pertussis toxin treatment is known to have no effect on TCTP-induced histamine release (Bheekha-Escura et al., 1999) but was found to decrease IL-8 secretion in response to TCTP by 50%. TCTP also induced elevated calcium levels in 25% of eosinophil samples, and induced eosinophil chemotaxis in vitro (Bheekha-Escura et al., 2000).

TCTP induced secretion of both IL-8 and GM-CSF from primary cultures of human bronchial epithelial cells (BECs) and from the transformed BEC line BEAS-2B. TCTP also enhanced secretion of IL-8 from BEAS-2B cells in response to IL-1β, TNFα or LPS. Hydrogen peroxide induced secretion of a 30 kDa TCTP isoform from BEAS-2B cells without increasing the intracellular 26 kDa TCTP isoform concentration; the authors suggested that oxidative stress may induce TCTP release. The researchers also found that bronchoalveolar lavage (BAL) samples from asthmatic patients had significantly higher levels of TCTP compared with healthy controls, TCTP levels were higher again in patients with idiopathic eosinophilic pneumonia. It was previously known that IL-8 levels in BAL samples of asthma patients were several-fold higher than healthy controls (Yoneda et al., 2004).
1.5.4 Actions of TCTP in stimulation of IL-4 release from basophils

Further investigations of the actions of TCTP on the immune response found that TCTP also induced IL-4 release from human IgE+ basophils passively sensitised with IgE+. A four-hour treatment with mouse or human TCTP or anti-IgE induced IL-4 synthesis (Schroeder et al., 1996).

1.5.5 TCTP secretion from macrophages

In the initial studies of the histamine-releasing activities of human TCTP, the protein was purified from macrophage culture (U937) supernatants (MacDonald et al., 1995). In further work, TCTP was found to be secreted from cultured macrophages and isolated peritoneal macrophages from ovalbumin (OVA)-sensitised mice following stimulation with macrophage colony stimulating factor (M-CSF). TCTP was secreted from cultured macrophages at a rate of 5-15 ng per hour. In vivo, repeated injections of M-CSF increased macrophage TCTP expression. An i.p. injection of M-CSF or TCTP resulted in eosinophil recruitment in OVA-sensitised mice but not in controls. M-CSF treatment also stimulated the secretion of an N-glycosylated TCTP with a MW of 30 kDa. Treatment with N-glycosidase F decreased the amount of the 30 kDa band present on Western blot, whereas the amount of the 25 kDa band present increased. Mouse TCTP has a potential N-linked glycosylation site (Asp-X-Ser) at amino acid residues 51-53 (Teshima et al., 1998).

TCTP may be also be involved in macrophage activation. A 2D SDS-PAGE study of the human monocytic U937 cell line examined proteins associated with macrophage activation. LPS, IFN-γ, and phorbol myristate acetate (PMA) are all known to activate macrophages but have different specific activities. TCTP was identified as one of the proteins upregulated by LPS and PMA, and was found at 28.5 kDa and a pI of 4.7 (Walsh et al., 1995).

1.5.6 TCTP involvement in B cell proliferation

Culture supernatants of mouse erythroleukemia cells have activities in B cell-stimulation. An investigation into proteins present in these supernatants identified TCTP. Recombinant TCTP expressed in both E. coli and kidney cells stimulated B cell proliferation in a dose-dependent manner. Recombinant TCTP enhanced B cell-activation induced by IL-2, IL-4, IL-5 and anti-CD40-antibodies, and up-regulated the expression of IL-1 and IL-6. This response was not due to an indirect effect caused by histamine secreted in response to TCTP, as the B-cell cytokine expression profile
induced by histamine is different to that induced by TCTP. Additionally, TCTP was shown to bind to B cells by FACS analysis (Kang et al., 2001).

1.5.7 Inhibition of T-cell activation by TCTP

Inhibition of IL-2 and IL-13 production was observed in purified primary T cells and the Jurkat T cell line incubated with TCTP then stimulated with PMA and the calcium ionophore A23187 compared with cells stimulated without TCTP incubation. The activity of the IL-2, IL-4 and IL-13 promoters was shown to be inhibited by up to 70% by TCTP incubation. Incubation of T cells with TCTP did not affect rates of apoptosis or cell proliferation (Vonakis et al., 2003).

1.5.8 Interaction of TCTP and SHIP

The group that has performed much of the research into the induction of histamine release by TCTP have now altered their hypothesis of the function of TCTP. Recent work has focused on characterisation of basophils with a hyper-releasable phenotype, by investigation of alterations in intracellular signalling molecules in IgE+ basophils. The signalling molecules studied were the Lyn and Syk tyrosine kinases, which are positive basophil activity regulators, and the SH2 domain-containing inositol 5'-phosphatase (SHIP), which is a negative activity regulator. SHIP is implicated in limiting basophil degranulation to incomplete stimuli; and TCTP was hypothesised to be an incomplete stimulus for basophils, along with IL-3 and D2O. A negative correlation was observed between the amount of SHIP protein present and the maximum histamine release to TCTP. The basophil mRNA levels of SHIP were not significantly different between the IgE+ and IgE- donors, implying post-transcriptional regulation of SHIP levels. SHIP regulates phosphatidylinositol (3,4,5) triphosphate (PIP3) levels, and a PIP3 kinase inhibitor prevented TCTP-induced histamine release in IgE+ basophils (Vonakis et al., 2001). In another study, knock-down of SHIP-1 expression in cultured basophils was performed using siRNA. Histamine release induced by TCTP was enhanced in the SHIP-1 knock-down basophils, with the amount of enhancement correlating to the percentage of expression inhibition (Langdon et al., 2008). No explanation was given as to how these new findings fit with the earlier stripped basophil assay experiments, where basophils could be made IgE+ with the addition of sera (MacDonald et al., 1991).
1.6 Parasite TCTP functions

1.6.1 Malarial TCTP induces histamine and IL-8 release

Malaria pathogenesis is complex, and the host immune system may contribute to pathology. Histamine, IL-8 and eosinophils are all reported to be elevated during malaria infection. Increased histamine has been associated with disease severity in both P. falciparum infections and in several animal models. Elevated IgE levels have also been associated with malaria severity (MacDonald et al., 2001).

At the amino acid level, P. falciparum TCTP shares 33% identity and 54% similarity with human TCTP. In IgE+ allergic donor basophils, recombinant Pf TCTP stimulated histamine release, and also induced IL-8 release from allergic donor eosinophils in vitro. Pf TCTP was a log less active than human TCTP (MacDonald et al., 2001).

Evidence of Pf TCTP secretion was observed in in vitro cultures and in malaria-infected individuals. Cultured trophozoite stages of P. falciparum contained 87±21 µg of TCTP per 10⁹ infected red blood cell (irbc). When cultured P. falciparum was harvested after schizogony, the culture supernatants contained 44±6 µg TCTP per 10⁹ irbc, therefore around half of the total parasite TCTP was released during schizogony. Pf TCTP was found at concentrations of around 0.6 µg/ml in the plasma of human volunteers with subpatent (<4 irbc/µl) infections, and at concentrations of 1.41 µg/ml in severely infected Malawian children. No PfTCTP was detected in the plasma of uninfected controls. There was no correlation between plasma PfTCTP levels and parasitemia. It was suggested that as P. falciparum parasites are sequestered, secreted Pf TCTP might have local rather than systemic effects. The induction of histamine release by malarial TCTP could assist the parasite by inducing vasodilation and increased expression of endothelial adhesion factors such as thrombomodulin, to which P. falciparum-infected erythrocytes adhere (MacDonald et al., 2001).

1.6.2 Malaria TCTP may competitively inhibit activation of B-cells by host TCTP

The 3D structure of P. knowlesi, a primate malaria, has been solved, and structural analysis revealed the presence of an extra helical segment that is a β-sheet in other TCTPs. This helix is present at amino acid residues 22-30, close to the GTPase binding pocket, and the researchers propose that this would hinder G protein binding. The researchers propose that Plasmodium TCTP may act as a dominant negative
mutant in the host, as it could potentially block the action of B cells due to its proposed inability to bind G proteins (Hinojosa-Moya et al., 2008). Therefore it would compete with the host endogenous TCTP, which has been shown to activate B cells in mice (Kang et al., 2001). This extra helical segment is also present in P. yoelii TCTP at residues 22-31.

1.6.3 Malarial TCTP may self-interact, and binds calcium

Analysis of P. falciparum TCTP using the Multicoil server showed that amino acids 75 to 106 had a possibility of forming a coiled coil, and thus being a potential site for self-interaction to form multimers (Chae et al., 2006). Like several other TCTPs, malarial TCTP was shown to bind calcium (Bhisutthibhan et al., 1999).

1.6.4 Other parasite TCTPs induce histamine release from basophils and induce host immune responses

The TCTP from the human filarial parasites Wuchereria bancrofti and Brugia malayi can induce histamine release in a basophil cell line in vitro. W. bancrofti and B. malayi cause human lymphatic filariasis, with symptoms including lymphoedema and elephantitis. These parasites can also induce a rare allergic condition known as tropical pulmonary eosinophilia, which involves elevations in levels of circulating eosinophils, IgE and IgG4.

Both B. malayi and W. bancrofti TCTP induced release of histamine in vitro. TCTP induced histamine release in an IgE-independent manner in peritoneal mast cell preparations from C57Bl/6 mice, and also induced histamine release from RBL-2H3 cells. When injected into the peritoneal cavity of ovalbumin-sensitised C57BL/6 mice, B. malayi TCTP induced an inflammatory infiltration of eosinophils. Additionally, both Bm and Wb TCTP bound calcium as tested with a 45Ca overlay assay (Gnanasekar et al., 2002).

Anti-BmTCTP antibodies recognised a 25 kDa protein in adult females, in microfilaria and in the ES of microfilaria, and recognised proteins around 48 and 96 kDa in adult males, adult females and in microfilaria, indicating the presence of TCTP multimers in these life stages. Recombinant filarial TCTP also formed multimers under non-reducing conditions. Evidence for the secretion of TCTP from some parasite life stages was observed, as B. malayi TCTP was found in abundance in excretory/secretions of microfilariae, despite lacking a signal sequence. TCTP found in excretory/secretion products was mostly monomeric. Differential expression of TCTP
mRNA and protein was observed in different *B. malayi* life stages. Only low transcript and protein expression of TCTP was detected in L3 stages, which are the infective stages transmitted by the cold-blooded mosquito vector. In the subsequent L4, adult and microfilariae stages found in the warm-blooded human host, abundant TCTP mRNA and protein expression was observed. As discussed in section 1.3.5, the upregulation of TCTP expression in response to a host body temperature increase was also observed in *Trichinella* nematodes, and may be part of a parasite adaptive response (Mak *et al.*, 2007).

Work by the same group revealed similar activities for the TCTP of *Schistosoma mansoni*, a human flatworm parasite. SmTCTP could induce the release of histamine from RBL-2H3 cells, and bound calcium. SmTCTP injected into OVA-sensitised C57Bl/6 mice induced eosinophil infiltration within 24 hours. SmTCTP formed monomers and dimers in different life stages, and differential expression was observed, in that high levels of TCTP mRNA and protein were only detected in the vertebrate host life stages (Rao *et al.*, 2002).

A recent report by this group stated that *B. malayi* TCTP functions as an antioxidant protein. TCTP chemically reduced by a number of agents was able to protect DNA from oxidative damage, and this activity was shown to involve the three cysteine residues. No further histamine-releasing activities of filarial TCTP were reported, and attempts were not made to relate this new finding to the earlier work (Gnanasekar and Ramaswamy, 2007).

A TCTP homolog from the dog tick species *Dermacentor variabilis* was identified as a secreted tick saliva protein. The tick TCTP was expressed in *E. coli* as a thioredoxin-fusion protein, and was able to induce histamine release from RBL-2H3 cells in a dose-dependent manner (Mulenga *et al.*, 2003). A more recent report by this group identified that TCTPs present in several tick species were immunologically cross-reactive, and did not bind calcium as demonstrated by an overlay assay (Mulenga and Azad, 2005).

*Cladosporium herbarum* is an allergenic fungal species, and between 5 and 30% of allergy patients have IgE reactive to the fungus. Specific IgE antibodies to fungal TCTP were found in nine individuals allergic to *C. herbarum* out of a total of 306 patients, and so TCTP was classed as a minor allergen. In five of the nine patients, IgE that could recognise fungal TCTP also recognised human TCTP. It was found that patients reactive
to TCTP had a higher occurrence of asthma. The fungal TCTP was able to induce histamine release from human basophils with around half the potency of the human TCTP (Rid et al., 2008).

Eumycetoma patients have cancerous swellings caused by the fungus *Madurella mycetomatis*. TCTP was identified as an immunogenic antigen from this fungus, and was secreted in *in vitro* cultures. A significant number of eumycetoma patients and some healthy controls had elevated levels of anti-TCTP antibodies. TCTP was expressed in newly forming fungal grain cells, which are in direct contact with host neutrophils, but was not expressed in fully formed grain, which forms a cement material in which neutrophils are not present. Eumycetoma patients with the largest lesions and the greatest disease duration had the highest anti-TCTP IgG antibody levels (van de Sande et al., 2006).

**1.6.5 The role of histamine and eosinophils in malaria**

Elevations in histamine in humans infected with *P. falciparum* have been observed, with greater elevations in patients with systemic complications (Srichaikul et al., 1976). One report observed a significant increase (p<0.025) in plasma histidine and a five-fold elevation (p<0.001) in plasma histamine in children infected with *P. falciparum* compared with uninfected controls (Enwonwu et al., 2000).

In one experiment, tricyclic antihistamine treatment prior to and during infection with sporozoites could completely prevent patent infection in mice inoculated with *P. yoelii nigeriensis*. At the doses used, the antihistamines did not have direct schizontocidal activity, meaning that the prevention of infection by these drugs was most likely due to their indirect activity in preventing histamine release in the mice (Singh and Puri, 1998). The antihistamines tested could also induce a reversal of chloroquine resistance in *P. falciparum* and *P. yoelii* (Peters et al., 1990; Singh and Puri, 1998).

In another study, C57BL/6 mice deficient in histamine due to disruption of the histidine decarboxylase gene were resistant to cerebral malaria development, instead succumbing to hyperparasitemia at around 20 days post-infection. Mice deficient in the H1 histamine receptor also had delayed mortality when infected with *P. berghei* sporozoites or irbc, and treatment with drugs that inhibit the H1 receptor could also delay mortality. In the histidine decarboxylase-deficient mice, the BBB integrity was preserved, and infected erythrocytes did not sequester in the brain blood vessels. The expression of
vascular adhesion molecules in the brain was also decreased in the mutant mice (Beghdadi et al., 2008).

Elevations in eosinophils have been observed in *P. falciparum* infections. Eosinophils may be protective in malaria infection by inducing parasite killing or they may contribute to pathology by the release of their granule proteins, including eosinophil cationic protein (ECP) and eosinophil protein X (EPX), as these are potent neurotoxins and may contribute to CM. Increased eosinophil counts were observed in Ghanian children with asymptomatic infections, whereas children who acquired symptomatic infections had a decrease in eosinophil counts. In a separate hospital-based study, children with cerebral malaria, severe anaemia, or uncomplicated malaria all had significantly low eosinophil counts during acute illness followed by eosinophilia 30 days after cure. Plasma levels of ECP and EPX are used as markers of eosinophil activation. Despite low eosinophil counts, plasma ECP levels were significantly higher in all patient groups prior to treatment than on day 30. EPX levels were significantly higher in patients with CM prior to treatment. In infected children at time of low eosinophil counts the bone marrow is rich in eosinophil precursors. Therefore, low eosinophil counts may be due to tissue sequestration and destruction (Kurtzhals et al., 1998).

In a small study of *P. falciparum*-infected Malawian children, IL-8 levels decreased in eight of ten patients following antimalarial treatment (MacDonald et al., 2001).
1.7 Artemisinin and TCTP:

1.7.1 Antimalarial action of artemisinin

Artemisinin (qinghaosu) is extracted from the sweet wormwood plant *Artemisia annua*. The brewed leaves of the plant have been used in traditional Chinese medicine for thousands of years to treat chills and fever. Due to the low solubility of artemisinin in oil and water, several semi-synthetic derivatives have been developed. These include artemether, arteether, and artesunate (Efferth, 2005). By the early 1990’s artemisinin derivatives were widely used in Thailand, Burma and Vietnam (Meshnick, 2002), and are now a major part of the recommended antimalarial therapy in many countries (http://www.rbm.who.int/wmr2005/). Artemisinin has a short half-life, which has helped to prevent the development of drug resistance by parasites but also led to recrudescence of parasitemia in early clinical trials. Artemisinin acts on the most parasite life stages of any antimalarial, from young rings to schizonts as well as gametocytes, this may also contribute to the slow development of drug resistance (Krishna et al., 2004).

The precise antimalarial action of artemisinin is disputed, with most but not all researchers agreeing that artemisinin interacts with intraparasitic haem as well as other parasite proteins (e.g. Meshnick, 2002; Olliaro et al., 2001; Robert et al., 2002). The malaria parasite is rich in haem as a by-product of haemoglobin digestion. Artemisinin derivatives are inactive against the RC strain of *P. berghei*, which doesn’t produce hemozoin (Meshnick, 2002).

In the argument against the haem-activation of artemisinin, it is stated that haem must be in the ferrous state for the interaction to occur. As the food vacuole is not a reducing environment, haem would not be present in this state for extended lengths of time. Also, artemisinin derivatives unable to react with haem but with potent antimalarial activity exist, and artemisinin is active against young ring stages, which do not contain haemozoin. Instead, it is argued that artemisinin binds to an active site of a parasite protein, specifically the PfATP6 Ca\(^{2+}\)-transporting ATPase (as reviewed in section 1.7.4), (Krishna et al., 2004).

The precise mechanism of the antimalarial action of artemisinin is not yet fully determined, however one hypothesis is that haem-mediated decomposition of the artemisinin endoperoxide bridge produces carbon-centred free radicals that then act as alkylating agents for specific parasite proteins (Meshnick, 2002).
1.7.2 Artemisinin and TCTP

Some evidence exists that TCTP is a target of artemisinin, although whether this interaction contributes to parasite killing is unknown. In a study of potential drug targets, *P. falciparum*-infected erythrocyte cultures were incubated with radio labelled dihydroartemisinin. Several malarial proteins became labelled, including TCTP. Labelled proteins were identified by isoelectric focusing followed by SDS-PAGE and autoradiography. TCTP was present as a strong band on the coomassie-stained gel and was the brightest band seen on autoradiography. Dihydroartemisinin reacted covalently with TCTP *in vitro* in the presence of hemin, and the association increased up to a concentration of 1 drug/TCTP molecule. More labelled dihydroartemisinin was retained *in vitro* when added to TCTP and hemin compared with artemisinin added to TCTP or hemin alone. Scatchard analysis showed that two hemin-binding sites of modest affinity existed on TCTP. The absorption spectra of TCTP+hemin were different to hemin alone, suggesting that binding occurs. Artemisinin was shown to bind to TCTP and not hemin by the fact that the TCTP was radio labelled (Bhisutthibhan *et al.*, 1998).

The same group then investigated the localisation of TCTP within malarial cells. By immunofluorescence and immuno-electron microscopy, TCTP was found to be mainly present in the cytoplasm and the parasitophorous vacuolar and/or parasite limiting membranes. Some TCTP was also present in the food vacuolar membrane (Bhisutthibhan *et al.*, 1999).

TCTP expression at the protein level was found to be up-regulated in an artemisinin-resistant strain of *P. yoelii*. The art-resistant strain was approximately four times less sensitive to artemisinin than the sensitive strain, and accumulated 43% less radio-labelled drug *in vitro*. Artemisinin appeared to react with the same parasite proteins in both strains, with artemisinin-associated bands at 14, 22.5, 36.5, 42 and >150 kDa. The TCTP DNA sequence was identical in both *P. yoelii* strains, but the art-resistant parasite strain expressed 2.5-fold more TCTP protein, as quantified by western blot (Walker *et al.*, 2000).

In further research, it was demonstrated that [*H*] dihydroartemisinin adducts could be immunoprecipitated using anti-TCTP antibodies. Cultured *P. falciparum* at the late ring and trophozoite stages were incubated with [*H*] dihydroartemisinin or [*S*] methionine. Isolated parasites were lysed, incubated with anti-TCTP or control
antibodies, and proteins were precipitated with protein A-sepharose. The proteins obtained were separated by SDS-PAGE, and visualised both by coomassie stain and by autoradiography. Radio labelled bands were seen at 22 and 45 kDa in artemisinin-incubated parasite lysates following anti-TCTP immunoprecipitation. It was suggested that the 45 kDa band might be a TCTP oligomer not reduced by SDS (Bhisutthibhan and Meshnick, 2001).

A molecular docking study using the computer program FlexiDock was used to study the interaction of heme, artemisinin and TCTP. A 3D model of \textit{P. falciparum} TCTP was created using the solved NMR structure of \textit{S. pombe} TCTP as a template. The peroxide bond of artemisinin was docked within a short distance (2.6Å) of the iron of heme. The C4-radical of the heme-activated artemisinin docked within a short distance (2.8Å) of the single cysteine residue (amino acid position 14) of the \textit{P. falciparum} TCTP (Chae \textit{et al.}, 2006). Earlier research had found that blocking the single cysteine of PfTCTP reduced artemisinin binding by 67% (Bhisutthibhan \textit{et al.}, 1998).

A recent study of potential drug targets was performed on artemisinin-resistant \textit{P. c. chabaudi} parasites. The drug resistance was genetically stable and mosquito-transmissible. Artemisinin resistance could only be generated in parasite clones already resistant to chloroquine, suggesting a potentiation mechanism in the development of drug resistance. The nucleotide sequences of potential drug targets, including the \textit{P. chabaudi} homologs of PfATP6, TCTP, \textit{pfmdr1} and \textit{pfcrt}, were analysed in the drug-resistant mutants. The nucleotide sequences of the mutant resistant parasites and the parental sensitive parasites were identical in both the coding sequences and the untranslated regions for all of the genes analysed. These genes were also not present in increased copy number in the resistant parasites (Afonso \textit{et al.}, 2006). However, as increased protein levels of TCTP were previously found in artemisinin-resistant \textit{P. yoelii} parasites (Walker \textit{et al.}, 2000), and TCTP expression is controlled at the translational level and is not proportionate to mRNA levels, this finding does not disprove the theory that TCTP is involved in the antimalarial action of artemisinin.

### 1.7.3 Other malarial targets of artemisinin

In \textit{P. falciparum}, artemisinin, but not quinine or chloroquine, was shown to inhibit PfATP6, a sarco/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) othologue. SERCAs are involved in transport of cytosolic calcium into the ER, and assist in the maintenance of high ER calcium levels (Xu \textit{et al.}, 1999). In this study, a fluorescent artemisinin derivative localised to membranes in the parasite cytoplasm and to tubovesicular...
membranes in the cytoplasm of the host erythrocyte, and did not localise to the parasite food vacuole. Thapsigargin, another SERCA inhibitor, antagonised the antimalarial activity of artemisinin. Chelation of iron inhibited the antimalarial activity of artemisinin but did not affect the activity of thapsigargin (Eckstein-Ludwig et al., 2003).

The effects of chloroquine and artemisinin treatment on the mechanisms involved in the parasite regulation of H\(^+\) and Ca\(^{2+}\) homeostasis were studied. Pools of calcium have been found in the acidic compartment of several malaria species. Both artemisinin and chloroquine were shown to act on the acidic compartment of the parasite, promoting alkalisation. The drugs also had a possible effect on the release of calcium from the acidic compartment, although this was not confirmed directly (Gazarini et al., 2007). This finding lends support to the theory of interaction of artemisinin with both PfATP6 and TCTP, as both are involved in calcium homeostasis (e.g. Arcuri et al., 2005; Bhisutthibhan et al., 1999; Eckstein-Ludwig et al., 2003; Feng et al., 2007; Sanchez et al., 1997).

**1.7.4 Artemisinin acts as an anti-cancer agent**

Artemisinin and its derivatives are cytotoxic to cancer cells at low concentrations *in vitro*, however higher concentrations are required for efficacy *in vivo*. It was found that yeast strains deficient in certain mitosis-regulating or proliferation-regulating genes were more sensitive to artesunate (Efferth et al., 2001), and artemisinin was found to potentiate proliferation of leukaemia cells (Kim et al., 2003).

A study to identify candidate genes that may contribute to the action of artemisinin on tumour cells was performed using mRNA expression analysis. Candidate genes identified included those involved in regulation of proliferation, angiogenesis, and apoptosis (including the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax). Artesunate treatment caused a decrease in the number of Bcl-2 positive hepatic carcinoma cells, and an increase in the number of Bax-positive cells (Wang et al., 2002). TCTP has been reported to interact with Bcl-2 to synergistically inhibit the action of Bax in promoting apoptosis (Li et al., 2001b), (reviewed in section 1.3.11).

The mRNA expression of TCTP correlated with sensitivity to artesunate in tumour cells. Cells expressing high levels of TCTP were more sensitive to artesunate, while cells with a low expression level of TCTP were resistant (Efferth, 2005). Direct comparisons between this work and the work of Walker et al. (2000) are difficult, as one study was based on mRNA levels and the other on protein, and it has been repeatedly
shown that TCTP protein and mRNA levels are not proportionate. However, this finding appears to be in contrast with the earlier finding of increased TCTP present in artemisinin-resistant parasites.

The work on the interaction of PfATP6 with artemisinin stated that artemisinin has specificity to malarial calcium pumps and does not work on mammalian protein homologs (Krishna et al., 2004). This suggests that artemisinin has a different mode of action on human cancer cells, possibly through interaction with TCTP. However, in a recent review, it was suggested that artemisinin has anti-cancer activity via its interaction with iron, which is present at higher concentrations in cancer cells than in normal cells (Nakase et al., 2008).
1.8 TCTP gene knockout or expression inhibition experiments

Expression of TCTP has been inhibited in several species, using several methods including gene disruption and siRNA. In an early experiment, a TCTP null mutant made in yeast had no mutant phenotype (Sanchez et al., 1997). In a recent experiment in Drosophila flies, TCTP expression was disrupted using RNAi and gene disruption. Targeted inhibition of TCTP expression by RNAi reduced the size of various tissues, caused by a decrease in both cell size and cell number. Ubiquitous inhibition of TCTP expression was incompletely lethal in the larval stage. Co-expression of complementary TCTP DNA rescued the mutant phenotypes. In Drosophila TCTP knockouts (made by targeted gene disruption), 100% lethality was observed at the larval stage (Hsu et al., 2007).

TCTP knockouts have been created in mice by gene disruption. Mice heterozygous for the TCTP deleted allele were fertile and morphologically normal, and heterozygous embryos developed normally. No homozygous mutant progeny were obtained from heterozygote crosses, indicating that the TCTP \(-/-\) mutation was embryonically lethal. Embryos at various life stages were examined to determine the gestation stage at which the TCTP deletion became lethal. Homozygote mutant embryos were present up to 10.5 days after fertilisation, however the phenotype appeared abnormal from 6.5 days onwards. The major abnormalities seen were severe growth retardation and cellular disorganisation. Increased cell death was observed in the TCTP mutant embryos from day 6.5 onwards. Conditional TCTP deletion experiments were also performed to investigate if TCTP was essential in mouse embryonic fibroblast (MEF) cells. MEFs were isolated from homozygous TCTP mutant mice and infected with Cre recombinase to generate knockouts. These knockout fibroblasts developed normally, and were not more sensitive to apoptotic stimuli than wild-type MEFs. This indicates that TCTP is essential only in some stages of mouse development, or in some cell types (Chen et al., 2007).
1.9 Malarial gene knockout experiments

Methods for the disruption of gene expression have been developed for several malarial species. Disruption of gene expression by homologous recombination of exogenous DNA is a commonly used technique in \textit{P. falciparum} and \textit{P. berghei}. In \textit{P. falciparum}, the target DNA used for recombination is a circular plasmid, in \textit{P. berghei} linearised target DNA is used (Waters \textit{et al.}, 1997a). These methods exploit the haploid nature of the parasite over much of its life cycle, meaning that the phenotype conferred by particular proteins can be studied by the disruption of a single gene copy.

The transfection of \textit{P. berghei} with exogenous DNA was first reported in 1995. Initially, circular plasmids were used to transf ect exoerythrocytic \textit{P. berghei} merozoites by electroporation, with a drug-resistant mutant (\textit{Ser}^{110} \rightarrow \text{Asn}) of the \textit{P. berghei} bifunctional dihydrofolate reductase-thymidylate synthase (DHFR/TS) enzyme used as a selectable marker. Parasites were injected into rats following electroporation, and transfected parasites were selected for by treatment of rats with an i.p. injection of the antifolate antimalarial pyrimethamine. Cloning by limiting dilution in mice was then used to separate drug-resistant parasites for genotype analysis. In this initial experiment, the plasmid DNA was found to not integrate at the designed sites of homologous recombination, but instead was maintained as an extrachromosomal plasmid (van Dijk \textit{et al.}, 1995). Later, it was reported that although \textit{P. berghei} parasites can maintain episomal plasmids over several generations, the plasmids are randomly segregated during schizogony and are lost during cell division in the absence of drug pressure (Waters \textit{et al.}, 1997a).

The method for \textit{P. berghei} transfection has since undergone several modifications. The successful disruption of target genes using linearised plasmids was first reported in 1997 for genes encoding both the circumsporozoite (CS) protein (Menard \textit{et al.}, 1997) and the thrombospondin-related anonymous protein (TRAP) (Sultan \textit{et al.}, 1997). Linearised plasmids were introduced into mature schizonts rather than free merozoites. In these experiments, the targeting plasmid was linearised with a single restriction endonuclease, and in the TRAP knockout experiment revertant parasites were obtained in which the linearised plasmid had been excised from the genome. A replacement construct was then designed to allow recombination by double crossover recombination, preventing reversion. The TRAP knockout parasites were 10,000 times...
less infective than wild-type parasites, and sporozoite gliding motility and mosquito salivary gland invasion were not observed (Sultan et al., 1997).

To reduce the likelihood of unwanted homologous recombination, a drug-resistant form of the *Toxoplasma gondii* DHFR/TS was developed for use as the selectable marker, with the 5’ and 3’ untranslated regions (UTRs) of the *P. berghei* DHFR incorporated (Waters et al., 1997a).

Depending on the desired outcome, *P. berghei* transfection is performed using either insertion constructs, with one continuous sequence of target gene, or with replacement constructs that contain two regions of the target gene separated by the selectable marker. Linearised insertion constructs integrate via a single crossover, with integration of the entire plasmid. Linearised replacement constructs are designed to integrate by double crossover, leading to disruption of the target gene by allelic exchange. These constructs are also often designed to delete the majority or all of the coding sequence of the target DNA. Thus the wild-type gene cannot be spontaneously re-created by excision of the replacement construct or gene rearrangements. However, the integration of replacement constructs by single crossover has been observed, in which the wild-type genotype can recur by excision of the target DNA. It is hypothesised that the replacement vectors are present as re-circularised plasmids prior to single crossover recombination (Menard and Janse, 1997).

Using electroporation techniques, regions of target DNA as small as 500 bp have been shown to be sufficient for recombination, with an upper size limit of around 1 kb recommended due to the instability of plasmids containing large regions of A/T-rich Plasmodium DNA (Waters et al., 1997a).

Repeated erythrocytic cycles of *Plasmodium* can lead to the accumulation of spontaneous genomic rearrangements, including large deletions of protein-coding sequences. Therefore the recommendation is to analyse the phenotype of several clones generated from independent experiments, and to complement the disrupted gene using a second selectable marker to see if the wild-type phenotype is restored (Menard and Janse, 1997).

The *P. berghei* transfection techniques have been used to introduce gene expression in parasite life stages. Using circular plasmids, the Pbs21 gene, encoding a stage-specific surface protein, was expressed in trophozoites, schizonts and
gametocytes. This expression of this protein is usually restricted to mosquito life stages (Margos et al., 1998). The *P. falciparum* AMA-1 gene has also been expressed in *P. berghei* by transfection with circular plasmids (Kocken et al., 1998). Green fluorescent protein (GFP) and luciferase have been expressed in *P. berghei* parasites, using stage-specific promoters, which could allow the selection of specific life stages by FACS analysis (de Koning-Ward et al., 1999; de Koning-Ward et al., 1998).

In *P. berghei* transfections, the human DHFR gene has also been used as a selectable marker, with a separate antifolate drug (WR99210) used for selection. This combination can be used for selection in both pyrimethamine resistant and sensitive parasites. This second selectable marker allows for complementation of disrupted genes to confirm that the wild-type phenotype can be reinstated, as it is possible that alterations in phenotype seen after transfection could be due to genetic disruptions to areas other than the target gene (de Koning-Ward et al., 2000). Using this second selectable marker system, the TRAP gene was re-introduced into TRAP-knockout parasites, and the wild-type phenotype was restored (Sultan et al., 2001).

These transfection techniques have also been used to disrupt genes in *P. yoelii*. The drug-resistant form of the *P. berghei* DHFR/TS gene was used as a selectable marker. The TRAP gene was disrupted, with the phenotype obtained identical to the *P. berghei* TRAP knockout. In this experiment, circular and linearised plasmids were used. Transfected circular DNA could be maintained under drug pressure as autonomously replicating plasmids. Linearised plasmids integrated at the TRAP locus by a single crossover, but the transfected DNA was found to be excised from the genome in blood-stage parasites, resulting in the restoration of the wild-type phenotype (Mota et al., 2001).

One application of the *P. berghei* transfection system is in the use of knockout parasites as a vaccine. In one experiment, the sporozoite-specific P36p protein was disrupted using a replacement vector. In mice, immunisation with the P36p-knockout sporozoites conferred protective immunity against subsequent challenge with wild-type sporozoites. Within hepatocytes, the P36p-knockout sporozoites failed to develop fully and were unable to form a mature parasitophorous vacuole (van Dijk et al., 2005).

The use of a more advanced transfection method for *P. berghei* was first reported in 2006 (Janse et al., 2006b). In this method, a nucleofector machine (Amaxa) was used in place of an electroporator. The nucleofector device is designed with specific electrical
parameters for the transfection of various cell types, allowing for efficient transport of DNA directly into the nucleus (http://www.amaxa.com/products/technology/). The transfection efficiency, as determined by the number of parasites surviving following pyrimethamine selection, was between $10^{-2}$ and $10^{-3}$ using the nucleofector, compared with transfection efficiencies of between $10^{-7}$ and $10^{-9}$ using electroporation. The nucleofector machine was used to generate parasites with a GFP or luciferase genes integrated by double crossover homologous recombination into the ssu-rna or p230p genes of *P. berghei*. Disruption of these genes has a negligible phenotypic effect. A drug-selectable marker was not introduced, instead transfected parasites were selected for by FACS sorting. Flow cytometry sorting can be performed immediately after transfection, without the need for drug selection. Transfection using the nucleofector allows homologous recombination of linear DNA with target fragments of around 300 bp (Janse et al., 2006a).

The Amaxa nucleofector has also been used for *P. yoelii* transfection. Circular plasmids containing human DHFR and TgDHFR/TS selectable markers were introduced, however the disruption of genes by the integration of linear DNA was not assessed (Jongco et al., 2006).

Stage-specific gene disruption techniques have been used in *P. berghei* for the examination of genes essential in blood-stage parasites. The Flp/FRT system of yeast was used for site-specific recombination after cross-fertilisation in the mosquito vector (Carvalho et al., 2004). However, further development of this system has not been reported, and requires the use of specific mosquito species.
1.10 Research Aims

The research aims of this thesis were:

1) To confirm the protective effect of *P. falciparum* TCTP immunisation previously observed in the *P. yoelii* YM challenge system

2) To produce recombinant *P. yoelii* and *P. berghei* TCTP of high yield and purity

3) To evaluate the protective effect of *P. yoelii* and *P. berghei* TCTP immunisation in several rodent malarial challenge models

4) To produce *P. berghei* parasites with the TCTP gene disrupted, to further assess the functions of malarial TCTP
# Chapter 2: Materials and Methods

## 2.1 Materials and equipment suppliers

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## Chapter 2: Materials and Methods

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## Chapter 2: Materials and Methods

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Chapter 2: Materials and Methods
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## Chapter 2: Materials and Methods

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<td>Whatman paper</td>
<td>Whatman-Rad, USA</td>
</tr>
<tr>
<td>X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside)</td>
<td>Progen</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>Oxoid, Australia</td>
</tr>
<tr>
<td>Yeast nutrient base without amino acids</td>
<td>BIO 101 Anachem, England</td>
</tr>
</tbody>
</table>
2.2 Preparation of materials

Unless otherwise specified, the following conditions were used: All materials were prepared using deionised water filtered through a Milli-Q® system (Milli-Pore). All pH adjustments were made using 12 M (concentrated) HCl or 10 M NaOH. Materials to be sterilised were autoclaved at 121°C for 20 min. All reactions were performed at room temperature (rt). Glassware was washed using Pyroneg detergent followed by rinsing in tap water with a final rinse in Milli-Q water.

2.3 General DNA Materials and Methods

2.3.1 DNA Materials

2.3.1.1 TE Buffer
10 mM Tris.HCl pH 8
1 mM EDTA

2.3.1.2 TEG/lysozyme
25 mM Tris.HCl pH 8
10 mM EDTA pH 8
50 mM glucose
Sterilised, 4 mg/ml lysozyme added. Stored at 4°C.

2.3.1.3 NaOH/SDS
0.2 M NaOH
1% w/v SDS

2.3.1.4 KOAc
2 M acetic acid
3 M KAc

2.3.1.5 Phenol/chloroform/isoamylalcohol (PCI)
50% v/v phenol (distilled, neutralised, saturated with TE)
46% v/v chloroform
4% v/v isoamylalcohol

2.3.1.6 Chloroform/isoamylalcohol (CI)
96% v/v chloroform
4% v/v isoamylalcohol
2.3.1.7 \( \text{H}_2\text{O/RNase A} \)
20 µg/ml pancreatic ribonuclease A in molecular grade water

2.3.1.8 \( \text{TE/RNase A} \)
20 µg/ml pancreatic ribonuclease A in TE buffer

2.3.1.9 \( 10X \text{ Loading dye} \)
40% w/v sucrose
0.2% w/v Orange G

2.3.1.10 \( \text{TAE Buffer} \)
40 mM Tris
20 mM acetic acid
2 mM EDTA

2.3.1.11 \( \lambda/\text{HindIII marker} \)
1x Buffer E
0.1 mg/ml BSA
20 µg λ DNA
40 U HindIII
Incubated at 37°C for 16 h, stored at -20°C.

2.3.1.12 \( \lambda/\text{PstI marker} \)
1x buffer H
0.1 mg/ml BSA
20 µg λ DNA
40 U PstI
Incubated at 37°C for 16 h, mixed with a final conc. of 1X loading dye, stored at 4°C.

2.3.1.13 \( \lambda/\text{NdeI marker} \)
1x buffer D
0.1 mg/ml BSA
5 µg λ DNA
10 U NdeI
Incubated at 37°C for 16 h, stored at -20°C.

2.3.1.14 Ethidium bromide staining bath
2 mg/L ethidium bromide in 2L water

2.3.2 DNA Methods

2.3.2.1 DNA extraction and purification methods

2.3.2.1.1 Plasmid DNA minipreparation – alkaline lysis method

Single *E. coli* DH5α colonies were taken from LB+Amp agar plates and grown for 16 h at 37°C in 5 ml LB+Amp broth, shaking at 200 rpm. 1.5 ml aliquots of culture were added to sterile 1.5 ml tubes and centrifuged at 10,000 x g for 2 min. Supernatants were discarded and cell pellets were resuspended in 100 µl TEG/lysozyme by repeated pipetting. 200 µl of NaOH/SDS was added; the tube contents were mixed by inversion (4-6 times) and were incubated on ice for 5 min. 150 µl of ice-cold KOAc was added and tube contents were mixed by inversion (4-6 times). Tubes were incubated on ice for 5 min before centrifugation at 16,000 x g for 10 min. Supernatants were transferred to fresh 1.5 ml tubes and 300 µl of PCI was added. Tube contents were mixed by vortexing for 10 s and supernatants were recovered by centrifugation at 16,000 x g for 2 min. Supernatants were transferred to new 1.5 ml tubes and 300 µl of CI was added. Tube contents were mixed by vortexing for 10 s, and supernatants were recovered as previously described.

Supernatants were transferred to new 1.5 ml tubes and 900 µl of 100% EtOH was added. Tube contents were mixed by vortexing for 10 s before incubation at rt for 5 min. DNA pellets were recovered by centrifugation at 16,000 x g for 5 min and washed with 1 ml of 70% EtOH before recovery by centrifugation as before. The DNA pellets dried by incubation at rt with the lids of the tubes open. DNA was resuspended in 50 µl of H₂O/RNase, except when long-term storage of plasmid DNA was required, in which case pellets were resuspended in TE/RNase. Resuspended DNA was stored at -20°C.

2.3.2.1.2 Plasmid midipreparation – Alkaline lysis method

Plasmid purification by midipreparation was performed as per the minipreparation method, with larger volumes of bacterial culture and extraction reagents. Plasmid DNA was extracted from 10 ml of o/night bacterial culture using 200 µl of TEG/lysozyme, 400 µl of NaOH/SDS and 300 µl of KOAc. Plasmid DNA from 600 µl of the supernatant was purified using 600 µl of PCI and CI. DNA was precipitated using 600 µl of isopropanol,
and washed with 1 ml of 70% EtOH. Purified plasmid DNA was resuspended in 100 µl of H$_2$O/RNase or TE/RNase.

2.3.2.2 PCR Methods

2.3.2.2.1 Primer reconstitution for 100 µM stock solutions

Lyophilised primers were reconstituted in TE to 100 µM. Primer solutions were incubated at rt for 5 mins and then mixed by vortexing for 10 s. Primers were pulse centrifuged and stored at -20°C.

2.3.2.2.2 Creation of primer working stocks

The 100 mM primer stock solutions were diluted in sterile TE or molecular grade water to create working stocks of 3 or 5 µM. These were stored at -20°C.

2.3.2.2.3 PCR using Taq polymerase

PCR using Taq polymerase was performed in 25 µl reaction volumes unless otherwise stated. Either AmpliTaq or AmpliTaq Gold was used, along with the appropriate 1X PCR buffer. A standard reaction contained 0.3 µM of each primer, 2.5 mM MgCl$_2$, 0.2 mM of dNTP mix and 1.25 U of Taq polymerase. PCR reactions were made to a final volume using Milli-Q or molecular-grade water. DNA template concentrations of between 10 and 500 ng were used. Aliquots were dispensed into sterile 0.2 ml PCR tubes. All experiments included a negative control reaction (master mix with no DNA template). Standard reaction parameters are described in Table 2.1.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial denaturation</td>
<td>95</td>
<td>2-5 min</td>
<td>1</td>
</tr>
<tr>
<td>2. Amplification:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Denaturation</td>
<td>95</td>
<td>30 s</td>
<td>30-35</td>
</tr>
<tr>
<td>b) Annealing</td>
<td>variable</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>c) Extension</td>
<td>72</td>
<td>1 min per kb</td>
<td></td>
</tr>
<tr>
<td>3. Final extension</td>
<td>72</td>
<td>7 min</td>
<td>1</td>
</tr>
<tr>
<td>4. Cooling</td>
<td>4</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>
2.3.2.2.4 PCR using Pfu polymerase

Pfu polymerase was used when proofreading ability was required or to amplify large products (>3 kb). Standard reactions were prepared in 50 µl volumes using 0.3 µM of each primer, 0.2 mM dNTP mix, 1 U of Pfu polymerase, 1X Pfu PCR buffer, and 0.5 µg of DNA template. PCR reactions were made to a final volume using Milli-Q or molecular-grade water. Aliquots of 50 µl were dispensed into sterile 0.2 ml PCR tubes. All experiments included a negative control reaction. Standard reaction parameters are described in Table 2.2.

### Table 2.2: Standard Pfu PCR parameters

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial denaturation</td>
<td>94</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>2. Amplification:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Denaturation</td>
<td>94</td>
<td>30 s</td>
<td>30-35</td>
</tr>
<tr>
<td>b) Annealing</td>
<td>variable</td>
<td>30s</td>
<td></td>
</tr>
<tr>
<td>c) Extension</td>
<td>68</td>
<td>2 min per kb</td>
<td></td>
</tr>
<tr>
<td>3. Final extension</td>
<td>68</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>4. Cooling</td>
<td>4</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

2.3.2.2.5 PCR using Expand polymerase

Expand polymerase was used to amplify large PCR products (>5 kb). Buffer 3 was used, which was supplied with the polymerase and contained 27.5 mM MgCl₂ and detergents. Each reaction also contained 0.5 mM dNTP mix, 0.3 µM of each primer and 3.75 U Expand polymerase. Typically, 0.5 µg of template DNA was used per reaction. PCR reactions were made to a final volume using Milli-Q or molecular-grade water, and 50 µl aliquots were dispensed into 0.2 ml PCR tubes. All experiments included a negative control reaction. Standard reaction parameters are described in Table 2.3.
Table 2.3: Standard Expand PCR parameters

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp. (°C)</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial denaturation</td>
<td>94</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>2. Initial amplification:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Denaturation</td>
<td>94</td>
<td>10 s</td>
<td>10</td>
</tr>
<tr>
<td>b) Annealing</td>
<td>50</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>c) Extension</td>
<td>68</td>
<td>4 min</td>
<td></td>
</tr>
<tr>
<td>3. Second amplification:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Denaturation</td>
<td>94</td>
<td>15 s</td>
<td></td>
</tr>
<tr>
<td>b) Annealing</td>
<td>50</td>
<td>30 s</td>
<td>25</td>
</tr>
<tr>
<td>c) Extension</td>
<td>68</td>
<td>4 min + 20 s each cycle</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>68</td>
<td>7 min</td>
<td>1</td>
</tr>
<tr>
<td>Cooling</td>
<td>4</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

2.3.2.2.6 DNA sequence analysis

Reactions to incorporate dye terminators by PCR were set up in 20 µl reaction volumes with 4 µl Terminator Ready Reaction mix, 2 µl BigDye Sequencing buffer, 0.3-0.5 µg of plasmid DNA and 3.2 pmol of a single primer. The reactions were made up to a final volume of 20 µl in molecular-grade water. The dye terminators were incorporated by PCR using the parameters described in Table 2.4.

Table 2.4: PCR to incorporate dye terminators for DNA sequencing

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp. (°C)</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial denaturation</td>
<td>96</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>2. Amplification:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Denaturation</td>
<td>96</td>
<td>10 s</td>
<td></td>
</tr>
<tr>
<td>b) Annealing</td>
<td>50</td>
<td>5 s</td>
<td>25</td>
</tr>
<tr>
<td>c) Extension</td>
<td>60</td>
<td>4 min</td>
<td></td>
</tr>
<tr>
<td>3. Cooling</td>
<td>4</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>
To remove unincorporated dye terminators, 2 µl of 3 M NaOAc and 50 µl of EtOH were added to completed sequencing reactions. Tubes were vortexed for ~10 s and left on ice for 12 min. DNA was pelleted by centrifugation at 16,000 x g for 20 min and washed twice with 250 µl of 70% EtOH by vortexing for 10 s followed by centrifugation at 16,000 x g for 5 min. The DNA pellet was dried by leaving the tube lids open for ~15 min. The dried DNA was sent for sequence analysis to the Micromon DNA sequencing facility, Department of Microbiology, Monash University, Melbourne, Australia.

2.3.2.3 Creation of cDNA

Purified *P. yoelii* mRNA was obtained from the laboratory of Prof. Ross Coppel, Department of Microbiology, Monash University, Melbourne, Australia. The Omniscript RT kit was used to produce cDNA from the *P. yoelii* mRNA, as per the manufacturer’s instructions. To each reaction was added 1 U of RNase inhibitor, 1 µM of 3'PyTCTP primer, and 2.5 µl of *P. yoelii* mRNA.

2.3.2.4 Restriction endonuclease digestion of DNA

Purified genomic or plasmid DNA was digested using concentrations of restriction endonuclease enzymes between 3 U/µg (for 16 h digestions) to 10 U/µg (for 2-4 h digestions). Digestion reactions were performed at 37°C in the presence of 0.1 µg/µl BSA and the appropriate buffer, as determined by the manufacturer. When multiple enzymes were used in a single reaction, a buffer was chosen that would give at least 50-75% activity for both enzymes, as determined by the manufacturer. At the completion of the digestion reaction, enzyme activity was stopped either by heating at 65°C for 15 min, or by separation of the reaction either by agarose gel electrophoresis or Qiaquick column purification.

2.3.2.5 Agarose gel electrophoresis

Gels containing 0.8 - 2% w/v agarose were used to separate DNA based on size. Gels were prepared using DNA grade agarose solubilised in TAE by microwave heating. DNA samples were mixed with a final concentration of 1X DNA loading dye prior to loading on the gel. One µg total of DNA marker was also loaded (λ/HindIII, λ/PstI, or λ/Ndel). Electrophoresis was performed in TAE buffer at voltages ranging from 50 to 110 V. Gels were stained in an ethidium bromide (EtBr) bath for 5 - 10 minutes and destained under running tap water for 30 - 60 minutes. DNA was visualised on a transilluminator using Quantity One software.
2.3.2.6 Quantification of DNA samples

2.3.2.6.1 Spectrophotometric quantification of DNA

DNA absorbance was measured using quartz cuvettes. For quantification, an optical density (OD) reading of 1 at 260 nm was taken to equal a concentration of 50 µg/ml DNA, while a 260/280 nm ratio of between 1.8 and 2 was indicative of acceptable DNA purity. The standard procedure to measure DNA absorbance was to dilute DNA 1:1000 using nuclease-free water, then convert the OD reading at 260 nm to a µg/ml concentration using the following equation: DNA concentration (µg/ml) = OD_{260} x 50 x 1000. Water was used as a blank.

2.3.2.6.2 Quantification of DNA by gel electrophoresis

DNA samples were separated by agarose gel electrophoresis alongside a DNA marker (λ/PstI) in which the quantity of DNA in each band was known. The fluorescence between the band of interest and the nearest sized (in bp) marker band was compared.

2.3.2.7 Cloning methods

2.3.2.7.1 TA cloning into pCR 2.1 vectors

Fresh products (<24 hours old) produced by Taq PCR were ligated into pCR 2.1 vectors following the manufacturer’s specifications. Briefly, the ligation reaction was set up in the supplied buffer using 1-2 µl of PCR product, 50 ng of pCR 2.1 vector and 4 U of T4 ligase. The reaction was incubated at 14°C for 16 hours and was transformed into OneShot™ competent cells by the addition of 1 µl of ligation reaction to one vial of cells. The cell and DNA mixture was incubated on ice for 30 min then at 42°C for 30 s. The cells were immediately placed on ice for 1-2 min, then 250 µl of SOC medium was added and the cells were incubated at 37°C for 1 hour, shaking at 225 rpm. Aliquots of 100 µl were plated onto LB+Amp+X-gal agar and incubated at 37°C for 16 h. Plates were then incubated at 4°C to allow full colour development. Plasmid DNA from white E. coli colonies was purified by minipreparation.

2.3.2.7.2 Standard ligation method

The following formula was used to calculate the relative amounts of insert and vector DNA for a ligation reaction:

\[
\text{Insert (ng)} = \frac{\text{Vector (ng)} \times \text{Insert size (kb)}}{\text{Vector size (kb)}} \times \text{ratio insert:vector}
\]
Where 100 ng of vector was standard and the insert:vector ratio was 3:1. The ligation reaction was set up using the calculated volumes of insert and vector DNA in T4 ligase buffer with 1U of T4 ligase per 10 µl reaction. The reactions were incubated overnight. The standard incubation temperature was 14°C.

2.3.2.7.3 Dextran purification of ligation reactions

Completed ligation reactions were made up to 20 µl in water. The following reagents were added: 1 µl of 10 mg/ml dextran sulphate, 2 µl of 3 M NaOAc (pH 5.2) and 50 µl of cold EtOH. The tube contents were mixed by vortexing and incubated on ice for 10 min. The DNA pellets were recovered by centrifugation at 16,000 x g for 15 min, the pellet was washed with 200 µl of 70% EtOH and recovered by centrifugation at 16,000 x g for 5 min. The pellet was dried with the tube lid open for ~15 min and resuspended in 10 µl of molecular grade water.

2.3.2.8 Ethanol/sodium acetate precipitation of DNA from solutions

A 1/10 volume of 3 M NaOAc (pH 5.2) was added to the DNA solution, together with 2.5 volumes of 100% EtOH. Tubes were inverted several times to mix. Precipitation reactions were incubated at -80°C for at least 20 minutes. DNA was pelleted at 16,000 x g for 20 minutes at 4°C. The DNA pellet was washed with 1 ml of 70% EtOH and recovered by centrifugation at 16,000 x g for 5 minutes. The DNA pellet was dried with the tube lid open for ~15 min. DNA was resuspended in 10 mM Tris.HCl pH 8.0, TE buffer, or molecular-grade water.
2.4 Bacteriological Materials and Methods

2.4.1 Bacteriological Materials

2.4.1.1 Luria-Bertani (LB) medium
1% w/v tryptone
0.5% w/v yeast extract
1% w/v NaCl
Made up in distilled water, sterilised

2.4.1.2 LB + Amp medium
Sterile LB medium
100 µg/ml ampicillin (filter-sterilised, 0.2 µm)

2.4.1.3 LB agar
LB medium
1.2% w/v bacteriological agar
Made up in distilled water, sterilised

2.4.1.4 LB + Amp agar
LB agar, sterilised, cooled to 55°C
100 µg/ml ampicillin (filter-sterilised, 0.2 µm)

2.4.1.5 LB + Amp + X-gal agar
LB + Amp agar
When agar set, 40 µl of a 40 mg/ml X-gal solution in DMSO added and spread evenly over the plate.

2.4.1.6 Super Optimal Catabolite (SOC) medium
2% w/v tryptone
0.5% w/v yeast extract
10 mM NaCl
10 mM KCl
Sterilised, then the following filter-sterilised materials added:
10 mM MgCl₂
10 mM MgSO₄
20 mM glucose
2.4.2 Bacterial strains used

Top10F', DH5α, and BL21 strains of E. coli were used as hosts for pCR2.1, pYEULCBX, b3D and pRSETA plasmids.

2.4.3 Bacteriological methods

2.4.3.1 Spectrophotometric quantification of bacterial cultures

Plastic disposable cuvettes were used. Sterile LB media was used as a blank. The OD was measured at 600 nm.

2.4.3.2 Electro-transformation of E. coli

2.4.3.2.1 Preparation of electro-competent cells

The DH5α strain of E. coli was resuscitated from glycerol stocks by streak dilution onto a LB agar plate and incubated for 16 h at 37°C. A single colony was subcultured into 5 ml of LB and incubated overnight (o/n) at 37°C, shaking at 160 rpm. A 1/100 volume of o/n culture was added to fresh LB (200-500 ml) and incubated at 37°C, shaking, until the bacterial culture reached an OD₆₀₀ reading of ~0.4. The culture was incubated on ice for 1 h, then divided into sterile 50 ml tubes and centrifuged at 4,500 x g for 15 min at 4°C. The cell pellet was resuspended in 25 ml cold sterile water by gentle mixing. The cells were recovered by centrifugation and were washed with 12.5 ml of cold sterile water. The cell pellets were resuspended in 1 ml of cold sterile 10% v/v glycerol by gentle repeated pipetting. The cell suspensions were transferred to sterile 1.5 ml tubes and centrifuged at 16,000 x g for 5 min at 4°C. The cell pellets were resuspended in 200 µl of cold sterile 10% v/v glycerol and stored at -80°C in 40 µl aliquots in sterile 1.5 ml tubes.

2.4.3.2.2 Transformation of electro-competent cells

Electro-competent DH5α E. coli cells were transformed by electroporation. Cell aliquots were thawed on ice before the addition of 0.5 – 1 µl (~5 to 50 ng) of dextran-purified DNA. Cell suspensions were gently mixed and incubated on ice for 1 - 2 min. A single cell suspension was transferred to a chilled 0.2 cm electroporation cuvette and placed in the chilled safety chamber slide of the Gene Pulser before electroporation at 25 µF, 2.5 kV, 200 Ohms. One millilitre of SOC medium was added to the electroporation cuvette and resuspended cells were transferred to a sterile 1.5 ml tube. The cells were incubated at 37°C for 1 h, shaking at 200 rpm. 100 µl cell aliquots were spread-plated
onto LB+Amp agar. The remaining suspension was centrifuged at 4,000 x g for 2 min, the supernatant was discarded, and 100 µl of the cell pellet was spread-plated onto a second LB+Amp agar plate. Plates were incubated at 37°C for 16 h. Plasmid DNA from the resulting colonies was purified by minipreparation.

2.4.3.3 Preparation of glycerol stocks of *E. coli*

Single colonies of *E. coli* were grown for 16 h in 5 ml LB+Amp broth. Equal volumes of culture and sterile glycerol were combined in sterile cryovials and stored at -70°C.
2.5 Yeast Materials and Methods

2.5.1 Yeast Materials

2.5.1.1 Yeast extract peptone dextrose (YEPD) medium

2% w/v yeast extract
1% w/v peptone
2% w/v D-glucose
Made up in distilled water, sterilised

2.5.1.2 Yeast nutrient broth (YNB) + glucose

0.67% w/v yeast nitrogen base without amino acids
2% w/v D-glucose
Made up in distilled water, sterilised

2.5.1.3 Complete-Ura agar

YNB + Glucose
2% w/v agar
0.2% w/v adenine sulfate, L-Tyrosine
1% w/v L-Tryptophan, L-Histidine HCl, L-Arginine HCl, L-Methionine, L-Leucine, L-Isoleucine, L-Lysine HCl, L-Phenylalanine, L-Glutamic acid
0.4% w/v L-Aspartic acid
3% w/v L-Valine
4% w/v L-Threonine
8% w/v L-Serine

2.5.2 Yeast methods

2.5.2.1 Transformation of \textit{S. cerevisiae} with pYEULCBX vectors

The w303.1a strain of \textit{S. cerevisiae} was used for recombinant protein expression. This yeast strain requires exogenous adenine, leucine, uracil, tryptophan and histidine for growth, and transformation with pYEULCBX eliminates the growth requirement for uracil and leucine. The transformation protocol was performed essentially as described previously (Gietz \textit{et al.}, 1995). Briefly, a single colony of \textit{S. cerevisiae} was used to inoculate 5 ml of YEPD and grown overnight at 30°C, shaking at 200 rpm. The cell density of the overnight culture was measured using a haemocytometer at 400X magnification. 50 ml of YEPD was inoculated to a density of 5 x 10^6 cells/ml. The 50 ml culture was incubated at 30°C, shaking at 200 rpm, until the cell density reached 2 x 10^7 cells/ml (typically 3-5 h). The culture was centrifuged at 2,500 x g for 5 min, and the cell
pellet was washed in 25 ml sterile distilled water. The cell pellet was resuspended in 1 ml 0.1 M Lithium Acetate (LiAc) and transferred to a sterile 1.5 ml tube. Cells were pelleted by centrifugation at 16,000 x g for 5 s. The cell pellet was resuspended to a final volume of 0.5 ml in 0.1 M LiAc, corresponding to a cell density of $2 \times 10^9$ cells/ml.

A sample of 10 mg/ml single-stranded (ss) carrier DNA was boiled for 5 min and quickly chilled in an ice-water bath. Meanwhile, the cell suspension was vortexed for 10 s then dispensed into 50 µl aliquots in sterile 1.5 ml tubes. The cells were pelleted at 16,000 x g for 5 s. To the pellet was added: 240 µl of 50% w/v PEG 3350, 36 µl of 1 M LiAc, 5 µl of 10 mg/ml ss carrier DNA and 1 µg of pYEULCBX plasmid DNA. The volume was made up to 350 µl in water, and tubes were vortexed for 1 min. Tubes were incubated for 30 min at 30°C, then heat-shocked at 42°C for 22 min. Tubes were spun at 16,000 x g for 15 s and the cell pellet was resuspended in 200 µl of water. The entire transformation mix was plated on Complete-Ura agar and incubated at 30°C for 2-3 days.

2.5.2.2 Preparation of glycerol stocks of \textit{S. cerevisiae}

Overnight cultures of single colonies of \textit{S. cerevisiae} were grown overnight in YEPD. Glycerol stocks were made up of 85% v/v culture, 15% v/v sterile glycerol in sterile cryovials and stored at -70°C.
2.6 Protein Purification Materials and Methods

2.6.1 Protein Purification Materials

2.6.1.1 PBS
1 PBS tablet per 100 ml water

2.6.1.2 E. coli lysis buffer
5 mM imidazole in PBS
4 mg/ml lysozyme

2.6.1.3 Phosphate buffer
80 mM Na$_2$HPO$_4$
20 mM KH$_2$PO$_4$
0.3 M NaCl
Made up in distilled water, pH adjusted to 7.3

2.6.1.4 5 mM imidazole wash buffer
5 mM imidazole in phosphate buffer or PBS

2.6.1.5 0.2 M/0.25 M imidazole elution buffer
0.2 M/0.25 imidazole in phosphate buffer or PBS

2.6.1.6 Mono-Q dialysis buffer 1
50 mM Tris.HCl pH 8.8
1 mM DTT
Made up in distilled water

2.6.1.7 Mono-Q dialysis buffer 2
1 mM CaCl$_2$ in Mono-Q dialysis buffer 1

2.6.1.8 Mono-Q buffer A
50 mM Tris.HCl pH 8.8
1 mM DTT
1 mM CaCl$_2$
Made up in distilled water, filtered (0.2 μM cut-off) before use
2.6.1.9 Mono-Q buffer B
0.5 M NaCl added to Mono-Q buffer A
Filtered (0.2 μM cut-off) before use
Made up in distilled water

2.6.1.10 Bradford Assay reagent
0.01% w/v Coomassie Blue G-250
5% v/v EtOH
10% w/v 85% Phosphoric acid
Coomassie powder was added to ethanol and mixed on magnetic stirrer, phosphoric acid was added once dissolved. Made up to final volume in water, filtered (0.45 μM cutoff) and stored at 4°C.

2.6.2 Yeast protein expression and purification methods
2.6.2.1 Confirming expression of TCTP in S. cerevisiae by Western blot
Single colonies of yeast from pYEULCBX.PfTCTP or pYEULCBX.PyTCTP transformations were picked from Complete-Ura plates and used to inoculate 10 ml YEPD in a sterile 50 ml tube. Cultures were grown at 30°C for 8 h, shaking at 200 rpm. A 1/10 volume of the pre-culture was used to inoculate 10 ml of YNB+Glucose supplemented with 0.002% w/v histidine and tryptophan and 0.004% w/v adenine for yeast growth, and with 0.5 mM CuSO₄ for induction of protein expression. Cultures were incubated at 30°C for 16 h, before centrifugation at 2,500 x g for 5 min at 4°C. A 30 μl sample of the culture supernatant was retained for Western blot, the cell pellet was washed once in 10 ml PBS. Samples of the washed cell pellet (~10 μl) were retained for Western blot analysis of TCTP expression.

2.6.2.2 TCTP expression in S. cerevisiae
Single colonies of PfTCTP- or PyTCTP-transformed yeast from Complete-Ura plates were used to inoculate 10 ml volumes of YEPD and grown at 30°C for 16 h, shaking at 200 rpm. A 1/100 volume of the pre-cultures was used to inoculate 0.5-1L of YNB+Glucose supplemented with 0.002% w/v histidine and tryptophan and 0.004% w/v adenine, and TCTP expression was induced with 0.5 mM CuSO₄ for 40 hours.

2.6.2.3 Protease-minimising protein expression method in S. cerevisiae
This protein expression method was adapted from the yeast expression method described in http://www.clontech.com/images/pt/dis_manuals/PT3081-1.pdf. Single
colonies of pYEULCBX.PyTCTP transformed yeast were grown in 10 ml of YEPD for 16 h at 30°C, shaking at 200 rpm. A 1/100 volume of the overnight cultures was used to inoculate 0.5 L of YNB+Glucose supplemented with 0.002% w/v histidine and tryptophan and 0.004% w/v adenine. The cultures were incubated at 30°C for 18 h, shaking.

Cultures was transferred to sterile centrifuge bottles and centrifuged at 600 x g for 5 min at RT. The supernatant was discarded and the cell pellets were resuspended in fresh supplemented YNB+Glucose medium as before to the original culture volume. The culture was incubated at 30°C, shaking, until the OD$_{600nm}$ was between 0.8 and 1.2. Protein expression was induced by the addition of 0.5 mM CuSO$_4$ and the culture was incubated at 30°C for 1 h, shaking.

2.6.2.4 Lysis of yeast cultures

2.6.2.4.1 Lysis of yeast by homogenisation

Yeast cultures were centrifuged at 2,500 x g for 5 min at 4°C. The supernatant was discarded and the cell pellet washed twice in PBS. The cell pellet was resuspended in one volume of PBS and the following protease inhibitors were added before cell lysis: 1 mM Pefabloc, 5 mM Benzamidine, 1 µg/ml E-64, 1 µg/ml Pepstatin, and 1 µg/ml leupeptin.

Yeast cells were lysed in 13 ml plastic homogenisation tubes using a Braun cell homogeniser. The tubes were filled with ~4 ml cell pellet + PBS and ~2 ml 0.45 mm glass beads. The cells were homogenised using 7 cycles of 25 s, using 5 s bursts of liquid CO$_2$ between and during the homogenisation cycles. The homogenate was decanted into a 50 ml tube and centrifuged at 2,500 x g for 5 min at 4°C. The supernatant was decanted into a centrifuge bottle and centrifuged at 21,000 x g for 25 min at 4°C. Protease inhibitors were added to the supernatant as before.
2.6.2.4 Yeast lysis by vortexing with glass beads

This method was used with yeast cultures grown via the protease-minimising expression method (2.6.2.3). Cells were pelleted by centrifugation at 3,000 x g for 5 min at 4°C. The pellet was washed twice with PBS. Cells were lysed in a mixture made up of equal volumes cell pellet, PBS and 0.45 mm glass beads in a 50 ml tube. Protease inhibitors were added before cell lysis: 1 mM Pefabloc, 5 mM Benzamidine, 1 µg/ml E-64, 1 µg/ml Pepstatin, and 1 µg/ml leupeptin. Cells were lysed by repeated cycles of vortexing and cooling; 20 s of vortexing followed by 20 s incubation on ice. This was repeated for 20 min in total. The percentage of cell lysis was analysed by analysing the percentage of intact yeast cells in 1 µl samples before and after lysis, using a haemocytometer under 400X magnification. The vortexing cycles were continued until at least 50% cell lysis was achieved (up to 40 min total). The lysis mixture was centrifuged at 3,000 x g for 10 min at 4°C.

2.6.2.5 Purification of His-tagged proteins expressed in *S. cerevisiae* using TALON™ resin

2.6.2.5.1 Talon resin preparation for use and storage

Before use, TALON™ metal affinity resin was washed to remove storage EtOH. A portion of resin slurry (usually ~ 2.5 – 5 ml) was decanted into a 50 ml tube and centrifuged at 2,500 x g for 10 min at 4°C. The supernatant was decanted and the resin was washed three times with a full tube of PBS.

After use in protein purifications, the Talon resin was centrifuged at 2,500 x g for 10 min at 4°C, the supernatant was decanted, and the resin was washed twice with 50 ml of 30% EtOH. The resin was resuspended in an equal volume of 30% EtOH and stored at 4°C.

2.6.2.5.2 Incubation of proteins with Talon resin

Yeast lysates were added to the prepared Talon slurry and incubated at 4°C for 16 h on a carousel shaker at low speed.

2.6.2.5.3 Elution of proteins from Talon resin

A Biologic Low Pressure system was used to measure absorbance of the eluate from the TALON™ resin. The system was washed with deionised water and equilibrated with phosphate buffer. The absorbance reader was set to zero and the incubated talon slurry was added to the column. The column flowthrough was collected, then the resin...
was washed with phosphate buffer until the absorbance reading had stabilised at around zero. Approximately 20 column volumes (CV) of 5 mM imidazole wash buffer were added to elute weakly-binding proteins from the resin, the eluate was retained. Approximately 10 CV of 0.2 M imidazole elution buffer was added to elute polyhistidine-tagged proteins. Small samples (~30 µl) of the collected eluates were retained for SDS-PAGE analysis; the remaining eluates were stored at 4°C for up to 24 h or at -20°C for longer periods. The resin was washed in phosphate buffer until the UV absorbance reading had dropped to around zero, then was washed in at least 20 CV of deionised water. The resin was decanted into a 50 ml tube and prepared for storage as described. The Biologic system was washed with ~20 ml water followed by ~20 ml of 30% EtOH.

2.6.2.6 Further purification of yeast-derived proteins using an anion-exchange column

2.6.2.6.1 Dialysis of protein samples prior to anion-exchange chromatography

TCTP samples from the 0.2 M imidazole eluate samples were combined and dialysed in at least 100 volumes of Mono-Q dialysis buffer 1 for between 4 - 16 h at 4°C, stirring. Samples were then dialysed in Mono-Q dialysis buffer 2 as before. Dialysis was performed using Spectra/Por tubing with a 3,500 MW cut-off.

2.6.2.6.2 Preparation of Mono-Q anion exchange column

Lines A and B were cleaned with RO water for 5 min. 100% Mono-Q Buffer B was run through the Mono-Q column for 3 min to elute any residual proteins, then 100% Mono-Q Buffer A was run through the column for 5 min to remove the salt.

2.6.2.6.3 Mono-Q column chromatography

The protein samples were filtered with a 0.22 µm syringe filter. The Biologic HP system was set to run at 1 ml/min. The system was set to load and 5 ml of protein sample was injected into a 5 ml loop. The samples were loaded onto a separate column attached to an Auto-Injection Valve. The system was set to inject. When multiple 5 ml samples were to be loaded, the sample was injected for 5 min then the system was set to load and another 5 ml of sample was injected into the loop. The system was set to run a 0-0.5 M NaCl gradient over 60 minutes. When the UV reading was <0.025, 2 ml fractions were collected. Fractions corresponding to UV peaks were subjected to SDS-PAGE.
2.6.2.7 N-terminal sequencing of PyTCTP

PyTCTP eluates from Mono-Q chromatography were subjected to N-terminal sequencing at CSIRO Health Sciences and Nutrition, Parkville, Australia (performed by Phil Strike).

2.6.2.8 Protein lyophilisation and reconstitution

Prior to lyophilization, TCTP samples previously dialysed in two changes of PBS were dialysed in two changes of at least 100 volumes of 50 mM ammonium bicarbonate pH 8, as previously described (2.6.2.6.1). Lyophilisation of proteins was performed by Dr Ross Fernley, CSIRO Health Science and Nutrition, Parkville, Australia. Lyophilised samples were reconstituted to a concentration of 0.5 mg/ml in PBS by vortexing for 10 s.

2.6.3 E. coli protein expression and purification methods

2.6.3.1 PbTCTP and PyTCTP expression in E. coli

A single colony of E. coli BL21 transformed with either pRSETA.PbTCTP or pRSETA.PyTCTP was used to inoculate 10 ml of LB+Amp media, and grown for 16 h at 37°C, shaking at 200 rpm. Larger volumes of LB+Amp (typically 0.5 L) were inoculated with a 1/100 volume of the 16 h pre-culture, and incubated at 37°C, shaking at 200 rpm, until the OD_{600nm} was approximately 0.5. Expression of TCTP was then induced by the addition of 1 mM IPTG, and cultures were grown a further 3 hours.

2.6.3.2 Lysis of E. coli

Cells were pelleted by centrifugation at 5,500 x g for 10 minutes. E. coli lysis buffer at a 1/25 volume of the original culture media volume was used to resuspend the pellet, cells were then incubated at 4°C for 20 min, shaking at 70 rpm. Four cycles of freeze/thaw were performed: pellets were frozen at -70°C for >20 min, followed by thawing in a 37°C water bath for >5 min. After the second freeze/thaw cycle, DNase was added to a final concentration of 10 U per 100 ml original culture volume. The pellets were incubated at 37°C for 15 min, shaking at 70 rpm. Two further freeze/thaw cycles were then performed. After completion of the freeze/thaw cycles, cell debris was removed by centrifugation at 5,500 x g for 20 minutes. Supernatants were passed through 0.2 µm syringe filters.
2.6.3.3 Py and PbTCTP purification using Profinity™ metal affinity chromatography

2.6.3.3.1 Preparation and storage of Profinity resin

Small volumes of Profinity™ resin slurry, typically 5 ml, were washed with 10 CV of water followed by 20 CV of 5 mM imidazole wash buffer prior to use, to remove storage EtOH and to equilibrate the column. After protein purification, Profinity™ resin was washed with 10 CV of 5 mM imidazole wash buffer, 10 CV of water, and 10 CV 30% EtOH, and stored at 4°C in two CV of 30% EtOH.

2.6.3.3.2 Profinity™ chromatography

Filtered PyTCTP and PbTCTP lysate supernatants (2.6.3.2) were added to prepared Profinity™ resin and incubated at rt for 15 min, shaking at 70 rpm. The resin was washed with 20 CV 5 mM imidazole buffer and a 1.5 ml eluate sample was retained for analysis. His-tagged proteins were eluted with 10 CV of 0.25 M imidazole elution buffer and 1.5 ml fractions were collected, 20 µl samples of which were analysed for the presence of TCTP by SDS-PAGE. The remaining volume of each fraction was stored at -70°C.

2.6.3.4 Endotoxin removal

One millilitre of resin was used in a 1 ml column per protein preparation. The resin was washed with 5 CV of 1% w/v sodium deoxycholate, then with 15 CV of PBS. The protein samples were added, and the column flow was stopped when the samples had entered the resin. The column was incubated at RT for 1 h, and the column flow was re-started. Protein samples were eluted in PBS.

2.6.4 Quantification of protein concentration

2.6.4.1 Quantification of protein concentration by spectrophotometry

Protein concentration was estimated by measuring absorbance at 280 nm, where a reading of 1 was taken to be equal to 1 mg/ml protein. The blank was the buffer used in the protein sample of interest.

2.6.4.2 Analysis of protein concentration by Bradford assay

All samples were diluted using 0.15 M NaCl. BSA was diluted to 1 mg/ml and the following protein concentration standards were prepared: 3, 6, 9, 12, 15, 18, and 21 µg/ml. Each standard was made up to a final volume of 200 µl in 1.5 ml tubes. Around 10 µg (as determined by spectrophotometry) of the protein samples of interest were
diluted to 200 µl in 0.15 M NaCl. 1 ml of Bradford Assay reagent was added to each tube. Tubes were vortexed and allowed to stand for 1 minute, and 200 µl duplicates of the samples were added to wells of a 96-well plate. Abs$_{595}$ was measured using an ELISA plate reader, and protein concentrations of samples of interest were calculated by plotting against the standard curve.

2.6.5 Preparation of proteins for vaccine use

2.6.5.1 Buffer exchange to PBS by dialysis

Purified TCTP samples were dialysed using Spectra/Por dialysis tubing (3,500 MW cut-off), in two changes of >100 volumes of PBS for between 4 and 16 h at 4°C, stirring.

2.6.5.2 Buffer exchange to PBS using concentration columns

Centricon columns were washed prior to use by adding 3.5 ml of 0.1 M NaOH and centrifuging at 3,000 x g for 15 min. The columns were washed 3 times with water. Purified TCTP samples were added to prepared concentration columns and centrifuged at 3,000 x g until the volume was reduced to 0.1X the initial volume (400 µl in a 4 ml column). The protein samples were resuspended to the original volume in PBS, and centrifuged as before. The cycles of PBS resuspension and centrifugation were repeated until the original buffer concentration had been diluted approximately 100X.

2.6.5.3 Storage of proteins prior to vaccine trial

Proteins in PBS were generally stored at -70°C prior to use in vaccine trials, some yeast-derived proteins were stored in 50% glycerol at -70°C. Lyophilised proteins were stored at -20°C.
2.6.6 SDS-PAGE and Western blot materials

2.6.6.1 15-4% acrylamide gel for SDS-PAGE, for 2 mini-gels

2.6.6.1.1 15% resolving gel
Reagents (final concentrations) were added in the following order:
water to 5 ml
0.375 M Tris.HCl pH 8.8
0.1% w/v SDS
12% v/v acrylamide
0.1% w/v APS
0.1% v/v TEMED

2.6.6.1.2 4% stacking gel
Reagents (final concentrations) were added in the following order:
water to 2.5 ml
0.125 M Tris.HCl pH 6.8
0.1% w/v SDS
4% v/v acrylamide
0.1% w/v APS
0.1% v/v TEMED

2.6.6.2 5X reducing sample buffer
0.2 M Tris.HCl pH 6.8
25% v/v glycerol
17.5% w/v β-mercaptoethanol
0.125% w/v bromophenol blue
5% w/v SDS
Made up in distilled water, stored at 4°C

2.6.6.3 10X SDS-PAGE buffer
14.4% w/v glycine
3% w/v Tris
2% w/v SDS
Diluted 1:10 in distilled water before use
Chapter 2: Materials and Methods

2.6.6.4 Coomassie staining solution
0.05% w/v Coomassie blue R-250
40% v/v methanol
10% v/v glacial acetic acid

2.6.6.5 Destaining solution
10% v/v acetic acid
10% v/v EtOH

2.6.6.6 10X Transfer buffer
3.025% w/v Tris
14.49% w/v glycine
Diluted 1:10 before use with water + 20% v/v methanol

2.6.6.7 Tris-buffered saline (TBS)
10 mM Tris
0.5 M NaCl
pH adjusted to 7.4

2.6.6.8 Blocking solution
5% w/v skim milk powder in TBS

2.6.6.9 Diluent solution
1% w/v skim milk powder in TBS

2.6.6.10 10X Citrate-EDTA buffer
10 mM tri-sodium citrate
10 mM EDTA
pH adjusted to 5.5, diluted 1:10 with distilled water before use

2.6.6.11 Western substrate 1
50 µl of 50 mg/ml TMB in DMSO
2 µl H₂O₂ (30%)
1% w/v dextran sulphate
Made up in 10 ml 1X Citrate-EDTA buffer
2.6.6.12 Western substrate 2
10% v/v of 3 mg/ml Chloro-1-naphthol in methanol
6% v/v of 30% H$_2$O$_2$
Made up in TBS

2.6.6.13 Detection buffer
0.1 M Tris.HCl pH 9.5
0.1 M NaCl

2.6.6.14 Western substrate 3
600 μl NBT/BCIP in 10 ml Detection buffer

2.6.7 SDS-PAGE method
2.6.7.1 Preparing protein samples
5 μl of 5X sample buffer was added to 20 μl of protein sample in PBS and samples were heated at 95°C for 5 min.

2.6.7.2 SDS-PAGE
Gels were prepared just before use. The 15% resolving gel solution was prepared, mixed by inverting the tube several times and ~4 ml of solution was pipetted into a prepared glass plate mini-gel system. The gel solution was overlaid with water using a pipette, and the gel was allowed to set for ~20 min. The water overlay was removed and the 4% stacking gel solution was then added with the well comb in place. The gel was allowed to set for ~20 min. Alternatively, 4-20% gradient pre-cast gels were used.

Gels were placed in the Protean II mini-gel system, with a buffer dam if only one gel was used. One litre of SDS-PAGE buffer was added to the gel tank and wells were washed with 30 μl of SDS running buffer twice before prepared protein samples were added. Eight microlitres of SeeBluePlus2 Pre-Stained marker, BenchMark marker or Mark12 Unstained Marker was added to one well. The samples underwent electrophoresis at 60 V for 30 min followed by 180 V for 45 min. If pre-cast gels were used, samples underwent electrophoresis at 175 V, 100 mA (150 mA if two gels were used) for 1 hour. Gels were washed briefly in distilled water before staining by shaking at 50 rpm in Coomassie stain for 1 h or in Gradipure stain for 2 h. Gels were destained by shaking incubation for 4-16 h in destaining solution.
2.6.8 Western Blot

At the completion of SDS-PAGE, gels were washed 2X in transfer buffer for 15 min, shaking at 50 rpm. Meanwhile, Whatman paper, Scotchbrite sponge and nitrocellulose membrane were soaked separately in transfer buffer for 30 min.

The blot was assembled as follows: The cassette was placed in a container filled with transfer buffer with the grey panel (anode) at the bottom. One piece of scotchbrite was placed on top of the grey panel, followed by Whatman paper, then the gel, then the nitrocellulose membrane. Air bubbles were removed by rolling a thin glass tube over the membrane, then Whatman paper was placed on top, followed by a piece of scotchbrite. The white panel of the Western cassette was placed on top and the sandwich was clipped together. The Western cassette was placed in a Protean II tank. A plastic mould filled with ice was placed in the tank. Proteins were transferred onto the nitrocellulose membrane by electrophoresis either at 200 V for 50 min using a flea and a magnetic stirrer; or at 70 V for 1 hour.

At the completion of the Western transfer, the membrane was removed from the sandwich and washed in TBS for 10 min, shaking at 50 rpm. The membrane was incubated in blocking solution for 1 h, shaking, then incubated with the primary antibody in diluent solution for between 1 and 20 h, shaking. The primary antibody was retained after incubation and stored at -20°C, for re-use up to 5 times. The membrane was washed twice in TBS for 10 min, shaking, and then incubated in secondary antibody in diluent solution for 1 h, shaking. The membrane was washed twice in TBS for 10 min, shaking, and then was incubated with 10-30 ml of substrate solution in the dark for 5-10 minutes. The blot was recorded either by scanning the image or by photography.

2.6.9 β-hexosaminidase assays

For further detail of the methods used and results obtained in the RBL-2H3 degranulation assays, please refer to the Honours thesis of Aya Taki, Department of Biotechnology and Environmental Biology, RMIT University, Melbourne, Australia (Taki, 2007).

β-hexosaminidase release assays using RBL-2H3 cells generally have an incubation with IgE specific to a particular antigen as the initial step. As a positive control, the specific antigen is added to stimulate IgE crosslinking on the surface of the
cells, prompting degranulation. In the assays performed using TCTP, filter-sterilised (0.2 \( \mu m \)) IgE specific to trinitrophenol (TNP) was used at a 100-fold dilution. RBL-2H3 cells were grown to confluence in 96-well plates and washed twice with 200 \( \mu l \) of freshly-prepared releasing buffer (1X HANKS, 0.14% w/v NaHCO\(_3\), 10 mM HEPES pH 7.2, 5.5 mM glucose, 0.05% w/v BSA, 0.73 mM MgSO\(_4\), 1.8 mM CaCl\(_2\), pH adjusted to 7.3). Cells were incubated with \( \alpha \)-TNP IgE for 16 h, washed twice in releasing buffer, and then resuspended in 150 \( \mu l \) releasing buffer.

Cells were stimulated with either ionomycin, PfTCTP, PbTCTP, PyTCTP or SmpB (negative control) at protein concentrations of 10, 5, 2.5, 1.25, 0.6 and 0.3 \( \mu g/ml \). As a positive control, concentrations of 30, 10 3, 1, 0.3 and 0.1 ng/ml of TNP were used to stimulate IgE-crosslinking on the RBL-2H3 cells. Each potential stimulant was used at a final volume of 50 \( \mu l \) per well, together with 50 \( \mu l \) of releasing buffer. In a separate well, 50 \( \mu l \) of lysis solution (0.1% Triton-X 100) was added to measure maximum degranulation. The cell mixtures were incubated for 30 min at 37\(^\circ\)C, and then 25 \( \mu l \) of supernatant from each well was transferred to a new 96-well plate. To assess the amount of \( \beta \)-hexosaminidase present, the supernatants were mixed with 25 \( \mu l \) of p-nitrophenyl N-acetyl-D-glucosaminide (pNAG) substrate. Plates were wrapped in foil and incubated at 37\(^\circ\)C for 90-120 min. The reaction was stopped with 50 \( \mu l \) of 0.4 M glycine and absorbance at 405 nm was measured.

Degranulation was measured as a percentage of total release. Cells incubated with releasing buffer alone served as a measure of spontaneous release. Three separate experiments were used for each data point. Both ionomycin and TNP were used as positive controls.
2.7 Malarial infection Materials and Methods

2.7.1 Materials for malarial infection

2.7.1.1 Citrate phosphate dextrose (CPD)
3% w/v tri-sodium citrate
0.015% w/v NaH$_2$PO$_4$.H$_2$O
0.2% w/v D-glucose
Filter-sterilised (0.2 µm) before use

2.7.1.2 Mouse-tonicity phosphate-buffered saline (MT-PBS)
20 mM Na$_2$HPO$_4$.H$_2$O
4 mM NaH$_2$PO$_4$.H$_2$O
150 mM NaCl
Filter-sterilised (0.2 µm) before use

2.7.1.3 Malaria freezing solution (MFS)
0.17 M sorbitol
0.11 M NaCl
28% v/v glycerol
Filter-sterilised (0.2 µm) before use, stored at 4°C.

2.7.1.4 Giemsa stain

2.7.1.4.1 Giemsa stock
50% v/v glycerol
50% v/v methanol
0.736% w/v Giemsa powder
Giemsa powder added to glycerol and incubated at 50°C for 30 min, stirring. Cooled when powder completely dissolved, methanol added. Filtered (0.45 µm)

2.7.1.4.2 Sorenson’s modified phosphate buffer
66.7 mM NaH$_2$PO$_4$
66.7 mM Na$_2$HPO$_4$
pH adjusted to 6.4
2.7.1.4.3 Giemsa stain

10% v/v methanol
10% v/v Giemsa stock
8% v/v Sorenson’s modified phosphate buffer
Made up just before use and filtered (0.45 µm)

2.7.2 Malaria infection methods

2.7.2.1 Marking of mice for identification

Mice were marked with between 0 and 4 stripes on the tail using black permanent (non-toxic) marker, allowing for differentiation of 5 mice. The markings were re-applied every 3-4 days for the duration of the experiment. When larger numbers of mice were to be identified, i.e. when mice were challenged with malaria parasites in the vaccine trials, box numbers were assigned randomly by one experimenter and the number was written on the stomach of each mouse using permanent marker. The malaria parasites were then injected into each mouse by a second experimenter, who was not aware of which box numbers corresponded with controls or immunised mice.

2.7.2.2 Preparation of malaria stabilates

Blood was taken from mice using retro-orbital puncture using capillary tubes into 2 volumes of CPD in 1.5 ml tubes. Blood was centrifuged at 100 x g for 10 min, and the erythrocyte pellet was washed once in 1 ml MT-PBS. The supernatant was discarded and 10 pellet volumes of MFS were added. The malaria stabilates were dispensed in 250-500 µl aliquots in sterile cryovials and snap frozen in dry ice or liquid nitrogen. Stabilates were stored at -70°C or in liquid nitrogen.

2.7.2.3 Injection of mice with malaria stabilates

Only a fraction of the malaria parasites in a stablate are viable, and so accurate numbers of parasitised erythrocytes cannot be calculated. For this reason, passaging mice were required when exact numbers of parasites were needed for infection. Two mice received between 50 and 200 µl of parasite stabilates by an i.p. injection using a 25-guage needle. Parasitemia was monitored daily. Blood was taken either by retro-orbital puncture, or by cardiac puncture following CO₂ asphyxiation, when parasitemia was between 2 and 10%. Parasitised blood was used for stablate creation or to infect mice with a specific number of parasites.
2.7.2.4 Infection of mice with specific doses of malaria parasites

When infection with a specific number of parasites was required, the parasitemia of blood taken from passage mice was calculated and blood was diluted in MT-PBS to the appropriate parasite dose as follows: Mouse blood contains $5 \times 10^9$ rbc/ml ($5 \times 10^6$ rbc/$\mu$l), therefore a mouse with a 10% parasitemia has a total of $5 \times 10^5$ parasitised erythrocytes/$\mu$l. Blood of known parasitemia was taken from passage mice by retro-orbital puncture, and was diluted in two volumes of CPD. The blood was further diluted in MT-PBS to give the correct concentration of parasitised erythrocytes/$\mu$l. Mice in the vaccine trials were injected with a dose of $1 \times 10^5$ parasitised erythrocytes in a total volume of 200 µl by an i.p. injection using a 25-guage needle.

2.7.2.5 Quantification of parasites in mouse blood

Parasite numbers were quantified in the morning (prior to 10 am) daily, starting at two days post-infection (dpi). One drop of blood was taken from the end of the tail of each mouse as follows: The very end of the tail (~1-2 mm) was removed using EtOH-sterilised scissors. The tail was squeezed gently and a single drop of blood was collected on a glass microscope slide. The blood was smeared across the slide using a second glass slide held at a 45° angle. The blood was allowed to air-dry, then was fixed to the slide by incubation in 100% methanol for 5 min. Excess methanol was removed and slides were incubated in Giemsa stain for 30 - 40 min. Slides were washed in tap water, allowed to dry fully and red blood cells were visualised on a light microscope under oil-immersion at a 1,000 X magnification. To ensure impartial counting, all identifying numbers on the slides were covered with opaque labels; slides were randomised and then numbered sequentially. Parasite numbers were quantified for all slides prior to the uncovering of slide identifications. Parasite numbers were quantified by the counting of 500 to 1,000 erythrocytes for each mouse at each time point. The numbers were expressed as percentage parasitemia. At least 1,000 erythrocytes were counted before mice were declared to have 0% parasitemia.

2.7.3 Blood collection methods

2.7.3.1 Blood collection by retro-orbital puncture

Blood was collected by retro-orbital puncture using heparinised capillary tubes into 1.5 ml tubes.

2.7.3.2 Blood collection by cardiac puncture

2.7.3.2.1 Anaesthesia of mice prior to blood collection
Prior to blood collection for parasite harvest, mice were anaesthetised using 100 mg/kg ketamine and 10 mg/kg xylazine. Unconsciousness was verified by the absence of pedal and eye responses, and blood was taken by cardiac puncture. Mice were killed by cervical dislocation following blood collection.

**2.7.3.2.2 Exsanguination of mice by CO\textsubscript{2} prior to blood collection**

Mice were placed in a container pre-filled with CO\textsubscript{2}. Mice remained in the container for an additional minute after breathing had ceased. Death was confirmed by the absence of pedal and eye responses, and blood was taken by cardiac puncture.

**2.7.3.2.3 Blood collection**

Once mice were anaesthetised or killed, an incision was made in the abdomen and diaphragm to visualise the heart, and blood was collected from the left ventricle using a 19-guage needle pre-washed with CPD. The blood was collected in two volumes of CPD. After blood collection, mice were killed by cervical dislocation.
2.8 Vaccine trial Materials and Methods

2.8.1 ELISA Materials

2.8.1.1 ELISA coating buffer
A: 0.2 M Na₂CO₃
B: 0.2 M NaHCO₃
Buffer made up of 8% A and 17% B in water, pH adjusted to 9.6

2.8.1.2 PBS Tween
0.05% v/v Tween 20 in PBS

2.8.1.3 ELISA Blocking solution
5% w/v skim milk powder in PBS Tween

2.8.1.4 ELISA Diluent buffer
1% w/v skim milk powder in PBS Tween

2.8.1.5 TMB Substrate
Equal parts TMB Solutions A and B combined just before use

2.8.1.6 2N H₂SO₄
5.5 ml concentrated H₂SO₄ added to 44.5 ml water in a dropwise fashion on ice

2.8.2 Vaccine trial methods

2.8.2.1 Preparation of vaccines
Mice were immunised either with sterile PBS (negative control) or with filtered (0.2 μm) TCTP in PBS. PBS or 0.5 mg/ml TCTP were combined with an equal volume of Freunds adjuvant. The vaccines were made up as double the number of doses required, e.g. if 6 x 100 μl doses were needed, 1.2 ml of vaccine was prepared. Vaccines were prepared in a Biosafety cabinet using sterile materials. An emulsion was created using one of two methods:

(1) The PBS/protein and Freunds adjuvant were added to 1.5 ml tubes and were emulsified using a 3 ml syringe and a 19-guage needle by syringing up and down repeatedly. An emulsion was obtained when a single drop of vaccine formed a stable
ball when dropped into a beaker of tap water. The vaccines were used within 2 h of preparation.

(2) An emulsion was made by mixing the adjuvant and protein/PBS using two glass syringes connected with a stopcock, using around 300 mixing repetitions. The emulsion was tested by injecting a single drop into a beaker of tap water as before.

Mice were immunised three times, the first immunisation was emulsified in Freunds complete adjuvant; subsequent immunisations were emulsified in Freunds incomplete adjuvant.

2.8.2.2 Mouse immunisations
Mice were given an intraperitoneal injection of a total vaccine volume of 100 µl using a 25-guage needle.

2.8.2.3 Serum collection
Blood was collected from mice for antibody titre determination prior to the first immunisation and 1-2 days prior to challenge. Approximately 150 µl of blood was collected by retro-orbital puncture into a 1.5 ml tube. Blood was centrifuged at 2,500 x g for 5 min and the sera supernatants were transferred to new 1.5 ml tubes. Serum was stored at -70°C.

2.8.2.4 Monitoring and scoring of mice during the immunisation period and following malaria challenge
All animal experiments were approved by the RMIT Animal Ethics Committee (AEC). After the commencement of all experiments, mice were monitored at least twice daily.

In the vaccine trial described in Chapter 3, mice infected with *P. yoelii* YM were monitored twice daily until 3 dpi, after which time they were monitored at 4-hourly intervals between 7 am and 7 pm, then at a maximum of 7-hour intervals between 7 pm and 7 am. Mice challenged with *P. yoelii* XNL were monitored twice daily unless malarial symptoms were observed, at which time the monitoring schedule was increased to that of a lethal infection.
In the vaccine trial described in chapter 4, mice infected with *P. yoelii* YM were monitored twice daily until parasitemia was >10%, after which time they were monitored at 4-hourly intervals between 8 am and 6 pm, then observed once between 12 and 1 am. Mice infected with *P. berghei* ANKA or *P. c. chabaudi* were monitored twice daily until malarial symptoms were observed, at which time the monitoring schedule was increased to 4-hourly intervals between 8 am and 6 pm, and one observation between 12 and 1 am. Mice able to clear parasites were monitored twice daily once parasite levels were decreasing.

Mouse symptoms were scored following the table provided by the RMIT AEC. A copy of this table is provided in *Appendix 2*, with the predicted scores for a lethal malarial infection provided. Mice were euthanised when they reached a score of 8, or when definite cerebral malaria symptoms were observed.

### 2.8.2.5 Euthanasia of mice

When mice reached a symptom score of 8 they were euthanised with an i.p. injection of an overdose of the barbiturate Euthal or Lethobarb.

### 2.8.2.6 Statistics

Comparison of parasitemia between TCTP-immunised and PBS-control groups was performed using the Student’s T-test (two-tailed).

### 2.8.2.7 Analysis of antibody titres by ELISA

Ninety-six well plates were coated with 2 µg/ml antigen in coating buffer, using 100 µl per well. Coating buffer alone was added to one well; this was used as the blank for absorbance readings. Plates were incubated at 4°C overnight. One well of the first row of the plate was used for each of the following controls: no antigen (coating buffer only), no blocking, no antisera, no secondary antibody. All other ELISA steps were performed on each of the control wells.

Coating buffer was removed by inverting the plate and tapping several times on paper towel. Wells were washed once with PBS, and were blocked with 200 µl of ELISA blocking buffer. Plates were incubated at 37°C for 1 h, shaking. Blocking buffer was removed by inverting and tapping the plate as before, and wells were washed 3 times in PBS Tween. Vaccine trial antiserum was diluted in ELISA diluent buffer in doubling dilutions, with an initial dilution between 1:50 and 1:4,000. Dilution of primary antisera
was performed in a separate 96-well plate, using 120 µl volumes prepared in duplicate. Volumes of 100 µl of the serum dilutions were added to the blocked plate and incubated at 37°C for 1 h, shaking. Unbound serum was removed as before and wells were washed 3 times in PBS Tween. Secondary antibody (goat-α-mouse IgG.HRP-conjugate) was diluted 1:5,000 in ELISA diluent buffer, 100 µl was added to each well, and plates were incubated at 37°C for 1 h, shaking. Unbound antibody was removed and wells were washed 3 times with PBS Tween, followed by one wash in PBS. A volume of 100 µl of freshly-prepared TMB substrate was added to each well and the colour development was stopped using 50 µl of 2 N H₂SO₄ once the reaction was saturated (usually after ~10 min incubation). The absorbance readings at 450 nm were plotted against the reciprocal serum dilution and a line was drawn between the points. The reciprocal titre was determined to be the point at which the serum dilution line crossed an OD of 0.2, which was 4X the background level.
2.9 Transfection and Knockout Analysis Materials and Methods

2.9.1 Malarial DNA and protein extraction materials

2.9.1.1 Ficoll gradient
5.7% w/v Ficoll in MT-PBS (2.7.1.2).

2.9.1.2 Buffer A
50 mM NaOAc pH 5.2
100 mM NaCl
1 mM EDTA

2.9.1.3 TNE
10 mM Tris
0.2 M NaCl
1 mM EDTA
pH adjusted to 7.4

2.9.1.4 TNET
10 mM Tris
0.15 M NaCl
5 mM EDTA
1% v/v Triton-X 100
pH adjusted to 7.4

2.9.1.5 Pyrimethamine water
Tap water from the RMIT animal house was pH-adjusted to between 3 and 5 with 1 M HCl and sterilised by autoclaving or by filtering through a 0.2 μm syringe filter. A freshly-made stock of 7 mg/ml pyrimethamine in DMSO was diluted 1:100 in the pH-adjusted water.

2.9.2 Malarial transfection and malarial DNA/protein extraction methods

2.9.2.1 Transfection of purified malarial schizonts with target DNA
All procedures involved in the transfection of malarial schizonts were performed by Dr. Tania de Koning Ward at the Walter and Eliza Hall Institute (WEHI), Melbourne, Australia, basically as per the method described (Janse et al., 2006b). Briefly, *P. berghei*
ANKA schizonts were purified from an overnight in vitro culture using a Nycodenz gradient. An Amaxa nucleofector™ machine was used for transfection. Per reaction, 10 µg of prepared linearised target DNA was used, and the transfected schizonts were injected into the lateral tail vein of one BALB/c mouse. The mouse was treated with 70 µg/ml pyrimethamine provided in the drinking water (pH adjusted to between 3.5 and 5) continuously from 24 - 30 hours post-injection. The pyrimethamine water was given as the sole water source in a light-protected water bottle for the mice to drink ad libitum. The pyrimethamine water was generally replaced with freshly made drug-treated water every 3 - 5 days until the end of the experiment. Parasitemia was monitored daily from 3 dpi. At a parasitemia of between 2 and 5%, mice were anaesthetised or killed using CO2 and blood was taken by cardiac puncture. The blood was used for DNA analysis and stabilate creation.

2.9.2.2 Infection of mice with stabilates obtained from transfection experiments

Stabilates created from the transfection experiments performed at WEHI were used to infect additional BALB/c mice, for secondary drug-selection and to obtain sufficient parasite material for DNA analysis. Each mouse received an i.p. injection of between 50 and 150 µl of stabilate. Mice were generally treated with pyrimethamine at between 24 and 30 hours post-infection.

2.9.2.3 Ficoll gradient separation of erythrocytes from blood

Blood collected from infected mice was centrifuged at 400 x g for 7 min, and the cell pellet was washed once with 10 ml of MT-PBS. The cell pellet was diluted in four volumes of MT-PBS. Meanwhile, a Ficoll gradient was prepared in a 10 ml centrifuge tube using 5.7% w/v Ficoll in MT-PBS, and erythrocytes diluted in MT-PBS were layered on top of an equal volume of prepared Ficoll. Erythrocytes were separated from leukocytes by centrifugation of the Ficoll gradient for 20 min at 400 x g with slow acceleration and no braking. After centrifugation, the blood components separated so that leukocytes and plasma were retained above the Ficoll gradient and erythrocytes pelleted at the bottom of the tube. The supernatant was removed and the erythrocyte pellet was washed twice in 10 ml MT-PBS by centrifugation at 400 x g for 10 min.
2.9.2.4 Malarial DNA extraction using SDS and PCI

The erythrocytes separated by Ficoll gradient centrifugation were resuspended in 4 volumes of Buffer A and 1 volume of 18% SDS, mixed by inversion and incubated for 2 min. An equal volume of PCI was added, and the solution was mixed by repeated inversion for 1 min, and centrifuged at 4,500 x g for 10 min. The upper phase was transferred to a new tube, and the PCI extraction was repeated twice. DNA was precipitated from the upper phase by the NaOAc/EtOH purification method (2.3.2.8) with a second 70% EtOH wash. The DNA pellet was resuspended in 250 µl of TE and stored at -20°C.

2.9.2.5 DNA extraction with SDS, PCI and Proteinase K

Leukocytes were removed from blood using a CF11 column: ~ 1 ml of CF11 powder (Whatman) was added to a 5 ml column. The powder was washed with 5 ml of PBS, the blood was layered over the CF11 and the column eluate was retained. The column was washed with ~25 ml of PBS until the eluate was clear, the preceding eluate was also retained. The total retained eluate was centrifuged at 4,000 x g for 10 min. The blood cell pellet was resuspended in 1.5 volumes of 0.15% w/v saponin in PBS. The tubes were incubated on ice for 15 min, shaking occasionally. The tubes were centrifuged at 5,000 x g for 10 min and the parasite pellet was washed in 10 ml TNE. The pellet was resuspended in 700 µl TNE + 1% w/v SDS and mixed by end-over-end tube inversion for 5 min. A total of 100 µg of RNase A was added and the tubes were incubated at 37°C for 30 min. 200 µg/ml Proteinase K was added and the tubes were incubated for between 40 min and 1 h at 37°C. An equal volume of PCI was added, tubes were mixed by vigorous shaking for 5 min, and spun at 16,000 x g for 4 min. The top layer was decanted into a new tube, and the PCI extraction was repeated. An equal volume of CI was added. The tubes were shaken and the top layer isolated as before. The DNA was precipitated overnight at -20°C with EtOH/NaOAc. Each DNA pellet was resuspended in 30 µl TE.

2.9.2.6 DNA extraction using Qiagen kits

Alternately, malarial DNA was extracted following leukocyte removal by Ficoll gradient separation using the blood protocol of the DNA mini kit (Qiagen).

2.9.2.7 Malarial protein extraction

Erythrocytes separated by Ficoll gradient were resuspended in two PCV of 0.15% saponin w/v in MT-PBS, and incubated on ice for 15 min. Free parasites were washed
twice in 10 ml of MT-PBS; cells were pelleted by centrifugation at 1,000 x g for 5 min. Pellets were resuspended in 0.5 ml of MT-PBS and centrifuged at 16,000 x g for 1 min. Parasite pellets were stored at -80°C if not immediately used. The parasite pellets were resuspended in 20 µl of TNET containing a protease inhibitor cocktail (Roche) and incubated on ice for 1 h. Five microlitres of 5X reducing sample buffer (2.6.6.2) was added, tubes were incubated at 95°C for 5 min, vortexed, and incubated at 95°C a further 5 min.

### 2.9.3 Southern Blot Materials

#### 2.9.3.1 Denaturation solution
0.5 M NaOH  
1.5 M NaCl

#### 2.9.3.2 Neutralisation solution
0.5 M Tris.HCl pH 7.5  
3 M NaCl

#### 2.9.3.3 20X SSC buffer
3 M NaCl  
0.3 M tri-sodium citrate  
Adjusted pH to 7, sterilised.

#### 2.9.3.4 Maleic acid buffer
0.1 M Maleic acid  
0.15 M NaCl  
Adjusted pH to 7.5 with solid NaOH, sterilised.

#### 2.9.3.5 10X Southern blocking reagent
10% w/v Blocking Reagent dissolved in maleic acid buffer, stirred on low heat for ~15 min, then sterilised. Stored at 4°C.

#### 2.9.3.6 Hybridisation buffer
5X SSC  
0.1% w/v N-laurylsarcosine  
0.02% w/v SDS  
1X Southern blocking reagent
2.9.3.7 Low stringency washing buffer
2X SSC
0.1% w/v SDS

2.9.3.8 High stringency washing buffer
0.5X SSC
0.1% w/v SDS

2.9.3.9 Washing buffer
0.3% v/v Tween 20 in Maleic acid buffer

2.9.3.10 Blocking solution
1X Blocking reagent in Maleic acid buffer

2.9.3.11 Antibody solution
A 1:10,000 dilution of Anti-Digoxigenin-AP antibody (75 mU/ml) was made in blocking solution when chemiluminescent substrate was used, a 1:5,000 dilution of antibody (150 mU/ml) was made in blocking solution when chromogenic substrate was used.

2.9.3.12 Detection buffer
0.1 M Tris.HCl pH 9.5
0.1 M NaCl

2.9.3.13 Chromogenic substrate solution
200 µl of NBT/BCIP in 10 ml Detection buffer

2.9.3.14 Chemiluminescent substrate solution
1:100 dilution of CSPD in detection buffer

2.9.3.15 Probe stripping solution
0.2 M NaOH
0.1% w/v SDS
2.9.4 Southern blot methods

2.9.2.1 Probe labelling reaction

30 µl of PCR product was either cleaned with the Wizard PCR purification kit (Promega), or was used following purification with the Qiaquick gel extraction kit. The cleaned PCR product was denatured in a boiling water bath for 10 min and quickly chilled on ice for 2 min. 4 µl of hexanucleotide mix, 4 µl of labelled dNTPs and 2 µl of Klenow enzyme were added, the tube contents were mixed by vortexing and incubated for 20 h at 37°C. The labelling reaction was stopped with the addition of 4 µl of 0.2 M EDTA pH 8.

2.9.2.2 Preparation of genomic DNA prior to Southern blot

Between 3 µg and 15 µg of purified parasite DNA was used in each sample. The concentration of genomic DNA was quantified by agarose gel electrophoresis and comparison to known quantities of lambda DNA. Prior to digestion, parasite DNA was heated at 55°C for 15 min to destroy nucleases and to aid solubilisation. Parasite DNA was digested for 16 h with 30 to 40 U of NdeI. When larger volumes were used, DNA was precipitated with NaOAc/EtOH following digestion and resuspended in 30 µl of water or 10 mM Tris.HCl pH 8.

2.9.2.3 Southern blot of *P. berghei* ANKA DNA

The digested genomic DNA was separated on a 1% w/v agarose gel at 50-65 V alongside a λ/PstI marker. The gel was stained and destained and photographed on a transilluminator with a ruler next to the marker. The gel was washed twice in denaturation solution for 15 min, shaking gently. The gel was rinsed in water and washed twice in neutralisation solution for 15 min, shaking gently. Pre-cut Whatman paper and Hybond nylon membrane was soaked in 20X SSC. The blot apparatus was set up as follows:

A pyrex dish was filled with 20X SSC. A glass plate was placed into the dish, above the level of the SSC. A piece of Whatman paper was placed on the glass plate so that both ends were in the SSC, forming a wick. The gel was placed over the Whatman paper. Parafilm was placed around the gel to cover exposed areas of the Whatman paper, to prevent short-circuiting. The Hybond membrane was placed on top of the gel, air bubbles were removed by rolling a thin glass tube over the surface, then two pieces of Whatman paper were placed on top and bubbles removed as before. A stack of paper
towels (~10 to 15 cm) was placed on top and secured with a glass plate and a medium weight (~200 g).

The Southern transfer was left overnight, then the membrane was removed and DNA fixed by UV crosslinking for 5 min in a transilluminator. The membrane was rinsed in water, then blocked for 2 h in hybridisation buffer at 65°C in a sealed plastic bag in a hybridisation oven. The labelled probe was denatured in a boiling water bath for 10 min then chilled on ice. The probe was added to 10 ml of pre-heated (65°C) hybridisation buffer. The membrane was incubated with the probe-containing hybridisation buffer at 63 - 68°C for 16 h in a hybridisation oven.

The probe-containing hybridisation buffer was retained at -20°C at the completion of hybridisation, for re-use up to five times. The membrane was washed two times for 5 min in low stringency washing buffer at RT, and was then washed two times for 15 min at 65°C with high stringency washing buffer.

The membrane was transferred to a new container and washed for 2 min at rt, shaking at 50 rpm, in 100 ml washing buffer. The membrane was then blocked in 100 ml blocking solution for 30 min at rt, shaking. The membrane was incubated in 20 ml of antibody solution for 30 min, shaking. The membrane was then washed twice for 15 min in 100 ml washing buffer, shaking.

The membrane was incubated for 3 min in 50 ml of detection buffer, and then the probe-hybridised DNA bands were detected using either chemiluminescent or chromogenic substrates. The membrane was incubated in the dark in 10 ml of chromogenic substrate solution for between 15 min and 16 h, until the desired band intensity was obtained. The reaction was then stopped by washing the membrane for 5 min in 50 ml of TE buffer. Alternatively, the membrane was placed between 2 transparent plastic sheets and 2 ml of chemiluminescent substrate solution was added. The membrane was incubated at rt for 5 min, then the sheets were sealed to prevent liquid escaping and the membrane was incubated for 10 min at 37°C. The probe-hybridised DNA bands were visualised either using X-ray film, with a development time of between 10 and 20 min, or were incubated for between 10 and 20 min on a GeneSnap imager using GeneSnap software (Syngene).
2.9.2.4 Stripping membranes prior to reprobing
To remove the chromogenic substrate, the membrane was incubated with DMF in a glass dish at 50°C for 1 h, the DMF was changed and the membrane was incubated at 50°C for several hours until all of the colour substrate was removed. The procedure to remove chemiluminescent substrates commenced at this point. The membrane was washed in two changes of water. The probe was stripped by two 20 min washes in probe stripping solution at 37°C, shaking. The membrane was rinsed and stored in 2X SSC at 4°C prior to reprobing.

2.9.2.5 Reprobing membranes
The stripped membrane was blocked for at least 2 h in hybridisation solution at 65°C then the Southern procedure was continued as previously described.

2.9.2.6 Re-use of probes
Probes were stored at -20°C after use. Prior to re-use, probes were heat-denatured in a boiling water bath for 10 min, cooled to 65°C and added to blocked membranes. The Southern procedure was continued as previously described.
CHAPTER 3: Evaluation of *P. yoelii* and *P. falciparum* TCTP as potential malaria vaccines

3.1 Introduction

TCTP was first trialled as a potential anti-malarial vaccine by our group in 2002. In the initial experiment, *P. falciparum* and human TCTP were expressed as histidine-tagged recombinant proteins in *S. cerevisiae* yeast. Groups of eight mice were immunised three times, with a four-week interval. Immunisations consisted of 25 µg Pf or human TCTP in Freund's adjuvant. Mice immunised with human TCTP did not produce antibodies against the recombinant protein. This was not unexpected, as the mouse and human TCTPs are highly conserved, sharing 95% amino acid identity. Mice immunised with PfTCTP had an average pre-challenge antibody titre of 1200, with individual titres ranging from 100 to 10,000 (Taylor, 2002).

Mice immunised with PfTCTP, and the PBS-immunised controls, were challenged three weeks after the final immunisation with 1 x 10⁵ *P. yoelii* YM-infected erythrocytes. Mice immunised with PfTCTP had significantly lower (P<0.01) parasitemia than the PBS controls at the early and middle stages of infection, until six days post-infection (dpi). Mice immunised with PfTCTP also had increased survival times compared with PBS-immunised controls. In these experiments, mice were euthanised when they reached a symptom score of eight, as required by the RMIT Animal Ethics Committee (AEC) (scoring system described in Appendix 2). A symptom score of eight in a *P. yoelii* YM infection usually corresponded to a parasitemia of between 60 and 80%. At six dpi there was a 100% survival rate for the PfTCTP-immunised mice compared with a 25% survival rate for the PBS controls. All control and PfTCTP-immunised mice had reached the experimental end-point by the end of seven dpi (Taylor, 2002).

These results indicated some promise for TCTP as a malarial vaccine. Although most research into TCTP has focused on the intracellular actions of the protein (reviewed in Bommer and Thiele, 2004), (described in section 1.3), some evidence exists of a second, extracellular role for TCTP (as described in section 1.5). Human TCTP induces histamine release from a subset of allergic human basophils, which have been termed IgE⁺, in an IgE-dependent manner (MacDonald *et al*., 1995). Human TCTP also induces IL-8 release from a subset of human allergic
donor eosinophils (Bheekha-Escura et al., 2000). There is evidence that *P. falciparum* TCTP is secreted during infection and can induce histamine and IL-8 release *in vitro* in human basophils and eosinophils, respectively (MacDonald et al., 2001). The TCTP from the parasites *S. mansoni* and *B. malayi* can also induce histamine release *in vitro* and can induce eosinophil recruitment in mice *in vivo* (Gnanasekar et al., 2002; Rao et al., 2002).

PfTCTP was evaluated as a potential malarial vaccine by our group due to its proposed role in inducing histamine release, and the evidence that it could be secreted from the parasite into the bloodstream of the host. Our hypothesis for the modest protection conferred by PfTCTP immunisation was that anti-TCTP antibodies participated in neutralising the extracellular actions of malarial TCTP. Histamine released by the host is thought to assist the malarial parasite, by causing vasodilation and increasing expression of endothelial adhesion factors that allow the parasites to sequester in organs such as the brain (as described in section 1.2.4). Complications during a malarial infection in humans are thought to be due, to some extent, to inappropriate inflammatory or immune responses by the host, and it has been shown in rodent models that inflammatory molecules (particularly TNF-α and IFN-γ) play a major role in malaria pathogenesis (Li et al., 2001a).

This results chapter describes a vaccine trial that was performed using *P. yoelii* and *P. falciparum* TCTP as antigens. *P. yoelii* TCTP was included in this trial as it was thought that a homologous vaccine might confer better protection to *P. yoelii* challenge. *P. falciparum* TCTP was included to confirm the results of the first vaccine trial. The two His-tagged recombinant proteins were expressed in the w303.1a mutant strain of *S. cerevisiae* using the pYEULCBX vector. This vector has both a yeast and bacterial origin of replication, two yeast selectable marker genes (URA3, encoding the production of uracil, and LEU2d, encoding the production of leucine), and an Amp’ gene for selection of *E. coli* transformants. The pYEULCBX vector also encodes a six-histidine tag, followed by a thrombin cleavage site, upstream of the multiple cloning site (MCS). Cloning of exogenous genes into the MCS allows for in-frame expression of recombinant protein with an N-terminal His tag. Recombinant protein expression is induced by the addition of copper, which activates the metallothionein *CUP1* promoter. The N-terminal His tag was not cleaved from either of the proteins used in this vaccine trial, as the first vaccine trial
also used His-tagged PfTCTP. A map of the pYEULCBX vector and the amino acid sequence upstream of the MCS are shown in Figure 3.1 (a) and (b).

This chapter describes the cloning of *P. yoelii* TCTP into the pYEULCBX vector, the expression and purification of PyTCTP and PfTCTP in yeast, and the vaccine trial of the two protein antigens using both a *P. yoelii* YM and XNL challenge model. The *P. yoelii* XNL strain, which produces a non-lethal infection in BALB/c mice (Kim *et al.*, 1980; Li *et al.*, 2001a; Ma *et al.*, 2007; Taylor *et al.*, 1986), was included as a challenge using a less virulent malarial strain, with a slower multiplication rate, may be a more appropriate model of human infection kinetics. Whilst the *P. yoelii* YM strain is often used in vaccine trials in mice, it produces much more rapidly ascending parasitemia than is seen in human infections and is uniformly lethal. In contrast, malarial infection in humans is generally non-lethal. The mortality rate from *P. falciparum* malaria, which is responsible for ~80% of human malarial deaths, is >1%, although this rises to 10-50% in severe infections (Dondorp and Day, 2007).

The methodology of the TCTP vaccine trials was based on vaccine trials of the *P. yoelii* merozoite surface protein 4/5 (MSP4/5), performed by Coppel's group (Goschnick *et al.*, 2004; Kedzierski *et al.*, 2000; Kedzierski *et al.*, 2002a; Kedzierski *et al.*, 2001). In their trials, MSP4/5 was expressed in *E. coli* and purified using Talon™ metal affinity resin. Groups of female BALB/c mice were immunised i.p. with 25 µg MSP4/5 in Freunds adjuvant, with two boosters of 25 µg at monthly intervals. Mice were challenged 12-14 days after immunisation with 1 x 10⁵ *P. yoelii* YM irbc. In the 33 immunised mice, 28 survived the *P. yoelii* infection (Kedzierski *et al.*, 2000).

The most effective malaria vaccine components tested so far have been proteins on exposed locations of the parasite and on the surface of infected erythrocytes. There is no evidence to suggest that malarial TCTP is expressed on the surface of exoerythrocytic parasites or on the surface of infected erythrocytes, however there is evidence that the protein is secreted (MacDonald *et al.*, 2001). The aim of TCTP immunisation was to induce antibodies that are designed to inhibit the extracellular actions of a secreted malarial protein, rather than direct recognition and inhibition of the malaria parasite.
The aims of the experiments outlined in this chapter were:

1: To clone the PyTCTP coding sequence into the pYEULCBX vector.

2: To express PyTCTP and PfTCTP as recombinant His-tagged proteins in the w303.1a S. cerevisiae strain.

3: To assess the protective effect of TCTP immunisation in BALB/c mice by challenge with a lethal (YM) or non-lethal (17XNL) strain of P. yoelii.

5: To assess the ability of TCTP antisera to recognise TCTP present in malarial lysates.

6: To assess the histamine release induced by TCTP injection in ovalbumin-sensitised C57BL/6 mice.
Figure 3.1: Map of the pYEULCBX vector
(a) Plasmid map of the 7.2 kb pYEULCBX vector;
(b) Sequence of the N-terminal six-histidine tag and thrombin cleavage site of the pYEULCBX vector. The BamHI cloning site is indicated.
Chapter 3: Evaluation of *P. yoelii* and *P. falciparum* TCTP as malaria vaccines

### 3.2 Materials and Methods

For more detailed information on the materials and methods used in this chapter, please refer to Chapter 2.

### 3.3 Results

#### 3.3.1 Amplification and cloning of *P. yoelii* TCTP

The strategy for cloning PyTCTP into the pYEULCBX yeast expression vector is depicted in Figure 3.2 (a). Briefly, the cloning strategy involved amplification of PyTCTP by PCR from *P. yoelii* YM cDNA and cloning into the pCR2.1 vector, followed by digestion of the PyTCTP coding sequence from the pCR2.1 vector and ligation into the pYEULCBX yeast expression vector.

##### 3.3.1.1 Primers used for amplification of *P. yoelii* TCTP

The primers used to amplify *P. yoelii* TCTP were designed based on the published sequence (GenBank accession number XM_720179). The 5′PyTCTP primer was designed to incorporate a *Bam*HI restriction site (CGCG GGA TCC ATG AAA GTA TAT AAA GAC ATT TTT ACA), and the 3′PyTCTP primer was designed to incorporate a *Pst*I restriction site (GCGC GAC GTC TTA GTA TTT TTC AAA TAA ACC ATC). This allowed for directional cloning into the pYEULCBX yeast expression vector.

##### 3.3.1.2 Transformation and purification of pYEULCBX vector

Purified pYEULCBX plasmid DNA was obtained from Prof. Ian Macreadie, CSIRO Health Sciences and Nutrition, Parkville, Victoria, Australia. Plasmid DNA was transformed into electrocompetent *E. coli* DH5α cells and minipreparations were performed to obtain sufficient plasmid for cloning (methods described in 2.4.3.2.2 and 2.3.2.1.1).

##### 3.3.1.3 Amplification of PyTCTP from cDNA

Purified *P. yoelii* mRNA was obtained from the laboratory of Prof. Ross Coppel, Department of Microbiology, Monash University, Melbourne, Australia. The Omniscript RT kit was used to produce cDNA as per manufacturer’s instructions. PyTCTP was amplified from *P. yoelii* cDNA using Taq polymerase as per the method described (2.3.2.2.3) with the following modifications and conditions: Each 50 µl reaction contained 0.5 µM of the 5′ and 3′ PyTCTP primers, 1.25 U Taq, and 0.5 µg of *P. yoelii* cDNA. The DNA was amplified by PCR with the following parameters:
Initial denaturation 95°C for 2 min, then 30 cycles of 95°C for 30 s, 50-55°C gradient for 30 s, and 72°C for 1 min followed by a final extension at 72°C for 7 min. The amplified products were separated by 1% agarose gel electrophoresis at 100 V (method described in 2.3.2.5). A single band of 516 bp was obtained from PCR at each of the annealing temperatures, as shown in Figure 3.2 (b). The PCR product obtained from the 55°C annealing temperature was cloned into the pCR2.1 vector.

3.3.1.4 TA cloning of PyTCTP into the pCR2.1 vector and ligation of PyTCTP into the pYEULCBX vector

Fresh PyTCTP product was ligated into the pCR 2.1 vector as per manufacturer’s instructions (method described in 2.3.2.7.1). Plasmid DNA was isolated from resulting white colonies. The presence of the PyTCTP insert was confirmed by restriction enzyme digestion with 20 U each of BamH I and PstI, 0.5 µg of pYEULCBX vector was also digested (method described in 2.3.2.4). An insert band at 516 bp was obtained from each of the pCR2.1 vector digests, as shown in Figure 3.2 (c) lanes 2-5. The restriction enzyme digestion also produced two other bands of plasmid backbone. A single band at 7.2 kb was obtained from digested pYEULCBX (Figure 3.2 (c) lane 7). Sequence analysis (2.3.2.2.6) of the 516 bp inserts using the 5’PyTCTP and 3’PyTCTP primers produced a sequence identical to the published sequence for P. yoelii TCTP (data not shown). Plasmids with the correct insert were designated pCR.PyTCTP.

Bands corresponding to the digested pYEULCBX vector and the PyTCTP insert were excised from the gel and DNA was purified using the Qiaquick Gel Extraction kit. Ligations were performed using a 3:1 insert to vector ratio (method described in 2.3.2.7.2). Completed reactions were cleaned using the dextran purification method (2.3.2.7.3), and were used to transform electro-competent E. coli DH5α. Plasmid DNA from resulting colonies was purified by minipreparation. The presence of TCTP insert was confirmed by digestion using PstI and BamHI (Figure 3.2 (d)). Sequence analysis of the insert confirmed that PyTCTP was in-frame with the N-terminal hexa-histidine tag. Plasmids were named pYEULCBX.PyTCTP. The sequence of the N-terminal portion of the expressed protein, including the His-tag, is depicted in Figure 3.3 (a). A sequence alignment of PfTCTP and PyTCTP, starting from the first methionine after the His-tag, is shown in Figure 3.3 (b). The two proteins share 88% identity, with 97% positives.
Chapter 3: Evaluation of \textit{P. yoelii} and \textit{P. falciparum} TCTP as malaria vaccines

(a)

Step 1: Amplification of PyTCTP from \textit{P. yoelii} cDNA, using primers engineered to contain BamHI and PstI restriction sites:

\[
\text{BamHI} \quad \text{PstI}
\]

\textit{P. yoelii} cDNA

---

Step 2: Cloning of PyTCTP into the pCR2.1® TA vector:

A \quad A

Fresh PyTCTP PCR product

T \quad T

pCR2.1 vector with TA overhangs

---

Step 3: Digestion of the pYEULCBX and pCR.PyTCTP vectors with \textit{BamHI} and \textit{PstI}

\[
\begin{array}{c}
\text{pYEULCBX vector digestion} \\
\text{pCR® Vector digestion}
\end{array}
\]


---

Step 4: Ligation of PyTCTP into the pYEULCBX vector

\[
\begin{array}{c}
\text{pYEULCBX.PyTCTP}
\end{array}
\]
Chapter 3: Evaluation of *P. yoeli* and *P. falciparum* TCTP as malaria vaccines

(b)

1 2 3 4 5 6 7

Size (bp)
1000 TCTP
500

(c)

1 2 3 4 5 6

Size (bp)
11501 pYEULCBX
5077
2838 pCR2.1
514 TCTP
Figure 3.2: Cloning of PyTCTP into the pYEULCBX yeast expression vector:
(a) Diagram of the PyTCTP cloning strategy
(b): Amplification of *P. yoelii* TCTP from cDNA by Taq PCR: Lane 1: 100 bp marker (full size range described in Appendix 1); Lanes 2-7: PCR products from annealing temperatures 50-55°C
(c): pCR2.1.PyTCTP and pYEULCBX vectors digested with BamHI and PstI: Lane 1: 1 µg λ/PstI marker (full size range described in Appendix 1); Lanes 2-5: pCR2.1.PyTCTP plasmid digests; Lane 6: pYEULCBX plasmid digest
(d): Confirmation digest of pYEULCBX.PyTCTP using BamHI and PstI: Lane 1: 1 µg λ/PstI marker; Lanes 2 & 3: pYEULCBX.PyTCTP plasmid digests
Chapter 3: Evaluation of *P. yoelii* and *P. falciparum* TCTP as malaria vaccines

(a) Sequence of the His-tag and the N-terminal portion of PyTCTP encoded by the pYEULCBX.PyTCTP vector

(b) Amino acid sequence alignment of PyTCTP and PfTCTP

---

**Figure 3.3:** Sequence analysis of the His-tag region of the pYEULCBX.PyTCTP vector, and sequence alignment of PyTCTP and PfTCTP

(a) Sequence of the His-tag and the N-terminal portion of PyTCTP encoded by the pYEULCBX.PyTCTP vector

(b) Amino acid sequence alignment of PyTCTP and PfTCTP
3.3.2 Expression of PyTCTP and PfTCTP in *S. cerevisiae*

3.3.2.1 Growth of pYEULCBX.PyTCTP and pYEULCBX.PfTCTP transformants of *S. cerevisiae*

The pYEULCBX.PyTCTP plasmid was transformed into the w303.1a strain of *S. cerevisiae* (method described in section 2.5.2.1). Transformation with pYEULCBX eliminates the growth requirement of the mutant yeast for uracil and leucine. The transformation mix was plated on Complete-Ura agar and incubated at 30°C for 2-3 days. A culture of *S. cerevisiae* w303.1a mutant yeast that had been transformed with the pYEULCBX.PfTCTP plasmid was obtained from Prof. Ian Macreadie, CSIRO, Parkville Australia, and grown on Complete-Ura agar for 2-3 days at 30°C.

3.3.2.3 Western blot to test for protein expression

Expression of Pf- and Py-TCTP in several colonies of transformed yeast was verified by Western blot (2.6.2.1). Ten millilitre cultures were induced with CuSO₄ for 16 hours. Both culture supernatants and cell pellets were analysed. SDS-PAGE was performed (2.6.7) followed by Western blot (2.6.8) with the following modifications and conditions: Casein (1% w/v in PBS) was used as a blocking solution and as an antibody diluent. To detect recombinant PfTCTP, the primary antibody used was an α-PfTCTP IgG antibody raised in rabbit, at a 1:4,000 dilution (obtained from Prof. Steven Meshnick, Department of Epidemiology, University of Michigan, USA). An α-rabbit IgG.HRP conjugate used at a 1:1,000 dilution as a secondary antibody. PfTCTP was detected in all the transformed colonies analysed, although expression levels varied. A major band at around 26 kDa was observed, as well as several smaller bands ranging from ~23 kDa to ~16 kDa (Figure 3.4 (a)). These smaller protein bands are most likely to be breakdown products of PfTCTP due to proteolysis. No bands were detected in untransformed yeast cultures probed with α-TCTP antibodies (results not shown). The TCTP from the induced yeast cultures had been subjected to SDS-PAGE prior to protein purification, indicating that some proteolysis of TCTP was occurring during yeast growth.

At the time of these experiments, it was unknown if PfTCTP antibodies would recognise PyTCTP, although cross-reactivity was likely given the high sequence similarity. Due to this, expression of PyTCTP in transformed yeast was verified using a Penta-His antibody raised in rabbit, at a 1:1,000 dilution. The α-rabbit secondary antibody was used as before. A major band was detected at around 28 kDa in all of the transformants analysed, as well as two to three smaller bands at around 24 and
22 kDa (Figure 3.4 (b)). The His-tag is present on the N-terminus of PyTCTP, so the smaller protein bands observed are most likely C-terminal truncates of PyTCTP.

A greater number of lower molecular-weight protein bands were detected using the α-PfTCTP antibody compared with the α-Penta-His antibody. This could be due to the presence of N-terminal truncates of TCTP, which would not be detected by the α-Penta-His antibody, or this result could indicate that more proteolysis occurred in the PfTCTP cultures compared with the PyTCTP cultures.
Figure 3.4: Confirmation of PfTCTP and PyTCTP expression in yeast

(a): PfTCTP transformants reacted with PfTCTP antibody: Lane 1: BenchMark pre-stained marker (full size range described in Appendix 1); Lanes 2-5: PfTCTP culture cell pellets

(b): PyTCTP transformants reacted with Poly-His antibody: Lanes 1, 2, 4: PyTCTP culture cell pellets; Lane 3: BenchMark pre-stained marker; Lane 5: His-tagged positive control protein.
3.3.2.5 Large-scale expression and purification of PyTCTP and PfTCTP

Yeast transformed with either pYEULCBX.PfTCTP or pYEULCBX.PyTCTP was used to inoculate 0.5-1L of supplemented YNB+Glucose, and expression was induced with 0.5 mM CuSO₄ for 40 hours (method described in 2.6.2.2). Yeast cells were lysed using a Braun cell homogeniser (2.6.2.4.1) in the presence of protease inhibitors (1 mM Pefabloc, 5 mM Benzamidine, 1 µg/ml E-64, 1 µg/ml Pepstatin, and 1 µg/ml leupeptin). The lysates were centrifuged at 21,000 x g for 25 min and protease inhibitors were added prior to incubation of the supernatants with prepared Talon™ resin at 4°C for 16 h. Metal affinity chromatography was performed using a wash step in phosphate buffer containing 5 mM imidazole, followed by elution of His-tagged proteins using 0.2 M imidazole (method described in 2.6.2.5). Samples of the eluates were subjected to SDS-PAGE. Figure 3.5 (a) shows the result of Talon affinity chromatography performed on PfTCTP yeast lysates. The 5 mM imidazole eluates (lanes 2 and 3) contain a small amount of low molecular weight proteins (<15 kDa). In the 0.2 M imidazole eluate (lane 4), a major protein band is present at around 26 kDa, as well as several lower and higher molecular weight proteins. Talon affinity chromatography of PyTCTP lysates yielded similar results, as shown in Figure 3.5 (b). Very low amounts of protein were detected in the 5 mM imidazole eluate (results not shown). A major band at around 28 kDa was present in the 0.2 M imidazole eluates, as well as several other higher and lower molecular weight proteins.
Figure 3.5: Purification of PfTCTP and PyTCTP by IMAC using Talon™ resin

(a) Purification of PfTCTP: Lane 1: BenchMark pre-stained marker; Lanes 2 and 3: 5 mM imidazole eluates; Lane 4: 0.2 M imidazole eluate

(b) Purification of PyTCTP: Lane 1: BenchMark pre-stained marker; Lanes 2 & 3: 0.2 M imidazole eluate fractions
3.3.2.6 Further purification of PyTCTP and PfTCTP

After IMAC, PfTCTP and PyTCTP were further purified using a Mono-Q anion-exchange column (method described in 2.6.2.7). A broad eluate peak was seen start of the NaCl gradient for both PyTCTP and PfTCTP, and a small shoulder peak was seen before the major narrow elution peak, as shown in Figure 3.6 (a) and (b). Fractions corresponding to each UV peak above 0.02 were collected and subjected to SDS-PAGE (results not shown). TCTP was present in the major, narrow UV peak that eluted at ~48 min, when the buffer B concentration was ~60% (~0.3 M NaCl). Fractions containing TCTP were combined and dialysed against PBS (method described in 2.6.2.6). Preparations of PfTCTP and PyTCTP following Mono-Q chromatography and PBS dialysis are shown in Figure 3.7 (a) and (b). The higher molecular weight bands seen in both Py and PfTCTP samples after Talon™ column purification (Figure 3.5) were absent following Mono-Q chromatography. These larger bands could have been contaminant proteins that were separated by the Mono-Q column, or could have been TCTP multimers that were not completely reduced on SDS-PAGE, but were reduced by the dithiothreitol (DTT) used during Mono-Q chromatography. Several smaller proteins, most likely TCTP breakdown products, were also present following Mono-Q purification.

3.3.2.7 Analysis of protein storage methods

Following Mono-Q anion exchange chromatography and PBS dialysis, centricron columns were used to concentrate proteins to at least 0.5 mg/ml, which was the concentration required for use in the vaccine trial. As previous experiments had indicated that TCTP was susceptible to degradation during prolonged storage, several methods of protein storage were assessed. A portion of PfTCTP was lyophilised (2.6.2.8), to assess the potential for the protein to be sent to overseas collaborators. Lyophilization of PfTCTP resulted in minimal loss of yield and minimal degradation, as seen in Figure 3.7 (a) by comparing lanes 2 and 5 (PfTCTP prior to lyophilization) with lanes 3 and 4 (PfTCTP post-lyophilization and reconstitution). As PyTCTP was purified shortly before the commencement of the vaccine trial and overseas transportation was not required, it was not lyophilised.

The other methods of protein storage evaluated were storage at -70°C, in PBS and in PBS+50% glycerol. These proteins were analysed by SDS-PAGE to assess the yield and proportion of full-length TCTP present after several months of storage, just prior to vaccine preparation. As seen in Figure 3.8 (a) and (b), storage
in 50% glycerol resulted in increased protein breakdown compared to proteins stored in PBS alone, as a greater proportion of lower molecular weight bands were seen. Bradford assays of the glycerol-stored proteins after PBS dialysis indicated that over 50% of total protein had been lost during storage. As the SDS-PAGE results showed essentially the same amount of protein immediately before and after PBS dialysis and concentration, it was determined that the loss of protein yield has occurred during the time the proteins were stored in 50% glycerol.

TCTP stored in 50% glycerol was not used in the subsequent vaccine trial due to the substantial breakdown, instead TCTP stored at -70°C in PBS was used. Whilst some protein breakdown was observed over several months using this storage method, SDS-PAGE indicated that a substantial amount of full-length TCTP remained.

Additionally, PfTCTP was observed to separate at a slightly lower molecular weight than PyTCTP when the two proteins were loaded alongside each other on the one gel. As the two proteins are both 171 amino acids in length, sharing 88% amino acid identity, the reason for the observed size difference is unknown. Pf and PyTCTP do not contain potential N-linked glycosylation sites.

3.3.2.8 Sequence analysis of TCTP following Mono-Q chromatography

N-terminal sequencing (as described in 2.6.2.7) of PyTCTP eluates from Mono-Q anion exchange chromatography revealed two major protein sequences. One corresponded to the N-terminal sequence of the His-tagged PyTCTP, with the sequence Ser-His-His-His-His-His. The other major sequence obtained was Glu-Asp-Ala-Val-Asp-Gly-Met-Gly-Ala. This sequence is identical to residues 55-63 of PyTCTP, as compared with the sequence of PyTCTP given in Figure 3.3 (b). Three residues were present prior to the Glu in the breakdown product, but the sequencing profiles were difficult to interpret. This result indicates that the lower molecular weight protein seen on SDS-PAGE of PyTCTP following Mono-Q chromatography is a breakdown product of PyTCTP as the result of cleavage between the Ala51 and Asp52 residues. Lower molecular weight proteins had previously been observed in PfTCTP samples following Mono-Q chromatography. N-terminal sequencing of these samples had shown that the major protein present other than full-length PfTCTP was a breakdown product due to cleavage between Met61 and Gly62. The minor peak eluting from Mono-Q chromatography prior to the major TCTP peak had previously been shown to contain proteins approximately corresponding in size to
Chapter 3: Evaluation of *P. yoelii* and *P. falciparum* TCTP as malaria vaccines

TCTP breakdown products (unpublished result). However, the major peak eluting from the Mono-Q column also contained proteins corresponding to TCTP breakdown products, as well as full-length TCTP.

![Elution profile of TCTP during Mono-Q purification](image)

**Figure 3.6: Elution profile of TCTP during Mono-Q purification**
(a) PfTCTP elution profile from Mono-Q purification
(b) PyTCTP elution profile from Mono-Q purification
Figure 3.7: TCTP following Mono-Q purification and PBS dialysis
(a) PfTCTP after Mono-Q purification: Lane 1: BenchMark Pre-stained marker; Lane 2: 2 µg PfTCTP following Mono-Q purification and PBS dialysis; Lane 3: 2 µg reconstituted PfTCTP post-lyophilization; Lane 4: 4 µg reconstituted PfTCTP post-lyophilization; Lane 5: 2 µg PfTCTP dialysed in ammonium bicarbonate, prior to lyophilisation.
(b) PyTCTP after Mono-Q purification and PBS dialysis: Lane 1: BenchMark Pre-stained marker; Lanes 2 & 3: Aliquots of 2 µg of PyTCTP following Mono-Q purification and PBS dialysis.
Figure 3.8: Comparison of protein yield and quality following storage in PBS or in 50% glycerol

(a) PyTCTP and PfTCTP stored in PBS:  Lane 1: SeeBluePlus2 marker (full size range described in Appendix 1); Lane 2: 1 µg PyTCTP; Lane 3: 2 µg PyTCTP; Lane 4: 0.5 µg PfTCTP; Lane 5: 1.5 µg PfTCTP

(b) PyTCTP and PfTCTP stored in 50% glycerol:  Lane 1: SeeBluePlus2 marker; lane 2: 2 µg PyTCTP in 50% glycerol; Lane 3: 2 µg PfTCTP in 50% glycerol
Chapter 3: Evaluation of *P. yoelii* and *P. falciparum* TCTP as malaria vaccines

3.3.2.9 Analysis of PfTCTP obtained by an alternate expression method

An alternate method of protein expression with a one-hour induction time (method described in 2.6.2.3) was evaluated following the vaccine trial, to assess if the modifications would reduce proteolysis. The method was adapted from [http://www.clontech.com/images/pt/dis_manuals/PT3081-1.pdf](http://www.clontech.com/images/pt/dis_manuals/PT3081-1.pdf) and was designed to minimise yeast protease activity by replacement of the culture medium prior to induction. The induction time was also decreased to 1 h. In this method, yeast cells were lysed using glass beads (2.6.2.4.2). This alternate method of protein expression improved protein yield; 25 mg/L of PfTCTP was obtained compared with 2-3 mg/L from cultures induced for 40 hours. **Figure 3.9** depicts Talon™-purified PfTCTP obtained using this alternate expression method. Less total protein is present compared with **Figure 3.5 (a)**, making comparisons of proteolysis difficult, however there appears to be equal or less proteolysis in the alternate expression method sample. This protein was used as an ELISA antigen, and was not further purified by Mono-Q chromatography.
Figure 3.9: Purification of PfTCTP following alternate expression method

Lane 1: BenchMark marker (full size range described in Appendix 1); Lanes 2 & 3: 0.2 M imidazole eluate fractions
3.3.3 Analysis of TCTP samples under reducing and non-reducing conditions

Smaller MW bands were always observed together with full-length TCTP on SDS-PAGE after IMAC and anion-exchange chromatography. At least some of these were TCTP breakdown products, as determined by western blot and N-terminal sequencing. It was thought that these breakdown products might be self-associating with full-length TCTP and co-eluting from the columns, as the self-interaction of TCTP had previously been reported (Yoon et al., 2000). An alternative explanation is that purified TCTP was subject to continual proteolysis and the breakdown products were newly formed after chromatography, prior to SDS-PAGE analysis.

To evaluate the self-interaction of malarial TCTP, PyTCTP and PfTCTP samples following Mono-Q purification were subjected to SDS-PAGE under reducing and non-reducing conditions. Samples containing 2 µg of either Py or PfTCTP were combined with reducing sample buffer or non-reducing sample buffer. The samples resuspended in non-reducing buffer were not boiled prior to loading. The reduced and unreduced samples were run on adjacent wells of a 15-4% SDS-PAGE gel as per the standard method (Figure 3.10). As in Figure 3.8, PfTCTP was observed to run at a slightly smaller size than PyTCTP.

Under reducing conditions, both PyTCTP and PfTCTP were present as a major band of ~28 kDa as well as several smaller molecular-weight bands. Under non-reducing conditions, higher molecular-weight bands of ~35 and 53 kDa were present together with a 28 kDa band in both PyTCTP and PfTCTP samples. Additionally, lower molecular-weight bands (~16 and 12 kDa) were present. The presence of a 35 kDa band suggests that some of the breakdown products could associate with full-length TCTP under non-reducing conditions. The presence of a 53 kDa band suggests that full-length TCTP could form dimers under non-reducing conditions. Anion exchange chromatography was performed under reducing conditions (in the presence of 1 mM DTT), and so self-interaction through cysteine bonds would not be expected. Also, a single cysteine is present in Py and PfTCTP at residue 14, and self-interaction was reported to involve residues 126-172. It is probable that this self-interaction occurred through another mechanism.
Figure 3.10: PyTCTP and PfTCTP separated by SDS-PAGE under reducing and non-reducing conditions

Lane 1: Mark12 marker (full size range described in Appendix 1); Lane 2: PyTCTP under reducing conditions; Lane 3: PyTCTP under non-reducing conditions; Lane 4: PfTCTP under reducing conditions; Lane 5: PfTCTP under non-reducing conditions
3.3.4 Evaluation of PfTCTP and PyTCTP as potential malarial vaccines

3.3.4.1 Mouse immunisation groups and schedule

Six groups of six BALB/c mice were used in the vaccine trial. All mice were female and were aged six to eight weeks at the commencement of the trial. Two groups of six mice received immunisations of the PBS control, two groups of six mice received immunisations of PyTCTP and two groups of six mice received immunisations of PfTCTP. The methodology of the TCTP vaccine trial was based on vaccine trials of the \textit{P. yoelii} merozoite surface protein 4/5 (MSP4/5) (Goschnick \textit{et al.}, 2004; Kedzierski \textit{et al.}, 2000; Kedzierski \textit{et al.}, 2002a; Kedzierski \textit{et al.}, 2001). In the MSP4/5 trials, mice were immunised with 25 \( \mu \text{g} \) of protein in Freund's adjuvant at monthly intervals.

Mice in each group were immunised three times, with PBS or 25 \( \mu \text{g} \) PyTCTP or PfTCTP in Freund's adjuvant. The methods of vaccine preparation and mouse immunisation are described in sections 2.8.2.1 and 2.8.2.2. The first immunisation utilised Freund's complete adjuvant, the second and third immunisations were prepared using Freund's incomplete adjuvant. Mice were immunised using an i.p. injection of 100 \( \mu \text{l} \) of emulsion. There was a three-week interval between immunisations. To assess antibody titres, serum was collected from mice prior to the first immunisation and just prior to parasite challenge (2.8.2.3). A summary of mouse vaccine groups is shown in Table 3.1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunisation</th>
<th>Challenge</th>
<th>No. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBS in adjuvant</td>
<td>\textit{P. yoelii YM}</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>\textit{P. yoelii 17XNL}</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>PyTCTP in adjuvant</td>
<td>\textit{P. yoelii YM}</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>\textit{P. yoelii 17XNL}</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>PfTCTP in adjuvant</td>
<td>\textit{P. yoelii YM}</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>\textit{P. yoelii 17XNL}</td>
<td>6</td>
</tr>
</tbody>
</table>
3.3.4.2 Health of mice during immunisation period

One mouse in the PBS control group died three days after the third immunisation. An autopsy was performed but no abnormalities were seen, and the cause of death was unknown. All mice showed slight clinical symptoms (lack of grooming, clinical score of 1, as described in Appendix 2) the first day following immunisations, but were otherwise healthy throughout the immunisation period.

3.3.4.3 Survival rates of groups challenged with \textit{P. yoelii} XNL and YM malaria

Mice were challenged with a dose of $1 \times 10^5$ infected erythrocytes of \textit{P. yoelii} 17XNL (non-lethal) or \textit{P. yoelii} YM (lethal) parasites three weeks after the final immunisation. The \textit{P. yoelii} 17XNL strain was obtained from the Austin Research Institute, Heidelberg, Australia. The \textit{P. yoelii} YM strain was obtained from the Department of Microbiology, Monash University, Melbourne, Australia. Stabiles of these strains were used to infect passage mice (method described in 2.7.2.3); blood from these mice was then used to infect immunised mice with the precise parasite dose (2.7.2.4).

After infection, immunised mice were closely monitored for the development of malarial symptoms. Blood was taken daily for parasitemia quantification (2.7.2.5). After 3 dpi, mice were monitored at intervals of between four and seven hours for malarial symptom development (2.8.2.4). Mouse symptoms were scored following the table provided by the RMIT AEC (Appendix 2). Mice were euthanised when they reached a score of 8. This corresponded to severe morbidity, with respiratory distress as a major symptom, as well as weight loss, lack of grooming, and decreased body temperature. These symptoms usually occurred at a parasitemia of 60 - 80%.

An unexpected result occurred after malarial challenge. \textit{P. yoelii} 17XNL, which is known to be non-lethal in BALB/c mice, was found to be uniformly lethal in both PBS-immunised controls and in Pf- and PyTCTP-immunised mice (Figure 3.11 (a)). All mice had rapidly ascending parasitemia and symptoms typical of a lethal \textit{P. yoelii} infection, all reached a symptom score of 8 and were sacrificed. It was thus assumed that the strain had become lethal due to increased virulence by repeated passage. The survival rates for PfTCTP-immunised mice and PBS-immunised controls were identical, whilst some mice immunised with PyTCTP survived longer than controls. At 7 dpi, 50% of PyTCTP-immunised mice were surviving, compared with 17% of PBS-immunised controls. At 8 dpi, all control mice had reached a
symptom score of 8, whereas one PyTCTP-immunised mouse survived until 10 dpi. The average survival time of the TCTP-immunised groups was compared to the PBS controls. The differences were non-significant (Table 3.2).

TCTP immunisation did not increase survival time in mice challenged with *P. yoelii* YM. As shown in Figure 3.11 (b), at 7 dpi 80% of PBS-immunised controls were surviving compared with 50% of PyTCTP-immunised mice and 17% of PfTCTP-immunised mice. All mice had reached a symptom score of 8 at 8 dpi. The average survival time of the TCTP-immunised groups was compared to the PBS controls. The difference in the PyTCTP-immunised group average survival time was not significantly different to the PBS controls, however the PfTCTP-immunised mice survived for significantly less time than the PBS group (Table 3.3).

Table 3.2: Average survival of mouse groups challenged with *P. yoelii* XNL

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Avg survival time</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>6.17</td>
<td>-</td>
</tr>
<tr>
<td>PyTCTP</td>
<td>7.17</td>
<td>0.129</td>
</tr>
<tr>
<td>PfTCTP</td>
<td>6.17</td>
<td>1</td>
</tr>
</tbody>
</table>

*Survival time compared with PBS control group

Table 3.3: Average survival of mouse groups challenged with *P. yoelii* XL

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Avg survival time</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>6.8</td>
<td>-</td>
</tr>
<tr>
<td>PyTCTP</td>
<td>6.5</td>
<td>0.343</td>
</tr>
<tr>
<td>PfTCTP</td>
<td>6.0</td>
<td>0.037</td>
</tr>
</tbody>
</table>

*Survival time compared with PBS control group
Figure 3.11: Survival of TCTP-immunised mice and controls following malaria challenge
(a) Survival of TCTP-immunised mice and PBS-immunised controls following challenge with *P. yoelii* XNL
(b) Survival of TCTP-immunised mice and PBS-immunised controls following challenge with *P. yoelii* YM
3.3.4.4 Comparison of parasitemia

No significant difference in parasitemia was observed between PBS controls and TCTP-immunised mice following either *P. yoelii* XNL or YM challenge. As shown in Figure 3.12 (a) and (b), large standard variations from the mean were generally observed for all groups.

A potentially interesting result was seen as the longest surviving mouse in the *P. yoelii* XNL trial (mouse 5 in PyTCTP #2) had a very low detectable parasitemia (<5%) until 7 dpi, when parasite levels rose to 25%. Parasite levels then continued to rise until 9 dpi, when the mouse reached a symptom score of 8 and was sacrificed. This mouse had the second-highest reciprocal antibody titre to PyTCTP of all individual mice (Figure 3.13 (b)). The two other longest surviving mice in the PyTCTP group #2, mouse 1 and 6, also had some of the highest reciprocal antibody titres to PyTCTP. However, mouse 4 in PyTCTP #2, which was the first mouse to succumb to *P. yoelii* XNL infection, also had high reciprocal titres to PyTCTP. A scatter plot of survival time vs. reciprocal titre for *P. yoelii* XNL-challenged control and PyTCTP-immunised mice was created, no significant correlation was observed (data not shown). There was also no significant correlation between reciprocal antibody titre and survival time in mice immunised with PfTCTP and challenged with *P. yoelii* XNL, or in mice immunised with PyTCTP or PfTCTP and challenged with *P. yoelii* YM.
Figure 3.12: Parasitemia in TCTP-immunised and control mice
(a) Parasitemia following challenge with *P. yoelii* XNL
(b) Parasitemia following challenge with *P. yoelii* YM
3.3.4.5 Analysis of antibody titres by ELISA

Pre-challenge TCTP reciprocal antibody titres were quantified by ELISA (method described in 2.8.2.7) with the following modifications and conditions: 96-well plates were coated with 2 µg/ml of PfTCTP or PbTCTP. Sera obtained from immunised mice were used as the primary antibody, with an initial dilution of 1:100 to 1:400. A goat-α-mouse IgG.HRP conjugate was used as a secondary antibody at a 1:5,000 dilution. The absorbance readings at 450 nm were plotted against the reciprocal serum dilution for pooled sera from the six vaccine groups, and for individual mice from the Py- and PfTCTP-immunised groups.

Sera from all TCTP-immunised mice, including those immunised with PfTCTP, bound more strongly to PyTCTP than PfTCTP (Figure 3.13 (a)). The average reciprocal titres for the pooled sera from PfTCTP groups #1 and #2 were 23,000 and 10,800 when tested for binding to PfTCTP, and 215,000 and 85,000 when tested for binding to PyTCTP. This could have been due to the fact that a different preparation of PfTCTP was used as an ELISA antigen than was used for immunisation. The PfTCTP used for ELISA was prepared using the alternative protein expression method described in section 3.3.2.9. This method produced less contaminant bands after IMAC, and so Mono-Q column purification was not performed. The two preparations gave similar banding patterns on SDS-PAGE. However, it is possible that some of the antibodies present in the polyclonal PfTCTP antisera were specific to epitopes only present in the initial Pf and PyTCTP preparations.

The average reciprocal titres for the pooled sera from PyTCTP groups #1 and #2 were 175,000 and 225,000 when tested for binding to PyTCTP and 9,100 and 5,800 when tested for binding to PfTCTP. Again, reciprocal titres were significantly higher to PyTCTP than to PfTCTP. Sera from PBS-immunised mice did not bind to PfTCTP or PyTCTP above background levels. The pooled sera from the PfTCTP-immunised mice from the 2002 vaccine trial (Taylor, 2002) was also included in these ELISAs, the average reciprocal titre was 14,800 against PyTCTP and 12,000 against PfTCTP.

As shown in Figure 3.13 (b), individual mouse reciprocal titres from PyTCTP groups #1 and #2 against PyTCTP ranged from 4,500 (mouse 3 PyTCTP #1) to 1,000,000 (mouse 6 PyTCTP #2). Individual mouse reciprocal titres from PfTCTP groups #1 and #2 against PfTCTP ranged from 5,000 (mouse 2 PfTCTP #1) to 127,500 (mouse 1 PfTCTP #1). The titration curves used to calculate each individual mouse
Chapter 3: Evaluation of *P. yoelii* and *P. falciparum* TCTP as malaria vaccines

reciprocal titre are included in Appendix 3.
Figure 3.13: Reciprocal titres of vaccine trial mice
(a) Reciprocal titres of pooled sera from immunised groups
(b) Reciprocal titres of individual mouse sera against homologous antigen
3.3.5 Recognition of *P. yoelii* TCTP by anti-TCTP antibodies present in immunised mouse sera

Western blot experiments were performed to assess the ability of the PyTCTP antiserum to recognise TCTP present in *P. yoelii* lysates. Parasitised blood taken from BALB/c mice was used to create *P. yoelii* YM lysates (method described in 2.9.2.4). Approximately 25 ng of recombinant PyTCTP was also included as a positive control. The total parasites obtained from the blood of one mouse with a parasitemia of 10% were lysed in a final volume of 50 µl; 20 µl and 10 µl of this lysate were separated by SDS-PAGE. The protein concentration of the parasite lysate could not be accurately quantified, due to the final lysis step being boiling for 10 min in reducing sample buffer. A western blot was performed with the following conditions: Antiserum from mouse 6 in the PyTCTP #2 vaccine group was used as a primary antibody at a 1:1,000 dilution. A goat-anti-mouse IgG.HRP conjugate was used as a secondary antibody at a 1:3,000 dilution.

Serum from mice immunised with PyTCTP could recognise TCTP present in *P. yoelii* YM parasite lysates, as shown in Figure 3.14. The recombinant PyTCTP separated at a higher molecular weight (~28 kDa) than the endogenous *P. yoelii* YM TCTP (~23 kDa), this is likely to be due to the presence of the hexa-histidine tag. A single band was detected in the malaria lysates, whereas TCTP breakdown products of around 12 kDa were detected in the recombinant PyTCTP preparation along with full-length protein.
Figure 3.14 Recognition of TCTP in *P. yoelii* YM lysates by PyTCTP anti-serum

Lane 1: ~25 ng recombinant PyTCTP; Lane 2: SeeBluePlus2 marker; Lanes 3 and 4: 20 µl *P. yoelii* YM lysate; Lane 5: 10 µl *P. yoelii* YM lysate
3.4 Discussion

Both Pf and PyTCTP were successfully expressed and purified from yeast. When loaded together on SDS-PAGE, PfTCTP was observed to migrate at a lower molecular weight than PyTCTP. The two proteins share a high degree of sequence similarity, are the same length, and were both cloned into the pYEULCBX vector in an identical manner, so the lower observed size of PfTCTP was unexpected. TCTP is generally acknowledged to migrate more slowly on SDS-PAGE than expected, as the theoretical mass of the protein is 19.5 kDa and several reports have observed TCTP migrating above 25 kDa (e.g. Bhisutthibhan et al., 1998; Gnanasekar et al., 2002; Rao et al., 2002). This is thought to be due to the known calcium-binding capacity of TCTP, as calcium-binding proteins are known to run abnormally on SDS-PAGE, and the addition of 1 mM Ca\(^{2+}\) caused faster migration of human TCTP (Sanchez et al., 1997). It is possible that Py and PfTCTP differ in their calcium-binding capacity, resulting in the size differences observed on SDS-PAGE.

Both Pf and PyTCTP were subject to substantial proteolysis during the purification procedure, as was previously observed during PfTCTP purification prior to the 2002 vaccine trial (Taylor, 2002). As demonstrated by SDS-PAGE, lower molecular-weight bands were present at all stages of TCTP purification. Some of these smaller bands were detected by western blot using anti-TCTP and anti-penta-His antibodies, and N-terminal sequencing demonstrated that full-length PyTCTP and an N-terminal truncate (52-171) were the major products present after anion-exchange chromatography. Therefore, at least some of the smaller bands present at all stages of purification were due to TCTP proteolysis.

Proteolysis of both PyTCTP and PfTCTP was also observed prior to purification. TCTP expression was induced in yeast cultures for 16 hours, and yeast cell pellets were boiled in sample buffer prior to SDS-PAGE. Western blots using both anti-His and anti-TCTP antibodies detected bands corresponding to full-length TCTP (26 kDa), as well as smaller molecular weight bands.

To assess if TCTP proteolysis was caused, in part, by the lengthy expression induction period (between 16 and 40 hours), an alternate method of protein expression was assessed. In this method, endogenous yeast protease activity was minimised by the replacement of culture medium, prior to induction of recombinant protein expression for one hour. Reduced protein breakdown and improved protein yield were observed in this
Chapter 3: Evaluation of *P. yoelii* and *P. falciparum* TCTP as malaria vaccines

The alternate expression method also used vortexing with glass beads for cell lysis in place of homogenisation, however it is unlikely that this alteration improved protein yield, as cell lysis was deemed to be less efficient by microscopic analysis. As the alternate expression method was not assessed until the completion of the vaccine trial, mice were immunised with TCTP preparations containing a substantial amount of breakdown products. PfTCTP with a substantial amount of degradation was also used in the 2002 vaccine trial. These protein preparations were able to induce a strong humoral response, as demonstrated by ELISA analysis.

It was assumed that the breakdown products were newly formed during each purification stage, as they were expected to elute separately from anion-exchange chromatography due to charge differences. However, self-interaction has previously been reported for rat TCTP, involving the amino acid residues 126-172 (Yoon *et al.*, 2000). As sequence analysis of the major breakdown product of PyTCTP after Mono-Q purification determined that it was an N-terminal 52-171 truncate, it is possible that this product could interact with full-length PyTCTP. To assess interaction, PyTCTP and PfTCTP samples were subjected to SDS-PAGE under reducing and non-reducing conditions. Some self-interaction of TCTP was observed, as bands of ~35 kDa and ~53 kDa were present under non-reducing conditions. Additionally, similar amounts of smaller proteins (16 and 12 kDa) were present in both reduced and non-reduced samples. This observed self-interaction suggests that under non-reducing conditions, e.g. during IMAC, proteolytic products could have co-eluted with full-length TCTP. However, it is not known if TCTP self-interaction would occur under the reducing conditions used for Mono-Q chromatography. The presence of similar amounts of breakdown products in reduced and non-reduced samples indicated that TCTP was also subject to continual proteolysis, despite the presence of protease inhibitors.

The further breakdown of TCTP during storage meant that a new preparation of PfTCTP was required to complete the ELISA experiments. An alternative method of expression and purification was used to prepare the PfTCTP. By ELISA analysis, a much greater antibody response was induced to PyTCTP than to PfTCTP. Additionally, sera from mice immunised with PfTCTP reacted more strongly to the PyTCTP than to homologous antigen. It is possible that some of the antibodies present in the polyclonal PfTCTP antisera were specific to epitopes only present the initial Pf and PyTCTP preparations, which could explain the higher titre generated to PyTCTP antigen. This hypothesis is supported by the relatively low titre of PyTCTP-immunised group sera when reacted with the alternate PfTCTP preparation. Western blot experiments using *P.
Chapter 3: Evaluation of *P. yoelii* and *P. falciparum* TCTP as malaria vaccines

*yoelii* YM lysates demonstrated that PyTCTP antisera could recognise malarial TCTP, and so the protective effect of TCTP immunisation could be assessed with some confidence.

Although a significant humoral response was generated to TCTP, no protection against *P. yoelii* infection was observed following TCTP immunisation. No significant differences were observed in parasitemia or survival time. Unlike in the previous TCTP vaccine trial (Taylor, 2002), there was no delay in the establishment of infection. The reasons for this are unknown. The lack of protective effect is unlikely to be due to insufficient anti-TCTP antibody production. Comparisons between ELISA experiments are difficult, due to variations in antigen preparation and in the reagents used. For this reason, the pooled serum from the 2002 trial PfTCTP vaccine group was included as a sample in the ELISAs, a reciprocal titre of 12,000 was obtained. The reciprocal titres of the pooled sera in mouse groups immunised with PfTCTP in the trial described in this chapter were similar or higher than the 2002 group sera (11,000 and 23,000), reciprocal titres of pooled sera in mouse groups immunised with PyTCTP were higher again (215,000 and 85,000).

As compared with the previous trial, the lack of protective effect following TCTP immunisation was unlikely to be due to the malarial strains used for challenge. The *P. yoelii* YM strain used was identical to that used in the 2002 trial, except that it had undergone one extra round of passage in mice. This may have increased the virulence of the parasite; however control mice survived at least as long as in the previous experiment. Whilst the second malaria strain used for challenge produced unexpected results, in that the strain was uniformly lethal, this did not render that challenge invalid as the protective effect of TCTP immunisation could still be assessed. It is possible that the strain obtained was actually a lethal strain of *P. yoelii*, such as XL or YM, or that a previously non-lethal strain of *P. yoelii* 17XNL had become lethal due to repeated passage in mice. In a typical *P. yoelii* 17XNL infection in BALB/c mice, parasitemia rises to a peak of around 20% at about ten dpi, and then mice rapidly clear the parasite load (eg Li et al., 2001a; Ma et al., 2007; Taylor et al., 1986). The strain used in this vaccine trial produced parasitemia that rapidly rose to between 55 and 75% at six dpi, with symptoms typical of a lethal infection. Additionally, it has previously been reported that non-lethal strains of *P. yoelii* preferentially invade reticulocytes, whereas lethal strains invade all erythrocytes (Mota et al., 2001). In the challenge experiment described, the strain was observed to invade both immature and mature erythrocytes.
TCTP was expressed in *S. cerevisiae* due to the previous protective effect observed using yeast-derived PfTCTP for immunisation. Additionally, it was thought that expressing TCTP in a eukaryotic system rather than in *E. coli* may result a protein with a conformation more similar to the native malarial TCTP. Although no protective effect was observed, the ability of antibodies generated to yeast-derived PyTCTP to recognise TCTP in *P. yoelii* YM lysates was demonstrated.

Yeast-derived PfTCTP has been reported to induce histamine release *in vitro* in a subset of allergic donor basophils (MacDonald et al., 2001). Additionally, filarial TCTP was observed to induce eosinophil infiltration *in vivo* in C57BL/6 mice sensitised by OVA injection (Gnanasekar et al., 2002; Rao et al., 2002). An experiment performed by our group assessed the induction of histamine release by TCTP injection *in vivo* in BALB/c mice, no elevations in histamine levels were observed (Taylor, 2002). It is possible that TCTP induces a localised increase in histamine that does not significantly raise systemic serum levels. During the establishment of a malarial infection, a localised release of histamine in response to TCTP may assist the parasite by increasing vasodilation, and/or by increasing the expression of host endothelial adhesion factors, as discussed in section 1.6.1. It is also possible that the labile histamine was degraded during serum collection and lyophilization.

It was thought that the expression of TCTP in a bacterial system might result in greater yields and more stable protein, due to a reduced amount of protease activity. However, it was not known if *E. coli*-derived TCTP would induce a protective immune response. TCTP from different host species has been expressed in *E. coli*, yeast, and insect cells. Although the overall function of TCTP has not been ascertained, protein with demonstrated histamine-releasing ability has been obtained from each of these expression systems. It is possible that TCTP expressed in *E. coli* may give better protection against malarial challenge than when expressed in yeast, it is also possible that the two expression systems would result in functionally equivalent proteins or that yeast-derived TCTP may be immunologically superior.

The protective effect of other malarial vaccine candidates expressed in yeast or *E. coli* systems has been compared, with varying results depending on the antigen tested. Less protection and significantly lower antibody titres were obtained using yeast-derived MSP4/5 as compared to that expressed in *E. coli*. The authors discounted the presence of endotoxin as the reason for the increased titres to *E. coli*-derived MSP4/5, as they performed another trial performed using endotoxin-resistant C3H/HeJ mice and
found identical results (Kedzierski et al., 2002a). Mice immunised with *E. coli*-derived MSP4/5 produced specific antibodies that were mainly IgG2a and IgG2b isotypes, whereas mice immunised with yeast-derived protein produced mainly IgG1 antibodies (Kedzierski et al., 2001). The proportion of antibody isotypes in the anti-TCTP antisera has not been studied. In other trials, immunisation with MSP1$_{19}$ expressed in yeast gave greater protection than when produced in *E. coli* (Hirunpetcharat et al., 1997), whereas expression of recombinant AMA1 in yeast or *E. coli* resulted in immunologically and functionally equivalent proteins (Giersing et al., 2005).

In conclusion, the reasons for the lack of protective effect observed following TCTP immunisation in this trial, as compared to our previous trial, are unknown. Higher levels of specific antibodies were generated by TCTP immunisation compared with previously, and the recognition of malarial TCTP by these antibodies was demonstrated. Although the protein preparations used for immunisation contained a significant amount of breakdown products, this was also the case in the initial vaccine trial. The *P. yoelii* YM strain used produced similar infections in the control mice in both challenge experiments. The protective effect observed in the initial vaccine trial was mild, with significant differences observed only in the early stages of infection. Due to this, it may be that minor variations between the two trials, e.g. in methodology or in the source of the experimental animals, would be sufficient to explain the lack of protection seen in the second trial. If this is the case, it does not augur well for the potential of TCTP as a malarial vaccine, as the human parasite is known to rapidly mutate to avoid host defences.
Chapter 4: Evaluation of P. yoelii and P. berghei TCTP as potential malaria vaccines using three malarial species

4.1 Introduction

Prior to the experiments outlined in this chapter, two vaccine trials using malarial TCTP had been performed. Although a significant protective effect following P. falciparum (Pf) TCTP immunisation was observed in the first trial (Taylor, 2002), (poster presentation at the Molecular Approaches to Malaria Conference, Lorne, Australia 2004) no protection was conferred by P. yoelii (Py) or Pf TCTP in the second vaccine trial (described in Chapter 3). This chapter describes a third vaccine trial of TCTP, using both Py and P. berghei (Pb) TCTP as antigens. P. yoelii is commonly used in mouse immunisation and challenge models, whereas P. berghei is commonly used as a model of human cerebral malaria (CM), a severe complication of P. falciparum infection (Li et al., 2001a). The susceptibility of mice to CM is strain-specific. Around 80% of C57BL/6 mice develop CM following P. berghei ANKA infection, whereas strains such as BALB/c do not develop cerebral symptoms. Non-CM-susceptible strains of mice succumb to infection 3-4 weeks after infection due to hyperparasitemia and anaemia (Lou et al., 2001).

As discussed in section 1.2.5, inflammatory molecules, especially TNF-α and IFN-γ, play a large role in the pathology of the mouse model of CM induced by P. berghei infection. Evidence exists that PfTCTP induces histamine release from human immune cells in vitro, and is secreted from parasites during infection (MacDonald et al., 2001). Therefore, it is possible that this molecule may contribute to host inflammatory responses during malarial infection. As the malarial TCTPs are highly conserved (Figure 4.1), it is likely that Py and PbTCTP have similar extracellular activities. Anti-TCTP antibodies could potentially neutralise the extracellular inflammatory actions of TCTP, and might confer significant protection against CM. For this reason, PbTCTP was used as an antigen in this vaccine trial, along with PyTCTP.

Following immunisation with either Py or PbTCTP, mice were challenged with P. yoelii YM, P. berghei ANKA, or P. chabaudi chabaudi AS. P. chabaudi AS was included as a challenge strain to provide a non-lethal malaria model. P. chabaudi (Pc) TCTP was not cloned and expressed for use in this vaccine trial, as the sequence shares
Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as malaria vaccines

98% amino acid identity with PyTCTP. Instead, mice challenged with *P.c. chabaudi* were immunised with PyTCTP. PbTCTP also shares 98% identity with PyTCTP, but was cloned and expressed for use in the vaccine trial. This decision was made due to the presence of a non-conservative substitution at residue 27 in PbTCTP from proline to alanine. A proline is present at this position in the TCTP of *P. falciparum, P. yoelii,* and *P. chabaudi,* while an alanine is present at this residue in the TCTP of *P. vivax* and the non-human primate malaria *P. knowlesi.* It is generally accepted that proline residues may introduce conformational changes in polypeptide chains (eg Cordes *et al.*, 2002), due to possessing inflexible phi-psi bond angles. The lack of a proline residue at position 25 may alter the structure of PbTCTP as compared with the structure of PyTCTP.

Initially, mice in this vaccine trial were to be immunised with TCTP expressed in *S. cerevisiae.* Both proteins were cloned into the pYEULCBX vector and were expressed using the alternate expression method described in section 3.3.2.6. This experiment was performed numerous times with various optimisation methods, including variations in induction time, column purification, and increased additions of protease inhibitors. The major problems encountered were low protein yield and substantial proteolysis. Due to the problems encountered using the *S. cerevisiae* expression system, it was decided to express Py and PbTCTP in *E. coli* using the pRSETA vector (*Figure 4.2*). TCTPs from numerous organisms have been expressed in bacterial systems, and filarial and schistosome TCTP with histamine-inducing activity has been expressed using pRSETA (Gnanasekar *et al.*, 2002; Rao *et al.*, 2002), indicating that a eukaryotic expression system is not required to obtain functional protein.
Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as malaria vaccines

The aims of the experiments outlined in this chapter were:

1: To clone the PyTCTP and PbTCTP coding sequences into pRSETA vectors, and to express the His-tagged recombinant proteins in *E. coli* BL21.

2: To use the recombinant proteins as vaccines in BALB/c and C57BL/6 mouse strains, to assess the protective effect of TCTP immunisation following challenge with *P. yoelii* YM, *P. c. chabaudi* AS, or *P. berghei* ANKA.

3: To determine if TCTP antisera generated to *E. coli*-derived protein could recognise TCTP present in malarial lysates.

4: To assess histamine release in response to PyTCTP and PbTCTP *in vitro* in the rat basophilic leukaemia cell line RBL-2H3.
Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as malaria vaccines

**Figure 4.1: Alignment of *P. yoelii, P. berghei, P. chabaudi* and *P. falciparum* TCTP amino acid sequences**

<table>
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<td>MKVYKDIPTNDEVCSYSIQEDPGNFEFREIAFEVKSNKRIGNDDYGIADNSEDAGVG</td>
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<td><em>P. chabaudi</em></td>
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<td>---------------N-----------------E-</td>
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<tr>
<td><em>P. chabaudi</em></td>
<td>---------------A-----------------I-----------------Y-KVA-Y------------------E-</td>
</tr>
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<td><em>P. falciparum</em></td>
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**Figure 4.2: pRSET vector diagram**

Reference:
Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as malaria vaccines

4.2 Materials and methods

For detailed information about the materials and methods utilised in this chapter, please refer to Chapter 2.

4.3 Results

4.3.1 Analysis of the modelled structure of PyTCTP and PbTCTP

Models of *P. yoelii* TCTP and *P. berghei* TCTP were constructed using the Swiss-Model program, with the solved crystal structure of TCTP from *P. knowlesi* used as the template in both cases. Despite the proline-alanine substitution at residue 27, the two TCTP models had the same topology, with no perturbation of the predicted 3D structure. However, as any true structural differences between *P. yoelii* and *P. berghei* TCTP could only be determined experimentally, using X-ray crystallography or nuclear magnetic resonance, both proteins were cloned and expressed for use in the vaccine trial.

4.3.2 Cloning of PyTCTP and PbTCTP into pRSETA vectors

4.3.2.1 Amplification of PbTCTP by PCR

The *P. yoelii* 5’ and 3’ primers (as described in section 3.3.1.1) were used to amplify *P. berghei* TCTP, as the DNA sequence of the two genes is identical in the regions where the two primers bind (*P. berghei* TCTP accession number XM_674443). The *P. berghei* TCTP gene contains no introns, and so the PCR product was amplified from *P. berghei* ANKA genomic DNA, obtained from Dr. Tania de Koning-Ward, The Walter and Eliza Hall Institute (WEHI), Melbourne, Australia. The *P. berghei* TCTP gene was amplified using Taq PCR (method described in 2.3.2.2.3) with 0.5 µg of genomic DNA per reaction. An annealing temperature of 55°C was used. The PCR products were subjected to 1.5% agarose gel electrophoresis (2.3.2.5), a single band of 516 bp was obtained (Figure 4.3 (a)).

4.3.2.2 Ligation of PyTCTP and PbTCTP into pCR2.1 and pRSETA vectors

Fresh PbTCTP PCR product obtained was ligated into the pCR2.1 vector as per manufacturer’s instructions (2.3.2.7.1). Plasmid DNA from several resulting white colonies was isolated by minipreparation (2.3.2.1.1) and digested using *Bam*HI and *Pst*I (2.3.2.4) to confirm the presence of TCTP insert. Sequence analysis (2.3.2.2.6) was performed on clones with inserts of the predicted size using the 5’ and 3’ PyTCTP primers, plasmids with the correct insert were designated pCR.PbTCTP.
PyTCTP had previously been cloned into the pCR2.1 vector, as described in section 3.3.1.4. Approximately 2 µg of both pCR.PyTCTP and pCR.PbTCTP were digested using 10 U of BamHI and PstI. Approximately 4 µg of purified pRSETA vector was linearised using the same method. The completed digest reactions were separated by 1.5% w/v agarose gel electrophoresis at 100 V (Figure 4.3 (b)). Bands corresponding to the digested pRSETA vector and TCTP inserts were excised from the gel with a scalpel and DNA was purified using the Qiaquick Gel Extraction kit. Ligation reactions were prepared using 100 ng of digested pRSETA vector and the appropriate amount of insert for a 3:1 insert:vector ratio (as per method in 2.3.2.7.2).

4.3.2.3 Transformation and analysis of pRSETA.PyTCTP and pRSETA.PbTCTP

Completed ligation reactions were cleaned using the dextran purification method (2.3.2.7.3) and transformed into electrocompetent E. coli DH5α (2.4.3.2). Plasmid DNA from resulting colonies was purified by minipreparation. To confirm the presence of the TCTP insert, plasmid DNA was digested using PstI and BamHI (Figure 4.3 (c) and (d)). Sequence analysis was performed on plasmid minipreparations with the correct-sized insert. Plasmids with the correct insert were named pRSETA.PyTCTP and pRSETA.PbTCTP.
Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as malaria vaccines

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PbTCTP

pRSETA

pCR2.1

TCTP
Figure 4.3 Cloning of PyTCTP and PbTCTP into pRSETA vectors
(a) Amplification of PbTCTP by PCR from *P. berghei* ANKA genomic DNA:
Lane 1: $\lambda/PstI$ marker; Lane 2: negative control; Lanes 3-5: TCTP amplified using *P. berghei* ANKA genomic DNA as template.
(b) Digestion of TCTPs and pRSETA with *BamH* and *PstI* prior to cloning:
Lane 1: $\lambda/PstI$ marker; Lanes 2 and 3: digestion of pCR.PbTCTP; Lane 4: digestion of pRSETA; Lanes 5 and 6: digestion of pCR.PbTCTP.
(c) and (d) Confirmation digests of pRSETA.PyTCTP and pRSETA.PbTCTP using *BamH* and *PstI*:
(c): Lane 1: $\lambda/PstI$; Lane 2: pRSETA.PyTCTP.
(d): Lane 1: $\lambda/PstI$; Lane 2: pRSETA.PbTCTP.
4.3.3 Py and PbTCTP expression in *E. coli* BL21

**4.3.3.1 Expression and purification of Py and PbTCTP from *E. coli* BL21**

Purified pRESTA.PyTCTP or pRSETA.PbTCTP vector DNA was transformed into the BL21 (DE3) strain of *E. coli* by electroporation. A single colony of transformed *E. coli* BL21 was used in an o/n culture of 10 ml of LB+Amp. Larger volumes of LB+Amp (typically 0.5 L) were inoculated with a 1/100 volume of the o/n culture, expression of TCTP was induced for 3 h by the addition of 1 mM IPTG once the OD$_{600nm}$ was ~0.5 (method described in 2.6.3.1). The cell pellets were resuspended in a 1/25 volume of lysis buffer, incubated at 4°C for 20 min, shaking, and then underwent four cycles of freeze/thaw. Ten units of DNase per 100 ml of original culture volume were added after the second freeze/thaw cycle, and cell pellets were incubated at 37°C for 15 min, shaking (2.6.3.2). Supernatants from lysed pellets were filtered (0.2 µM) prior to incubation with prepared Profinity resin (2.6.3.3.1).

Column purification was performed with a wash step using 20 column volumes (CV) of 5 mM imidazole wash buffer, followed by elution of His-tagged proteins with 5 CV of 0.25 M imidazole elution buffer (2.6.3.3.2). The eluates were collected as 1.5 ml fractions, 20 µl samples were analysed for the presence of TCTP by SDS-PAGE (2.6.7). As shown in **Figure 4.4 (a) and (b)**, a predominant band at around 13 kDa was observed in both the Pb and PyTCTP 5 mM imidazole eluate fractions but not in the 0.25 M imidazole eluate fractions. Additionally, a higher amount of protein was observed in the PbTCTP 5 mM imidazole eluate compared with the PyTCTP 5 mM imidazole eluate, this is likely due to the samples being taken at different wash stages. Py and PbTCTP of high purity were obtained in the 0.25 M imidazole eluates, as a single band at around 29 kDa was observed in both samples.
Figure 4.4: Purification on PyTCTP and PbTCTP from *E. coli* BL21 lysates using Profinity™ resin

(a) Purification of PbTCTP from BL21 *E. coli* using Profinity™ resin:
Lane 1: Mark12 marker; Lane 2: 5 mM imidazole eluate sample; lanes 3-6: fractions from the 0.25 M imidazole eluate.

(b) Purification of PyTCTP from BL21 *E. coli* using Profinity™ resin:
Lane 1: Mark12 marker; Lane 2: 5 mM imidazole eluate sample; Lanes 3-7: fractions from 0.25 M imidazole eluate.
4.3.3.2 Concentration and buffer exchange of Py and PbTCTP

Following SDS-PAGE analysis, fractions containing Py or PbTCTP were pooled, and the 0.25 M imidazole was replaced with PBS by buffer exchange (2.6.5.2). The final concentration of imidazole remaining after buffer exchange was between 10 µM and 30 µM. Two micrograms of PyTCTP and PbTCTP were analysed by SDS-PAGE to assess purity. As expected, a predominant band at ~29 kDa was observed for both proteins, as well as very faint lower molecular weight bands, most likely TCTP breakdown products (Figure 4.5 (a)). *E. coli*-derived TCTP was stored at -70°C in aliquots. No degradation was observed over time when aliquots were thawed for use in the vaccine trial.

4.3.3.3 Western blots of Py and PbTCTP

The recognition of *E. coli*-derived TCTP (EcTCTP) by sera from mice immunised with yeast-derived PyTCTP (ScPyTCTP) was assessed. Approximately 1 µg of Py and PbTCTP in PBS was separated by SDS-PAGE and transferred to a nitrocellulose membrane for Western blotting (2.6.8). The primary antibody used was the high reciprocal titre (1,000,000) PyTCTP antisera obtained from a single mouse in the Chapter 3 vaccine trial (described in section 3.3.4.5). The primary antibody was used at a 1:1,000 dilution; the secondary antibody used was a 1:3,000 dilution of a goat-anti-mouse IgG.HRP. The antisera raised against ScPyTCTP could detect a strong band at ~29 kDa in EcPyTCTP and EcPbTCTP, small amounts of lower molecular-weight breakdown products were also detected for both Py and PbTCTP (Figure 4.5 (b)).
Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as malaria vaccines

Figure 4.5: Western blot of Py and PbTCTP

Western blot was probed with antisera obtained from mice immunised with yeast-derived PyTCTP.

(a) Coomassie-stained gel of Py and PbTCTP after buffer exchange to PBS: Lane 1: SeeBluePlus2 Marker; Lane 2: 1 µg PyTCTP; Lane 3: 1 µg PbTCTP

(b) Western blot of Py and PbTCTP using anti-ScPyTCTP antiserum: Lane 1: SeeBluePlus2 Marker; Lane 2: 1 µg PyTCTP; Lane 3: 1 µg PbTCTP
**Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as malaria vaccines**

### 4.3.4 Immunisation of mice with Py or PbTCTP

#### 4.3.4.1 Preparation of vaccines, mouse immunisation groups and schedule

Mice were immunised either with PBS (negative control) or with 25 µg of PyTCTP or PbTCTP in PBS. The initial immunisation was emulsified in Freund’s complete adjuvant; subsequent immunisations were emulsified in Freund’s incomplete adjuvant. Equal volumes of PBS or TCTP in PBS at 0.5 mg/ml concentrations were combined with Freund’s adjuvant, emulsions were made using two glass syringes connected with a stopcock (method described in 2.8.2.1).

Eight groups of six mice were used in the vaccine trial, two groups of C57BL/6 mice and six groups of BALB/c mice. All mice were female and were aged six to eight weeks at the commencement of the trial. One C57BL/6 group and three BALB/c groups received immunisations of the PBS control, two groups of BALB/c mice received immunisations of 25 µg PyTCTP, and one BALB/c and one C57BL/6 group received immunisations of 25 µg PbTCTP. Three i.p. immunisations were given, with a three-week interval between immunisations (2.8.2.2). Mice were challenged with 1 X 10⁵ *P. yoelii* YM, *P. berghei* ANKA, or *P. c. chabaudi* AS parasites within three weeks of the final immunisation (2.7.2.4). A summary of mouse vaccine and challenge groups is shown in Table 4.1. Some BALB/c mice in TCTP and PBS groups had slightly rough coats and were mildly inactive on the day following immunisations (symptom score of 1, as measured using the method described in 2.8.2.4). The C57BL/6 mice had no noticeable symptoms.

#### Table 4.1: Mouse immunisation groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Mouse strain</th>
<th>Immunisation</th>
<th>Challenge</th>
<th>No. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BALB/c</td>
<td>PBS in adjuvant</td>
<td><em>P. c. chabaudi</em> AS</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>BALB/c</td>
<td>&quot;</td>
<td><em>P. yoelii</em> YM</td>
<td>&quot;</td>
</tr>
<tr>
<td>3</td>
<td>BALB/c</td>
<td>&quot;</td>
<td><em>P. berghei</em> ANKA</td>
<td>&quot;</td>
</tr>
<tr>
<td>4</td>
<td>C57BL/6</td>
<td>&quot;</td>
<td><em>P. berghei</em> ANKA</td>
<td>&quot;</td>
</tr>
<tr>
<td>5</td>
<td>BALB/c</td>
<td>PyTCTP in adjuvant</td>
<td><em>P. c. chabaudi</em> AS</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>BALB/c</td>
<td>&quot;</td>
<td><em>P. yoelii</em> YM</td>
<td>&quot;</td>
</tr>
<tr>
<td>7</td>
<td>BALB/c</td>
<td>PbTCTP in adjuvant</td>
<td><em>P. berghei</em> ANKA</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>C57BL/6</td>
<td>&quot;</td>
<td><em>P. berghei</em> ANKA</td>
<td>&quot;</td>
</tr>
</tbody>
</table>
4.3.4.2 Analysis of antibody titres

To assess pre-challenge antibody titres, serum was collected from mice prior to the first immunisation and one day prior to parasite challenge (2.8.2.3). Levels of anti-PyTCTP or anti-PbTCTP antibodies were quantified by ELISA (2.8.2.7) with the following modifications and conditions: 96-well plates were coated with 2 µg/ml of PyTCTP or PbTCTP. Sera obtained from immunised mice were used as primary antibody, with an initial dilution of 1:100 to 1:4,000. Goat-α-mouse IgG.HRP conjugate was used as a secondary antibody at a 1:5,000 dilution. Pooled sera from mice in each of the eight vaccine groups were tested for binding against PyTCTP and PbTCTP antigens. Additionally, serum from individual mice in the PyTCTP- and PbTCTP-immunised groups (groups 5-8) was tested for binding against homologous antigen. The absorbance readings at 450 nm were plotted against the reciprocal serum dilution.

All PBS-immunised controls had titres at background levels, and pre-immunisation sera did not react with TCTP over background levels (data not shown). Reciprocal titre results were similar for the pre-challenge sera from immunised mice (groups 5-8) when binding against PyTCTP and PbTCTP antigen was tested, indicating that the two proteins are immunologically cross-reactive, as expected. The reciprocal titres obtained from the TCTP-immunised groups against homologous and heterologous TCTP antigen are displayed in Table 4.2. C57Bl/6 mice produced far lower reciprocal anti-TCTP titres than the BALB/c mice. This is in contrast to other published reports, in which similar IgG antibody responses have been observed in C57BL/6 and BALB/C mice immunised with recombinant malarial protein in Freund's adjuvant. For example, a trial of a GST-MSP1 fusion protein in which mice were immunised by i.p. injection three times over six weeks, high levels of IgG antibodies were generated in both mouse strains (Ahlborg et al., 2002). In a vaccine trial of MSP-2, similar reciprocal antibody titres were obtained in both C57BL/6 and BALB/C mice (Lougovskoi et al., 1999). The reasons for the much lower antibody responses observed following TCTP immunisation in C57BL/6 mice in this trial are unknown.

Pooled antiserum from a mouse group immunised with yeast-derived PyTCTP (PyT #2, as described in 3.3.4.5) was also analysed in this ELISA experiment. The reciprocal titre of the pooled antiserum from this group was 83,000 against EcPyTCTP, compared with 85,000 against ScPyTCTP, indicating that the proteins were immunologically similar (results not shown). The titration curves used to calculate reciprocal titre are included in Appendix 3.
Table 4.2 Reciprocal titres of pooled TCTP sera to homologous and heterologous antigen

<table>
<thead>
<tr>
<th>Group</th>
<th>Mouse strain</th>
<th>Immunisation</th>
<th>Reciprocal titre against PyTCTP</th>
<th>Reciprocal titre against PbTCTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>BALB/c</td>
<td>PyTCTP</td>
<td>63,000</td>
<td>81,000</td>
</tr>
<tr>
<td>6</td>
<td>BALB/c</td>
<td>PyTCTP</td>
<td>233,000</td>
<td>295,000</td>
</tr>
<tr>
<td>7</td>
<td>BALB/c</td>
<td>PbTCTP</td>
<td>138,000</td>
<td>240,000</td>
</tr>
<tr>
<td>8</td>
<td>C57BL/6</td>
<td>PbTCTP</td>
<td>365</td>
<td>1,650</td>
</tr>
</tbody>
</table>

4.3.5 Challenge and monitoring of immunised mice

4.3.5.1 Assessment of parasite strains in pre-challenge trial

Due to the unexpected behaviour of the *P. yoelii* XNL strain used in the trial described in Chapter 3, the *P. berghei* ANKA and *P. c. chabaudi* parasite strains were trialled in mice before commencement of the vaccine trial. Stabilates of both of these strains were obtained from the laboratory of Prof. Alan Cowman, WEHI, Parkville, Australia. Two BALB/c and two C57Bl/6 mice were infected with 100 µl of *P. berghei* ANKA stabilate by i.p. injection. Another two BALB/c mice were infected with 100 µl of *P. c. chabaudi* AS stabilate by i.p. injection. Parasitemia was monitored from 2 d.p.i., and malarial symptoms were closely monitored for the duration of the infections.

The general pattern of infection and disease progression was as expected for each of the parasite strains assessed. However, in BALB/c mice infected with *P. berghei* ANKA, disease symptoms were observed at 6 dpi, when mice had low parasitemia (6 to 8%). At this time, mice developed symptoms of mild respiratory distress, had slightly rough coats, and were mildly inactive. The symptom score reached was 4-5. The mice recovered from these symptoms by 8 dpi, and parasite levels continued to increase without obvious malarial symptoms being displayed until 14 dpi, at a parasitemia of 42%.

In the mice given *P. c. chabaudi* AS, the infection progression was basically as expected, however the peak parasitemia (~50%) was higher than generally reported (e.g. Hensmann *et al.*, 2004; Li *et al.*, 2001a). One mouse had a peak parasitemia of 46% on two consecutive days. The day after this extended peak, the parasitemia had decreased to 18%, however the mouse reached a symptom score of 8 and was culled. The high
symptom score was most likely due to severe anaemia caused by the extended peak parasitemia, compared to the rapid parasite clearance observed in the other *P.c. chabaudi*-infected mouse.

### 4.3.5.2 Malaria challenge of immunised mice

Two passage mice were used for preparation of each of the parasite strains prior to challenge. BALB/c mice were used for passage of *P. yoelii* YM (obtained from the laboratory of Prof. Ross Coppel, Department of Microbiology, Monash University, Clayton, Australia), *P. berghei* ANKA and *P.c. chabaudi* AS. C57Bl/6 mice were also used for passage of *P. berghei* ANKA. All mice received an i.p. injection of 100 µl of thawed stabilate and parasitemia was monitored daily starting at two dpi (2.7.2.3).

Malaria-infected blood of known parasitemia taken from passage mice was used to challenge TCTP-immunised mice and controls (2.7.2.4). Thin blood smears were prepared and Giemsa-stained (2.7.2.5) for quantification of parasite numbers in the morning (prior to 10 am) daily, starting at two d.p.i (one d.p.i. for mice challenged with *P. yoelii* YM). Mice were monitored for the development of malaria symptoms as per the method described in 2.8.2.4.
4.3.6 Trial results in PyTCTP-immunised mice challenged with *P. c. chabaudi* AS

4.3.6.1 Parasitemia and infection progression in control and immunised mice

A delay in infection progression was observed in PyTCTP-immunised mice, as a significant reduction (p<0.05) in mean parasitemia compared with PBS-immunised controls was observed at each observation point from 4 dpi until 9 dpi, when parasite levels peaked (Table 4.3). The delay observed was similar to that seen in the vaccine trial of PfTCTP using a *P. yoelii* YM challenge model (Taylor, 2002). A reduced mean peak parasitemia was also observed for PyTCTP-immunised mice (32.9 at 9 dpi) compared with PBS controls (43.6 at 8 dpi). The mean parasitemia for control and TCTP-immunised mice at each dpi is depicted in Figure 4.6 (a).

Variation was observed in the peak parasitemia between individual mice in the control and TCTP-immunised groups. The peak parasitemia ranged from 31.2% (mouse #6) to 61.6% (mouse #3) in the PBS controls and from 22.6% (mouse #30) to 63.2% (mouse #25) in the PyTCTP-immunised mice. A small recrudescence was only observed in two control mice (#2 and #3) and one PyTCTP-immunised mouse (#26). The peak parasitemia in these recrudescences was between 0.6 and 2%. The graphs of individual parasitemia for PBS control and PyTCTP-immunised mice is depicted in Figure 4.6 (b) and (c).

Table 4.3: Mean parasitemia for PyTCTP-immunised mice and PBS controls challenged with *P. c. chabaudi* AS

<table>
<thead>
<tr>
<th>D.P.I.</th>
<th>PBS parasitemia</th>
<th>Standard deviation</th>
<th>PyTCTP parasitemia</th>
<th>Standard deviation</th>
<th>Statistical difference (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.13</td>
<td>0.24</td>
<td>0.07</td>
<td>0.16</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
<td>0.28</td>
<td>0.2</td>
<td>0.34</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>4</td>
<td>0.83</td>
<td>0.29</td>
<td>0.2</td>
<td>0.18</td>
<td>p&lt;0.002</td>
</tr>
<tr>
<td>5</td>
<td>2.07</td>
<td>0.97</td>
<td>1.03</td>
<td>0.39</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>6</td>
<td>9.9</td>
<td>2.66</td>
<td>3.13</td>
<td>1.85</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>7</td>
<td>21.9</td>
<td>8.53</td>
<td>9</td>
<td>6.19</td>
<td>p&lt;0.02</td>
</tr>
<tr>
<td>8</td>
<td>43.57</td>
<td>12.30</td>
<td>18.43</td>
<td>11.36</td>
<td>p&lt;0.005</td>
</tr>
<tr>
<td>9</td>
<td>33.02</td>
<td>7.48</td>
<td>32.9</td>
<td>19.34</td>
<td>p&gt;0.05</td>
</tr>
</tbody>
</table>
Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as malaria vaccines

(a)

![Graph showing parasitemia (% vs. D.P.I.) for PBS and PyTCTP](image)

- **Y-axis:** Parasitemia (%)
- **X-axis:** D.P.I.
- Lines for PBS and PyTCTP are shown, with error bars indicating variability.
Figure 4.6: Malarial progression in BALB/c mice challenged with *P. c. chabaudi* AS.
(a) Average parasitemia at each day post-infection (D.P.I.) for PyTCTP-immunised mice and PBS controls.
(b) Individual parasitemia at each D.P.I. for the PBS control group #1.
(c) Individual parasitemia at each D.P.I. for the PyTCTP-immunised group #5.
4.3.5.2 Survival of mice following P. c. chabaudi AS challenge

Although *P. c. chabaudi* AS is documented as a non-lethal malaria strain in BALB/c mice (e.g. Hensmann *et al.*, 2004; Lamb *et al.*, 2007), in this trial several *P. c. chabaudi*-infected mice reached symptom scores of eight and were euthanased. These high symptom scores were due to severe anaemia induced by a prolonged peak parasitemia. One PBS-control mouse (#4) required euthanasia at 9 dpi, it had reached a peak parasitemia of 56% at 8 dpi and was 40% at 9 dpi. Two PyTCTP-immunised mice were also euthanased; mouse #25 at 10 dpi, (peak parasitemia of 63% at 9 dpi, was 39% at 10 dpi) and mouse #27 at 11 dpi (peak parasitemia of 45% at 9 dpi, the parasitemia was 43% at 10 dpi and 21% at 11 dpi). In all of these mice the parasitemia had peaked before the highest symptom score was reached, but the parasite load was not cleared quickly, leading to severe malarial symptoms. The comparative survival rate of the PBS controls and the PyTCTP-immunised mice is depicted in Figure 4.7.

4.3.5.3 Individual reciprocal antibody titres in mice immunised with PyTCTP and challenged with *P. c. chabaudi* AS

Variations in reciprocal antibody titre were observed between individual mice in the PyTCTP-immunised group #5. As shown in Figure 4.8, mouse #25 and #27 had the highest pre-challenge reciprocal antibody titres against PyTCTP (98,000). Mouse #26 had the lowest reciprocal antibody titre to PyTCTP (18,000). No correlation was found between reciprocal antibody titre and peak parasitemia (data not shown). The titration curves used to calculate reciprocal titre are included in Appendix 3.
Figure 4.7: Cumulative survival of mice challenged with *P.c. chabaudi* AS.

Figure 4.8: Individual reciprocal antibody titres for PyTCTP-immunised mice prior to challenge with *P.c. chabaudi* AS.
4.3.7 Trial results in PyTCTP-immunised mice challenged with \( P.\ yoelii \) YM

4.3.7.1 Parasitemia and infection progression in control and immunised mice

The \( P.\ yoelii \) YM challenge infections progressed as expected in the majority of the mice, with mean parasitemia increasing rapidly (Figure 4.9 (a)) and mice developing severe malaria symptoms by seven dpi. A delay in infection progression was observed, as PyTCTP-immunised mice had significantly lower (\( p<0.05 \)) mean parasitemia compared with PBS-immunised controls at 3 and 4 dpi (Table 4.4).

In three mice (#7, a PBS control, and #34 and #35, two PyTCTP-immunised mice) the \( P.\ yoelii \) YM challenge did not progress as expected. These mice were eventually able to clear the parasites and survive the lethal challenge. The parasite levels in these mice presented as two waves of parasitemia, with the second increase in infection being almost completely limited to reticulocytes. There were differences seen in the progression of infection in the three mice able to clear the parasite load. In the PBS-immunised mouse, parasite levels were lower compared with other \( P.\ yoelii \) YM-challenged mice at all stages of infection. This mouse had an initial peak parasitemia of 21% at 6 dpi, followed by a second peak parasitemia of 28% at 10 dpi. In the two PyTCTP-immunised mice, parasitemia initially rose to a peak of 28.8% (#34) and 38.1% (#35) at 7 dpi, before a second peak parasitemia of 45.6% at 11 dpi (#34) and 36.2% and 13 dpi (#35). Parasitemia had decreased to undetectable levels in mouse #7 at 16 dpi, in mouse #34 at 18 dpi, and in mouse #35 at 19 dpi. Graphs of infection progression for each individual mouse challenged with \( P.\ yoelii \) YM are depicted in Figure 4.9 (b) and (c).

<table>
<thead>
<tr>
<th>D.P.I.</th>
<th>PBS</th>
<th>Standard deviation</th>
<th>PyTCTP</th>
<th>Standard deviation</th>
<th>Statistical difference (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.2</td>
<td>0.17</td>
<td>0.05</td>
<td>0.06</td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>3</td>
<td>1.383</td>
<td>0.64</td>
<td>0.517</td>
<td>0.35</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>4</td>
<td>9.968</td>
<td>4.07</td>
<td>4.812</td>
<td>2.37</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>5</td>
<td>29.11</td>
<td>13.20</td>
<td>22.53</td>
<td>8.31</td>
<td>P&gt;0.05</td>
</tr>
</tbody>
</table>
Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as malaria vaccines

(a)

![Graph showing parasitemia (%) over days for PBS and PyTCTP groups.](image)
Figure 4.9: Malarial progression in PBS controls and PyTCTP-immunised mice challenged with *P. yoelii* YM.
(a) Average parasitemia at each day post-infection for PyTCTP-immunised mice and PBS controls.
(b) Individual parasitemia at each dpi for the PBS control group #2.
(c) Individual parasitemia at each dpi for the PyTCTP-immunised group #6.
4.3.7.2 Survival of mice challenged with \textit{P. yoelii YM}

The expected mortality following \textit{P. yoelii YM} infection is 100\%, according to the literature (e.g. de Souza and Playfair, 1995; De Souza \textit{et al.}, 1997; Wykes \textit{et al.}, 2005). Using a different stabilate from the same source (Laboratory of Prof. Coppel, Department of Medicine, Monash University, Australia), we observed a 100\% mortality rate in both controls and in PfTCTP- and PyTCTP-immunised mice in both of the previous vaccine trials. Researchers in the group from which the \textit{P. yoelii YM} was sourced have also previously observed survival of a control mouse following \textit{P. yoelii YM} infection, in that instance the parasitemia reached 40\% on day 6 before parasite clearance (Kedzierski \textit{et al.}, 2000).

Due to the survival of one PBS control and two PyTCTP-immunised mice, the mortality rate following \textit{P. yoelii YM} infection was 83.33\% for PBS controls and 66.67\% for PyTCTP-immunised mice. In the mice that succumbed to the infection, the average time taken to reach a symptom score of 8 was 6.9 days for PBS controls and 7.1 days for PyTCTP-immunised mice, which was not significantly different \((p=0.124)\). The three mice that survived the infection were eventually sacrificed at 35 dpi, having been parasite-free for more than 14 days. A graph of comparative mortality for the controls and PyTCTP-immunised mice is depicted in Figure 4.10.

4.3.7.3 Individual reciprocal antibody titres of mice immunised with PyTCTP and challenged with \textit{P. yoelii YM}

In the PyTCTP-immunised mice of group 6, a large variation in reciprocal antibody titre was observed, from 41,000 for mouse #33 to 680,000 for mouse #31. The individual reciprocal antibody titres for the six mice in this group are given in Figure 4.11. The titration curves used to calculate reciprocal titre are included in Appendix 3. No correlation was observed between reciprocal antibody titre and disease severity. The serum from the control mouse (#7) that survived infection was tested to ensure there was no reactivity to PyTCTP. None was observed (result not shown).
Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as malaria vaccines

Figure 4.10: Cumulative survival of PyTCTP-immunised and PBS control mice.

Figure 4.11: Individual reciprocal antibody titres for PyTCTP-immunised mice prior to challenge with *P. yoelii* YM.
4.3.8 Trial results in BALB/c mice immunised with PbTCTP and challenged with *P. berghei* ANKA

4.3.8.1 Infection progression in control and immunised mice

No delay in parasitemia progression or malarial symptom development was observed for BALB/c mice infected with *P. berghei* ANKA, compared with PBS controls. Instead, all BALB/c mice infected with *P. berghei* ANKA developed severe malaria symptoms at relatively low parasitemia. Mice developed symptoms usually associated with severe malaria, including respiratory distress, inactivity and rough coats, during the early stages of infection. Some *P. berghei*-infected BALB/c mice first showed mild disease symptoms at five dpi, with rough coats. At this time, the parasitemia was between nine and 17% for controls and six and 19% for PbTCTP-immunised mice. At six dpi, some *P. berghei*-infected mice exhibited mild respiratory distress, with all PBS and PbTCTP-immunised mice exhibiting respiratory distress at seven dpi. Typically, respiratory distress is observed in malarial infections at high parasitemia (>50%), most likely as a result of severe anaemia. In the BALB/c mice infected with *P. berghei* ANKA, other symptoms of anaemia, including haemolytic urine, were not observed.

At seven dpi, parasitemia was between 12.5% and 25% for controls and between 11.5% and 15% for immunised mice. One PbTCTP-immunised mouse (#37) was found dead at eight dpi. The parasitemia for this mouse had been 15% on day seven. All remaining *P. berghei* ANKA-challenged mice reached a symptom score of eight at between eight and ten days post-infection and required euthanasia. The average time to reach a symptom score of eight was 8.8 dpi for PBS control mice and 8.6 dpi for PbTCTP-immunised mice, which was not significant (p=0.710). Graphs of averaged and individual parasitemia for PBS controls and PbTCTP-immunised mice are given in Figure 4.12 (a)-(c). The cumulative survival of PBS controls and PbTCTP-immunised mice is shown in Figure 4.13.

4.3.8.2 Individual reciprocal titres of BALB/c mice immunised with PbTCTP

As in the other immunised mouse groups, variation in reciprocal antibody titre was observed in the PbTCTP group seven. Reciprocal titres ranged from 110,000 for mouse #41 to 470,000 for mouse #37. The reciprocal titres for the individual mice in PbTCTP group #7 are given in Figure 4.14. The titration curves used to calculate reciprocal titre are included in Appendix 3.
Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as malaria vaccines

(a)

![Graph showing parasitemia (%) over time for PBS and PbTCTP groups. The x-axis represents days post-infection (D.P.I.), and the y-axis represents parasitemia (%). The graph shows a peak in parasitemia for both groups on Day 7, with PBS showing higher parasitemia compared to PbTCTP.](image-url)
Figure 4.12: Malarial progression in BALB/c mice challenged with *P. berghei* ANKA.

(a) Average parasitemia at each day post-infection for PbTCTP-immunised mice and PBS controls.

(b) Individual parasitemia at each dpi for the PBS control group #3.

(c) Individual parasitemia at each dpi for the PbTCTP-immunised group #7.
Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as malaria vaccines

Figure 4.13: Cumulative survival of BALB/c mice challenged with *P. berghei* ANKA

Figure 4.14: Individual reciprocal antibody titres for mice in PbTCTP group #7
4.3.9 Trial results in C57BL/6 mice immunised with PbTCTP and challenged with *P. berghei* ANKA

4.3.9.1 Infection progression and cerebral symptom development in control and immunised mice

The *P. berghei* ANKA infection progressed as expected in C57BL/6 mice, with all mice except one PBS control (mouse #20) developing cerebral malaria symptoms at relatively low parasitemia. No significant delay in malarial symptom progression and no significant difference in parasitemia were observed between PbTCTP-immunised mice and PBS controls. The average time of cerebral symptom onset was 6.6 dpi for PBS controls and 6.74 dpi for PbTCTP-immunised mice. There was no significant difference between the two groups (p=0.799). The parasitemia to the point of onset of cerebral symptoms is depicted in Figure 4.15. The cumulative survival of PbTCTP-immunised and PBS control mice is depicted in Figure 4.16.

The PBS control mouse that did not develop cerebral malaria reached a peak parasitemia of 52% at 17 days post-infection, and was culled at 19 dpi with symptoms of anaemia. It has previously been reported that CM occurs in less than 100% of C57BL/6 mice infected with *P. berghei* ANKA (eg Amani *et al*., 1998; Bagot *et al*., 2002; Pierrot *et al*., 2003; Renia *et al*., 2006b).

4.3.9.2 Individual reciprocal antibody titres in C57BL/6 mice immunised with PbTCTP

Variation in reciprocal antibody titre was observed in C57BL/6 mice immunised with PbTCTP. All reciprocal antibody titres were much lower than reciprocal titres observed in PbTCTP-immunised BALB/c mice, and two immunised mice (#44 and #46) did not produce detectable levels of PbTCTP antibodies. The highest reciprocal titre observed was 38,000 for mouse #48. The individual reciprocal titres for the mice in group #8 are given in Figure 4.17. The titration curves used to calculate reciprocal titre are included in Appendix 3.
Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as malaria vaccines

Figure 4.15: Malarial progression in C57BL/6 mice challenged with *P. berghei* ANKA

Figure 4.16: Survival of C57BL/6 mice challenged with *P. berghei* ANKA

Figure 4.17: Individual reciprocal antibody titres for mice in PbTCTP group #8
4.3.10 Recognition of *P. yoelii* and *P. berghei* TCTP in parasite lysates by anti-TCTP antibodies

The excess blood taken from mice used for passage of *P. yoelii* YM, *P. c. chabaudi* and *P. berghei* ANKA was used as a source of malarial protein. The total blood obtained from two passage mice was pooled and used a single sample for the *P. berghei* and *P. yoelii* lysates, the total blood from one mouse was used to create the *P. chabaudi* lysate. The detection of *S. cerevisiae*-derived (Sc) PfTCTP and PyTCTP by antisera raised to *E. coli*-derived (Ec) PyTCTP was also assessed. Proteins were separated by SDS-PAGE and Western blots were performed with the following modifications: the primary antibody used was a 1:500 dilution of antisera from either mouse #31 for PyTCTP detection or mouse #37 for PbTCTP detection. A Zymax Goat-anti-mouse IgG (H+L) alkaline phosphatase conjugate was used as a secondary antibody at a 1:2,000 dilution. A BCIP/NBT substrate was used in a 1:1 dilution in detection buffer.

The antiserum from mouse #31 was the highest reciprocal titre antiserum (680,000) of the PyTCTP-immunised mice. This antiserum could detect recombinant yeast-derived (Sc) PfTCTP and PyTCTP, as well as 10 and 100 ng of *E. coli*-derived (Ec) PyTCTP (Figure 4.18 Lanes 1, 2, 4, 5). Using the alkaline phosphatase detection system, multiple bands of both higher and lower molecular weights were detected in the two ScTCTP samples and in the 100 ng sample of EcPyTCTP. Additionally, the major band detected in the ScPfTCTP sample (Lane 1) was of a lower molecular weight than the other recombinant TCTPs. The reduced size of rPfTCTP on SDS-PAGE had previously been observed, as described in Chapter 3.

The αPyTCTP antiserum from mouse #31 could also detect very faint bands approximately corresponding in size to malarial TCTP (Figure 4.18 Lanes 6, 7, 8). Recombinant PyTCTP has previously been observed to resolve at a higher molecular weight than the TCTP present in *P. yoelii* lysate (see Figure 3.16), due to the presence of the N-terminal extension in the recombinant protein. A faint band corresponding in size to malarial TCTP was detected in the *P. chabaudi* lysate (Lane 8). Very faint, non-distinct bands were detected in the *P. yoelii* lysates (Lanes 6 and 7). Greater detection of malarial TCTP in *P. yoelii* lysates had previously been observed using antiserum raised against ScPyTCTP in a less sensitive detection system (Figure 3.16). This was most likely due to the higher titre of antisera previously used, or to variations in sample processing.
Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as malaria vaccines

The antiserum from mouse #37 was used to detect PbTCTP in malaria lysates made from BALB/c and C57BL/6 mice infected with *P. berghei* ANKA. This was the highest reciprocal titre antiserum (470,000) obtained from mice immunised with PbTCTP. In the blot depicted in **Figure 4.19**, higher and lower molecular weight bands were detected in the sample containing 100 ng of PbTCTP (Lane 1), and distinct bands corresponding to malarial TCTP were detected in *P. berghei* ANKA lysates as well as faint higher molecular-weight bands, possibly corresponding to TCTP multimers (Lanes 4 & 5). Much stronger bands were detected in *P. berghei* ANKA lysates compared with those from *P. yoelii* and *P. chabaudi* samples.

The reason for the variation in TCTP detection between malarial lysates is unknown. The blood used to create the *P. berghei* ANKA lysates did not contain a higher number of parasites. The method used for malarial protein extraction had a final purification stage of boiling the isolated, partially lysed parasites in reducing sample buffer, making total protein determination difficult. Alternate methods of malarial protein extraction were assessed but did not produce detectable levels of malarial TCTP as analysed by western blot.
Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as malaria vaccines

Figure 4.18: Recognition of PyTCTP in malarial lysates, and cross-reactivity of Sc- and Ec-TCTP

Western blot was probed with PyTCTP antisera from mouse #31.
Lane 1: 100 ng ScPyTCTP; Lane 2: 100 ng ScPfTCTP; Lane 3: SeeBluePlus2 marker; Lane 4: 10 ng EcPyTCTP; Lane 5: 100 ng EcPyTCTP; Lanes 6 and 7: *P. yoelii* lysates; Lane 8: *P. chabaudi* lysate

Figure 4.19: Recognition of PbTCTP in *P. berghei* lysates

Western blot was probed with PbTCTP antisera from mouse #37.
Lane 1: 100 ng EcPbTCTP; Lane 2: SeeBluePlus2 marker; Lane 4: *P. berghei* ANKA lysate from BALB/c mice; Lane 5: *P. berghei* ANKA lysate from C57BL/6 mice.
4.3.11 RBL-2H3 degranulation assays using malarial TCTP

The *E. coli*-derived PyTCTP and PbTCTP were used in degranulation assays using the rat basophilic leukaemia cell line RBL-2H3. The rationale for performing these degranulation assays was based on previous studies of the histamine-release inducing properties of TCTP from schistosome and filarial parasites (Gnanasekar *et al.*, 2002; Rao *et al.*, 2002). In these studies, TCTPs from the filarial parasites *B. malayi* and *W. bancrofti*, and the flat worm *S. mansoni*, were expressed as His-tagged recombinant proteins in *E. coli*, residual endotoxin was removed. Histamine release from RBL-2Hs cells induced by the recombinant parasite TCTPs was assessed using a commercial ELISA kit (Beckman-Coulter). The calcium ionophore 48/80 (Sigma, USA) was used as a positive control; an unrelated recombinant parasite protein was used as a negative control. Significant amounts of histamine (600 to 800 nM) were released after incubation with TCTP in a dose-dependent manner up to 20 µg/ml, however higher doses inhibited histamine release. The ionophore 48/80 also induced histamine release of around 800 nM, and spontaneous histamine release was 100 - 200 nM.

Histamine is labile and is difficult to measure directly, requiring the use of relatively expensive methods (e.g. ELISA kits). In contrast, assays that measure degranulation via the release of the much more stable β-hexosaminidase are relatively cheap to perform, and are commonly used (e.g. Chan *et al.*, 2007; Hoffmann *et al.*, 1999; Morikawa *et al.*, 2002; Pittertschatscher *et al.*, 2002; Tanaka *et al.*, 2006; Yamada *et al.*, 2007). β-hexosaminidase is stored in the secretory granules of mast cells and basophils along with histamine, and both substances are released upon degranulation. Therefore, it is generally accepted that β-hexosaminidase is a degranulation marker that can be used to indirectly assess histamine release (Morikawa *et al.*, 2002; Yamada *et al.*, 2007).

Prior to use in the degranulation assays, residual endotoxin was removed from Py- and PbTCTP protein preparations (4.3.3.2) using Detoxi-gel resin (method described in 2.6.3.4). Fractions were analysed for protein concentration and purity by Bradford assay and SDS-PAGE. The protein yield decreased around 50% following endotoxin removal, and protein purity was essentially unchanged for both PyTCTP and PbTCTP (results not shown).

Aya Taki (RMIT) expressed and purified PfTCTP using the *E. coli* pRSET expression system. SmpB (cloned from *Brachyspir a hyodysenteriae*) was expressed
and purified by Amber Mitton (RMIT). This was used in the degranulation assays as a negative control, as it had been expressed and purified in an identical manner to the TCTPs. Residual endotoxin was removed from PfTCTP and SmpB. The degranulation assays were performed by Aya Taki, in collaboration with Dr. Graham Mackay, at the Department of Pathology, Melbourne University, Parkville, Australia (Taki, 2007). The method is described briefly in section 2.6.9.

β-hexosaminidase release assays using RBL-2H3 cells generally include an incubation with IgE as the initial step. As a positive control, the antigen specific for this IgE is added, prompting degranulation. In the assays performed using TCTP, IgE specific to trinitrophenol (TNP) was used. Confluent cultures of RBL-2H3 cells were incubated with α-TNP IgE for 16 h, then stimulated with either ionomycin, PfTCTP, PbTCTP, PyTCTP or SmpB (negative control) at several protein concentrations ranging from 10 to 0.3 µg/ml. Two positive controls were used (TNP or ionomycin), and cells lysed with 0.1% Triton-X 100 were used to measure maximum degranulation.

No significant (p>0.05) degranulation was observed at any concentration of TCTP or SmpB. In a separate experiment, degranulation by TCTP following antigen enhancement was measured, as it was thought that TCTP may act as an incomplete secretagogue. RBL-2H3 cells were grown to confluency and were incubated with anti-TNP IgE. BSA-conjugated TNP at a concentration of 0.3 ng/ml was added to all wells, along with TCTP, ionomycin or SmpB at the same concentration range as previously tested. This concentration of TNP induced a low level of degranulation, meaning that any increased degranulation induced by TCTP could be measured. Cells stimulated with low levels of TNP had around 22% degranulation. The addition of malarial TCTP did not significantly increase degranulation above this baseline.
4.4 Discussion

The *E. coli* pRSET expression system gave higher TCTP yield and less proteolysis compared with the *S. cerevisiae* system previously used. Only a very small proportion of the TCTP produced in *E. coli* was present as lower molecular weight bands when analysed by SDS-PAGE, whereas a high proportion of lower molecular weight bands were consistently observed in TCTP samples expressed in yeast. The addition of protease inhibitors was not required for purification of TCTP from *E. coli*, whereas the addition of a protease inhibitor cocktail at each stage of TCTP purification from *S. cerevisiae* was required to obtain a sufficient proportion of full-length protein.

Antisera raised against yeast–derived (Sc) and *E. coli*-derived (Ec) PyTCTP were cross-reactive, as expected. EcPyTCTP and EcPbTCTP could be detected using antiserum from a mouse immunised with ScPyTCTP, and antiserum raised to EcPyTCTP could detect ScPfTCTP and ScPyTCTP. Using ELISA analysis, the reciprocal titre of the pooled antiserum from mice immunised with ScPyTCTP was 85,000 against homologous antigen, and was 83,000 against EcPyTCTP, indicating that the TCTPs expressed in yeast and bacterial systems were immunologically similar.

Antiserum raised against EcPyTCTP and EcPbTCTP could recognise TCTP present in malarial lysates of *P. yoelii* YM, *P. c. chabaudi* AS and *P. berghei* ANKA. The recognition of malarial TCTP by this antiserum was not as strong as when antiserum raised to ScPyTCTP was used (as shown in Figure 3.16, chapter 3). This is most likely due to the lower reciprocal titre of EcTCTP antiserum (680,000 compared with 1,000,000) and to variations in sample processing. As the protein extraction method used does not allow accurate quantification of malarial protein, due to a final step of boiling samples in SDS-PAGE sample buffer, variation between samples is inevitable. The variations in TCTP detection between the different malarial samples could also possibly indicate that the expression of TCTP varies in different parasite life stages or between different rodent malaria species. However, this result is very preliminary and further experiments would need to be performed to confirm differential expression of TCTP in different rodent malarial species and/or in different life stages. It has been previously reported that more TCTP is present in trophozoite stages of *P. falciparum*, compared with rings (Bhisutthibhan et al., 1998). As *P. yoelii* and *P. berghei* infections are relatively asynchronous, it would be difficult to assess the relative proportions of TCTP in different life stages. Quantification of TCTP in *P. c. chabaudi* AS life stages
Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as malaria vaccines

would be achievable, but would require a different method of malarial protein extraction such that total protein concentration could be reliably estimated.

TCTP immunisation resulted in a significant delay in infection progression in mice challenged with *P. chabaudi* and *P. yoelii*. The longest delay was seen in mice challenged with *P.c. chabaudi* AS, in that TCTP-immunised mice had reduced mean parasitemia from 4 dpi to 9 dpi. This reduced parasitemia led to a delay in peak parasitemia of one day compared with the control group. Additionally, the peak parasitemia was reduced (33%) in TCTP-immunised mice compared with control mice (44%), however this reduction in peak parasitemia was not significant (p>0.05). In mice challenged with *P. yoelii* YM, a statistically significant reduction in parasitemia was observed in PyTCTP-immunised mice only at the early stages of infection, at 3 and 4 dpi. Although parasite numbers were not significantly different, the peak parasitemia again occurred one day later in TCTP-immunised mice (day 7 compared with day 6) (Figure 4.12 (a)). Although an increased number of TCTP-immunised mice challenged with *P. yoelii* YM had not reached the infection endpoint at 7 dpi, by 8 dpi most control and immunised mice had been killed due to reaching a symptom score of 8. The reduction and delayed increase in parasitemia seen in the EcPyTCTP-immunised mice was not as strong as in the initial *P. yoelii* YM challenge trial, in which mice were immunised with ScPfTCTP (Taylor, 2002). In that trial, the parasitemia was significantly reduced in immunised mice on days 3-5 post-infection, peak parasitemia was delayed by one day, and immunised mice survived a full day longer than PBS controls. However, in both trials some delay in disease progression was observed.

Unlike in either of the previous TCTP vaccine trials using *P. yoelii*, in this trial two TCTP-immunised mice and one control mouse survived the usually lethal infection, with a peak symptom score of 6 or below. In the mice that survived, two parasitemia peaks were observed. Survival of a proportion of control mice following *P. yoelii* YM challenge has been reported (Goschnick et al., 2004; Kedzierski et al., 2000). A second peak parasitemia has previously been reported in *P. yoelii* YM infections (Goschnick et al., 2004; Kedzierski et al., 2001; Kedzierski et al., 2002b), and in infections using the closely related strain *P. yoelii* 17XL (Mello et al., 2004; Rotman et al., 1999). In most of these reports, a second peak parasitemia was only observed in immunised mice, however an observation of two parasitemia peaks in controls following challenge with *P. yoelii* YM and *P. yoelii* 17XL has also been reported (Goschnick et al., 2004; Mello et al., 2004). The survival of two PyTCTP-immunised mice cannot be confidently stated to be due to the effects of the TCTP vaccine, as one control mouse also survived, with a lower peak
Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as malaria vaccines

parasitemia. Further immunisation and *P. yoelii* YM challenge experiments would be required to observe if mice immunised with EcPyTCTP consistently had higher survival rates than controls.

TCTP immunisation did not result in any significant differences in disease progression in C57BL/6 or BALB/c mice challenged with *P. berghei* ANKA. Malarial TCTP may contribute to inflammation during malarial infection by inducing histamine release from the host (MacDonald et al., 2001). As cerebral malaria (CM) development is associated with inflammatory cytokines, particularly TNFα and IFNγ (e.g. de Kossodo and Grau, 1993; Jennings et al., 1997; Lou et al., 2001), it was thought that TCTP immunisation might confer protection against CM in susceptible C57BL/6 mice. However, no significant differences in parasitemia or cerebral symptom development were observed in PbTCTP-immunised C57BL/6 mice compared with PBS controls. All mice except one PBS control developed cerebral symptoms and were sacrificed at between five and eight days post-infection, with a mean time to cerebral symptom onset of six days for both control and immunised mice. The mouse that did not develop CM was eventually sacrificed three weeks post-infection with fulminating parasitemia. It has previously been reported that CM occurs in less than 100% of C57BL/6 mice infected with *P. berghei* ANKA (e.g. Amani et al., 1998; Bagot et al., 2002; Pierrot et al., 2003; Renia et al., 2006b).

In BALB/c mice challenged with *P. berghei* ANKA, both PbTCTP-immunised and PBS control mice reached the disease endpoint (a symptom score of 8) at low parasitemia, relatively early in the infection. It is generally reported that BALB/c mice infected with *P. berghei* ANKA succumb to disease at around three weeks post-infection (e.g. Lou et al., 2001). A literature search of *P. berghei* ANKA infections revealed that a few studies have previously reported deaths of a small proportion of BALB/c mice at relatively low parasitemia, with a 10-20% mortality rate observed prior to 10 dpi (Hansen et al., 2005; Hansen et al., 2003; Pierrot et al., 2003). However, reports of severe morbidity and/or deaths of all *P. berghei* ANKA-challenged mice at low parasitemia have not been published.

It is known that variability occurs in malaria challenge experiments, due to virulence differences between isolates. Increased virulence was observed in mice challenged with *P.c. chabaudi* AS and with *P. berghei* ANKA. The average peak parasitemia for the *P. chabaudi* AS-infected control mice was higher (44%) than is generally reported (~20-30%) (e.g. Helmby et al., 2000; Lamb et al., 2007; Stephens et
Chapter 4: Evaluation of *P. yoelii* and *P. berghei* TCTP as malaria vaccines

Also, some *P. chabaudi*-infected mice reached a symptom score of eight and were sacrificed, with a peak parasitemia exceeding 45%. BALB/c mice infected with *P.c. chabaudi* AS are generally reported to have a 0% mortality rate (e.g. Hensmann *et al.*, 2004; Lamb *et al.*, 2007). It is likely that the increase in virulence of the parasite strains was due to serial passage in mice. It has been reported that serial passage of malaria rapidly increases virulence, as defined by damage to the host (Mackinnon and Read, 2004). In contrast, the survival of several mice following *P. yoelii* YM challenge suggests that this strain may have had lower virulence than strains previously tested.

In all TCTP vaccine trials that have produced significant differences between immunised and control groups, the greatest observed difference is a reduced parasitemia in the early stages of infection, a delayed peak parasitemia, and increased survival time. Taken together, these results suggest that malarial TCTP may have a role in the early stages of infection progression, and is able to be recognised by the host immune system. If malarial TCTP were only found in the cytoplasm of malarial parasites, specific antibodies would be unable to recognise and interact with the protein. TCTP has no recognised secretory signal sequence or transmembrane domains, as determined using the MalSig and SecretomeP programs (http://bioserve.latrobe.edu.au/cgi-bin/pfsigseq.py, http://www.cbs.dtu.dk/services/SecretomeP/). However, evidence of TCTP secretion exists, as reviewed in section 1.4. Extracellular functions of malarial TCTP have also been identified, including the induction of histamine release from basophils and IL-8 release from eosinophils *in vitro* (MacDonald *et al.*, 2001). The protective effect observed following TCTP immunisation is most likely due to an induced immune response that inhibits the extracellular actions of the protein. Further determination of the specific functions of malarial TCTP is required to better understand the mechanisms of the modest protection conferred by TCTP immunisation.

Although histamine-releasing activities of malarial TCTP have been reported, no degranulation was induced by malarial or human TCTP in the RBL-2H3 cell line. Reports have demonstrated histamine release from RBL-2H3 cells in response to filarial, flatworm, and tick TCTP (Gnanasekar *et al.*, 2002; Mulenga *et al.*, 2003; Rao *et al.*, 2002). These studies all used a commercial kit to detect histamine release; the β-hexosaminidase assay is less sensitive than the commercial kit. To determine if the lack of induction of basophil degranulation by malarial TCTP was due to experimental differences between laboratories, the TCTPs previously shown to induce RBL-2H3 degranulation would need to be assessed together with malarial TCTP under identical assay conditions.
TCTP immunisation generated only a very moderate protective effect against some of the rodent malaria strains tested, and is therefore a much less promising malarial vaccine candidate than other proteins already assessed. For example, in a trial in which BALB/c mice were immunised with native MSP1_{19} and challenged with *P. c. chabaudi* AS, four of five mice did not develop detectable parasitemia (Hensmann *et al.*, 2004). This vaccine candidate is now in clinical trials (Shi *et al.*, 2007). Other promising malarial vaccine candidates able to completely prevent the establishment of malaria infection in mice include AMA-1 (Anders *et al.*, 1998), also in clinical trials (Ballou *et al.*, 2004), and MSP4/5 (Kedzierski *et al.*, 2000). For this reason, it is unlikely that TCTP could be viewed as a potential human malaria vaccine candidate.
Chapter 5: Development of TCTP-knockout strains of \textit{P. berghei}

5.1 Introduction

The precise function of malarial TCTP is unknown. It is known to be a target of the antimalarial drug artemisinin (Bhisutthibhan and Meshnick, 2001), and there is evidence that it is secreted from parasites during infection and can induce histamine release in cultured human basophils (MacDonald \textit{et al.}, 2001). TCTPs from other eukaryotes are known to be involved in a variety of functions, including cell growth.

This chapter describes the development of a \textit{P. berghei} ANKA strain with the TCTP gene disrupted. A TCTP ‘knockout’ malaria strain may be useful in elucidating the precise functions of TCTP in the parasite. As discussed in section 1.8, TCTP knockouts have been created in mice and \textit{Drosophila} flies. In \textit{Drosophila}, disruption of the TCTP gene led to 100\% lethality at the larval stage (Hsu \textit{et al.}, 2007). Mouse embryos heterozygous for the TCTP deleted allele developed normally, however homozygous mutant embryos died at around 6.5 days. Mouse fibroblast cells with the TCTP gene disrupted by conditional mutagenesis developed normally, indicating that TCTP is essential only in some stages of mouse development, or in some cell types (Chen \textit{et al.}, 2007).

The methods for the creation of gene knockouts in \textit{P. berghei} ANKA are well established, as discussed in section 1.9. Targeted gene disruption in involves homologous recombination of a linearised target DNA sequence into the genome. Both single and double crossover events can occur, depending on the target DNA design. Early transfection experiments used electroporation to introduce exogenous DNA into purified schizonts (e.g. Menard and Janse, 1997). Transfection technology using the Amaxa nucleofector™ machine (http://www.amaxa.com/products/technology/) has since been developed, and has increased transfection efficiencies from \( \sim 10^{-8} \) to \( \sim 10^{-3} \) (Janse \textit{et al.}, 2006a).

The selectable marker used in \textit{P. berghei} transfections is the dihydrofolate reductase-thymidylate synthase (DHFR/TS) gene from \textit{P. berghei}, \textit{Toxoplasma gondii}, or humans. This gene confers resistance to either the antimalarial drug pyrimethamine (\textit{P. berghei} and \textit{T. gondii} DHFR) or to both pyrimethamine and the antifolate drug WR99210.
Chapter 5: Development of TCTP-knockout strains of *P. berghei*

(human DHFR). Using these transfection techniques, several malarial genes have been disrupted, including those encoding the circumsporozoite (CS) protein (Menard *et al.*, 1997), the thrombospondin-related anonymous protein (TRAP) (Sultan *et al.*, 1997), and the macrophage migration inhibitory factor (MIF) (Augustijn *et al.*, 2007).

As a protective effect following TCTP immunisation was not observed in PbTCTP-immunised mice, it might have been more relevant to disrupt the TCTP gene in *P. yoelii*. Transfection of *P. yoelii* has been accomplished using electroporation techniques, and the TRAP gene has been disrupted (Mota *et al.*, 2001). These experiments were performed using insertion plasmids rather than replacement plasmids, and gene disruption occurred by single-crossover. Gene analysis of TRAP-disrupted *P. yoelii* parasites indicated that gene reversion had occurred and wild-type TRAP was present, most likely from the excision of the linearised TRAP plasmid from the genome. At the time the TCTP disruption experiments were performed, transfection of *P. yoelii* using the nucleofector™ machine had been reported, however gene disruption experiments had not been attempted (Jongco *et al.*, 2006). One such experiment was recently published (Mikolajczak *et al.*, 2008). TCTP knockout experiments were performed in *P. berghei* ANKA due to the more developed transfection methods, and the ability to perform these experiments together with collaborators at the Walter and Elisa Hall Institute (WEHI).

The experiments described in this chapter utilised the b3D vector (Figure 5.1) encoding the *T. gondii* DHFR/TS gene with a two multiple cloning sites (MCS) present adjacent to the 5' and 3' UTRs. A section of either the 5' or 3' TCTP flanking region was cloned into each MCS. In successful transfection experiments, the two TCTP flanking regions would undergo homologous recombination with the TCTP gene on the genome, resulting in the interruption of chromosomal TCTP with the DHFR gene. The target DNA was also designed so that either 140 bp or 507 bp of the 516 bp TCTP coding region would be excised during homologous recombination, to prevent wild-type protein expression if the DHFR gene was excised from the chromosome at a later stage. Knockout parasites could be selected for by treatment of infected mice with pyrimethamine. Confirmed TCTP-knockout parasites could then be tested for phenotypic differences to wild-type, including altered growth morphology and altered resistance to artemisinin.
Chapter 5: Development of TCTP-knockout strains of \textit{P. berghei}

The aims of the experiments outlined in this chapter were:

1: To assess the feasibility of the TCTP knockout experiments, by:

   a) Investigating whether multiple copies of the TCTP gene are present in \textit{P. berghei} ANKA

   b) analysing the TCTP flanking regions on the chromosome to assess if any nearby genes might be disrupted during the creation of TCTP knockouts

2: To create vectors suitable for the disruption of TCTP in the \textit{P. berghei} ANKA genome

3: To perform transfection experiments using these vectors, in order to obtain TCTP knockout mutant parasites

4: To verify the disruption of TCTP in the \textit{P. berghei} genome by PCR and Southern blot analysis

5: To analyse the phenotype of TCTP-knockout mutant parasites
Chapter 5: Development of TCTP-knockout strains of *P. berghei*

Figure 5.1: The b3D vector
(http://www.lumc.nl/1040/research/malaria/plas01.html)
Chapter 5: Development of TCTP-knockout strains of *P. berghei*

5.2 Materials and Methods

For the materials and methods used in this study, please refer to chapter 2.

5.3 Results

5.3.1 Feasibility assessment of the TCTP knockout experiments by analysis of the *P. berghei* ANKA genome

The *P. berghei* genome sequence has not been fully assembled, and is available as a series of contigs. Using the plasmoDB database ([http://www.plasmodb.org/plasmo/](http://www.plasmodb.org/plasmo/)), the nucleotide sequence of *P. berghei* TCTP was entered into a BLAST search against the *P. berghei* genome to assess if multiple copies of TCTP were present, in which case the transfection experiment would be unlikely to result in parasites with no TCTP expression. No other sequences with high similarity to the TCTP coding sequence were found.

The sequence of the *P. berghei* contig containing TCTP was entered into an open reading frame (ORF) finder program (NCBI) to analyse if other nearby genes might be disrupted in the knockout experiment. The results obtained showed that two very small ORFs (35 and 44 amino acids) are present at around 1 kb either side of the TCTP coding region. The closest genes to TCTP with an identified function are a steroyl-CoA desaturase protein (accession number XP_679533) 2.8 kb upstream and a WD-repeat protein (accession number XP_679536) 3.3 kb downstream of TCTP.

The short and long integration fragment sequences to be cloned into the b3D vector were subjected to a BLAST search using NCBI to ensure that they were unlikely to recombine in other areas of the *P. berghei* genome. Using the Amaxa nucleofector™, the minimum length sequence required for homologous recombination in malaria transfection is ~300 bp (Janse *et al.*, 2006a). No other similar sequences approaching this size were found in the search.
5.3.2 Cloning of the short integration construct

5.3.2.1 Rationale for the creation of the short target b3Dint1int2 construct

The standard protocol in *P. berghei* transfection is to design replacement constructs so that the target gene is truncated or deleted after homologous recombination, so that the wild-type gene cannot be restored if the exogenous target DNA is excised from the genome. In designing the first integration construct, it was decided to use mostly TCTP coding region, due to the higher GC content. Thus the fragments were designed such that the middle 141 bp portion of the TCTP coding region would be removed following homologous recombination. This meant that a 5’ coding region of 199 bp and a 3’ coding region of 175 bp would still present following a successful transfection, but would be interrupted with the DHFR gene. PCR analysis would be able to detect if the target DNA were excised from the genome at a later stage, as the TCTP gene in revertants would be truncated.

The lower limit in size of the homologous DNA used for each integration fragment is 300 bp. As *P. berghei* TCTP is a small gene (516 bp) containing no introns, the integration fragments were designed to incorporate part of the 5’ and 3’ UTRs of TCTP. The first integration fragment (int1) was 336 bp in length, from -121 bp upstream of the TCTP start codon to position 199 in the TCTP gene. The second integration fragment (int2) was 344 bp in length, from position 340 on the TCTP gene to +152 bp downstream of the stop codon.

A schematic diagram for the steps involved in creating the short integration construct (b3D.int1.int2) is given in Figure 5.2 (a). A diagram of the disruption of the TCTP region of the *P. berghei* ANKA chromosome by homologous recombination of the int1.int2 target DNA is given in Figure 5.2 (b). A schematic representation of the *P. berghei* ANKA TCTP region following disruption with the int1.int2 target is given in Figure 5.2 (c).
Chapter 5: Development of TCTP-knockout strains of *P. berghei*

(a)

**PCR amplification of int1 and int2 fragments:**

<table>
<thead>
<tr>
<th></th>
<th>Int1</th>
<th>Int2</th>
</tr>
</thead>
<tbody>
<tr>
<td>-121</td>
<td>199</td>
<td>340</td>
</tr>
<tr>
<td>+152</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P. berghei* genome  
TCTP

**Cloning of int1 and int2 into pCR2.1 vectors:**

- **pCR.int1**
- **pCR.int2**

**Cloning of int1 and int2 sequentially into the b3D vector:**

- **pCR.int1**
- **b3D**
- **b3D.int1**
- **pCR.int2**
- **b3D.int1**
- **b3D.int1.int2**
Chapter 5: Development of TCTP-knockout strains of *P. berghei*

Figure 5.2 Creation of the b3D.int1.int2 vector, and disruption of the TCTP gene with the int1.int2 target

(a) Creation of the b3D.int1.int2 vector by PCR amplification of int1 and int2 from *P. berghei* ANKA genomic DNA, followed by cloning of the int1 and int2 products into pCR2.1 vectors, then sequential digestion and ligation of int1 and int2 into the MCS1 and MCS2 of the b3D vector

(b) Schematic diagram of recombination via double cross-over between int1 and int2 on the linearised int1.int2 target DNA and the homologous regions on the *P. berghei* ANKA chromosome following transfection, the box represents the TCTP coding sequence

(c) Schematic diagram of the TCTP region of the *P. berghei* chromosome following homologous recombination in a successful transfection experiment with the int1.int2 target DNA, the boxes represent TCTP coding sequence remaining
5.3.2.2 PCR amplification of the short integration fragments and cloning into pCR2.1 vectors

The sequence of the primers used to amplify the short integration fragments were designed based on the sequence of the *P. berghei* contig containing the TCTP gene (PlasmoDB contig accession number PB_RP1763). The first fragment primers were designed to incorporate *Cla*I and *Hin*III restriction enzyme sites (as underlined) to allow for directional cloning into the first MCS of the b3D vector. The second fragment primers were designed to incorporate *Bam*HI and *Xba*I restriction enzyme sites (as underlined) to allow for directional cloning into the second MCS of b3D. The sequence of the four primers was:

First for: GCATCGATGAAGCCTTTTAATTATATAGTA  
First rev: GCAAGCTTGTTCAACATCGCTCCATTCC  
Second for: GCGGATCCAAACAAAGGCACAACCTTAAT  
Second rev: GCTCTAGACATTGGACCATTITTAAGACA

The first (int1) and second (int2) fragments were amplified by Taq PCR (as per the method described in 2.3.2.2.3) from *P. berghei* ANKA genomic DNA (obtained from Dr. Tania de Koning-Ward, WEHI, Australia). Each reaction contained 0.5 μM concentrations of the first for and first rev or the second for and second rev primers and 25 ng of *P. berghei* ANKA genomic DNA. An annealing temperature gradient of 50 to 65°C was used. The PCR products were separated by electrophoresis at 100 V (2.3.2.5). A strong band was amplified at ~320 bp for the int1 and int2 products (Figure 5.3 (a)).

The int1 and int2 PCR products from the 65°C annealing temperature reactions were cloned into separate pCR 2.1 vectors as per manufacturer’s instructions (2.3.2.7.1). Plasmid DNA from resulting white colonies was purified by minipreparation (2.3.2.1.1). Restriction enzyme digests (2.3.2.4) using either *Cla*I and *Hin*III (int1) or *Bam*HI and *Xba*I (int2) confirmed that the int1 and int2 products had been successfully cloned into pCR2.1 vectors (Figure 5.3 (b)). Sequence analysis was performed (as per the method described in 2.3.2.2.6); pCR2.1 vectors containing inserts with the correct DNA sequence were named pCR.int1 and pCR.int2.
Figure 5.3: Amplification and pCR2.1 cloning of the int1 and int2 fragments
(a) PCR amplification of the int1 and int2 products: Lane 1: 100 bp marker; Lane 2: int1 PCR neg control; Lanes 3-8: int1 PCRs with annealing temp 50-65°C; Lane 9: int2 PCR neg control; Lanes 10-15: int2 PCRs with annealing temp 50-65°C.
(b) Confirmation of int1 and int2 in pCR2.1 cloning vectors: Lane 1: λ/PstI marker; Lanes 2-5: pCR.int1 digested with Clal and HindIII; Lanes 6-9: pCR.int2 digested with Xbal and BamHI.
5.3.2.3 Cloning of the int1 and int2 products into the b3D vector

Overnight cultures of single colonies of *E. coli* DH5α transformed with the b3D plasmid (obtained from Dr. Tania de Koning-Ward) were grown in LB+Amp broth, and plasmid was extracted by minipreparation. The int1 and int2 fragments were cloned into the two multiple cloning sites of the b3D vector sequentially. First, 1 µg each of the pCR.int1 and the b3D vectors were digested with 10 U each of ClaI and HindIII. The completed digestion reactions underwent electrophoresis at 50 V on a 1% agarose gel (*Figure 5.4 (a)*). Bands of the correct size were excised with a scalpel, and DNA was purified from the agarose using the Qiaquick Gel Extraction kit following manufacturer’s instructions.

The ligation reaction was prepared as standard (2.3.2.7.2) with the modification that 50 ng of b3D vector was used, with the appropriate amount of insert for a 3:1 insert:vector ratio. The completed ligation reaction was used to transform electrocompetent *E. coli* (2.4.3.2); plasmid DNA from resulting colonies was purified by minipreparation. Plasmid preparations were screened for the presence of the int1 insert by digestion with ClaI and HindIII. The completed digestion reactions were separated by 1.5% agarose gel electrophoresis at 100 V (*Figure 5.4 (b)*).

One microgram of the pCR.int2 and b3D.int1 vectors were digested with 10 U each of BamHI and XbaI. Completed digestion reactions were separated by 1.5% agarose gel electrophoresis at 60 V (*Figure 5.4 (c)*). Desired bands were excised with a scalpel and DNA was purified using the Qiaquick Gel Extraction kit. Ligation reactions of the int2 insert and the b3D.int1 vector were prepared using 100 ng vector and the appropriate amount of insert DNA for a 3:1 ratio. The completed ligation reaction was used to transform electrocompetent *E. coli*. Plasmids from resulting colonies were purified by minipreparation and screened for confirmation of the presence of both the int1 and the int2 insert by digestion with 10 U each of either ClaI and HindIII or BamHI and XbaI (*Figure 5.4 (d)*). Plasmid preparations containing both the int1 and int2 inserts were named b3D.int1.int2.
Figure 5.4: Creation of the b3D.int1.int2 construct

(a) Digestion of pCR.int1 and b3D vectors with Clal and HindIII: Lane 1: 100 bp marker; Lanes 4 & 7: pCR.int1; Lanes 10 & 13: b3D.

(b) Confirmation digest of b3D.int1 using Clal and HindIII enzymes: Lane 1: 100 bp marker; Lane 3: b3D.int1; Lane 7: pCR.int1.
Figure 5.4: Creation of the b3D.int1.int2 construct

(c) Digestion of b3D.int1 and pCR.int2 with XbaI and BamHI: Lane 1: λ/HindIII marker; Lane 4: b3D.int1; Lanes 6 & 8: pCR.int2.

(d) Confirmation digests of b3D.int1.int2: Lane 1: λ/HindIII marker; Lanes 3 and 5: b3D.int1.int2 digested with ClaI and HindIII for confirmation of int1 cloning; Lane 4 and 6: b3D.int1.int2 digested with XbaI and BamHI for confirmation of int2 cloning; Lane 8: 100 bp marker.
5.3.3 First transfection of *P. berghei* ANKA parasites using the short target b3D.int1.int2 vector

5.3.3.1 Preparation of target DNA prior to transfection

Transfection of *P. berghei* ANKA requires linearised target DNA. Residual intact plasmid can transform *P. berghei* parasites, resulting in episomes, which can be transferred to daughter parasites during schizont formation. This can result in drug-resistant parasites without the target gene disrupted. Therefore, target DNA must be completely digested prior to transfection.

The short target DNA used in the first transfection experiment was prepared from the b3D.int1.int2 construct. The target DNA was prepared by excising the region spanning the int1 fragment and int2 fragments out of the b3D plasmid backbone, using *Cla*I and *Xba*I. To further ensure that no intact plasmid remained, the b3Dint1int2 construct was digested with a third restriction enzyme that cut within the plasmid backbone (*Bgl*I).

A final concentration of 10 µg of target DNA was required per transformation experiment. A total of 120 µg of the b3D.int1.int2 plasmid was digested with 200 U each of *Cla*I, *Xba*I and *Bgl*I in Buffer C for 15 h. Aliquots of 20 µl of the digest reaction were separated by 1% agarose gel electrophoresis at 60 V (Figure 5.5 (a)). The target DNA bands (5270 bp) were excised from the gel using a scalpel and DNA was purified using the Qiaquick Gel Extraction kit. All preparations of gel-purified short target DNA were combined and DNA was precipitated with EtOH/NaOAc (2.3.2.8). The target DNA was resuspended in 10.5 µl of TE. A small amount of prepared short target DNA was quantified before transfection by comparison with known quantities of *Pst*I-digested-λ DNA (Figure 5.5 (b)).
Figure 5.5: Preparation of short integration target DNA prior to first transfection
(a) Representative sample of b3D.int1.int2 digestions with Clal, BamHI and BglII:
Lane 1: λ/HindIII marker (full size range described in Appendix 1); Lanes 4,6,8,10,12,14: b3D.int1.int2 digests
(b): Quantification and purity assessment of purified target DNA preparations prior to transfection: (b) Lane 1: λ/PstI marker; Lanes 3-6: dilutions of target DNA.
5.3.3.2 Growth of parasites and secondary infection of mice following first transfection

The initial transfection, drug treatment and parasitemia monitoring experiments were performed at WEHI by Dr. Tania de Koning-Ward. Briefly, *P. berghei* ANKA schizonts were purified from an overnight *in vitro* culture using Nycodenz columns. An Amaxa nucleofector™ machine was used for transfection. Per reaction, 10 µg of purified short integration target DNA was used, and the transfected schizonts were injected into the lateral tail vein of one BALB/c mouse. The mouse was treated with 70 µg/ml pyrimethamine provided in the drinking water (pH adjusted to between 3.5 and 5) continuously from 24-30 hours post-injection. Parasitemia was monitored at regular intervals. Detectable parasitemia was only observed in the mouse at 18 days post-injection, at this time the mouse was sacrificed and blood was used for stabilate creation. Successful transfection experiments usually yield a parasitemia of 0.1-5% at 4-7 dpi, and it has previously been noted that parasites are often detected between 13 and 15 dpi in unsuccessful transfection experiments. These parasites are typically non-drug resistant parasites that have survived pyrimethamine treatment (http://www.lumc.nl/1040/research/malaria/model.html).

Therefore, it was unknown whether the finding of detectable parasitemia at 18 dpi was due to an unsuccessful transfection experiment, with the parasites present being wild-type, or if the result obtained was due to a slow-growth phenotype in parasites with the TCTP gene successfully disrupted. For this reason, the parasite stabilate from the first transfection experiment was used to inject two mice at RMIT, to obtain sufficient DNA for genotype analysis by Southern blot and PCR. These mice were designated as 1.1 and 1.2. Each mouse received 100-150 µl of stabilate by i.p. injection (method described in 2.7.2.3). As it was thought that the parasites obtained were most likely wild-type, mice received no pyrimethamine treatment. At the same time, four mice received an i.p. injection of 75 µl of *P. berghei* ANKA stabilate, to obtain sufficient wild-type (wt) parasite DNA.

In all mice, a parasitemia of between 2 and 4% was reached at between 3 dpi and 6 dpi. Mice were anaesthetised and blood was taken by cardiac puncture (2.7.3.2). Blood was used to create stabilates (2.7.2.2) and for malarial DNA extraction using SDS and PCI (2.9.2.4).
5.3.3.3 Analysis of the genotype of the transfected parasites by PCR

Primers were designed to assess disruption of the TCTP gene by integration of the target DNA in the *P. berghei* ANKA genome. Two primer sets were used that should only amplify products in parasite DNA with the TCTP gene disrupted. These were the 5’PyTCTP (CGCGGGATCCATGAAAGTATATAAAGACATTTTTACA) and Pf1rev (AAAATAAAAGGGAAATCAATG) combination and the Pf2for (TCTTCAATGATTCATAAATAG) and 3’PyTCTP (GCGCGACGTCTTAGATTTTTTCTTCAAAATAACCATC) combination. In successfully transfected parasites, the 5’PyTCTP and Pf1rev primer combination would amplify a region from the remaining 5’ section of the PbTCTP to just inside the 5’UTR of the DHFR gene used for disruption, producing a product of 309 bp designated as TCTP1. Likewise, the Pf2for and 3’PyTCTP primer combination would amplify a region from the end of the DHFR 3’UTR to the end of the remaining 3’ portion of the PbTCTP, resulting in a 348 bp product designated as TCTP2. The 5’ and 3’PyTCTP primer set was also used. These primers would give a PCR product of 5007 bp (long TCTP) in parasite DNA with the TCTP gene successfully disrupted, due to the insertion of the DHFR gene within the TCTP gene. A diagram of the primer binding sites in wild-type and TCTP-knockout parasites is given in Figure 5.6.

![Diagram of primer binding sites in wild-type and TCTP-knockout parasites](image)

**Figure 5.6: Amplification products of primers initially used to confirm disruption of TCTP with the DHFR gene**

1: **TCTP1** - 5’PyTCTP and Pf1rev
2: **TCTP2** - Pf2for and 3’PyTCTP
3: **long TCTP** - 5’PyTCTP and 3’PyTCTP
The integration of the short target DNA into the genome of transfected parasites was assessed by Taq PCR analysis. In all PCRs, a one-minute extension time was used. Wild-type *P. berghei* ANKA DNA was used as a control.

In transfected parasite DNA obtained from mouse 1.1, a 516 bp wild-type TCTP band was not amplified, but was present in wt *P. berghei* ANKA DNA. Using the 5’PyTCTP and Pf1rev primer combination, a TCTP1 product of ~300 bp was obtained from the mouse 1.1 parasite DNA but not from wt DNA, indicating that the TCTP gene may have been successfully disrupted. However, no TCTP2 product was obtained using the Pf2for and 3’PyTCTP primer combination, so it was unclear whether the transfection experiment had been successful (Figure 5.7 (a)). The annealing temperature was reduced to 45°C in an attempt to amplify the TCTP2 product using the Pf2for and 3’PyTCTP primers. Again, no product was amplified (results not shown).

In transfected parasite DNA obtained from mouse 1.2, a faint wt TCTP band was obtained, as well as a strong 300 bp TCTP1 band (Figure 5.7 (b)). Again, no TCTP2 product was amplified. This indicated that a mix of parasite genotypes might be present in the blood taken from this mouse.

To confirm the presence of the TgDHFR/TS gene in the transfected parasite DNA, the TgDHFRf (GGCATCGGCATCAACAACGG) and TgDHFRr (GCTAGACAGCCATCTCCATC) primers were used. These primers were designed to amplify a 1,790 bp product corresponding to the majority of the coding region. PCRs were performed using Pfu polymerase with a 10 min extension time. A strong 1,800 bp DHFR product was amplified in transfected DNA from both mouse 1.1 and 1.2. Very faint non-specific bands were also observed. No distinct products were observed in *P. berghei* ANKA wt DNA (Figure 5.7 (c)). The presence of a strong band in the transfected parasite DNA suggested that the *T. gondii* DHFR gene was present.

Under appropriate PCR conditions, the 5’PyTCTP and 3’PyTCTP primer set should have theoretically amplified a 5,007 bp band in successfully transfected parasites. The 5’PyTCTP and 3’PyTCTP primer set was used in PCRs using Pfu or Expand long template polymerase and a 10 min extension time, no products were amplified in either sample of transfected parasite DNA (results not shown).
Chapter 5: Development of TCTP-knockout strains of *P. berghei*

(a)

(b)
Chapter 5: Development of TCTP-knockout strains of *P. berghei*

Figure 5.7: PCR analysis of parasite DNA obtained from the first transfection experiment

(a) PCR analysis of transfected parasites from mouse 1.1: Lane 1: $\lambda/PstI$ marker; Lanes 2-5: TCTP PCR – (2) neg control; (3-4) dilutions of mouse 1.1 parasite DNA; (5) wt DNA; Lanes 6-9: TCTP1 PCR – (6) neg control; (7-8) dilutions of mouse 1.1 parasite DNA; (9) wt DNA

(b) PCR analysis of transfected parasites from mouse 1.2: Lane 1: $\lambda/PstI$ marker; Lanes 3-7: TCTP PCR - (3) neg control; (4-5) dilutions of wt DNA; (6-7) dilutions of mouse 1.2 parasite DNA; Lanes 8-12: TCTP1 PCR – (8) neg control; (9-10) dilutions of wt DNA; (11-12) dilutions of mouse 1.2 parasite DNA

(c) PCR amplification of TgDHFR/TS from transfected parasite DNA from mouse 1.1 and 1.2: Lane 1: $\lambda/PstI$ marker; Lanes 3-8: TgDHFR/TS PCR – (3) neg control; (4) parasite DNA from mouse 1.1; (5) parasite DNA from mouse 1.2; (7) DNA from *P. berghei* wt.
5.3.3.4 Genetic analysis of parasite DNA from the first transfection experiment by Southern blot

The PCR results obtained indicated that the target DNA was present in the transfected parasite DNA obtained from mouse 1.1 and 1.2, as the TCTP1 and TgDHFR/TS products were amplified. However, integration of the target DNA in the correct genomic location could not be confirmed from these results, as the TCTP2 product was not amplified. To clarify the location of the integrated target DNA, a Southern blot was performed, using both TCTP and TgDHFR/TS probes. The TCTP probe would hybridise with both wt and transfected DNA, as only the middle 141 bp of the TCTP coding region would have been removed in a successful integration experiment, leaving 375 bp remaining, interrupted by the ~5 kb TgDHFR/TS gene. The TgDHFR/TS probe should only hybridise with transfected parasite DNA.

5.3.3.4.1 PCR amplification of the TCTP probe

PbTCTP was amplified from *P. berghei* ANKA genomic DNA by PCR using AmpliTaq polymerase with a final concentration of 0.3 µM 5’ and 3’PyTCTP primers and 50 ng DNA per reaction. An annealing temperature of 55°C was used. The PCR products were analysed by agarose gel electrophoresis (*Figure 5.9 (a)*) prior to hexanucleotide labelling (as per the method described in 2.9.4.1).

5.3.3.4.2: PCR amplification of the TgDHFR/TS probe

The TgDHFR/TS probe was amplified by PCR using ~ 2 ng of b3D vector as the template. An annealing temperature gradient of 55 - 65°C was tested. As shown in *Figure 5.9 (b)*, faint non-specific bands were also amplified at all annealing temperatures. For this reason, the band corresponding to the TgDHFR/TS product was excised from the gel and purified prior to hexanucleotide labelling.
Figure 5.8: Preparation of probes for Southern blot of parasite DNA from the first transfection experiment

(a) Amplification of TCTP probe: Lane 1: λ/PstI marker; Lane 2: TCTP PCR neg control; Lanes 3 & 4: TCTP PCR products

(b) Amplification of DHFR probe: Lane 1: λ/PstI marker; Lane 2: DHFR PCR neg control; Lanes 3-7: DHFR PCR using an annealing temperature from 55-65°C.
5.3.3.4.3: Southern blot of transfected parasite and wt berghei DNA

A DNA concentration of \( \sim 10 \mu g \) per sample is recommended by the manufacturer for Southern blotting using the DIG system with a chromogenic substrate ([http://www.roche-applied-science.com/](http://www.roche-applied-science.com/)). The transfected parasite DNA obtained from mouse 1 and 2 was pooled to obtain a total of 10 \( \mu g \). The DNA from two mice infected with wt \( P. berghei \) ANKA was also pooled. The genomic DNA was digested with 30 U of NdeI for 16 h (as per the method described in 2.9.4.2). This digest would result in a 3.5 kb band containing wt TCTP in non-transfected parasites and a band of 8 kb in parasite DNA with the TCTP gene successfully disrupted. The digested genomic DNA was separated on a 1% w/v agarose gel at 60 V alongside a \( \lambda/PstI \) marker. The DNA gels and Southern membranes were photographed next to rulers, so that the band sizes could be compared. Figure 5.10 (a) depicts the digested wt and transfected parasite DNA separated by agarose gel electrophoresis.

The DNA was transferred to a nylon membrane and probed with DIG-labelled TCTP at a 65°C hybridisation temperature, as per the method described in 2.9.4.3. When hybridised DNA was detected using anti-DIG antibody and a chromogenic substrate, undigested parasite DNA was detected in the well positions, at 2 cm from the top of the membrane. This allowed the distance migrated from the wells to be measured for other bands. A band approximately 3.8 cm from the wells, corresponding to a size of \( \sim 3.5 \) kb, was observed in the wt \( P. berghei \) ANKA DNA. A band approximately 2.5 cm from the wells, corresponding to a size of \( \sim 8 \) kb, was observed in the transfected parasite DNA (Figure 5.10 (b)). This indicated that the TCTP gene had been disrupted.

To confirm this result, the blot was then stripped and re-probed (2.9.4.4) with the TgDHFR/TS probe at a 65°C hybridisation temperature. No bands were detected in the wt \( P. berghei \) ANKA DNA, as expected. A single band at \( \sim 2.5 \) cm from the wells was again observed in the transfected parasite DNA (Figure 5.10 (c)). From these results, it was determined that the TCTP gene had been successfully disrupted by the integration of the TgDHFR/TS gene in at least the majority of transfected parasites.
Figure 5.9: Southern blot of transfected and wild-type (wt) parasite DNA using TCTP and DHFR probes

(a) Separation of *NdeI*-digested genomic DNA by agarose gel electrophoresis: Lane 1: wt *P. berghei* ANKA DNA; Lane 2: transfected parasite DNA; Lane 3: λ/PstI marker.

(b) Southern blot using TCTP probe: Lane 1: wt *P. berghei* ANKA DNA; Lane 2: transfected parasite DNA.

(c) Southern blot using DHFR probe: Lane 1: wt *P. berghei* ANKA DNA; Lane 2: transfected parasite DNA.
5.3.3.5 Secondary drug-selection of parasites obtained from the first transfection experiment

5.3.3.5.1 Growth of parasites during drug selection

Due to the successful transfection result as indicated by Southern blot, the parasites obtained from the first transfection experiment were passaged into new mice for further drug selection using pyrimethamine. Three mice received an i.p. injection of 100 µl of parasites, these mice were designated 2.1, 2.2 and 2.3. Only ~125 µl of the initial transfected parasite stabilate (obtained from WEHI) remained, 100 µl of this was used to infect mouse 2.1. Mouse 2.2 and 2.3 received the stabilate made from the second passage of the original stabilate, obtained from non-drug treated mice (mouse 1.1, and 1.3, as described in 5.3.2.2). The mice were treated with pyrimethamine continuously, starting at 28 h post-infection (as per the method described in 2.9.2.2). A tree diagram of the parasite sources and recipients is shown in Figure 5.10.

1: Parasites following transfection
   ↓
2: Used to infect one mouse at WEHI, drug treated, parasite stabilate created
   ↓
3: Stabilate used to infect mouse 1.1 and 1.2, not drug treated
   ↓
4: Stabilate used to infect mouse 2.1, drug treated
   ↓
5: Stabilates used to infect mouse 2.2 and 2.3, drug treated

Figure 5.10: Tree diagram of parasite recipients from the first transfection experiment
Mouse 2.1, which received the original transfected parasite stabilate, reached a parasitemia of 5% at 9 dpi. At this point, the mouse was sacrificed and blood was taken for stabilate creation and malarial DNA extraction. Mouse 2.2, which received the non-drug treated stabilate made from the original stabilate passage, reached a parasitemia of 8% at 7 dpi. At this time the mouse was sacrificed and blood was taken for stabilate creation and malarial DNA extraction. Mouse 2.3, which also received the non-drug treated stabilate, developed low but detectable parasitemia (<0.1%) at 6 dpi. The parasite numbers remained at ~0.1% until 11 dpi, then dropped below the detection limit. Mouse 2.3 was eventually sacrificed at 28 dpi, having had no detectable parasitemia since 11 dpi.

### 5.3.3.5.2 Analysis of the transfected parasite DNA from the secondary drug-selection by PCR

PCR was performed to confirm the integration of the short target DNA in the parasites that had undergone secondary drug selection, using the 5’PyTCTP+Pf1rev and Pf2for+3’PyTCTP primer combinations; PCRs were also performed to assess the presence of wt TCTP in transfected parasites using the 5’ and 3’PyTCTP primers.

A band corresponding to wt TCTP (516 bp) was amplified in both the wt DNA control and in the drug-selected parasites from both mouse 2.1 and 2.2. A TCTP1 integration product was only obtained in the DNA from mouse 2.2 (Figure 5.11). No TCTP2 integration products were amplified in any of the DNA samples (results not shown).
Figure 5.11: PCR analysis of parasite DNA from secondary drug selection

Lane 1: λ/PstI marker; **Lanes 3-6: TCTP PCR**: (3) neg control; (4) wt DNA; (5) parasite DNA from mouse 2.1 (received original transfected parasite stabilate); (6) parasite DNA from mouse 2.2 (received passaged, non-drug treated parasite stabilate); **Lanes 7-10: TCTP1 PCR**: (7) neg control; (8) wt DNA; (9) parasite DNA from mouse 2.1; (10) parasite DNA from mouse 2.2.
5.3.3.5 Analysis of the transfected parasite DNA from the secondary drug-selection by Southern blot

A Southern blot was performed on the transfected parasite DNA following secondary drug selection. Ten micrograms of genomic DNA was digested with 30 U of Ndel for 16 h. The DNA was separated by 1% agarose gel electrophoresis (Figure 5.12 (a)). The migration distance of the DNA bands in the λ/PstI marker was calculated by placing a ruler with the 1 cm mark at the position of the wells, and measuring the distance to the bands relative to the wells.

In this experiment, sufficient DNA was obtained from mouse 2.1 and 2.2 to allow them to be analysed as separate samples. Unfortunately, no wt *P. berghei* DNA was available for use as a control in this experiment. The TCTP and TgDHFR/TS probes were re-used from the first Southern blot experiment (as per the method described in 2.9.4.5). Hybridisation was performed at 68°C. As previously observed (Figure 5.9), bands corresponding to the well position on the original agarose gel were present on the membrane when reacted with the substrate, due to the presence of undigested parasite DNA. The presence of these bands allowed the distance from the wells of other bands to be calculated.

As shown in Figure 5.12 (b), a single band at about 3.7 cm from the wells, corresponding to a size of ~3.5 kb, was detected in the DNA from both mice using the TCTP probe. In comparison, a single band at ~1.8 cm from the wells was detected using the TgDHFR/TS probe in both samples (Figure 5.12 (c)). By comparison with Figure 5.12 (a), the largest band (11.5 kb) in the λ/PstI marker migrated to just over 2 cm from the wells. This combination of results indicated that two events had occurred: 1 - TCTP was present as a non-disrupted wt gene in the majority of parasites present after the second round of drug selection, and 2 - the TgDHFR/TS gene was present in the drug-selected parasites, but in an incorrect location that was not digested to less than 11.5 kb using Ndel.

The TgDHFR/TS probe should react with an identical band as the TCTP probe in parasites with the target DNA correctly integrated. The result obtained indicated that secondary drug treatment had selected for parasites containing non-disrupted wild-type TCTP together with the TgDHFR/TS gene in an incorrect genomic location.
Figure 5.12: Genetic analysis by Southern blot of transfected parasites following secondary drug selection
(a) Transfected parasite DNA digested with Ndel from mouse 2.1 (infected with original transfected parasite stabilate) and mouse 2.2 (infected with passaged non-drug treated stabilate). Lane 1: Mouse 2.2 DNA; Lane 2: Mouse 2.1 DNA; Lane 3: λ/PstI marker.
(b) Southern blot using TCTP probe: Lane 1: mouse 2.1 DNA; Lane 2: mouse 2.2 DNA.
(c) Southern blot using TgDHFR/TS probe: Lane 1: mouse 2.1 DNA; Lane 2: mouse 2.2 DNA.
5.3.3.5.4 Southern blot using HindIII-digested DNA

To further analyse the genotype of the transfected parasites, DNA from parasites prior to and following secondary drug selection was digested with HindIII. When reacted with the TCTP probe, this should result in the detection of a 5 kb band in TCTP-knockout parasites, and a 1 kb band in wild-type parasites. A faint 5 kb band was detected in the parasites prior to secondary drug selection, whereas no distinct bands were observed in the parasite DNA following secondary drug selection when probed with either TCTP or TgDHFR/TS (results not shown).
5.3.3.6 Repeat of secondary drug selection of parasites from the first transfection experiment

The secondary drug selection was repeated on the original parasites obtained from the first transfection experiment, to assess if parasites with the desired, TCTP-disrupted genotype would again be selected against.

5.3.3.6.1 Growth of parasites during drug selection

Three mice were infected with various transfected parasite stabilates, in an identical regimen as was shown in the tree diagram in Figure 5.10. Mouse 3.1 received an i.p. injection of the last of the original transfection stabilate (from WEHI). Mouse 3.2 and 3.3 received stabilates from the passage of the original stabilate (from mouse 1.1 and 1.2), in which mice were not drug-treated and the majority of parasites contained the desired integration event. The mice received 100 µl of stabilate by i.p. injection and were treated continuously with pyrimethamine, starting at 28 hours post-injection. The pyrimethamine water was replaced at five, seven and 15 dpi. Mouse 3.2 and 3.3 reached a parasitemia of 3-4% at 9 dpi. Mouse 3.1 reached a parasitemia of 4% at 16 dpi; this was most likely due to the lower dose of stabilate received. Mice were sacrificed and blood was taken for stabilate creation and malarial DNA extraction.

5.3.3.6.2 Genetic analysis of drug-selected parasites by Southern blot

A total of 10 µg of DNA from each of the three mice was digested with NdeI as previously described. Again, no wt *P. berghei* ANKA control DNA was available. The DNA was separated on a 1% agarose gel (Figure 5.14 (a)). In this experiment, the membrane was placed on the gel such that the top of the membrane corresponded to the well position on the agarose gel. The DNA was transferred to a membrane and reacted with the TCTP probe; then the membrane was stripped and reacted with the TgDHFR/TS probe. When probed with TCTP, a single band, corresponding to wt TCTP, was detected at approximately 3.7 cm (~3.5 kb) in all of the DNA samples. When probed with TgDHFR/TS, a single band was detected at ~1.8 cm from the wells, corresponding to a size of >11.5 kb. The results are shown in Figure 5.14 (b) and (c). This is an identical result to that obtained in the parasites from the initial secondary drug-selection (section 5.3.3.5.3), and gave further indication that parasites containing non-disrupted wild-type TCTP together with the TgDHFR/TS gene integrated in an incorrect location were selected for during the secondary drug treatment.
Chapter 5: Development of TCTP-knockout strains of *P. berghei*

![Image of gel and Southern blots](image)

**Figure 5.14:** Southern blot on repeated secondary drug-selection of transfected parasites

(a) Separation of *Nde*I-digested DNA by agarose gel electrophoresis: Lane 1: λ/*Pst*I marker; Lane 2: Mouse 3.3 parasite DNA (passage of non-drug treated parasites from mouse 1.2); Lane 3: Mouse 3.2 parasite DNA (passage of non-drug treated parasites from mouse 1.1); Lane 4: Mouse 3.1 parasite DNA (infected with original transfection stabilate)

(b) Southern blot using TCTP probe, and (c): Southern blot using TgDHFR/TS probe: Lane 1: mouse 3.1 parasite DNA; Lane 2: mouse 3.2 parasite DNA; Lane 3: Mouse 3.3 parasite DNA.
5.3.4 Second transfection experiment

5.3.4.1 Design of the long target b3D.Lf1.Lf2 construct

The overall result obtained from the first transfection experiment was that initially, the TCTP gene was disrupted with the TgDHFR/TS gene in the majority of parasites, however further drug selection resulted in the majority of parasites containing wt TCTP together with the TgDHFR/TS gene integrated in another location. Due to this, another targeting construct was designed, with longer stretches of DNA for homologous recombination, in the hope that this longer target might result in more stable integration.

The first integration construct (referred to as the short integration construct or b3D.int1.int2) used homologous sequences of ~340 bp, which is at the lower limit of fragments that have resulted in successful integration. It has been observed that longer stretches of homology result in higher transformation efficiencies, however an upper size limit of ~1 kb has been recommended for integration fragments, due to the known instability of vectors containing large stretches of A/T rich Plasmodium DNA (Menard and Janse, 1997).

The second integration construct was referred to as the long integration construct, or b3D.Lf1.Lf2. The first integration fragment (Lf1) was 906 bp in size, and corresponded to a region from -878 bp upstream to position 9 of the TCTP coding sequence. The second integration fragment (Lf2) was 759 bp in size, and corresponded to a region from +19 to +761 downstream of the TCTP stop codon. This long target DNA was designed such that almost the entire TCTP coding sequence, except for the first nine bases, would be replaced by the DHFR/TS gene in successfully transfected parasites. The steps involved in creating the b3D.Lf1.Lf2 were essentially identical to the steps involved in creating the b3D.int1.int2 vector, as represented in Figure 5.2. A schematic representation of the disruption of the TCTP region of the P. berghei ANKA chromosome by homologous recombination of the Lf1.Lf2 target DNA is given in Figure 5.15 (a). A schematic representation of the P. berghei ANKA TCTP region following disruption with the Lf1.Lf2 target is given in Figure 5.15 (b).
Figure 5.15: Disruption of TCTP on the *P. berghei* ANKA genome with the Lf1.Lf2 target DNA

(a) Schematic diagram of recombination via double cross-over between Lf1 and Lf2 on the linearised Lf1.Lf2 target DNA and the homologous regions on the *P. berghei* ANKA chromosome following transfection, the box represents TCTP coding sequence

(b) Schematic diagram of the TCTP region of the *P. berghei* chromosome following homologous recombination in a successful transfection experiment with the Lf1.Lf2 target DNA
5.3.4.1.1 PCR amplification of the long integration fragments and cloning into pCR2.1 vectors

The sequences of the primers used to amplify the long integration fragments were designed based on the sequence of the *P. berghei* contig containing the TCTP gene. The first long fragment primers (Lf1for and Lf1rev) were designed to incorporate *Apa*I and *Kpn*I restriction enzyme sites (as underlined), to allow for directional cloning into the first MCS of the b3D vector. The second long fragment primers (Lf2for and Lf2rev) were designed to incorporate *Bam*HI and *Xba*I restriction enzyme sites (as underlined), to allow for directional cloning into the second MCS of b3D. The sequence of the four primers was:

Lf1for: GCGGTACCTATACCTTTTGAAATGTCCAAGAT
Lf1rev: GCGGGCCCATACTTTTCATTGGCTATGC
Lf2for: GCGGATCCTATACCTACGATGGACGTGTTATTAC
Lf2rev: GCTCTAGACGACAATGGTTGGTCAAGTTT

The Lf1 and Lf2 fragments were amplified using Taq polymerase, under standard conditions using ~25 ng of *P. berghei* ANKA genomic DNA. Thirty amplification cycles were performed. Lf1 was amplified using a 50°C annealing temperature; Lf2 was amplified at a 55°C annealing temperature. A single product was amplified for each PCR at these annealing temperatures (Figure 5.16 (a)).

The Lf1 and Lf2 integration fragments obtained by PCR amplification were cloned into pCR2.1 vectors. Plasmid DNA from resulting white colonies was isolated using the Qiaprep Spin Miniprep kit, and restriction digests were performed to confirm insert size. Multiple bands, most likely due to star activity of the restriction enzymes, were observed when pCR.Lf1 plasmids were digested with both *Apa*I and *Kpn*I (results not shown). To avoid this, the plasmid preps were digested sequentially with 20 U of *Apa*I and then *Kpn*I in the appropriate buffer, with the reactions cleaned using the Qiaprep Spin Miniprep kit in between the first and second digest. Potential pCR.Lf2 plasmid preps were digested with *Bam*HI and *Xba*I. Analysis of digest reactions by electrophoresis indicated that the inserts had been successfully cloned into the pCR vectors (results not shown). Sequence analysis was performed on both inserts, and confirmed that the correct sequences had been ligated into the pCR2.1 vectors.
5.3.4.1.2 Cloning of the Lf1 and Lf2 products into the b3D vector

The pCR.Lf2 and b3D vectors were digested with 20 U each of BamHI and XbaI. DNA was separated by 1% agarose gel electrophoresis, correct-sized bands were excised with a scalpel and DNA was purified using the Qiaquick Gel Extraction kit as per manufacturer’s directions. Standard ligations of prepared DNA were performed using 50 ng vector and the appropriate amount of insert as calculated. The completed ligation reaction was used to transform electro-competent *E. coli*. Plasmid DNA from resulting colonies was isolated by minipreparation and was screened for the presence of the Lf2 insert by digestion of 10 µl of DNA with 10 U each of *Bam*HI and XbaI (*Figure 5.16 (b)*). Plasmid preparations with the correct-sized insert were named b3D.Lf2.

To avoid star activity, pCR.Lf1 and b3D.Lf2 plasmids were digested with *Apa*I then *Kpn*I sequentially, using 20 U of enzyme per reaction. The DNA was cleaned between digest reactions using the Qiaprep Spin Miniprep kit. The digested DNA was separated on a 1% w/v agarose gel, the bands were excised from the gel and DNA purified using the Qiaquick Gel Extraction kit. Ligation reactions of digested pCR.Lf1 and b3D.Lf2 plasmid DNA were performed using 50 ng vector and the appropriate amount of insert. Dextran-cleaned ligation product was used to transform electro-competent *E. coli*. Plasmid DNA from resulting colonies was isolated by minipreparation. Plasmids were analysed for the presence of the Lf1 insert by sequential *Apa*I and *Kpn*I enzyme digestion (*Figure 5.16 (c)*). Plasmid preps with the correct-sized insert were named b3D.Lf1.Lf2.
Figure 5.16: Creation of the b3D.Lf1.Lf2 construct

(a) PCR amplification of the Lf1 and LF2 integration fragments: Lane 1: λ/PstI marker; Lanes 2 & 3: Lf1; Lanes 4 & 5: Lf2

(b) Confirmation of insertion of Lf2 fragment into the b3D vector by digestion with *XbaI* and *BamHI*: Lane 1: λ/PstI marker; Lanes 2-5: b3D.Lf2

(c) Confirmation of insertion of Lf1 into the b3D.Lf2 vector by digestion with *ApaI* and *KpnI*: Lane 1: λ/PstI marker; Lanes 2-4: b3D.Lf1.Lf2
5.3.4.2 Rationale for the second transfection experiment

In the second transfection experiment, both the short integration target and the long integration target were used in separate transfections. The short integration target was included to see if unstable integration would again be observed.

5.3.4.2.1 Preparation of short and long target DNA prior to transfection

A final concentration of 10 µg of target DNA was required per transformation experiment. The short target DNA was prepared by digestion of the b3D.int1.int2 vector as previously described in section 5.3.3.1. Several preparations of the digested short target DNA prior to gel excision and purification are shown in Figure 5.17 (a); the purified short target DNA prior to transfection is shown in Figure 5.17 (c).

The long target DNA was prepared by digestion of the b3D.Lf1.Lf2 plasmid with XbaI and BgII, followed by digestion with Kpnl to avoid star activity. As with preparation of the short integration target, BgII was used to cut within the plasmid backbone, to minimise the likelihood of parasite transfection with residual intact plasmid.

Multiple aliquots containing 15 µg of the b3D.Lf1.Lf2 plasmid were digested with 25 U each of XbaI and BgII for 16 h. The completed digest reactions were each cleaned using the Qiaprep Spin Miniprep kit and digested for 5 h with 20 U of Kpnl. The digested DNA was separated on a 1% w/v agarose gel (Figure 5.17 (b)). The resulting target DNA of 6,280 bp was excised from agarose gels and DNA was purified using the Qiaquick Gel Extraction kit. The purified DNA preps were combined, precipitated using EtOH/NaOAc, and resuspended in a total volume of 10.5 µl in TE. A small aliquot of several preparations of prepared long target DNA was quantified before transfections by gel electrophoresis of 0.5 µl of target DNA using known quantities of PstI-digested λ DNA for comparison. Samples of the purified long target DNA prior to transfection are depicted in Figure 5.17 (c).
Chapter 5: Development of TCTP-knockout strains of *P. berghei*

Figure 5.17: Preparation of short and long integration target DNA prior to second transfection

(a) Preparation of short target: Lane 1: λ/PstI marker; Lanes 3-15: Digestion of b3D.int1.int2 with *ClaI*, *BamHI* and *BglII*.

(b) Preparation of long target: Lane 1: λ/PstI marker; Lanes 3-14: Digestion of b3D.Lf1.Lf2 with *XbaI*, *KpnI* and *BglII*.

(c) Quantification of target DNA prior to transfection: Lane 1: 1 µg λ/PstI marker; Lanes 2-4: long target; Lanes 5-7: short target.
5.3.4.2 Parasite transfection using the short and long target DNA

The prepared long target DNA was used in one transfection experiment, the prepared short target DNA was used in another transfection experiment. The transfection experiments were performed at WEHI by Dr. Tania de Koning-Ward. Each completed transfection mix was injected into the tail vein of one mouse. Both mice were treated with pyrimethamine water, commencing at between 24 and 30 hours post-infection, and parasitemia was monitored at regular intervals. At 10 dpi, parasitemia was 1% in the mouse that had received parasites from a short target transfection (designated mouse 4.1) and in the mouse that had received parasites from a long target transfection (designated mouse 4.2). These mice reached a parasitemia of ~5% at 11 dpi, and were sacrificed for blood collection. The blood was used for stabilate creation and for malarial DNA extraction.

5.3.4.3 Analysis of parasites obtained from second transfection experiments

5.3.4.3.1 Initial PCR analysis of transfected parasites

A PCR using Taq polymerase was performed to analyse the presence of the DHFR gene in the parasites from the short and long target transfection experiments. A product corresponding to the size of the DHFR gene was amplified in both the short and long target-transfected parasite DNA. Faint bands were also amplified in wt *P. berghei* ANKA DNA, however none of these bands was at the correct size. This result is shown in Figure 5.18 (a).

The parasite DNA obtained from transfections using the short (int1.int2) target was analysed using the same primer sets used to assess integration in the first transfection experiment (5'PyTCTP+Pf1rev and Pf2for+3'PyTCTP). Bands were observed for both the TCTP1 and TCTP2 integration PCRs in the transfected parasite DNA, but not in wt *P. berghei*. However, a strong wt TCTP band was also amplified in the transfected parasite DNA and in the wt DNA (Figure 5.18 (b)). This indicated that a mix of wild-type and TCTP-disrupted parasites might be present.

A strong wild-type TCTP band was detected in parasites from mouse 4.1, which had received parasites transfected with long target DNA (results not shown). This primer combination should not amplify a band in parasite DNA successfully transfected with the long target, as the primer binding sites should have been removed.
Figure 5.18: Initial genetic analysis of parasites from second transfection experiment by PCR

(a) Presence of TgDHFR/TS gene in transfected parasites: Lane 1: λ/PstI marker; Lanes 2-4: TgDHFR/TS PCR: 2: wt *P. berghei* ANKA DNA; 3: parasite DNA from short target transfection (mouse 4.2); 4: parasite DNA from long target transfection (mouse 4.1).

(b) Presence of TCTP1 and TCTP2 products, as well as wt TCTP, in parasites from short transfection experiment: Lane 1: λ/PstI marker; Lanes 3-5: TCTP1 PCR: 3: neg control; 4: parasite DNA from short transfection; 5: wt DNA; Lanes 6-8: TCTP2 PCR: 6: neg control; 7: parasite DNA from short transfection; 8: wt DNA; Lanes 9-11: TCTP PCR: 9: neg control; 10: parasite DNA from short transfection; 11: wt DNA.
5.3.4.3.2 Further PCR analysis of the short and long target-transfected parasites

The primer sets used to assess integration in parasites transfected with the short target could not be used to assess integration in parasites transfected with the long target, as the binding sites for the 5’ and 3’PyTCTP primers would have been removed.

Furthermore, it was realised that the primer sets previously used to assess integration in short target (5’PyTCTP+PF1rev and Pf2for+3’PyTCTP) could not distinguish between integration of the target at the right genome location or in other areas of the genome, as the 5’ and 3’PyTCTP primers did not bind outside the integration target region. Also, if short target DNA was present as extrachromosomal episomes in the transfected parasites, these primer sets would give false positive results as all primers had binding sites present on the b3D.int1.int2 plasmid.

To resolve this problem, new primer sets were designed. Initially, the primer pair of Lf1for and Pf1rev was used to check for integration of the first fragment, and the primer pair of Pf2for and Lf2rev was used to check for integration of the second fragment. These primer sets could be used to confirm correct integration in the parasites transfected with the short target, as the Lf1for and Lf2rev primers would bind outside the target sequence, but couldn’t be used to confirm integration in parasites transfected with the long target. These primer sets should have produced bands of ~1.1 kb in parasites from successful transfection experiments using the short target DNA. The PCRs were repeated several times at different annealing temperatures, no products were amplified. It is unknown whether this result was due to unsuccessful transfections, or was a PCR artefact due to the formation of primer dimers, etc. At a later stage, another set of primers was designed to detect integration; these PCRs are described in section 5.3.5.5.
5.3.4.4 Secondary drug-selection of parasites obtained from second transfection experiment

The initial PCR analysis results gave some indication that the transfections had been successful, due to the presence of the TgDHFR/TS gene product in parasites transfected with both the short and the long target. However, the presence of this gene in the correct location on the genome could not be determined by PCR, and further genetic analysis of parasites transfected with the short target indicated that wild-type TCTP was present. For this reason, parasites underwent secondary drug selection. The parasite stabilates from mouse 4.1 (infected with parasites from short target transfection) and mouse 4.2 (infected with parasites from long target transfection) were each used to infect three mice for secondary drug selection (mouse 5.1-5.6). After drug selection, the parasite DNA obtained from the three mice was pooled, such that there was one sample of secondary drug-treated parasites from the initial short target (mouse 5.1-5.3) and long target (mouse 5.4-5.6) transfection experiments. Mice received an i.p. injection of 75 µl of stabilate, and were treated with pyrimethamine provided in the drinking water from 26 hours post-infection. Parasitemia was around 2.5% in mice at 5 dpi, after this time parasite levels slightly decreased. Mice were sacrificed and blood was taken at 7 dpi, with parasitemia between 1 and 2%. Blood was used for stabilate creation and malarial DNA extraction.

5.3.4.5 Genetic analysis of initial and secondary drug-selected parasites from the second transfection experiment by Southern blot

5.3.4.5.1 Preparation of the long TCTP probe

Various primer combinations that would amplify a ‘long TCTP’ probe, containing the flanking regions of TCTP, were evaluated. This probe should bind to the TCTP flanking regions present in both wild-type parasites and in parasites transfected with the short or long target. Of the primer sets evaluated, the combination of First for (described in 5.3.2.2) and Lf2rev (described in 5.3.4.1.1) produced a strong band at the expected size of ~1,300 bp from wt *P. berghei* ANKA DNA, as well as several other faint non-specific bands. Entire PCR reactions were split in two and separated on adjacent lanes of a 1% agarose gel, to obtain sufficient DNA (Figure 5.19). The PCR products were excised from the gel and purified prior to hexanucleotide labelling.
Figure 5.19: Separation of whole PCR reactions used to amplify long TCTP probe

Lane 1: λ/PstI marker; Lanes 5, 6, 8, 9, 11, 12, 14, 15: PCR products used to create the long TCTP probe.
5.3.4.5.2 Southern blot

A Southern blot was performed using DNA both from the initial parasites obtained from the second transfection experiments (from mouse 4.1 and 4.2), and from the combined DNA of parasites following secondary drug selection (from mouse 5.1-5.3 and 5.4-5.6). Preparations of 10 µg of genomic DNA were digested with NdeI. The digested DNA was separated by 1% agarose gel electrophoresis (Figure 5.20 (a)). The DNA was transferred to a nylon membrane and probed with the long TCTP probe. In NdeI-digested DNA from successfully transfected parasites, the long TCTP and DHFR probes should detect a band of 7,988 bp in parasites transfected with short target, a band of 7,643 bp in parasites transfected with the long target, and a 3.5 kb band in parasites containing wild-type TCTP. The size of the bands detected in the Southern blot was calculated by placing a ruler next to the wells on the stained agarose gel and next to the well mark on the Southern blot. The wells were visible on the Southern blot due to the presence of undigested parasite DNA.

A strong background was observed in blots probed with the long TCTP probe (Figure 5.20 (b)). A band at ~3.6 cm from the wells, corresponding to a size of ~3.5 kb, was detected in the wt *P. berghei* ANKA sample (Lane 1). This band was detected faintly in parasites from both transfection experiments following secondary drug selection (from mouse 5.1-5.3 and 5.4-5.6) (Lanes 3 and 5). The parasite DNA obtained from the initial short and long target transfection experiments (mouse 4.1 and 4.2) gave a slightly different binding pattern to either wild-type or secondary drug selected-parasites. A strong band at ~3.7 cm from the wells, corresponding to a size of ~3.2 kb, was detected (Lanes 2 & 4). It is probable that these bands also correspond to wild-type TCTP, and the size difference is due to the different migration of the digested genomic DNA. The DNA corresponding to lanes 2 and 4 on the Southern blot appears to have migrated more rapidly on the agarose gel (Figure 5.20 (a), lanes 3 and 5).

The membrane was stripped and reprobed with the DHFR probe (Figure 5.20 (c)). Only two DNA samples produced distinct bands. One of these samples was from the initial parasites transfected with the long target, prior to secondary drug selection (from mouse 4.2). In this sample, a band was detected at ~3.3 cm from the wells, with a size of 4.7 kb. Indistinct bands above 11.5 kb were also detected (Lane 4). An indistinct band at >11.5 kb was also detected in parasites transfected with the short target prior to secondary drug selection (from mouse 4.1) (Lane 2).
Figure 5.20: Southern blot of parasite DNA from second transfection experiment

(a) Separation of Ndel-digested parasite DNA: Lane 1: λ/PstI marker; Lane 3: wt *P. berghei* DNA; Lane 4: initial parasite DNA from short target transfection (mouse 4.1); Lane 5: parasite DNA from short target transfection following secondary drug selection (mouse 5.1-5.3); Lane 7: initial parasite DNA from long target transfection (mouse 4.2); Lane 8: parasite DNA from long target transfection following secondary drug selection (mouse 5.4-5.6).

(b) Southern blot using long TCTP probe, and (c) Southern blot using TgDHFR/TS probe: Lane 1: wt *P. berghei* DNA; Lane 2: initial parasite DNA from short target transfection; Lane 3: parasite DNA from short target transfection following secondary drug selection; Lane 4: initial parasite DNA from long target transfection; Lane 5: parasite DNA from long target transfection following secondary drug selection.
5.3.5 Third transfection experiment

5.3.5.1 Rationale for final transfection experiment

A final transfection experiment was performed, using long target DNA (from b3D.Lf1.Lf2). As well as the initial drug selection following transfection, the secondary drug selection was also performed at WEHI, as it was thought that the unexpected results obtained following secondary drug selection in the previous transfection experiments may have been due to differences in the drug selection procedure or in the mice used between WEHI and RMIT.

5.3.5.2 Preparation of long target DNA prior to transfection

The long target DNA was prepared by digestion of the b3D.Lf1.Lf2 plasmid by XbaI and BglI, followed by purification of the DNA and subsequent digestion with KpnI. Representative samples of the digested target DNA prior to purification are shown in Figure 5.21 (a), samples of long target DNA after purification are shown in Figure 5.21 (b). Two final samples of 10 µg long target DNA were prepared.

5.3.5.3 Transfection of parasites with long target DNA

The purified long target DNA was used in two separate transfection experiments. Transfection experiments were performed at WEHI by Dr. Tania de Koning-Ward as previously described, and each transfection experiment was used to infect one mouse (mouse 6.1 and 6.2). At a parasitemia of ~5%, mice were sacrificed and blood was taken for malarial DNA extraction. An alternate DNA extraction method was evaluated. In this method leukocytes were removed by passing blood over a CF11 column, erythrocytes were lysed with saponin and the parasites were lysed with SDS (as per the method described in 2.9.2.4.2). Two malarial DNA preparations were performed on the blood of each of the two mice.

5.3.5.4 Initial genetic analysis of parasite DNA

PCRs were performed using the 5’ and 3’PyTCTP primers. A wild-type TCTP band was amplified in all transfected parasite DNA preparations, as well as in wild-type P. berghei ANKA DNA. This result is shown in Figure 5.22.
Chapter 5: Development of TCTP-knockout strains of *P. berghei*

Figure 5.21: Preparation of long target DNA prior to third transfection experiment
(a): Digestion of b3D.Lf1.Lf2 with *Xba*I, *Kpn*I and *Bgl*I: Lanes 1, 3, 5, 6, 8, 9: b3D.Lf1.Lf2 digests
(b) Samples of purified long target DNA prior to third transfection: Lane 1: 1 µg λ/PstI marker; Lane 2-4: long target samples from three separate preparations; Lane 6 & 7: 0.5 µg λ DNA.

Figure 5.22: TCTP PCR analysis of parasite DNA from 3rd transfection experiment
Lane 1: neg control; Lanes 2 & 3: DNA obtained from mouse 6.1; Lanes 4 & 5: obtained from mouse 6.2; Lane 6: wt *P. berghei* ANKA DNA; Lane 7: λ/PstI marker.
5.3.5.5 Further genetic analysis of parasite DNA obtained from the 1st, 2nd and 3rd transfection experiments

As previously discussed (section 5.3.4.3.2), the PCRrs previously used to assess integration in short target transfections could not distinguish between correct integration and integration of the target DNA elsewhere on the genome, or the presence of the target DNA as episomes. Also, previous attempts to analyse integration in parasites transfected with the long target did not result in PCR products, but it was not known whether this was due to a PCR artefact. Due to this, another set of primers was designed to assess correct integration of the short and long target DNA. The Intf1f primer (CGTGGGCGTAGCATATATTTTG) was designed to bind upstream of the Lf1 integration fragment in the *P. berghei* genome, so it could be used together with the Pf1rev primer to assess for correct integration of the first homologous region in both the short and long target DNA. The Intf2r primer (TTTTTACCCCTTAATAACTAGT) was designed to bind downstream of the Lf2 integration fragment in the *P. berghei* genome, so it could be used together with the Pf2for primer to assess for correct integration of the second homologous region in both the short and long target DNA. The Pf1rev and Pf2for primers bind within the TgDHFR/TS UTRs, and therefore should not bind to wild-type parasite DNA. PCRs were performed on DNA obtained from each of the three knockout experiments.

The Intf1f+Pf1rev primer combination should have resulted in 1.6 kb and 1.4 kb products in parasites successfully transfected with the short and long target DNA, respectively. These potential products were designated longTCTP1. The Pf2for+Intf2r primer combination should result in 1.15 kb and 0.97 kb products in parasites successfully transfected with the short and long target DNA, respectively. These potential products were designated longTCTP2. The smaller size of products in parasites transfected with the long target DNA is due to the deletion of more of the TCTP coding sequence.

The Intf1f and Intf2r primers were used to assess correct integration in parasite DNA from the first, second and third transfection experiments. PCR experiments were performed using Taq polymerase, and the Intf1f+Pf1rev or Pf2for+Intf2r primer sets. The longTCTP2 PCR product was amplified from parasite DNA obtained from the first transfection experiment, both in the initial non drug-treated parasites (from mouse 1.1 and 1.2) and in the parasites subjected to secondary drug selection (from mouse 2.1 and 2.2) (Figure 5.23, lanes 2-5). The longTCTP2 product was also amplified from both parasite preparations obtained in the third transfection experiment (from mouse 6.1 and
6.2) (Figure 5.23, lanes 6-9). No products were obtained from the wt *P. berghei* DNA or from parasites obtained in the second transfection experiment (results not shown).

Several attempts were made to amplify the longTCTP1 products, using annealing temperatures as low as 45°C. No products were obtained. This was somewhat unexpected, as in the earlier genetic analyses the TCTP1 product was amplified from parasites extracted from mouse 1.1, 1.2 and 2.2, whereas the TCTP2 product was not. As the longTCTP2 fragment was amplified in these parasites, the TCTP2 product should also have been amplified in the earlier PCRs. This combination of results suggests that a negative PCR result could not be taken as confirmation of an unsuccessful transfection reaction. The longTCTP2 products obtained indicated that, in the first and third transfection experiments, the target DNA had integrated in the correct location by at least a single crossover event. Due to this somewhat positive result, parasites obtained from the third transfection experiment were used to infect mice at WEHI for secondary drug selection.
Figure 5.23: Analysis of target DNA integration in parasites from the 1\textsuperscript{st} and 3\textsuperscript{rd} transfection experiments

Lane 1: $\lambda$ PstI marker; Lanes 2, 4, 6, 8: Long TCTP1 PCR; Lanes 3, 5, 7, 9: LongTCTP2 PCR: (2 & 3) non-drug selected parasites from 1\textsuperscript{st} transfection experiment (combined DNA from mouse 1.1 and 1.2); (4 & 5) parasites from 1\textsuperscript{st} transfection after secondary drug selection (combined DNA from mouse 2.1 and 2.2); (6 & 7) parasites from 3\textsuperscript{rd} transfection experiment extracted from mouse 6.1; (8 & 9) parasites from 3\textsuperscript{rd} transfection experiment extracted from mouse 6.2.
5.3.5.6 Secondary drug selection of parasites from 3rd transfection experiment

5.3.5.6.1 Secondary drug selection of parasites from mouse 6.1 and 6.2

The two parasite stabilates obtained from the third transfection experiment (two separate transfections, both using the long target) were each injected into two mice for secondary drug selection (designated mouse 7.1 - 7.4). The second round of drug selection was performed at WEHI. At a parasitemia of ~5%, mice were sacrificed and blood was collected for stabilate creation and parasite DNA extraction.

5.3.5.6.2 Genetic analysis of parasites from 3rd transfection experiment after secondary drug selection

PCRs were performed on the malarial DNA obtained from each of the four mice following secondary drug selection. The Intf1f + Pf1rev and Pf2for + Intf2r primer combinations were used to assess for correct integration of the Lf1 and Lf2 fragments in the *P. berghei* genome. An annealing temperature of 50°C was used. As observed in the parasites from the first round of drug selection, only longTCTP2 products (0.97 kb) in were obtained in all of the transfected parasite samples. No integration products were amplified in wt *P. berghei* DNA. These results are shown in Figure 5.24 (a). A strong wt TCTP band of 516 bp was also amplified in all of the transfected parasite samples, as well as in wt *P. berghei* DNA. This result is shown in Figure 5.24 (b).
Figure 5.24: Genetic analysis of parasites from 3rd transfection experiment following secondary drug selection

(a) Analysis of long target integration by PCR: Lane 1: λ/PstI marker; Lanes 2, 4, 6, 8, 10, 12: longTCTP1 PCR using Intf1f and Pf1rev primers; Lanes 3, 5, 7, 9, 11, 13: longTCTP2 PCR using Pf2for and Intf2r primers: (2 & 3): neg control; (4 & 5): wt P. berghei DNA; (6 & 7): secondary drug-selected parasites from mouse 7.1; (8 & 9): secondary drug-selected parasites from mouse 7.2; (10 & 11): secondary drug-selected parasites from mouse 7.3; (12 & 13): secondary drug-selected parasites from mouse 7.4.

(b) Analysis of presence of wild-type TCTP by PCR: Lane 1: λ/PstI marker; Lane 2: neg control; Lane 3: wt P. berghei DNA; Lanes 4-7: parasite DNA from mouse 7.1, 7.2, 7.3 and 7.4, respectively.
5.3.5.7 Genetic analysis of parasites from 3rd transfection experiment by Southern blot

The PCR results were identical for the initial and secondary drug-selected parasites obtained from the third transfection experiment. In all of these parasite preparations, a strong wt TCTP product was amplified, as well as a longTCTP2 product. This indicated that a mix of parasite genotypes were present in the preparations. To gain a better understanding of the relative proportion of wild-type and TCTP-disrupted parasites present, a Southern blot was performed.

5.3.5.7.1 Preparation of Lf2 and TgDHFR/TS probes

The Lf2 and TgDHFR/TS probes were prepared by digestion of the b3D.Lf1.Lf2 plasmid, rather than by PCR. The Lf2 probe was prepared by digestion of b3D with BamHI and XbaI, and the TgDHFR/TS probe by digestion with Apal and BamHI. The completed digest reactions are shown in Figure 5.25 (a). The bands of the correct size for the probes were excised from the gel and purified. A sample of the purified probes was separated by agarose gel electrophoresis for DNA quantitation prior to hexanucleotide labelling. The purified probes are shown in Figure 5.25 (b).

5.3.5.7.2 Preparation of λ/Ndel marker

A λ/Ndel marker was prepared, to allow for better determination of bands detected by Southern blot. A total of 10 µg of λ DNA was digested with 10 U Ndel for 16 h, 2 µg was loaded per gel lane. Theoretically, the fragment sizes obtained would be: 27,633, 8,371, 3,801, 2,433, 2,253, 1,689, 1,174, and 556 bp. This marker would allow for differentiation between target DNA integration in the genome (a band of 7.6 kb) and the presence of intact b3D.Lf1.Lf2 plasmid as episomes. The intact b3D.Lf1.Lf2 plasmid would be 9.16 kb in size, and contains a single Ndel site.
Figure 5.25: Preparation of Lf2 and DHFR probes

(a) Digestion of b3D.Lf1.Lf2 plasmids for Lf2 probe and TgDHFR/TS probe. Lane 1: λ/PstI marker; Lanes 3 & 4, 6 & 7: b3D.Lf1.Lf2 digested with BamHI and XbaI for Lf2 probe; Lanes 9 & 10, 12 & 13: b3D.Lf1.Lf2 digested with ApaI and BamHI for TgDHFR/TS probe.

(b) Purified Lf2 and TgDHFR/TS probes. Lane 1: λ/PstI marker; Lane 2: Lf2 probe; Lane 3: TgDHFR/TS probe.
5.3.5.7.3 Southern blot of parasite DNA from 3\textsuperscript{rd} transfection experiment

Seven DNA samples were analysed: wt \textit{P. berghei} ANKA, each of the initial transfected parasite preparations (obtained from mouse 6.1 and 6.2) and the parasites following secondary drug selection (obtained from mouse 7.1, 7.2, 7.3 and 7.4). Approximately 10 $\mu$g of genomic DNA from each of the samples was digested with 30 U of \textit{Nde}I, and separated on a 1\% agarose gel at 70 V (\textit{Figure 5.26 (a)}).

The DNA was transferred to a nylon membrane, and hybridised with the Lf2 probe, which should detect a 3.5 kb band in wt DNA, and a 7.6 kb band in parasites successfully transfected with the long target. The probe binding reaction was detected using the chemiluminescent CSPD substrate, for enhanced sensitivity. Hybridisation was performed at a hybridisation temperature of 63°C. The reaction was detected by autoradiography using X-ray film, as depicted in \textit{Figure 5.26 (b)}, and also by chemiluminescent detection using the GeneSnap machine, as shown in \textit{Figure 5.26 (c)}. The membrane was placed such that the top edge was flush with the wells on the gel, so a ruler starting from the top of the membrane could be used to compare band distances with those of the markers on the agarose gel.

Several incubation times of X-ray film were tested, from 10 to 20 minutes. The banding pattern was similar for all incubation times. To clarify the sizes of the bands obtained, the blot was visualised using a GeneSnap™ imaging machine capable of chemiluminescent detection (\textit{Figure 5.26 (c)}). A ruler could not be used in this detection method. The same banding pattern was observed for each of the samples as on the X-ray film.

Two bands were detected in the wt DNA sample (\textit{Figure 5.26 (b) and (c), Lane 1}). A major band was observed at $\sim$3.5 cm from the wells, corresponding to a size of $\sim$3.8 kb. Another band was detected at approximately 1.9 cm from the wells, corresponding to a size of greater than 11.5 kb, which was most likely due to the presence of residual undigested parasite DNA.

In all transfected parasite samples, multiple bands were present (\textit{Figure 5.26 (b) and (c), lanes 2-7}). A band was present at 3.5 to 3.7 cm from the wells, at around $\sim$3.8 kb. This corresponded to wild-type TCTP. The minor differences in size of this low band were most likely due to differences in migration of the digested genomic DNA through the agarose gel, as previously observed.
Several higher molecular-weight bands were detected in each of the transfected parasite samples. In successful transfection experiments, a single band of 7.6 kb, corresponding to a distance of ~2.5 cm from the wells was expected. Instead, a major band at ~2.8 cm from the wells, corresponding to a size of ~6 kb, was present in all samples, as well as several other bands above this size. In the parasites obtained initially from the transfection experiment (from mouse 6.1 and 6.2), and in the secondary drug-selected parasites from mouse 7.3 and 7.4, the 6 kb band was the most prominent band present (Figure 5.26 (b) and (c), Lanes 2, 3, 6, 7).

In the parasite DNA obtained from mouse 7.2, the major band present was at ~2.5 cm from the wells, corresponding to a size of ~8 kb. This was also the major band detected in the parasites from mouse 7.1 (Figure 5.26 (b) and (c), Lanes 4 & 5). It is possible that this major band represents successfully transformed parasites. A band at around this size was also present, at a much lower proportion, in the other transfected parasite preparations. As this band was detected at a far greater proportion in the secondary drug-selected parasites from mouse 7.2 than in the parental parasites from the initial transfection (from mouse 6.1, Lane 2) it is possible that in this case, the secondary drug treatment was selecting for the correctly transformed parasites. However, it is clear that a number of other parasite genotypes were still present, including a proportion of parasites that contained wild-type, non-disrupted TCTP.

It is possible, but unlikely, that the lower hybridisation temperature used with the Lf2 probe (63°C) compared with the TCTP and TgDHFR/TS probes used previously (65-68°C) contributed to the detection of multiple bands. However, hybridisation of this probe to the wild-type \textit{P. berghei} ANKA sample produced the expected banding pattern.

In the transfected parasite samples a bright band was observed on the stained agarose gel, corresponding to a size of ~2.4 kb. It was thought that this might be a plasmid, but no DNA of that size should have been produced with any of the restriction enzymes used. This band was not detected with the Lf2 probe. It is assumed that if this band is a contaminating plasmid, it is too small to confer drug resistance, as the TgDHFR/TS gene with the 5'UTR is around 4 kb. It is possible that this band corresponds to a ribosomal RNA gene, transcripts of \textit{P. berghei} rRNA genes at around 2.7 kb have been identified (Waters \textit{et al.}, 1997b).
Figure 5.26: Southern blot of parasite DNA obtained from 3rd transfection experiment

(a) Separation of Ndel-digested parasite DNA: Lane 1: λ/PstI marker; Lane 2: *P. berghei* ANKA DNA; Lane 3: initial parasite DNA from 3rd transfection (from mouse 6.1); Lane 4: initial parasite DNA from 3rd transfection (from mouse 6.2); Lanes 5-8: parasite DNA after secondary drug selection (from mouse 7.1 to 7.4, respectively). Lane 9: λ/Ndel marker.

(b) Detection of Lf2-hybridised DNA bands using X-ray radiography: Lane 1: *P. berghei* ANKA DNA; Lane 2: initial parasite DNA from 3rd transfection (from mouse 6.1); Lane 3: initial parasite DNA from 3rd transfection (from mouse 6.2); Lanes 4-7: parasite DNA after secondary drug selection (from mouse 7.1 to 7.4, respectively)

(c) Detection of Lf2-hybridised bands using chemiluminescence reader: Lane 1: *P. berghei* ANKA DNA; Lane 2: initial parasite DNA from 3rd transfection (from mouse 6.1); Lane 3: initial parasite DNA from 3rd transfection (from mouse 6.2); Lanes 4-7: parasite DNA after secondary drug selection (from mouse 7.1 to 7.4, respectively)
In an attempt to further characterise the genotype of the parasites obtained from the third transfection experiment, the membrane was stripped and re-probed using the TgDHFR/TS probe. Unexpectedly, the initial Lf2 probe could not be stripped from the membrane. This was determined due to the fact that identical bands were obtained using the TgDHFR/TS probe as for the Lf2 probe, including in the wild-type *P. berghei* ANKA sample (results not shown). It is possible that the nylon membrane dried sufficiently for the DIG-labelled Lf2 probe to become permanently attached. Therefore, the possible correct integration of the target DNA in some of the parasite samples could not be confirmed, as the location of the TgDHFR/TS gene could not be identified.

There was insufficient DNA remaining from the original parasite samples to perform another blot. The parasite stabilates could have been used to infect new mice, however from previous observations this would have most likely resulted in further genomic rearrangements.
5.4 Discussion

Prior to the start of the transfection experiments, the genomic sequence of *P. berghei* ANKA was analysed to assess the feasibility of developing a TCTP knockout malaria strain. The genome sequence of *P. berghei* ANKA is not yet assembled, however a contig sequence of ~11.5 kb containing the TCTP gene was available on the PlasmoDB database (www.plasmodb.org/plasmo). A BLAST search using the TCTP coding sequence indicated that only a single gene copy was present in the genome. The Southern blot results using wild-type *P. berghei* ANKA DNA also indicated that only a single copy of the TCTP gene was present. By analysis of the TCTP flanking regions of the contig, the nearest identified genes were around 3 kb from the TCTP coding sequence. Therefore it was feasible that TCTP could be knocked out without disruption of adjacent genes.

The first transfection construct (b3D.int1.int2) was designed to include mainly TCTP coding sequence, due to the higher GC content. The entire TCTP sequence could not be contained within the insertion fragments, as this could result in transfected parasites with wild-type TCTP if the TgDHFR/TS gene was excised from the genome. As TCTP is a small gene (516 bp), and the minimum fragment size that has previously resulted in successful integration in Amaxa transfections is ~300 bp (Janse *et al.*, 2006b; Jongco *et al.*, 2006), around 150 bp of the 5’ and 3’ TCTP UTRs were also included in the integration fragments. In successful transfection experiments, this should lead to the permanent excision of the middle 141 bp of TCTP. It was thought that this would be sufficient to result in non-active protein in the event of TgDHFR/TS gene excision from the genome following transfection.

In the initial transfection experiment, parasites grew very slowly, with the recipient mouse only developing detectable parasitemia at 18 dpi. It has been stated that wild-type parasites can survive and slowly develop drug resistance during pyrimethamine treatment, and are typically observed at day 13-15 following unsuccessful transfection experiments (Dr Tania de Koning-Ward, pers. comm., http://www.lumc.nl/rep/cod/redirect/1040/research/malaria/malaria.html). Due to this previous finding, it was assumed that the parasites obtained at 18 dpi were wild-type. In order to obtain sufficient DNA for Southern blot analysis, two mice were injected with stablate, drug treatment was not performed as this would have killed wild-type parasites.
However, genetic analysis indicated that the initial knockout experiment had been successful. By PCR analysis, no wild-type TCTP was amplified, and the Southern blot results indicated that the majority of parasites present contained the TCTP gene disrupted with TgDHFR/TS. PCRs using primers that should only give products in transfected parasites gave mixed results. The TCTP1 integration fragment was amplified, this involved primers that would bind to the start of the TCTP coding sequence and the 5'UTR of the TgDHFR/TS. However, the TCTP2 fragment was never amplified, this involved primers that bound in the 3'UTR of the TgDHFR/TS and the end of the TCTP coding sequence. It was thought that the target DNA might have integrated incorrectly in the second crossover region.

It was later apparent that the primer combinations used to amplify TCTP1 and TCTP2 would not differentiate between correct integration and the presence of the target DNA in other areas of the genome, including as episomes. To properly assess integration of the int1.int2 target in the parasite DNA from the first transfection, the Int1f and Int2r primers, which bound outside of homologous recombination regions, were used along with primers that bound in the TgDHFR/TS UTRs. Only the longTCTP2 product was amplified, indicating that the target DNA had integrated correctly in this region. The inability to amplify the longTCTP1 fragment could have been due to suboptimal PCR conditions, or could possibly have been due to incorrect integration such that the int1f primer binding site was not present in the correct location.

Primers were designed using the CloneManager program (SECentral), which analyses potential primer stability and the possibility of primer dimer formation. Despite this, it is possible that the inconclusive PCR results were due solely to PCR failure, or they could indicate that a more complex integration reaction had occurred, rather than straightforward homologous recombination. Southern blot results were relied on for a more accurate determination of the parasite genotype following transfection.

Due to the positive result obtained on the initial Southern blot, the parasites from the first transfection were used to infect three mice for further drug selection. In two of the mice, the parasitemia increased to between 5-8% at 7-9 dpi. In the third mouse, detectable parasitemia (~0.1%) was observed at 6 dpi, but the parasites then decreased and were not detectable from 11 dpi onwards. Initial PCR analysis indicated that a mixture of parasite genotypes were present, in that products indicative of integration (TCTP1 and longTCTP2) were amplified, as well as wild-type TCTP. This suggested that
TCTP-knockout parasites might be at a selective disadvantage, despite the pyrimethamine drug pressure.

To further analyse the mixture of parasite genotypes present, Southern blots were performed. The parasite DNA from each drug-treated mouse was analysed individually. The Southern result was identical for each mouse, and indicated that the majority of the parasites present following drug selection consisted of wild-type, uninterrupted TCTP, with the TgDHFR/TS gene present in another, incorrect genomic location.

The secondary drug selection was repeated with three more mice. The same Southern result was obtained, indicating that secondary drug treatment was selecting for parasites containing the TgDHFR/TS gene integrated in an incorrect location, with the wild-type TCTP gene also present. The presence of the wild-type TCTP gene was most likely not due to reversion correctly transfected parasites leading to excision of the target DNA, as this should have resulted in a TCTP PCR product 141 base pairs shorter (375) than the wild-type TCTP gene (516 bp). Instead, it is hypothesised that the secondary drug treatment strongly selected against parasites with the TCTP gene disrupted. The genotype that was obtained following secondary drug treatment could have been the result of strong selection of a very small initial population of parasites containing incorrectly-integrated TgDHFR/TS, which were not detected by the initial TCTP PCRs.

It was thought that transfection with a second construct containing longer integration fragments might result in higher transfection efficiencies and more stable integration, therefore the b3D.Lf1.Lf2 construct was created. The two fragments designed for homologous recombination were between 750 and 900 bp in size, compared with the 340 bp fragments used in the first integration construct. This construct was also designed such that almost the entire TCTP coding sequence (excluding the first 9 bp) would be removed from the genome in a successful transfection experiment.

In the second transfection experiment, target DNA prepared from both the short (b3D.int1.int2) and long (b3D.Lf1.Lf2) constructs was used in two mice each. In both transfections, parasites appeared more rapidly than in the first transfection experiment (~10 dpi compared with 18 dpi), but were still somewhat slow to appear compared to other published reports using the Amaxa nucleofector (3-7 days), (Janse et al., 2006b).
In the parasites transfected with the short target in the second knockout experiment, the TCTP1 and TCTP2 integration products, the TgDHFR/TS product, and a strong wild-type TCTP band were amplified. These results indicated that either a mixture of parasite genotypes were present, or that the entire short target DNA had integrated in the wrong area of the genome. The longTCTP1 and longTCTP2 products were not amplified, supporting the assumption that the short target DNA was either present as episomes or had integrated in an undesired area of the genome. In parasites transfected with the long target, a wild-type TCTP band was amplified, as well as a TgDHFR/TS product. The longTCTP1 and longTCTP2 products were not amplified.

Secondary drug selection was performed on parasites from both the short and long target transfections. The genotype of these parasites was analysed by Southern blot, as well as the genotypes of the parasites prior to secondary drug selection. A band corresponding to wild-type TCTP was detected in all parasite preparations. The TgDHFR/TS gene was only detected by Southern blot in the parasite preparations initially transfected with both the short and long target, and was in an incorrect genomic location. In the parasites transfected using the short target DNA, the TgDHFR/TS probe detected a band at >11.5 kb. In the parasites transfected using the long target DNA, the TgDHFR/TS probe detected a band at 4.7 kb. Unexpectedly, the TgDHFR/TS probe did not detect a band in either the short- or long-target transfected parasites following secondary drug selection. These parasites were pyrimethamine-resistant, as parasitemia had rapidly increased to ~3% at five days post-infection. The reason for the lack of detection of a TgDHFR/TS gene in the secondary drug-selected parasites is unknown. If pyrimethamine resistance was conferred by the presence of episomal b3D.int1.int2 or b3DLf1.Lf2, detection of a band corresponding to the size of the linearised construct would be expected, as both plasmids contain one NdeI site.

A third transfection experiment was performed using long target DNA, in which both the initial and secondary drug selection was performed at WEHI. In both the initial parasites obtained following transfection and in the parasites following secondary drug selection, wild-type TCTP and longTCTP2 products were amplified by PCR. LongTCTP1 products were never amplified. This indicated that in a proportion of parasites, at least the second homologous fragment of the long target DNA had integrated in the correct location on the genome. It was unknown whether the inability to amplify the LongTCTP1 product might be due to a PCR artefact, and so Southern blots were relied on for more accurate genotyping of the transfected parasites.
Southern blot analysis was performed, using a more sensitive method of detection (chemiluminescence) together with a new probe (Lf2) prepared by digestion of the b3DLf1.Lf2 plasmid rather than PCR. Both X-ray film and a chemiluminescent imager were used to detect DNA bands reacted with the Lf2 probe. Multiple bands were detected in all parasite samples. Two bands were detected in the wild-type \textit{P. berghei} sample; one corresponding to wild-type TCTP, the other band was most likely undigested parasite DNA. A band corresponding in size to wild-type TCTP was present in of the transfected parasite samples. Additionally, multiple bands ranging in size from \(~6\) kb to over \(11.5\) kb were detected. It is unlikely that these multiple bands were due to incomplete digestion of the parasite DNA, as bright smears had been present on the agarose gel for all samples, and the wild-type \textit{P. berghei} sample had given the expected banding pattern. Also, only faint bands corresponding to undigested parasite DNA were present. As the Lf2 probe gave the expected banding pattern in wild-type parasites, it was clear that in all of the transfected parasites, a mixture of genotypes was present, and a proportion of parasites possessed a non-disrupted TCTP gene.

A band possibly indicative of correct long target DNA integration was present at \(~7.6\) kb in several of the transfected parasite samples. This \(7.6\) kb band appeared to be selected for by repeated pyrimethamine treatment, as it was present in a higher proportion of secondary-drug selected parasites than in the parental parasites. The Lf2 probe could not be stripped from the membrane, so the presence of the TgDHFR/TS gene at this same location could not be assessed. Insufficient parasite DNA remained to repeat the Southern blot using the TgDHFR/TS probe. Obtaining more parasite DNA would have required further drug selection in mice, which would be likely to further alter the proportions of parasite genotypes present, based on the results of earlier experiments. Multiple rounds of drug selection to obtain a single transfected genotype are not generally required, according to published reports. These transfection experiments were performed at a time when the Amaxa transfection protocols were still being developed for \textit{P. berghei} ANKA, it has been since reported that no secondary drug selection is necessary for Amaxa transfections of malaria parasites, due to the high transfection efficiencies obtained (Janse et al., 2006b).

In the majority of parasites obtained from the transfection experiments, the Southern blot results indicated that the target DNA used for transfection had integrated incorrectly in the genome. Bioinformatics analysis had indicated that only a single copy of the TCTP gene was present in \textit{P. berghei} ANKA, and that no additional highly similar sequences to any of the integration fragments were present. However, as the \textit{P. berghei}
Chapter 5: Development of TCTP-knockout strains of *P. berghei*

genome has not been fully assembled, it is possible that other sequences highly similar to TCTP may exist, allowing for homologous recombination in undesired areas of the genome. As the wild-type *P. berghei* DNA used in the Southern blots produced a single band when reacted with TCTP probes, this is not the most likely explanation for the results observed.

Pyrimethamine-resistant non-targeted parasite clones may arise by replacement of the endogenous DHFR/TS with the mutant TgDHFR/TS, as the 5' and 3'UTRs on the b3D plasmid are obtained from *P. berghei* DHFR/TS and may act as target sequences for homologous recombination (Menard and Janse, 1997). Analysis of the contig containing *P. berghei* DHFR/TS indicated that digestion with *Nde*I would result in a band of over 5.5 kb, the exact size could not be determined as a second *Nde*I site was not present on the contig downstream of the DHFR/TS gene. It is possible that the >11.5 kb bands sometimes observed on Southern blots probed with TgDHFR/TS could have been due to homologous recombination between the exogenous and endogenous DHFR/TS genes. However, in a published report a drug-resistant mutant version of the *P. berghei* DHFR/TS gene together with UTRs was used as a selectable marker, and integration in the endogenous *P. berghei* DHFR/TS region did not occur (Menard and Janse, 1997).

The confirmed correct integration of target DNA was observed to occur in the majority of parasites in the first transfection experiment, however these parasites were selected against during further passage and drug treatment. One possible explanation is that TCTP contributes in some way to growth in the presence of pyrimethamine, such that parasites with the gene disrupted are selected against by repeated drug treatment. The facts supporting this hypothesis are that the occasion in which the majority of transfected parasites contained a disrupted TCTP gene was when secondary selection was performed in the absence of drug pressure. When the identical parasite stablate was subjected to secondary pyrimethamine treatment, parasites with the TgDHFR/TS gene integrated elsewhere in the genome were strongly selected for. However, parasites with the TCTP gene disrupted were able to survive the initial round of pyrimethamine treatment following the transfection experiment. The proportion of these parasites in the initial stablate obtained is unknown, as direct genetic analysis was not performed. Although TCTP is known to interact with the antimalarial drug artemisinin (Bhisutthibhan and Meshnick, 2001; Meshnick, 2002), there is no published literature to support the involvement of TCTP in antifolate drug resistance, which has a separate mode of action. An alternate hypothesis is that parasites with the TCTP gene grew much more slowly
and were out-competed by other parasites containing the TgDHFR/TS gene, which were more strongly selected for when drug pressure was applied.

Other methods to inhibit the expression of TCTP in malaria parasites, such as through the use of RNAi, could be attempted. Such methods would abolish the need for long periods of drug selection, which appeared to lead to the selection of parasites with undesired integration events in the majority of these experiments. The PCR results from the first and third transfection experiments, together with the first Southern blot result, suggested that a proportion of the parasites contained the desired integration event. Therefore it cannot be concluded that TCTP is an essential gene in *P. berghei* malaria asexual life stages. It is concluded that a stable TCTP knockout parasite could not be generated by the transfection methods described.
TCTP from several malarial species was successfully expressed in both *S. cerevisiae* yeast and in *E. coli*. Initially, TCTP was expressed in yeast due to the protective effect observed when yeast-derived TCTP was used as a vaccine against *P. yoelii* YM (Taylor, 2002). Also, in published reports yeast-derived TCTP had induced histamine release *in vitro* in purified allergic donor basophils (MacDonald *et al.*, 2001). However, purification of TCTP from yeast was hampered by substantial amounts of protein breakdown and relatively low yields. Alternative methods of expression and purification were trialled. A yeast expression system in which protease activity was minimised, by the replacement of culture media prior to expression induction and by substantial reduction of the induction time, resulted in greater protein yields. However, expression of TCTP in *E. coli* gave the best protein yields with minimal breakdown. Purification of TCTP from *E. coli* was also achieved more rapidly than from yeast, and protease inhibitors were not required.

Yeast-derived and *E. coli*-derived TCTP were cross-reactive, as determined by western blot and ELISA. A similar protective effect to malaria challenge was observed using *E. coli*-derived TCTP as in the first trial when yeast-derived TCTP was used (Taylor, 2002), indicating the proteins were immunologically similar. Antibodies generated against both yeast-derived and *E. coli*-derived PyTCTP could react with malarial TCTP by western blot. Antibodies generated against *E. coli*-derived TCTP reacted less strongly to the malarial protein, this could have been due to several factors including lower antibody titre or variations in malaria lysate preparation.

Malarial and recombinant TCTP migrated more slowly on SDS-PAGE than expected from their theoretical mass. This has previously been observed in TCTPs from other species, and is thought to be due to the calcium-binding activity of the protein (e.g. Arcuri *et al.*, 2004; Bhisutthibhan *et al.*, 1999; Sanchez *et al.*, 1997). In *P. yoelii* YM lysates, TCTP migrated at around 23 kDa, the theoretical mass of the protein is 19.8 kDa (http://ca.expasy.org/tools/pi_tool.html). Recombinant TCTP migrated more slowly again, at around 28 kDa, due to the presence of the N-terminal hexa-his tag.

In the yeast protein purification experiments, PfTCTP migrated faster than PyTCTP, and was present on SDS-PAGE at around 26 kDa. The reasons for this are unknown, but could indicate differences in calcium-binding activities between the two.
proteins, as they were cloned into the same vector and expressed using identical methods. One possible area of future research would be to determine the calcium-binding activities of TCTPs from different species.

Self-interaction of rat TCTP has been reported (Yoon et al., 2000), and multimers of yeast-derived Pf and Py TCTP were observed when proteins were separated by SDS-PAGE under non-reducing conditions, however the predominant band present was the monomeric form. A possible future experiment would be to perform western blots on malarial lysates separated under reducing and non-reducing conditions, to establish the dominant form of TCTP present in the parasite.

A mild protective effect against malaria challenge was generated by TCTP immunisation in two of the three trials. The reasons for the lack of protection in the second vaccine trial (described in Chapter 3) are unknown. Although the proteins used for immunisation contained a substantial amount of breakdown products, this is unlikely to be the reason for the lack of protection observed, as the proteins used in the first vaccine trial contained similar amounts of breakdown products. Also, the proteins used in the Chapter 3 vaccine trial generated high titre antibodies capable of malarial TCTP recognition. The difference in protective effect between the first and second vaccine trial is unlikely to be due to increased virulence of the malarial strains used for challenge, as the control mice challenged with *P. yoelii* YM survived for similar amounts of time in both vaccine trials. The lack of protection observed in the second vaccine trial, along with the difficulties encountered in purification of yeast-derived TCTP, prompted the decision to evaluate the vaccine potential of TCTP expressed in a bacterial system in the third trial.

Immunisation with *E. coli*-derived TCTP conferred a mild protective effect in *P. yoelii* YM and *P.c. chabaudi* AS-challenged mice, but not in mice challenged with *P. berghei* ANKA. In PyTCTP-immunised mice challenged with *P. chabaudi*, mean parasitemia was significantly reduced from 4 to 9 dpi as compared with PBS-immunised controls. In *P. yoelii* YM-challenged mice, PyTCTP immunisation resulted in a significantly reduced mean parasitemia at 3 and 4 dpi. In both groups peak parasitemia was delayed by one day. In mice challenged with *P. yoelii* YM, two PyTCTP-immunised mice and one control mouse survived the usually lethal infection. However, the mean survival times of immunised and control mice were not significantly different.

In the initial vaccine trial using yeast-derived TCTP (Taylor, 2002), immunised mice survived a day longer than controls following *P. yoelii* YM challenge, and the
reduction in parasitemia in the early stages of infection was more pronounced than was observed in the vaccine trial using \textit{E. coli}-derived TCTP. This could indicate that immunisation with yeast-derived TCTP has the potential to generate stronger protective immune responses, or the differences could be due to minor variations in methodology or in parasite virulence between trials.

Treatments that specifically inhibit histamine release by the host have been shown to have a protective effect against malarial infection in mice. Tricyclic antihistamine treatment could prevent patent infection in mice challenged with \textit{P. yoelii nigeriensis} (Singh and Puri, 1998). Additionally, C57BL/6 mice deficient in histamine responses via several methods (through disruption of the histidine decarboxylase gene or the H1 histamine receptor, or by drug treatment to inhibit of the H1 receptor) were resistant to cerebral malaria development, instead succumbing to hyperparasitemia at around 20 dpi. In these mice, integrity of the BBB was preserved, sequestration of infected erythrocytes in brain blood vessels did not occur, and the expression of vascular adhesion molecules was reduced (Beghdadi \textit{et al.}, 2008).

It was thought that TCTP immunisation might result in significant protection against cerebral malaria development in C57BL/6 mice challenged with \textit{P. berghei ANKA}. However, no differences in cerebral malaria development were observed between control and TCTP-immunised C57BL/6 mice. Although unexpected morbidity and mortality was observed at low parasitemia in all BALB/c mice challenged with \textit{P. berghei ANKA}, it could be determined that PbTCTP immunisation did not reduce parasitemia or increase survival time.

In summary, the results so far have indicated that TCTP is not a good malarial vaccine candidate compared to others previously reported, such as AMA-1, MSP-1 or MSP4/5 (e.g. Anders \textit{et al.}, 1998; Crewther \textit{et al.}, 1996; Daly and Long, 1996; Hirunpetcharat \textit{et al.}, 1997; Kedzierski \textit{et al.}, 2000). TCTP immunisation generated only a very modest protective response, and sterile immunity or significant reduction in disease symptoms was not observed. Additionally, the protective result obtained was not consistent in all of the vaccine trials or in all of the challenge models used. This is a further argument against the further assessment of TCTP as a malarial vaccine candidate, as a human malaria vaccine would be required to generate a strong protective effect with broad specificity.
The major effects generated by TCTP immunisation have been a reduced parasitemia early in infection and a delayed peak parasitemia. Taken together, these results indicate that malarial TCTP may have a role in the early stages of infection progression. The protective effect seen also indicates, but does not confirm, that malarial TCTP can be recognised and inhibited by the specific host antibodies during malarial infection. This supports the hypothesis that malarial TCTP is secreted into the bloodstream of the host during infection, as previously reported (MacDonald et al., 2001). Secretion of TCTP from other species, including other human parasites, has been reported (Amzallag et al., 2004; Gnanasekar et al., 2002; MacDonald et al., 1995; Oikawa et al., 2002). TCTP has no extracellular signal sequence or transmembrane domains, however cell culture experiments have indicated that around half of the total *P. falciparum* TCTP is released during schizogony (MacDonald et al., 2001). It has not been proven that this is due to an active secretion process, and future experiments could evaluate the secretion of malarial TCTP. For example, culture supernatants of *P. falciparum* could be analysed for the present of TCTP by western blot, using antibodies specific to intracellular malarial proteins as a negative control. The presence of malarial TCTP in the sera of infected rodents could also be analysed by western blot. However, a high-titre antibody would be required, as only faint bands of malarial TCTP were detected in purified *P. yoelii* and *P. berghei* lysates using the antibodies generated as described in chapters 3 and 4.

It is unknown if the expression of TCTP in yeast and *E. coli* generated proteins with functional differences. No active protein, as measured by the induction of histamine release, was generated using either expression system. Yeast-derived TCTP did not induce an *in vivo* response in mice; it was first thought that this could have been due to TCTP possessing local rather than systemic effects (Taylor, 2002). However, *in vitro* experiments using rat basophilic leukaemia (RBL-2H3) cells also showed no degranulation induced by *E. coli*-derived TCTP. Hexosaminidase assays were used in place of direct measurement of histamine release as it was thought that the earlier negative results could also have been due to the labile nature of histamine.

A previous report stated that human TCTP did not stimulate histamine release from RBL-2H3 cells, and that they are considered to be a mast cell lineage rather than a basophil model (Wantke et al., 1999). However, other researchers have demonstrated the capacity of filarial, schistosome and tick TCTP to induce histamine release from RBL-2H3 cells (Gnanasekar et al., 2002; Mulenga et al., 2003; Rao et al., 2002). Based on these results, malarial TCTP should have induced histamine release from RBL-2H3 cells.
as the methods for protein purification and basophil degranulation were highly similar to those previously reported for filarial TCTP (Gnanasekar et al., 2002). To determine if the lack of induction of basophil degranulation by malarial TCTP was due to true differences in histamine-releasing activities of the proteins, or are due to experimental differences between laboratories, the TCTP from several different species would need to be assessed together in both in vivo and in vitro assays.

In all malarial TCTP immunisation and activity experiments, the N-terminal His-tag was not removed from the recombinant proteins. It is possible that the inclusion of the His-tag could have affected the secondary structure of the recombinant proteins and their activity. Many vaccine trials use recombinant proteins with His-tags attached, as they appear to have little or no effect on immunogenicity. Further TCTP vaccine evaluations could include recombinant protein with the His-tag removed. However, recognition of TCTP present in malarial lysates by antibodies raised to recombinant His-tagged malarial TCTP was detected by western blot. In all published reports of the histamine-releasing activity of TCTP, recombinant protein has been used with the purification tag left intact. For example, His-tagged PITCTP and GST-tagged human TCTP were used in the histamine assays of human basophils (Macdonald et al., 2001). Histamine release from RBL-2H3 cells was induced by filarial worm TCTP with the His-tag present (Gnanasekar et al., 2002; Rao et al., 2002). Tick TCTP with an intact thioredoxin tag induced histamine release from RBL-2H3 cells (Mulenga et al., 2003). Therefore, the negative results obtained in the activity assays described in this thesis are unlikely to be due to the inclusion of the His tag in the recombinant malarial TCTPs.

Experiments aimed at generating a TCTP-knockout malaria strain were performed in an attempt to more fully characterise the function of the protein. These experiments involved the transfection of purified P. berghei ANKA schizonts with linearised DNA designed to disrupt TCTP with the TgDHFR/TS gene, removing part of the TCTP coding sequence in the process. Successfully transfected parasites could then be selected for by treatment of infected mice with the antifolate drug pyrimethamine. Genetic analysis of the parasites obtained was performed using PCR and Southern blot.

In the parasites initially obtained from the first knockout experiment, determination of the transfected parasite genotype by both PCR and Southern blot indicated that the TCTP gene had been successfully disrupted with the TgDHFR/TS gene. However, prior to any genotype analysis these parasites had undergone a second passage in mice, to obtain sufficient DNA for Southern blot analysis. These mice were not drug-treated, as
the extended time (18 days) required for detectable parasitemia immediately following transfection had indicated that the parasites obtained were most likely wild-type.

When the initial parasite stablate was used to infect six mice which were then drug treated, in all mice the majority of parasites obtained had a genotype in which the TCTP gene was non-disrupted, together with the TgDHFR/TS gene integrated in an incorrect location that was not digested to less than 11.5 kb using Ndel. The presence of the wild-type TCTP gene was most likely not due to excision of the target DNA from the correctly transfected parasites, as this should have resulted in a shorter TCTP PCR product. This result suggested that parasites containing a disrupted TCTP gene were out-competed by parasites containing the TgDHFR/TS gene integrated in another location during secondary drug selection. It is unknown if this was due to a previously unreported function of TCTP in resistance to pyrimethamine, such that TCTP-knockout parasites were at a selective disadvantage during drug treatment. However, the negative selection of TCTP-disrupted parasites during drug treatment may have been due to a more general selective disadvantage, in that TCTP-knockout parasites grew much more slowly and were out-competed by other parasites containing the TgDHFR/TS gene, which were more strongly selected for when drug pressure was applied.

Due to the overall unsuccessful results of the first knockout experiment, further transfections were performed. In some of these experiments target DNA with longer integration fragments were used, in an attempt to generate higher transfection efficiencies and more stable integration. Southern blot and PCR analysis revealed that in all of these experiments wild-type TCTP was present in the transfected parasites following both the initial and secondary rounds of drug selection. In the final transfection experiment, multiple bands of unknown identity were also detected using the TCTP probe, ranging in size from 6 kb to >11.5 kb. The TgDHFR/TS probe could only be used in genotype analysis of some of the transfected parasite samples. When used, the TgDHFR/TS probe detected bands at several locations that did not correspond with TCTP disruption.

Although none of the transfection experiments were completely successful, genotype analysis by Southern blot and PCR indicated that a proportion of the parasites contained the desired integration event. From these results, it cannot be stated that malarial TCTP is an essential gene. One potential future experiment would be to use RNA interference technology to inhibit TCTP expression in specific malaria life stages. Whereas the creation of homozygous TCTP mutants by targeted gene disruption was
100% lethal in the *Drosophila* larval stage, inhibition of TCTP expression by RNAi allowed for the mutant phenotype to be studied in several adult tissues. Reduced size, caused by a decreased cell size and number, was observed in several tissues, including eye and wing (Hsu *et al.*, 2007). This supports the hypothesis that disruption of malarial TCTP expression leads to slower cell growth.

The strong selection pressure against parasites with the TCTP gene disrupted, together with the ability of TCTP immunisation to generate mild protective responses against several rodent malaria species, indicates that TCTP has an important role in malaria. As the most significant effect was observed early in infection, it is hypothesised that the protein has a role in the parasite establishment within the host. It remains to be proven whether this is due to the induction of histamine release, or through other extracellular functions of TCTP.
References


References


References


Appendix 1: Markers used

Full sizes of DNA ladders:

- **λ/PstI:**
  - λλ λλ
  - λλ λλ
  - λλ λλ
  - λλ λλ
  - λλ λλ

- **λ/HindIII:**
  - λλ λλ
  - λλ λλ
  - λλ λλ
  - λλ λλ

- **100 bp ladder:**

  - [Link to 100 bp ladder](http://www.fermentas.com/catalog/electrophoresis/convlambdamarkers.htm)
  - [Link to 100 bp ladder](http://www.neb.com/nebecomm/products/productN3231.asp)
Full sizes of protein markers:

**SeeBlue® Plus2**

- Myosin (188 kDa)
- Phosphorylase B (98 kDa)
- BSA (62 kDa)
- Glutamic dehydrogenase (40 kDa)
- Alcohol dehydrogenase (38 kDa)
- Carbonic anhydrase (28 kDa)
- Myoglobin-Red (17 kDa)
- Lysosome (14 kDa)
- Aprotinin (6 kDa)
- Insulin B chain (3.5 kDa)
- Insulin A chain (2.5 kDa)

(4-12% Bis-Tris gel)

**Mark12™**

- Myosin (200 kDa)
- β-Galactosidase (116.3 kDa)
- Phosphorylase b (97.4 kDa)
- BSA (66.3 kDa)
- Glutamic dehydrogenase (55.4 kDa)
- Lactate dehydrogenase (36.5 kDa)
- Carbonic anhydrase (31 kDa)
- Trypsin inhibitor (21.5 kDa)
- Lysosome (14.4 kDa)
- Aprotinin (6 kDa)
- Insulin B chain (3.5 kDa)
- Insulin A chain (2.5 kDa)

(4-12% Bis-Tris gel)

**pre-stained BenchMark™**

- ~190 kDa
- ~120 kDa
- ~85 kDa
- ~60 kDa
- ~50 kDa
- ~40 kDa
- ~25 kDa
- ~20 kDa
- ~15 kDa
- ~10 kDa

(4-20% Tris-Glycine gel)

**unstained BenchMark™**

(12.5% SDS-polyacrylamide gel)

gory&catKey=94101)
# Appendix 2: Quantifying Pain/Distress/Suffering

<table>
<thead>
<tr>
<th>Variable</th>
<th>Predicted Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Body Weight Changes</strong></td>
<td></td>
</tr>
<tr>
<td>0 Normal</td>
<td></td>
</tr>
<tr>
<td>1 &lt;10% weight loss</td>
<td>1</td>
</tr>
<tr>
<td>2 10-15% weight loss</td>
<td></td>
</tr>
<tr>
<td>3 &gt;20% weight loss</td>
<td></td>
</tr>
<tr>
<td><strong>B. Physical Appearance</strong></td>
<td></td>
</tr>
<tr>
<td>0 Normal</td>
<td></td>
</tr>
<tr>
<td>1 Lack of grooming</td>
<td>1</td>
</tr>
<tr>
<td>2 Rough coat, nasal/ocular discharge</td>
<td></td>
</tr>
<tr>
<td>3 Very rough coat, abnormal posture, enlarged pupils</td>
<td></td>
</tr>
<tr>
<td><strong>C. Measurable Clinical Signs</strong></td>
<td></td>
</tr>
<tr>
<td>0 Normal</td>
<td></td>
</tr>
<tr>
<td>1 Small changes of potential significance</td>
<td>2</td>
</tr>
<tr>
<td>2 Temp change 1-2°C, cardiac and respiratory rates increased up to 30%</td>
<td></td>
</tr>
<tr>
<td>3 Temp change &gt;2°C, cardiac &amp; respiratory rates increased up to 50%, or markedly reduced</td>
<td></td>
</tr>
<tr>
<td><strong>D. Unprovoked Behaviour</strong></td>
<td></td>
</tr>
<tr>
<td>0 Normal</td>
<td></td>
</tr>
<tr>
<td>1 Minor changes</td>
<td>2</td>
</tr>
<tr>
<td>2 Abnormal/reduced mobility, decreased alertness, inactive</td>
<td></td>
</tr>
<tr>
<td>3 Unsolicited vocalisations, self-mutilations either very restless or immobile</td>
<td></td>
</tr>
</tbody>
</table>
| **E. Behavioural Responses to External Stimuli** |         |**
| 0 Normal                                |                 |
| 1 Minor depression / exaggeration of response | 2             |
| 2 Moderately abnormal responses         | 2               |
| 3 Violent reactions, or comatose        |                 |

**Total predicted score:** 8

Adapted from Morton & Griffiths 1985 Vet Rec 116:431-436.
Appendix 3: Titration curves used to calculate reciprocal titres:

Titration curves for chapter 3:

Pooled group sera against PyT (1st)

Pooled group sera against PyT (2nd)
Appendix 3

Individual mice from PyT1 (1st)

Individual mice from PyT1 (2nd)
Appendix 3

Individual mice from PfT1 (1st)

Individual mice in PfT1 (2nd)
Appendix 3

Titration curves for chapter 4:

Reciprocal titres pooled group sera against PyTCTP 1st

pooled group sera against PyTCTP 1st
Reciprocal titres pooled group sera against PyTCTP 2nd

Abs (450 nm)

Reciprocal titre

PBS #1
PBS #2
PBS #3
PBS #4
B6 prebleed
PbT #8
Balb/c prebl
04 trial PyT
PyT #5
PyT #6
PbT #7

pooled group sera against Pyt 2nd

PBS #1
PBS #2
PBS #3
PBS #4
B6 prebleed
PbT #8
Balb/c prebl
Appendix 3

Reciprocal titres pooled group sera against PbTCTP 2

Reciprocal titres pooled group sera against PbTCTP 2