The Potential of Triclabendazole in Combination with Praziquantel for the Treatment of *Schistosoma mansoni* Infections

by

Bong Sze How BSc. (Hons.)
This thesis is presented for the degree of
Doctor of Philosophy

of

School of Veterinary Sciences
Murdoch University
Western Australia

2007
I declare that this thesis is the account of my own research, and contains as its main content work which has not previously been submitted for a degree at any tertiary institution.

Bong Sze How
Abstract

Previous work has suggested that triclabendazole (Tcbz), a member of the benzimidazole group of compounds, possessed efficacy against Schistosoma mansoni (S. mansoni). In view of recent indications in praziquantel (Pzq) treatment failures and loss of sensitivity, it is imperative that new anti-schistosomals are developed as contingent treatment options, while resistance alleles, if any, remain at low frequencies. While recent studies have indicated that Tcbz monotherapy exert weak anti-schistosomal effects, the combined application of Tcbz with Pzq has not been explored. To assess this hypothesis, triclabendazole and its metabolites were initially assessed against the many life-stages of S. mansoni in vitro. Combination drug and isobologram analyses against adult S. mansoni was also performed, and subsequently assessed against other parasite species to assess the specificity of such effects. Subsequently, the drug combinations were assessed against S. mansoni in vivo. A cost-effectiveness model was then developed to predict the feasibility of administering Pzq-Tcbz drug combinations in Senegal. It was concluded that triclabendazole and its metabolites possessed good efficacy against immature schistosomula, although weak efficacy was observed against adult S. mansoni. Upon combination with Pzq, however, a strong synergistic effect against adult worms were observed in vitro. Praziquantel and Tcbz were also shown to possess unique and independent ovicidal modes of action that can be clinically significant. More importantly, in vivo drug trials concluded that the combinations exerted additive effects against S. mansoni harbored in mice. Economic modeling and cost-effectiveness analysis further demonstrated the feasibility of this drug combination and showed that the drug combinations may represent a new line of treatment against mansonial schistosomiasis.
# Table of Contents

Thesis declaration .............................................. ii  
Abstract ........................................................ iv  
Table of Contents ............................................. v  
List of Figures and Tables .................................. viii  
Abbreviations .................................................... xiv  
Acknowledgements ............................................. xvi  
Publications and Presentations ............................. xvii  
Dedication ....................................................... xviii

## Chapter 1  Introduction

1.0 General introduction .................................. 1  
1.1 Life cycle .................................................. 1  
1.2 Pathology ................................................... 3  
1.3 Immunology ................................................ 3  
1.4 Genomics and biochemistry .............................. 4  
1.5 Transmission and Epidemiology ....................... 5  
1.6 Diagnosis .................................................... 5  
1.7 Control ....................................................... 6  
1.7.1 Praziquantel control ................................... 7  
1.7.1.1 Pharmacodynamics and metabolism ............... 8  
1.7.1.1 Mechanism of action ................................ 8  
1.7.2 Triclabendazole control ............................... 10  
1.7.2.1 Pharmacodynamics and metabolism ............... 11  
1.7.2.2 Mechanism of action ................................ 12  
1.7.2.3 Cytoskeletal proteins as *Schistosoma* drug targets 13  
1.8 Mass chemotherapeutic regimens for the treatment of schistosomiasis 15  
1.9 Refractoriness to praziquantel treatment ............ 16  
1.10 Combination chemotherapy ............................. 16  
1.11 Cost-effectiveness of praziquantel for control ........ 19  
1.12 Aims and hypothesis .................................... 23

## Chapter 2  General Materials and Methods

2.1 Growth medium .......................................... 24  
2.1.1 *Schistosoma* medium 169 ............................ 24  
2.1.2 *Giardia* medium ....................................... 24  
2.1.3 *Haemonchus* medium composition ................. 24  
2.2 *Biomphalaria glabrata* maintenance ..................... 25  
2.3 *Biomphalaria glabrata* infection with miracidium .... 25  
2.4 Cercariae shedding ......................................... 26  
2.5 Infection of definitive mouse host ....................... 26  
2.6 *In vitro* cultivation of immature schistosomulum .... 26  
2.7 *Schistosoma* egg extraction from liver ............... 27  
2.8 *Haemonchus* egg extraction from faeces .............. 28  
2.9 *In vitro* drug evaluation ................................ 28  
2.9.1 *In vitro* drug evaluation against immature schistosomulum 28  
2.9.2 *In vitro* drug evaluation against mature worms .... 29  
2.10 Evaluation of combination drug treatment ............ 30  
2.10.1 Evaluation of drug combinations against *Schistosoma mansoni* 30
2.10.2 Evaluation of drug combinations against *Giardia duodenalis* 30
2.10.3 Evaluation of drug combinations against *Haemonchus contortus* 31
2.11 Evaluation of drug action against *Schistosoma* eggs 32
  2.11.1 Assessment of praziquantel-induced hatching of eggs 32
  2.11.2 Assessment of triclabendazole and metabolites on egg viability 32
2.12 Dose response and statistical analysis 33

Chapter 3 Evaluation of the effects of triclabendazole and metabolites against *Schistosoma mansoni* in vitro

3.1 Introduction 34
3.2 Materials and Methods 36
  3.2.1 *Schistosoma mansoni* maintenance 36
  3.2.2 Drug treatment 36
  3.3 Drug efficacy scores 37
  3.4 Dose response and statistical analysis 37
3.3 Results 38
  3.3.1 Efficacy of triclabendazole against immature schistosomulum 38
  3.3.2 Efficacy of triclabendazole and metabolites against adult worms 42
  3.3.3 Drug efficacy scores 46
3.4 Discussion 53

Chapter 4 Evaluation of the ovicidal activity of praziquantel, triclabendazole and its metabolites against *Schistosoma* eggs

4.1 Introduction 59
4.2 Materials and Methods 61
  4.2.1 Preparation of *Schistosoma* eggs 61
  4.2.2 Evaluation of the effects of praziquantel on egg hatching 61
  4.2.3 Evaluation of triclabendazole and its metabolites on egg viability 62
  4.2.4 Evaluation of calcium blockers on eggs 62
  4.2.5 Dose response and statistical analysis 63
4.3 Results 64
  4.3.1 Effects of praziquantel-induced hatching of *Schistosoma* eggs 64
  4.3.2 Morphological effects observed in eggs exposed to praziquantel 65
  4.3.3 Effects of calcium blockers and inducers on eggs 66
  4.3.3 Effects of triclabendazole and its metabolites on egg viability 69
4.4 Discussion 77

Chapter 5 Evaluation of the combinatorial effects of praziquantel and triclabendazole against *Schistosoma mansoni*, *Giardia duodenalis* and *Haemonchus contortus*

5.1 Introduction 81
5.2 Materials and Methods 83
  5.2.1 *Schistosoma* maintenance 83
  5.2.2 *Giardia* maintenance 83
  5.2.3 *Haemonchus* maintenance 83
  5.2.4 Experimental design 83
  5.2.5 *In vitro* efficacy of single and combination treatments of praziquantel triclabendazole-sulphoxide 84
    5.2.5.1 *Schistosoma mansoni* 84
    5.2.5.2 *Haemonchus contortus* 85
    5.2.5.3 *Giardia duodenalis* 85
  5.2.6 Statistical analysis 86
  5.2.7 Combination dose response and isobologram analysis 86
Chapter 6 The efficacy of a combination of praziquantel and triclabendazole for the treatment of mice infected with Schistosoma mansoni

6.1 Introduction
6.2 Materials and Methods
   6.2.1 Schistosoma maintenance
   6.2.2 Drug efficacy
   6.2.3 Oogram pattern and tissue egg load analyses
   6.2.4 Statistical analysis
6.3 Results
   6.3.1 Tissue egg load analysis
   6.3.2 Oogram pattern analysis
   6.3.3 Worm burden reduction
6.4 Discussion

Chapter 7 Economic analysis: feasibility study of praziquantel-triclabendazole drug combinations for the treatment of schistosomiasis

7.1 Introduction
7.2 Materials and Methods
   7.2.1 Data sources
   7.2.2 General assumptions
   7.2.3 Drug and operational costs
   7.2.4 Patient number projections
   7.2.5 Cost-effectiveness analysis
   7.2.6 Stochastic model development
7.3 Results
   7.3.1 Production cost of praziquantel and triclabendazole
   7.3.2 Sale price of praziquantel and triclabendazole
   7.3.3 Sale price of praziquantel and triclabendazole drug combinations
   7.3.4 Patient number projections
   7.3.5 Drug costs, operational costs and total costs of programme
   7.3.6 Cost-effectiveness analysis
7.4 Discussion

Chapter 8 General Discussion

References
Key terminology and definitions
List of figures and tables

Chapter 1

Figure 1.1  Life-cycle of *Schistosoma mansoni*  2
Figure 1.2  Praziquantel chemical structure  7
Figure 1.3  Triclabendazole chemical structure  11

Chapter 2

Table 2.1  Composition of culture medium 169  24
Table 2.2  Composition of *Giardia* medium  24
Table 2.3  Composition of *Haemonchus* medium  24

Chapter 3

Figure 3.1  Dose response curve of triclabendazole evaluated against immature schistomulum at Week four stage and assessed for seven days after exposure  39
Figure 3.2  Morphological changes observed in four week old immature schistosomula exposed to varying concentrations of triclabendazole *in vitro.*  41
Figure 3.3  Dose response curve of praziquantel assessed against mature worms at 24, 48 and 72 hrs post exposure.  44
Figure 3.4  Dose response curve of triclabendazole assessed against mature worms at 24, 48 and 72 hrs post exposure.  44
Figure 3.5  Dose response curve of triclabendazole-sulphoxide assessed against mature worms at 24, 48 and 72 hrs post exposure.  45
Figure 3.6  Dose response curve of triclabendazole-sulphone assessed against mature worms at 24, 48 and 72 hrs post exposure.  45
Figure 3.7  Morphological changes observed in *Schistosoma mansoni* after exposure to triclabendazole for 24 hrs.  48
Figure 3.8  Morphological changes observed in *Schistosoma mansoni* after exposure to triclabendazole-sulphoxide for 24 hrs.  49
Figure 3.9  Morphological changes observed in *Schistosoma mansoni* after exposure to triclabendazole-sulphone for 24 hrs.  50
Figure 3.10  Morphological changes observed in *Schistosoma mansoni* after exposure to praziquantel for 24 hrs.  51
Figure 3.11  Untreated 49-day old mature worms in DMSO drug vehicle.  52
Table 3.1  The concentration of triclabendazole and praziquantel required to kill 50% of Wk four immature schistosomulum *in vitro*.  40
Table 3.2  Effective concentration required to kill 50% (EC$_{50}$) of adult *Schistosoma mansoni* worms at four hour time intervals.  43
Table 3.3  Percentages of worms (%) scored according to efficacy criteria (contraction and motility).  47

Chapter 4

Figure 4.1  Representative images of *Schistosoma mansoni* (left column) and *Schistosoma japonicum* (right column) eggs after exposure to 10µM of praziquantel.  67
Figure 4.2: *Schistosoma mansoni* (left column) and *Schistosoma japonicum* (right column) eggs treated with praziquantel showing hatching of miracidium.  68
Figure 4.3  Dose response curves of *Schistosoma mansoni* eggs exposed to triclabendazole, triclabendazole-sulphoxide and triclabendazole-sulphone for 24hrs  71
Figure 4.3  Dose response curves of *Schistosoma japonicum* eggs exposed to triclabendazole, triclabendazole-sulphoxide and triclabendazole-sulphone for 24hrs  71
Figure 4.5  Microstructural effects of *Schistosoma japonicum* eggs exposed to triclabendazole, triclabendazole-sulphoxide and triclabendazole-sulphone.  72
Figure 4.6  Microstructural effects of *Schistosoma japonicum* eggs exposed to various concentrations of triclabendazole, triclabendazole-sulphoxide and triclabendazole-sulphone.  73
Figure 4.7  Microstructural effects of detached vitelline membrane and miracidium of *Schistosoma japonicum* eggs after exposure to
triclabendazole, triclabendazole-sulphoxide and triclabendazole-sulphone.

Figure 4.8 Representative images of *Schistosoma mansoni* eggs after exposure to triclabendazole, triclabendazole-sulphoxide and triclabendazole-sulphone for 24 hours.

Figure 3.9 Representative images of the microstructural effects of discharged *Schistosoma mansoni* miracidium following 24 hours exposure to triclabendazole, triclabendazole-sulphoxide and triclabendazole-sulphone.

Table 4.1 Mean length of time following the addition of praziquantel before the activation of eggs were observed.

Table 4.2 Mean length of time required for *Schistosoma mansoni* and *Schistosoma japonicum* eggs to hatch.

Table 4.3 Percentage of *Schistosoma mansoni* and *Schistosoma japonicum* eggs hatched after exposure to triclabendazole, triclabendazole-sulphoxide and triclabendazole-sulphone at 10nM, 100nM, 1µM, 10µM, 100µM and 1mM.

Table 4.4 Concentration of triclabendazole, triclabendazole-sulphoxide and triclabendazole-sulphone required to kill 50% of *Schistosoma mansoni* and *Schistosoma japonicum* eggs.

**Chapter 5**

Figure 5.1 Microstructural effects of adult *Schistosoma mansoni* treated with an EC$_{50}$ + EC$_{50}$ combination of praziquantel and triclabendazole-sulphoxide.

Figure 5.2 Proportion of *Schistosoma mansoni* worms killed by triclabendazole-sulphoxide plotted against the praziquantel EC$_{50}$, EC$_{25}$ and EC$_{10}$ concentrations.

Figure 5.3 Proportion of *Schistosoma mansoni* worms killed by praziquantel plotted against the triclabendazole-sulphoxide EC$_{50}$, EC$_{25}$ and EC$_{10}$ concentrations.

Figure 5.4 EC$_{90}$ isobologram of praziquantel and triclabendazole-sulphoxide combinations assessed against *Schistosoma mansoni*. 
Figure 5.5  Proportion of *Haemonchus contortus* worms inhibited by triclabendazole-sulphoxide plotted against the praziquantel EC\(_{50}\), EC\(_{25}\) and EC\(_{10}\) concentrations.

Figure 5.6  Proportion of *Haemonchus contortus* worms inhibited by praziquantel plotted against the triclabendazole-sulphoxide EC\(_{50}\), EC\(_{25}\) and EC\(_{10}\) concentrations.

Figure 5.7  EC\(_{70}\) isobologram of praziquantel and triclabendazole-sulphoxide combinations assessed against *Haemonchus contortus*.

Figure 5.8  Proportion of *Giardia* trophozoites inhibited by triclabendazole-sulphoxide plotted against the praziquantel EC\(_{50}\), EC\(_{25}\) and EC\(_{10}\) concentrations.

Figure 5.9  Proportion of *Giardia* trophozoites inhibited by triclabendazole-sulphoxide plotted against the praziquantel EC\(_{50}\), EC\(_{25}\) and EC\(_{10}\) concentrations.

Figure 5.10  EC\(_{50}\) isobologram of praziquantel and triclabendazole-sulphoxide combinations assessed against *Giardia duodenalis* trophozoites.

Table 5.1  Combinations of the EC\(_{50}\), EC\(_{25}\) and EC\(_{10}\) values of praziquantel and triclabendazole-sulphoxide used in this study.

Table 5.2  Concentrations of praziquantel and triclabendazole-sulphoxide required to kill 50% (EC\(_{50}\)), 25% (EC\(_{25}\)) and 10% (EC\(_{10}\)) of *Schistosoma mansoni* worms after 24 hours exposure *in vitro*.

Table 5.3  Percent mortality of adult *Schistosoma mansoni* treated with combinations of praziquantel and triclabendazole-sulphoxide.

Table 5.4  Dose response analysis of praziquantel, triclabendazole-sulphoxide and albendazole exposed to larval stages of *Haemonchus contortus* showing the EC\(_{50}\), EC\(_{25}\) and EC\(_{10}\) values.

Table 5.5  Percent inhibition of L3 development of *Haemonchus contortus* treated with combinations of praziquantel and triclabendazole-sulphoxide.

Table 5.6  Concentration of praziquantel, triclabendazole-sulphoxide and albendazole required to inhibit 50%, 25% and 10% of *Giardia* adherence after 24 hours exposure *in vitro*.

Table 5.7  Inhibition of adherence of *Giardia duodenalis* trophozoites.
following exposure to combinations of praziquantel and triclabendazole-sulphoxide

Chapter 6

Table 6.1 Mean hepatic egg loads in eggs per gram of mice infected with *Schistosoma mansoni* and treated with Pzq (80mg/kg), Tcbz (120mg/kg), Pzq-Tcbz combination (80mg/kg +120mg/kg) and Pzq-Tcbz combination (250mg/kg +250mg/kg).

Table 6.2 Oogram pattern of mice infected with *Schistosoma mansoni* and treated with Pzq (80mg/kg), Tcbz (120mg/kg), Pzq-Tcbz combination (80mg/kg +120mg/kg) and Pzq-Tcbz combination (250mg/kg +250mg/kg).

Table 6.3 Worm burden of mice infected with *Schistosoma mansoni* and treated with Pzq (80mg/kg), Tcbz (120mg/kg), Pzq-Tcbz combination (80mg/kg +120mg/kg) and Pzq-Tcbz combination (250mg/kg +250mg/kg).

Chapter 7

Table 7.1 Sources of operational costs for the delivery of praziquantel treatments.

Table 7.2 Current availability of praziquantel (600mg) tablet and the current proportion of number with access to treatment.

Table 7.3 Demographic, epidemiology and economic data for Senegal.

Table 7.4 Distribution parameters of variables used in CostMod for the estimation of drug production price, operational costs and days lost due to schistosomiasis.

Table 7.5 Production costs and sale price of one 600mg tablet of praziquantel and triclabendazole in 2007.

Table 7.6 The mean costs to treat a child and an adult per year derived from Monte Carlo simulations.

Table 7.7 Global and Senegalese population distribution of children and adults from 2007 to 2030 in five year increments.
Table 7.8  Projections of the number of children and adults receiving praziquantel treatment from 2007 to 2030 in all countries.  124

Table 7.9  Projected drug costs of a combination of praziquantel-triclabendazole at different drug ratios compared to the drug costs for deliver praziquantel alone in 2013.  125

Table 7.10  Projected total costs for treatment programmes using a combination of praziquantel and triclabendazole at different drug ratios compared to the cost to deliver praziquantel alone in 2013.  125

Figure 7.11  The mean gross national income (GNI) saved per capita per year for ever US$1 spent, the total number of days lost despite treatment and the total number of days saved with praziquantel (Pzq) treatment at 70, 80, 90 and 100% efficacy and a combination of Pzq and triclabendazole (600mg-600mg).  126
Abbreviations

~   approximately  
<   less than  
>   greater than  
%  percent  
°C   degree celcius  
Abz   albendazole  
b.w.   body weight  
Bz   benzimidazole  
CaCO₃   calcium carbonate  
cm   centimetres  
DDI H₂O   double deionised water  
DMSO   dimethysulphoxide  
DNA   deoxyribonucleic acid  
EC₅₀   concentration required to cause 50% worm mortality  
EC₂₅   concentration required to cause 25% worm mortality  
EC₁₀   concentration required to cause 10% worm mortality  
EDTA   ethylenediaminetetraacetic acid  
EGTA   ethylene glycol-bis(β-aminoethyl ether) N,N',N'-tetraacetic acid  
et al.   and others  
FCS   fetal calf serum  
g   gram  
g   unit of gravitational field  
G   gauge  
HCl   hydrochloric acid  
hr   hour  
IMF   International Monetary Fund  
IRR   internal rate of return  
kg   kilogram  
L   litre  
µ (prefix)   micro (10 x -⁶)  
m (prefix)   milli (10 x -³)  
M   mole

xiv
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>m</td>
<td>million</td>
</tr>
<tr>
<td>x mag</td>
<td>magnification</td>
</tr>
<tr>
<td>met</td>
<td>metrifonate</td>
</tr>
<tr>
<td>µM</td>
<td>micromoles</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>mL</td>
<td>millilitres</td>
</tr>
<tr>
<td>mM</td>
<td>millimoles</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>M.W.</td>
<td>molecular weight</td>
</tr>
<tr>
<td>n (prefix)</td>
<td>nano ((10 \times 10^{-9}))</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NBCS</td>
<td>newborn calf serum</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NPV</td>
<td>net present value</td>
</tr>
<tr>
<td>Oxm</td>
<td>oxamniquine</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>pH</td>
<td>minus log of hydrogen ion concentration</td>
</tr>
<tr>
<td>Pzq</td>
<td>praziquantel</td>
</tr>
<tr>
<td>QIMR</td>
<td>Queensland Institute of Medical Research</td>
</tr>
<tr>
<td>R&amp;D</td>
<td>research and development</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>Tcbz</td>
<td>triclabendazole</td>
</tr>
<tr>
<td>Tcbz-Sx</td>
<td>triclabendazole sulphone</td>
</tr>
<tr>
<td>Tcbz-Sp</td>
<td>triclabendazole sulphoxide</td>
</tr>
<tr>
<td>US$</td>
<td>United States dollars</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>Wk</td>
<td>week</td>
</tr>
</tbody>
</table>
Acknowledgements

Foremost, my sincere gratitude and appreciation to Professor Andrew Thompson for his vision, direction and trust. To Dr Simon Reid, for his faithful guidance, motivation and unconditional support. To Dr Wayne Best for his invaluable insights.

I would also like to acknowledge Dr Malcolm Jones, Dr Rob Dobson, Ms Mary Duke, Dr Dieter Palmer and Mr Jeff Mitchell for their direct intellectual and research contributions. I look forward to our further collaborations.

Especially to Dr Patrizia Washer, Professor Jim Reynoldson and Mr Tim Morrison of the Murdoch University Commercialization Office for the opportunity to be part of such a dynamic enterprise. A special note of appreciation to the Intern analysts, Ms Erin McGuigan, Ms Sandra Depelsanaire and Mr Henry Lee for their infectious and unbridled enthusiasm.

Also to Mr Richard McCulloch, Ms Elizabeth Liu and Mr Tim Colwill of MurdochLink, and a special mention for Mr Rob Newman, Mr Matt Callahan, Mr Ian Callahan, Mr Robin Lees, Ms Debbie Harrison and Ms Susan Holland of the Murdoch Westscheme Enterprise Partnership Fund. Special thanks to Mark Gummer and Helen Cheeseman from AusBiotech, WA.

A special acknowledgement to Dr Howard Carr, for being an inspirational mentor.

To my partner, Tegan, for your boundless love.

To my best friend Jill, friendship and selflessness. Also for Jeff, and my god-son, little Jacob.

For my best mates, Amy, Cain, Adina, Kenneth, Emily, Niko, Jem, Eugene, Nicky, Shaun, Hui, Jimmy, Cindy, Sharon, Ondy and Adrian. To Carol, for your love and splendid cooking.

For all my friends and colleagues in the Division of Health Sciences, including Trish, Nevi, Miko, Annika, Peter, Yazid, Nyree, Tom, Bahman, Reza, Andrew and Joyce, Rebecca, Una, Stan, Cassie, Rob, Will, Moira, Ryan, Susannah, Natalie, Josh, Susannah Linda, Linda D, Jo, Scott and Hanna, Louise, Tanya, Mark, Jess, Olivier, Gail, Timmhay, Margaret, Simon and Toby.

Most of all, to my family, for their infinite love, support and strength.
Presentations

Oral

‘Evaluation of triclabendazole in combination with praziquantel for the treatment of mansonial schistosomiasis’
Postgraduate Seminar Programme
Division of Health Sciences, Murdoch University
June, 2007

‘Identification of novel targets and compounds for anti-schistosomal chemotherapy’
Postgraduate Seminar Programme
Division of Health Sciences, Murdoch University
June, 2004

Poster

‘Synergistic potential of praziquantel and triclabendazole in combinatorial chemotherapy’
Postgraduate Poster Day
Division of Health Sciences, Murdoch University
November, 2006

‘In vitro combinatorial effects of triclabendazole and praziquantel against Schistosoma mansoni’
Postgraduate Poster Day
Division of Health Sciences, Murdoch University
November, 2005

‘A novel compound for the treatment of schistosomiasis’
Postgraduate Poster Day
Division of Health Sciences, Murdoch University
November, 2004

Awards

‘Novel compounds for anti-schistosomal chemotherapy’
AusBiotech 2006 National Biotechnology Conference
Awarded Student Excellence Award, Western Australian State Winner
Sydney
September, 2006

‘Evaluation of the synergistic potential of a combination of triclabendazole and praziquantel for the treatment of systemic and gastrointestinal parasites’
Awarded Science, Technology and Economic Progress Forum Scholarship
ARC Research Network for a Secure Australia, University of Canberra
November, 2006
Nil sine labore
1.0 General Introduction

Schistosomiasis (bilharziasis or snail fever) is the most widely distributed parasitic disease of humans. Endemic in 75 countries, 600 million people are at risk of infection, with approximately 200 million people infected with schistosomiasis annually (Yosry, 2006). The causative agents are dioecious parasites of the blood-vascular system belonging to the genus *Schistosoma*. At present, 19 species of schistosomes have been identified, with five species primarily capable of human transmission, namely: *Schistosoma mansoni* (*S. mansoni*), *Schistosoma japonicum*, *Schistosoma haematobium*, *Schistosoma intercalatum* and *Schistosoma mekongi*. Of these, *S. mansoni* remains the main causative agent of human schistosomiasis.

1.1 Life cycle

The life cycle of *S. mansoni* follows a common pathway of all schistosome species, from the sexual generation of adult schistosomula within the vascular system of the definitive host through to asexual reproduction within intermediate aquatic snails, and eventually a return to the definitive host by infective cercariae (Kojima, 1998; see Figure 1.1). Adult schistosomes are approximately 7-20mm in length, featuring two terminal suckers, a complex tegument, a blind digestive tract and reproductive organs. Paired adult schistosomes remain permanently *in copula*, residing in the mesenteric venous plexus (Gryseels *et al*., 2006). Female schistosomes can produce hundreds of eggs per day. Each egg contains a ciliated miracidium, the first free swimming larval stage of the parasite. The proteolytic enzymes secreted by the miracidium help the egg migrate through intestinal parenchyma into the lumen to be excreted in the faeces.

*Schistosoma mansoni* spends a proportion of its life-cycle developing in the intermediate pulmonate molluscs of the genus *Biomphalaria* (Southgate *et al*., 1998). The emergence of the miracidium is induced by a response to physical stimuli such as sunlight and fresh water. Upon contact, penetration of the intermediate snail host occurs via the foot, tentacle or edge of the mantle. The miracidium then multiply asexually into multicellular sporocysts and subsequently into cercarial larvae. Typically circadian, cercarial shedding for *S. mansoni* occurs during the
photophase (Pitchford et al., 1969). Upon finding the definitive mammalian hosts, the cercariae penetrates the skin, migrate in the blood via the lungs to the liver and transforms into immature schistosomulae (Wheater & Wilson, 1979; Miller & Wilson, 1980; Wilson & Coulson, 1986). Once the trapped schistosomula have transformed into liver-stage worms, the mature worms begin to pair with the opposite sex. The worm pairs eventually migrate into their mesenteric destination where the cycle begins again (Gryseels et al., 2006).

![Life-cycle of Schistosoma mansoni](Image reproduced from the WHO/TDR website at <www.who.int/tdr/diseases/schisto/lifecycle.htm> (accessed on 1/3/2007).

**Figure 1.1:** Life-cycle of *Schistosoma mansoni.*

- **(A)** Egg laying and subsequent excretion of eggs in feces,
- **(B)** Hatching of miracidium in the presence of fresh water,
- **(C)** Infection of freshwater snail host and subsequent development into mother and daughter sporocysts within the host,
- **(D)** Shedding of human-infective cercariae into fresh water,
- **(E)** Cercarial infection and penetration of definitive host and
- **(F)** Development of lung-stage worms followed by migration into the liver and pairing of male and female worms. Sexual reproduction then leads to egg laying and excretion of eggs in feces.
1.2 Pathology

The pathology of schistosomiasis results from parasite migration through different tissue types in the mammalian hosts (Kojima, 1998). Initial clinical manifestation following cercarial penetration includes dermatitis, coughing and mild fever as the schistosomulum migrates to the lungs. Following a latent period of a month, the acute phase of disease, known as ‘Katayama fever’, coincides with the onset of egg laying in the portal veins of the intestinal mesentery, accompanied by dysentery or diarrhoea as the eggs are discharged into the intestinal canal. The eggs are eventually deposited in the liver as the disease progresses.

The formation of granulomas to schistosome eggs signals the onset of chronic schistosomiasis, characterized by cellular and granulomatous inflammation of the liver tissues surrounded by deposited eggs, with subsequent tissue fibrosis. Granulomatous inflammation also occurs in the intestines as a consequence of T-cell mediated hypersensitivity to soluble egg antigens. Chronic schistosomiasis, often referred to as hepatosplenic schistosomiasis, is characterised by the formation of polyps in the intestines, with egg granulomas in the liver eventually replaced by fibrotic tissues. The liver decreases in size but increases in hardness as fibrosis extends to the parenchyma. The severity of the disease is related to the intensity of the infection as well as the immune responses of the host (Gryseels et al., 2006).

1.3 Immunology

Schistosomiasis is a multifaceted, immunopathological disease characterised mainly by host hypersensitivity to eggs trapped within tissues. Generally, the intensity of infection declines with age, conditioned by behavioural and immunological development of the host. Once challenged, the host mounts both cellular and humoral immune responses against antigens derived from the various developmental stages of schistosome development. While the significance of complement fixing antibodies is yet to be established, protective immunity by antibodies is likely to result in the cytotoxic destruction of schistosomulum targets, with antibody dependent cellular toxicity acting as the main mechanism for parasitic death in the human host (Capron, 1998).
A critical feature of the transformed schistosomulum is its ability to mimic host molecules, rendering it resistant to antibody-mediated defences. Initially susceptible to host immunity, the young schistosomula gradually become highly resistant with subsequent transformation. It has been suggested that the modulation in the expression of schistosomulum surface antigens may be an important mechanism of immune escape (Simpson, 1992). Furthermore, the continual shedding of the tegumental surface (Pearce et al., 1986) and the role of immunomodulatory proteases (Fishelson, 1995) may enhance immune evasion. The physical resistance of the tegumental inner bilayer membrane to host complement is also believed to play critical role in evasion and protection (Kusel & Gordon, 1989).

1.4 Genomics and biochemistry

Sequencing of the entire genome of *S. mansoni* was completed in 2004. The 270Mb haploid *Schistosoma* genome is contained in eight pairs of chromosome with a total GC content of approximately 34% (LoVerde et al., 2004). *Schistosoma* was shown to possess seven pairs of autosomes and one pair of sex chromosomes for each of the sexes, with an estimated total of 15,000 to 20,000 expressed genes. A range of highly and moderately repetitive elements has been identified (4–8% highly repetitive and 30–35% moderately repetitive), with 60% of the genome being single copy sequences. The anaerobic schistosomes derived most of their energy from ATP synthesised through oxidative phosphorylation. Glycolysis appears to generate the bulk of the ATP required, with lactate production dependent on the availability of high concentrations of pyruvate. Although schistosomes generally undergo homolactic fermentation with the production of lactate as a metabolite, oxygen utilisation by adult schistosome is not uncommon (Rumjanek, 1987). The ability of schistosomes to consume large amounts of glucose exceeded that of red blood cells, and as such, schistosomes are often described as fast-growing tumours (Rumjanek, 1987). Paired schistosomes absorb copious amount of glucose through their tegument while residing in the blood stream, consuming their dry weight in glucose every five hours (Beuding, 1950). Adult schistosomes are also capable of ingesting red blood corpuscles with subsequent utilization of haemoglobins and serum globulins following enzymic cleavage (Kojima, 1998).
1.5 Transmission and epidemiology

Intestinal schistosomiasis is currently endemic in 54 countries in the Middle East and most of the African continent, including Madagascar and Mauritius (Chitsulo et al., 2000). Due to cultural and social biases in behaviour and occupation, men are generally more at risk of infection than women. In areas where intestinal schistosomiasis is endemic, the highest prevalence of infection occurs in individuals of age 10 to 24 years and the most heavily infected individuals are those aged between 10 to 14 years (Schmunis & López Antuñano, 1998). The economic cost of schistosomiasis is measured as a reduction in work capacity as a result of morbidity. The prevalence of schistosomiasis has increased in many endemic countries due to the impoundment of water for large-scale irrigation and electricity production to support growing human populations (Hunter, 2003). Current control strategies are designed to reduce the morbidity associated with schistosomiasis rather than the eradication of infections as the costs of eradication is beyond the financial means of most endemic countries.

1.6 Diagnosis

Mircoscopic examination of excreta is the cornerstone for the diagnosis of schistosomiasis (Feldmeier & Poggensee, 1993). The technique currently recommended by the World Health Organisation (WHO) for definitive and semi-quantitative diagnosis of intestinal schistosomiasis is the Kato-Ktaz smear technique. The Kato Katz method is a simple and inexpensive procedure, which has led to its widespread use in the classical endemic areas of intestinal schistosomiasis. However, its low sensitivity is compounded by high intra- and inter-specimen variations in egg counts (Utzinger et al., 2001; Teesdale, 1985; Feldmeier & Poggensee, 1993). This is particularly evident in areas recently introduced with schistosomiasis or areas where control efforts have reduced parasitic burdens (Alarcon de-Noya et al., 1997; Graeff-Teixeira et al., 2004; Noya et al., 2002). Current antibody-based assays are sensitive but are incapable of distinguishing the history of exposure from active infections and are not easily applied in the field (Feldmeier & Poggensee, 1993). Immunoassays tools for the detection of *S. mansoni* antigens may not have the expected high sensitivity and specificity when employed for diagnosis in regions of low endemicity or for light infections in travellers (van Lieshout et al.,
Molecular diagnostic methods are sensitive alternatives, but require more extensive validation in areas of low endemicity. Other methods such as urine-based antigen detection field strips have considerable potential, but may be less sensitive (van Dam et al., 2004). The use of differential fecal concentration methods, increased amounts of fecal samples and mathematical modelling have been attempted to improve the sensitivity of parasitological diagnoses, but have not been convincing enough to warrant extensive field trials (Eberl et al., 2002; Polman et al., 2000). Diagnoses of the disease in hospital settings are currently achieved using cytoscopy and endoscopy. In hepatic schistosomiasis, contrast radiography, myelography, computed tomography imaging and magnetic resonance imaging are routinely used for detailed imaging, particularly for neuro-schistosomiasis (Palmer & Reeder, 2006; Lambertucci et al., 2000).

1.7 Control

The control of schistosomiasis is an ongoing and evolutionary process. While successful treatment of individuals can be achieved, reinfection by the parasite continues to be a perennial challenge. Control measures for intestinal schistosomiasis are often complicated by the maintenance of the parasite in reservoir hosts such as domestic animals and rodents. As a result, the commitments of schistosomiasis control programs are long term. Concerted efforts in snail control, health education, diagnosis as well as effective chemotherapy measures must be systematically maintained.

Antimony potassium tartrate, a trivalent antimonial, was the first compound used to treat schistosomiasis in the early 1900s (Marshall, 1987). The effectiveness of different drugs varies depending on the species, sex and developmental stages of the parasite. There is also evidence that different parasite strains vary in their susceptibility to drugs (Keiser et al., 2006). The antimonials were gradually replaced by compounds such as oxamniquine and metrifonate. Despite being the cornerstone of anti-schistosomal treatment for the last 20 years, resistance to oxamniquine has been indicated (Lambertucci et al., 2000; Saconato & Atallah, 2000) and is gradually being replaced by cheaper alternatives. Metrifonate is no longer commercially
available due to insufficient market demand (Fenwick et al., 2003). The subsequent discovery of the anthelmintic properties of praziquantel in the 70s has dramatically revolutionised the
treatment of schistosomiasis.

Recently, the antimalarial artemisinin and derivatives were shown to have putative anti-
schistosomal activities against both immature and mature worms (Chen et al., 1980, Xiao et al.,
2000; Xiao et al., 2002, Utzinger et al., 2001; Li et al., 1996). More recent studies into the
effects of artemisinins and other synthetic compounds such as the trioxolanes demonstrate broad
spectrum activities against trematodes such as *S. japonicum*, *S. mansoni*, *Clonorchis sinensis*, *F.
hepatica* and *Opisthorchis viverrini* (Keiser & Utzinger, 2007; Xiao et al., 2007). Keiser et al. (2007) further demonstrated the *in vivo* effects of tribendimidine, a broad spectrum anthelmintic
against *S. mansoni* and other trematodes.

**1.7.1 Praziquantel control**

The pyrazinoisoquinolone praziquantel (Pzq) has been registered for human and veterinary use
since the early 1980s. Praziquantel is 2-cyclohexylcarbonyl-1,2,3,6,7,11b-
hexahydropyrazino{2,1-a}isoquinolin-4-one (see Figure 1.2), with the R or (−) form as the
active enantiomer. Available as a white crystalline racemate, the compound is stable, melting at
136 to 140°C with decomposition. Praziquantel is sparingly soluble in ethanol, chloroform and
dimethylsulphoxide (DMSO) and is slightly soluble in water.

![Praziquantel chemical structure](image)

**Figure 1.2: Praziquantel chemical structure**
1.7.1.1 Pharmacodynamics and metabolism

Praziquantel is rapidly and well absorbed following oral administration; with peak plasma levels achieved one to two hours post-ingestion. With a half-life of 0.8 to 2.0 hours, the drug is 80% reversibly bound to plasma proteins in animals and man (Dayan, 2003). Rapid metabolism is achieved in the liver with extensive first pass hydroxylation of the parent compound into inactive, conjugated metabolites (Marshall, 1987). Eighty percent of the drug dose is excreted in the urine mainly as 4-hydroxycyclo-hexylcarbonyl with a small amount of the parent compound.

1.7.1.2 Mechanism of action

Praziquantel is highly effective against all human schistosomes. Its utility has been repeatedly demonstrated in large-scale control efforts in many endemic countries. Active against a range of platyhelminths, it has been proposed that drug action was limited to organisms within this phylum (Andrews et al., 1983).

At lower therapeutic concentrations, the drug induces an increase in worm motor activity, followed by muscular contraction and subsequently, spastic paralysis. At higher and more effective concentrations, Pzq induce tegumental damage, leading to the activation of host immunity which ultimately results in the elimination of parasite (Fallon et al., 1992; Linder & Thors, 1992; Brindley, 1994). Praziquantel was shown to cause increased membrane permeability to monovalent and divalent cations, particularly to Ca$^{2+}$ (Pax et al., 1978; Blair et al., 1992), causing disruption in membrane calcium homeostasis (Kohn et al., 2001). Removal of Ca$^{2+}$ from the medium blocked both responses, indicating that the action was calcium-dependent. Tegumental damage includes severe swelling, vacuolisation, fusion of the tegumental ridges, loss and shortening of spines on tubercles with collapse and peeling (Shuhua et al., 2000). Alterations in glycogen content and energy metabolism of the schistosome appeared to be a secondary effect (Dollery, 1999).
Praziquantel’s molecular mechanism of action is unclear, although several potential mechanisms have been suggested based on the elucidation of novel sequences with no clear homology to genes from other phyla (Hu et al., 2004; LoVerde et al., 2004; McManus et al., 2004; Verjovski-Almeida et al., 2004). GSH S-Transferase Sj26, a molecular target for praziquantel action was recently challenged by Milhon et al. (1997). Similarly, direct action of Pzq on (Na$^+$, K$^+$)-ATPase and (Ca$^{2+}$, Mg$^{2+}$)-ATPase activity has been disproved (Nechay et al., 1980; Cunha & Noel, 1997). The apparent relationship between Pzq and calcium redistribution in worm tissues suggested that the target site of the drug could very well be a calcium-permeable membrane channel (Köhler, 2001). Studies by Chubb et al. (1978) have demonstrated that 50µM of Pzq prolonged the Ca$^{2+}$-dependent plateau phase of cardiac action potential in rats. In addition, Fetterer et al. (1980) demonstrated that methoxyverapamil (D-600), an inhibitor of L-type mammalian Ca$^{2+}$ channels, did not block the Pzq dependent Ca$^{2+}$ influx in schistosomes but blocked tonic contraction resulting from increased K$^+$ concentrations. Although Ca$^{2+}$ contractility induced by Pzq has been demonstrated in isolated muscle cells from S. mansoni, direct incontrovertible evidence for native Ca$^{2+}$ currents from schistosome cells have not been observed.

Recently, Kohn et al. (2001) proposed the β-subunit of voltage-gated calcium channels as a potential target of Pzq. Voltage-gated calcium channels are members of the voltage-gated ion channel superfamily that are regulators of Ca$^{2+}$ homeostasis in excitable cells. Voltage-gated calcium channels are heteromultimeric transmembrane protein complexes that contribute to impulse propagation by opening in response to changes in membrane potential (Greenberg, 2005). These channels are composed of a pore-forming, voltage-sensing α-subunit that is modulated by auxiliary subunits such as α$\delta$, β and γ. The α-subunit is composed of four domains, each consisting of six transmembrane regions (S1-S6). The fifth and sixth transmembrane regions (S5 and S6) along with the P-loop form a tetrameric structure that is the building block of ion channels. The residues residing within the P-loop are responsible for much of the pharmacology and ionic selectivity of the channel (Greenberg, 2005). The intracellular β-
subunits (Ca,βs) are critical modulators of the α₁ subunits by increasing current densities and ligand binding when co-expressed with the α₁ subunits. Homology modelling has indicated that Ca,βs were members of the membrane-associated guanylate kinase family of proteins (Hanlon et al., 1999). Membrane-associated guanylate kinase proteins are usually concentrated at synapses and are responsible for clustering at ion channels and neurotransmitter receptors (Dimitratos et al., 1999).

In view of this purported mode of action, it appeared likely that the mode of resistance can be acquired through a simple structural mutation in the variant subunit. However, Valle et al. (2003) were unable to demonstrate any quantifiable differences between the expression of the subunit between sensitive and insensitive isolates. In addition, Valle et al. (2003) found that adult and immature schistosomes demonstrated no appreciable differences in the level of β-subunit mRNA expression, despite significant differences in Pzq sensitivity between the various life stages. It is likely that the β-subunit of calcium channels was not the mode of resistance to Pzq in schistosomes. It has been further proposed that the anatomical localization of the calcium channel subunits may be a contributing factor in conferring Pzq sensitivity. However, the lack of evidence for β-subunit generated resistance demonstrated by Valle et al. (2003) must be critically reviewed as the mechanism of drug action is not necessarily similar to those involved in the mechanism of resistance. Furthermore, the drug structure or drug availability may be modified in the insensitive schistosomes even in the absence of target molecule modifications (Valle et al., 2003).

### 1.7.2 Triclabendazole control

Recent studies on the efficacy of the benzimidazole (Bz) class of compounds have generated renewed interest on the use of these compounds for the treatment of human schistosomiasis. Benzimidazoles are classically broad-spectrum anthelmintics used for the control of nematodes, cestodes and trematodes. Triclabendazole (Tcbz) is principally used to treat fascioliasis in humans and livestock. Recent reports have also suggested that the drug is effective against S.
Using scanning microscopy, el Sayed & Allam (1997) examined the effects of Tcbz on the tegument. When adult worms was exposed to Tcbz at 15µg/mL and 75µg/mL for a period of 24-hrs, there were rapid disruptions to the tegument with contraction and subsequent immobilization of the worm within one hour of Tcbz uptake. One hundred percent mortality was observed following 24-hrs exposure. Further *in vitro* Tcbz challenge studies on adult worms showed disruptions to the teguments and oral sucker (Mansoury, 1997). Khalil (2000) compared the *in vivo* efficacy of both Pzq and Tcbz in mice based on egg excretion, number, sex and distribution of worms, oogram changes and tissue egg load. Triclabendazole was shown to be more efficacious on male worms. A return of surviving worms from the hepatic to the mesenteric veins was also noted. More importantly, there was evidence of a cessation of eff-laying with Tcbz-treated mice.

The halogenated Tcbz is distinct from other Bz-carbamates because it possesses chloride atoms and a thiomethyl group in place of a carbamate moiety (see figure 1.2). In particular, computational chemistry has demonstrated that the methylthio substituent (as well as active metabolites bearing sulphoxide and sulphone substituents) at the 2-position imparts distinct shape and electronic distribution when compared to other Bzs. While most Bzs are L-shaped, the U-shape of Tcbz may be responsible for its select spectrum of activity (Lipkowitz & McCracken, 1991).

![Figure 2: Triclabendazole chemical structure](image)
1.7.2.1 Pharmacodynamics and metabolism

Mottier et al. (2004) characterised the in vitro biotransformation of Tcbz in sheep liver and Fasciola hepatica (F. hepatica) microsomal preparations. Sheep liver microsomes metabolised Tcbz into its active sulphoxide and sulphone metabolites in 30 min while F. hepatica metabolised Tcbz into its active metabolites following 60 min of incubation at a metabolic rate of 0.09 nmol min⁻¹ mg protein⁻¹. The high correlation between drug lipophilicity expressed as octanol-water partition coefficients and drug availability measured within the parasite indicated that the parent compound as well as its active metabolites are absorbed by F. hepatica.

The maximum concentrations of Tcbz-sulphoxide (Tcbz-Sx) and Tcbz-sulphone (Tcbz-Sp) observed in the plasma reached maximum concentrations at > 13µg/mL at 18 and 36 hr respectively (Hennessey et al., 1987). Active Tcbz metabolites were specifically bound to plasma albumin. In bile, major Tcbz metabolites were hydroxylated in the 4’ position and were secreted as sulphate esters and to a lesser extent, glucuronide conjugates. Conjugated hydroxy Tcbz-Sx is the major biliary metabolite and can reach concentrations in excess of 40µg/mL in bile.

1.7.2.2 Mechanism of action

By binding directly to β-tubulin, Bzs cause the depolymerization of cytoplasmic microtubules, subsequently leading to the disruption of microtubule-based processes. Microtubules are cytoskeletal elements involved in mitosis, intracellular transport and axonemal motility. The 100-KDa subunit of microtubule is tubulin, comprising of a heterodimer of α and β polypeptides (Dustin, 1984). The specificity of Bzs appear to be due to its higher affinity for helminth tubulin compared to mammalian tubulin (Lacey, 1988). Triclabendazole demonstrates high efficacy against both adult and juvenile flukes of F. hepatica, F. gigantica and Fascioloides magna, but lacks activity against nematodes, cestodes and other trematodes (Boray et al., 1983; Smeal & Hall, 1983). Despite its widespread use and high levels of efficacy, the
exact mechanism of action is unclear. The active sulphoxide of Tcbz was shown to effectively inhibit the movement of tegumental secretory bodies from the cell body to the apical surface of the tegument (Stitt & Fairweather, 1994), leading to progressive and total loss of worm tegument following 24 hrs of exposure *in vitro* (Stitt & Fairweather, 1993). In addition, Tcbz-induced inhibition of proteolytic enzyme secretion in *F. hepatica* has been attributed to microtubule-based secretory processes (Bennet & Köhler, 1987). The active metabolite of Tcbz also inhibits mitosis in the spermatogenic cells in the testis and the stem vitelline cells of *F. hepatica*. While the effect of Tcbz on energy-producing pathways is yet to be established, a stimulation of acetate and propionate production was observed to coincide with reduced worm motility. Carr *et al.* (1993) also demonstrated that Tcbz and its metabolites have the ability to uncouple oxidative phosphorylation in rat liver mitochondria. The changes induced by Tcbz are typical of microtubule inhibition and are consistent with changes induced by tubulozole-C and colchicine, both potent microtubule inhibitors. Much of the data presented from morphological studies suggested that Tcbz caused microtubule disruption, although additional action against protein synthesis may also occur.

Fetterer (1986) and Bennet & Köhler (1987) demonstrated the potential interaction of Tcbz with tubulin from *F. hepatica* by showing competitive binding between Tcbz and colchicine. In addition, many studies investigating the effects of colchicine and tubulozole-C on *F. hepatica* have demonstrated similar morphological changes to those observed with worms challenged by Tcbz (Stitt et al., 1992; Stitt & Fairweather, 1993; Stoitsova & Gorchilova 1997), suggesting that Tcbz acted via microtubule inhibition. Interestingly, studies have shown that Tcbz and tubulozole-C may bind to the same site on β-tubulin compared to the preferred colchicine binding site of other Bzs (Fairweather & Boray, 1999).

Consequently, further work with tubulozole-C will be useful for determining not only the mode of action of Tcbz against *Fasciola* but also the mechanism of resistance. This is because Bz resistance in *Haemonchus contortus* is associated with the substitution of tyrosine at position
200 of β-tubulin isotype-1. This alters the three-dimensional conformation of the protein and reduces the affinity of BZs for the target molecule (Kwa et al., 1994; Lubega & Prichard, 1991). Tubulin isolated from TcBz-sensitive and TcBz-resistant *F. hepatica* possesses a tyrosine residue at position 200 (Robinson et al., 2002), which suggests that TcBz has a unique binding site and mode of action that is distinctive to other Bzs. It is possible that TcBz resistance in *Fasciola* may arise from a similar β-tubulin isotype variant selection mechanism as many eukaryotic species encode more than one β-tubulin isotype (Brennan et al., 2007). However, it has also been suggested that adult *F. hepatica* has the ability to convert triclabendazole-sulphoxide into its relatively inert sulphone metabolite. Robinson et al. (2004) demonstrated that the production of triclabendazole-sulphone was greater within TcBz-resistant *F. hepatica* compared with susceptible flukes. The study indicates that more than one mechanism of resistance may be conferred.

1.7.2.3 Cytoskeletal proteins as *Schistosoma* drug targets

The schistosome body wall is an essential host-interactive layer involved in nutrition, immune evasion and modulation, excretion, osmoregulation, sensory reception and signal transduction (Jones et al., 2004). While the significance of cytoskeletons from eukaryotic cells has been emphasised in recent studies, schistosome cytoskeletal proteins are often overlooked as potential targets for chemotherapy or vaccine research. Intuitively, cytoskeletal molecules do not represent good targets for immune therapy due to their internal localization. Despite this, some schistosome cytoskeletal molecules can be involved in host humoral responses (Jones et al., 2004) and may represent potential drug and/or vaccine targets.

The high transcriptional activity of cytoskeletal and motor proteins in all life stages of schistosomes has been demonstrated through expressed sequence tag (EST) datasets (Prosdocimi et al., 2002; Verjovski-Almeida et al., 2003; Hu et al., 2003). dbESTs indicated that schistosomes possess major components of a microtubular and actin-based cytoskeletal system, with alpha, beta and epsilon tubulin transcripts well represented in the tegument.
(Verjovski-Almeida et al., 2003; Hu et al., 2003; Duvaux-Miret et al., 1991). Other proteins such as microtubule-associated motors, actin, microfilamented associated motors, myosins, kinesins and dyneins were also highly expressed in the tegument.

The lack of information on the tegumentary distribution of schistosome cytoskeletal proteins is largely due to the unavailability of antisera against schistosome proteins as well as a poor understanding of the multi and syncytial nature of schistosome tissues (Jones et al., 2004). Microtubules appeared to be poorly presented within the distal cytoplasm of the tegument (Duvaux-Miret et al., 1991; MacGregor & Shore, 1990). The cytoplasmic bridges of the tegumentary cytoplasm, on the other hand, are shown to possess a peripheral ring of microtubules. Stabilized microtubules are common in Platyhelminthes, particularly in the nervous system, cilia and flagella, and sperm (Iomini et al., 1998; Hartenstein & Jones, 2003). It has thus been suggested that this microtubular array was involved in anterograde transport through the cytoplasmic bridges as the disruption of the microtubular skeleton of schistosomes also disrupted surface expression of major components including some carbohydrates (Weist et al., 1988; Schmidt, 1998).

The potential for the exploitation of cytoskeletal molecules in anthelmintic design and vaccinology was suggested by Jones et al. (2004). While data concerning microtubule disruption by microtubule inhibitors such as the Bzs are fragmentary, these compounds appeared to disrupt anterograde transport in the tegument of cestodes and other digeneans but not in schistosomes (Schmidt, 1998). Following exposure to albendazole, the tegument of the trematode F. hepatica showed a marked increase in tubulin immunofluorescence, but lacking the ordered array of untreated microtubular cytoskeleton (Buchanan et al., 2003). This may have been due to the exposure of otherwise hidden antigenic epitopes on tubulin by albendazole. Alternatively, this increase could be attributed to a compensatory microtubule-associated secretory burst (Jones et al., 2004). Such studies are yet to be replicated on schistosomes.
1.8 Mass chemotherapy regimes for the treatment of schistosomiasis

Significant advances in control of schistosomiasis has been achieved through education, snail control and sanitation management. However, the long-term maintenance of these programmes is cost-prohibitive. The introduction of single-dose Pzq mass treatment appeared to be an ideal option. In 1984, the WHO implemented mass treatment regimes with Pzq as the major operational component due to its cost-effectiveness and availability. There is also indication that different generic brands of Pzq may have varying levels of efficacy, and as such, may contribute to the development of resistance as a result of sub-optimal dosing (Guyatt & Chan, 1998). Furthermore, most international agencies still procure Pzq on the basis of cost, despite growing concerns that insufficient credence is given to the efficacy evaluation of generic Pzq brands (Guyatt & Chan, 1998). Treatment and control is now largely reliant on the use of Pzq. The relative success of this treatment regime has prompted efforts to expand the use of cheap, generic Pzq in endemic areas with high levels of parasite transmission (Hagan et al., 2004). However, the absolute reliance of a single compound for control may have indeterminate consequences on the epidemiology of disease (Colley et al. 2001; Raso, et al. 2004; Anon, 2004). Indeed, there are significant shortcomings in disease treatment with Pzq. Despite the previous successes of Pzq control programs, schistosomiasis is spreading into new areas (McManus & Loukas, 2008). Praziquantel is also marketed as a racemate of the R and S isomers of which the R isomer is the only active enantiomer. The unnecessary administration of inactive isomers may increase the likelihood of drug side-effects. Praziquantel is also ineffective against immature schistosomula (4-6 weeks) and can result in treatment failures and does not protect against re-infections (Lawn et al. 2003; Cioli et al. 2003, McManus & Loukas, 2008). In addition, Pzq treatment is based on body weight and required distribution by trained personnel. This can increase operational costs of drug regimens, and ultimately limits its application in mass chemotherapeutic regimes. Moreover, early suggestions of praziquantel resistance (or tolerance) and treatment failures have surfaced (Stelma et al., 1993, 1995; Guisse et al., 1997; Picquet et al., 1998; Alonso et al., 2006; Silva et al., 2005).
1.9 Refractoriness to praziquantel treatment

*Schistosoma* resistance is defined as a significant decrease in the response of a susceptible population to a schistosomicide (Coles & Kinoti, 1997). Specifically, the classic phenotype of drug resistance is defined as a significant increase in the 50% effective dose value of isolates retrieved from patients not responding to the drug (Ismail *et al.*, 1999). Strong evidence exists to suggest that schistosomes have the potential to evade the therapeutic action of anti-schistosomals (Rodger & Bueding, 1971). Fallon *et al.* (1996) and Kusel & Hagan (1999) demonstrated the possibility of inducing laboratory resistance by selecting for parasites refractory to Pzq treatment. The first indication of Pzq resistance appeared in northern Senegal, where low cure rates were observed (Gryseels *et al.*, 1994; Stelma *et al.*, 1995). Additional evidence was presented in Egypt, in which isolates obtained from patients unsuccessfully treated with Pzq was established in the laboratory. These isolates were subsequently shown to have a lower susceptibility to Pzq *in vitro* (Ismail *et al.*, 1999) and *in vivo* (Bennett *et al.*, 1997). There have also been reports demonstrating treatment failures with standard doses of 40mg/kg Pzq. Dabo *et al.* (2000) showed consistent reinfection of children with *S. mansoni* and *S. hematobium*, despite repeated Pzq treatment. Wolfe (2003) reported treatment failure for a returned traveller infected with *S. mansoni* at 40mg/kg of Pzq. Silva *et al.* (2005) also reported the therapeutical failure of Pzq in the treatment of several *S. haematobium* infections. More recently, Alonso *et al.* (2006) reported repeated treatment failures of two travellers with genitourinary schistosomiasis using standard 40mg/kg of Pzq.

1.10 Combination chemotherapy

Drug combinations are increasingly being used for the treatment of parasitic diseases. Where resistance is conferred as a result of mutations in genes encoding drug targets, the application of combination therapy has sound scientific rationale (Nduati & Kamau, 2005). Provided that the mutations resulting in resistance reside in unlinked genes, the probability that a single parasite simultaneously carrying mutations to each of the drugs is remote, and is the product of the mutation rates to resistance for each individual drug in combination (White & Olliaro, 1996). In
effect, the application of combination therapy enables the mutual protection of one inhibition mechanism by another, thereby extending the therapeutic lives of the components (White et al., 1999). By exerting efficacy through independent mechanisms of action, drug combinations have the capacity to produce a quantitative effect that is greater than the sole additive effect of the combined drugs (Yeo et al., 1997).

Drug combinations with independent mechanisms of action were first developed against tuberculosis and have since been successfully adapted for anti-cancer, AIDS/HIV and antimalarial control (White et al., 1999). The aims of combinatorial treatments are to exert amplified effects at lower doses, which in turn, minimize drug side-effects. Combination drug therapy can also enable the mutual protection of one inhibition mechanism by another, thereby extending the therapeutic lives of the components (White et al., 1999).

A major strategy in the global effort to eliminate lymphatic filariasis is through the introduction of new drug combinations such as diethylcarbamazine, ivermectin and albendazole (Sunish et al., 2006). In particular, drug combinations have been widely adopted for the control of malaria, where multidrug resistance has rendered existing monotherapies useless in many parts of the world (Kremsner & Krishna, 2004). Drug combinations are a routine practice in the animal health sector. A combination of febantel, pyrantel and Pzq was shown to be highly effective for the treatment of giardiasis in dogs (Payne et al., 2002) and kittens (Scorza et al., 2005). In human parasitic infections, a combination of albendazole and ivermectin or diethylcarbamazine, and between mebendazole and levamisole or pyrantel has been indicated for the treatment of soil-transmitted helminths (Albonico, 2003; Utzinger & Keiser, 2004).

The use of drug combinations against schistosomiasis has been explored more than two decades ago (Utzinger et al., 2003). A combination of Pzq and oxamniquine (Oxm) has been shown to reduce worm and egg burden in mice infected with S. mansoni and S. japonicum infections in two separate studies (Pugh & Teesdale, 1983; Shaw and Brammer, 1983). However, inconsistencies in experimental methodologies suggested that therapeutic efficacies may have
been overestimated (Utzinger et al., 2003). It was further determined that the similarity in activities, half-lives and stage-specific susceptibilities of both Pzq and Oxm was unlikely to lead to improved outcomes. More recently, Mattos et al. (2007) successfully demonstrated the efficacy of this combination on the intramolluscan phase of *S. mansoni*. The WHO has recently established protocols for Phase 1 clinical trials for a Pzq and Oxm combination (Mattock & Pink, 2005). A separate proposal has been established for the assessment of triclabendazole against *Schistosoma* and *Fasciola* co-infections (TDR, 2005). Combinations of Pzq and artemisinin derivatives have also been extensively studied (Xiao et al., 2000; Utzinger et al., 2001; DeCelrio et al., 2000; Borrman et al., 2001). An *in vivo* study by Shu-hua et al. (2006) further demonstrated the utility of artemisinin-Pzq combinations in the treatment of mice co-infected with *P. berghei* and *S. mansoni*, suggesting that artemisinin-based combination therapies may have ancillary benefits against schistosomiasis. However, it has been suggested that artemisinin-based combinations should not be administered in areas where both schistosome and plasmodium co-exist to prevent the development of resistance to malaria (Utzinger et al., 2003; Menar et al., 2005). This is particularly evident in light of reports that helminth infections (soil-transmitted helminths and *S. mansoni*) can adversely affect clinical outcomes as well as enhances the incidence and severity of malaria infections (Nacher et al., 2002, Spiegel et al., 2003, Sokhna et al., 2004). It is clear that rigorous monitoring of the effects of artemisinin is warranted when used for malaria control in settings where malaria and schistosoma co-exist (Shu-hua et al., 2006). Other limiting factors include high costs, limited knowledge and public awareness, limited knowledge on efficacy and safety in pregnancy, operational issues such as indiscriminate use, lack of suitable formulations, lack of post-marketing surveillance as well as an imbalance between supply and demand (Mutabingwa, 2005).

### 1.11 Cost-effectiveness of praziquantel for control

Cost-effectiveness analysis is the predominant type of economic evaluation for the assessment of health care interventions relating to parasitic diseases (Walker & Fox-Rushby, 2000). Cost-
effectiveness analyses play critical roles in assessing the most optimal option for the control of schistosomiasis through the evaluation of resource, social, ethical and political constraints. By assessing the gains (effectiveness) and resource input requirements (costs) of different methods of interventions in common natural units, the effectiveness of the objective can be evaluated. In light of budgetary limitations and logistical inadequacies in endemic and developing countries, cost effectiveness analyses can serve to determine the most effective means to deliver control strategies and ultimately, to strengthen political will to commit these funds. While cost-effectiveness information primarily serves to guide policy and decision makers at the earliest stage of programme implementation, this information can also support the rational allocation of funds to more closely match the real funding need over time (Marseille et al., 2002).

Guyatt et al. (1994) developed a framework for the evaluation of delivery strategies in the Kilombero District of Tanzania by comparing the primary health care system, the mobile team mass treatment system and the reagent strip testing system. Korte et al. (1986) compared the cost-effectiveness of metrifonate regimens and Pzq regimens in the Peoples Republic of Congo and Mali. The study demonstrated that the feasibility and cost-effectiveness of a single dose Pzq programme compared to a three course metrifonate regimen was largely due to lower operational costs. Yu et al., (2002) evaluated the feasibility of three Pzq treatment schemes and determined that the treatment of patients with high risk activities and those displaying symptoms are more cost effective than mass treatment programmes and treatment of positively screened patients by Kato-Katz examination. Carabin et al. (2000) on the other hand, outlined the importance of maximizing treatment potential by demonstrating that the treatment of children not enrolled in schools can also be cost-effective.

While some cost effectiveness data are available for conventional drug treatment regimes such as Pzq and metrifonate, there is only small body of information available that assess the cost-effectiveness of applying new anti-schistosomal compounds for treatment. Metrifonate, an organophosphorus cholinesterase inhibitor, is administered in a three dose regime in two week intervals. In response to the surmounting market dominance of Pzq, narrow spectrum of
activity and inadequate demand, metrifonate has been withdrawn from commercial production (Korte et al., 1986). Oxamniquine is the only other drug for treating schistosomiasis and is regularly administered in regions of high endemicity. However, its narrow spectrum of activity is confined to S. mansoni and is not effective against S. haematobium. A synergistic effect has also been noted when the Pzq and oxamniquine were applied in combination. However, resistance and potential toxicity has resulted in increasing tepidity by health authorities to administer Oxamniquine.

The anti-schistosomal properties of artemisinin and its derivatives were first described in the 80s (Le et al., 1981). The two main derivatives, artemether and artesunate, showed high efficacies against the juvenile stages of the parasite (Xiao, 2005). Recent studies have demonstrated the feasibility of applying Pzq and the artemisinins in combinations, which results in significant worm burden reductions (Utzinger et al., 2003; Utzinger et al., 2001). The prophylactic properties of the artemisinins have also been demonstrated at high doses with little side effects (Wu et al., 2003). However, the widespread application of these compounds is unlikely due to prohibitive costs, multiple dosing regimens and possible selection for resistance for malaria through indiscriminate use in regions co-endemic with malaria and schistosomiasis (White et al., 1999; Utzinger et al., 2001).

More than 100 million doses of Pzq have been administered in China and Egypt alone (Chen, 2005; Fenwick et al., 2003). Dynamic models of transmission and morbidity have been successfully applied to assess the effects of Pzq treatment programmes against target groups, coverage and frequency of treatments. Using a dynamic model of schistosomiasis transmission and morbidity to assess the correlation between drug efficacy and price, Guyatt & Chan (1998) demonstrated that the relative cost-effectiveness of different combinations of drug efficacy and drug price did not appear to differ for drugs that possess moderate to high efficacies. Guyatt & Chan (1998) also emphasized the highly non-linear relationship between drug efficacy and cost-effectiveness. When drug efficacies are > 50-60%, the cost effectiveness ratios were lower and similar. Lower drug efficacies on the other hand, produced high and variable cost effectiveness.
ratios, particularly when programme costs related to distance travelled were high (Guyatt & Chan, 1998). This suggested that drugs possessing <50% efficacy should not be recommended for control, in view of drug resistance that are fostered by sub-therapeutic use (Warren et al., 1993). The non-linear relationship suggested that an increase of drug efficacy for low efficacy drugs had a greater impact on cost-effectiveness than an equivalent percentage reduction in the prices of more costly brands of Pzq.

The price of Pzq varies widely between US$0.15 to US$0.60 per 600mg tablet (Guyatt & Chan, 1998). The cost of a single course of treatment at 40mg/kg in 2003 was approximately US$0.30 for an adult and US$0.20 for a child. Through school-based or community-directed systems, the average treatment cost for a child was an estimated US$0.50 per annum, including operational costs (Fenwick et al., 2003). The attractiveness of competitive prices and the successes of Pzq treatment regimes in the field have resulted in the adoption of large-scale treatment programmes in other endemic regions. This will have a significant impetus on the demand for Pzq. The market for Pzq is expected to rise substantially, with consumption increased to 40 million tablets per annum provided that the price is maintained and resistance to the compound is avoided.

The delivery of mass Pzq regimes is based on a ‘blue-print’ approach by adapting treatment programmes to different endemic areas. While this approach is advantageous in its simplicity and cost-effectiveness, such strategies may result in the selection for drug resistance. This is best exemplified by the selection for anthelmintic resistance in veterinary health. It is important to note that while resistance to Pzq has not been demonstrated in the field, resistance to the drug has been successfully induced in the laboratory (Ismail et al., 1999; Bennett et al., 1997). The potential for the application of combination chemotherapy against schistosomiasis has not been explored. Utzinger et al. (2003) suggested that a combinational approach for schistosomiasis is feasible if partner drugs possess different modes of action to reduce the likelihood of resistance development.
1.13 Aims and Hypothesis

The aims of this study are:

- To determine the *in vitro* and *in vivo* efficacy of Tcbz against *S. mansoni*,
- To evaluate the effects of combinations of Pzq and Tcbz against *S. mansoni*.

The hypotheses of this study are that:

- Triclabendazole and its metabolites are active against all life stages of the parasite,
- Triclabendazole and its metabolites show synergism when applied in combination with Pzq,
- Drug synergism between Pzq and Tcbz is reproducible in other parasite species,
- Combinations of Pzq and Tcbz are cost-effective for treatment of human schistosomiasis.
Chapter 2

2.1 Growth Medium

2.1.1 Schistosoma medium 169 composition

Table 2.1: Composition of culture medium 169 for the *in vitro* cultivation of immature schistosomulum and maintenance of adult worm adapted from Basch (1981).

<table>
<thead>
<tr>
<th>Composition</th>
<th>Storage</th>
<th>Stock []</th>
<th>Final []</th>
<th>100mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>BME</td>
<td>4°C</td>
<td>Liquid</td>
<td>-</td>
<td>To 100mL</td>
</tr>
<tr>
<td>Lactalalbumin hydrolysate</td>
<td>4°C</td>
<td>50x</td>
<td>1g/L</td>
<td>2mL</td>
</tr>
<tr>
<td>Glucose</td>
<td>-20°C</td>
<td>100x</td>
<td>1g/L</td>
<td>1mL</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>-20°C</td>
<td>0.5mM</td>
<td>0.5µM</td>
<td>100µL</td>
</tr>
<tr>
<td>Serotonin</td>
<td>-20°C</td>
<td>1mM</td>
<td>1µM</td>
<td>100µL</td>
</tr>
<tr>
<td>Insulin (crystalline)</td>
<td>-20°C</td>
<td>8mg/ml</td>
<td>8µg/ml</td>
<td>100µL</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>-20°C</td>
<td>1mM</td>
<td>1µM</td>
<td>100µL</td>
</tr>
<tr>
<td>Triliodothyronine</td>
<td>-20°C</td>
<td>0.2mM</td>
<td>0.2µM</td>
<td>100µL</td>
</tr>
<tr>
<td>MEM vitamins</td>
<td>-20°C</td>
<td>100x</td>
<td>0.5x</td>
<td>500µL</td>
</tr>
<tr>
<td>Schneider’s medium</td>
<td>4°C</td>
<td>1x</td>
<td>5%</td>
<td>5mL</td>
</tr>
<tr>
<td>Human Serum/FCS</td>
<td>20°C</td>
<td>1x</td>
<td>10%</td>
<td>10mL</td>
</tr>
<tr>
<td>HEPES</td>
<td>20°C</td>
<td>1M</td>
<td>10mM</td>
<td>1mL</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>20°C</td>
<td>2.2g/L</td>
<td>26mM</td>
<td>-</td>
</tr>
<tr>
<td>NaOH</td>
<td>20°C</td>
<td>-</td>
<td>pH 7.4</td>
<td>-</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>-20°C</td>
<td>10,000U/mL</td>
<td>300U/ml</td>
<td>3mL</td>
</tr>
<tr>
<td>L-glutatmine</td>
<td>-20°C</td>
<td>100x</td>
<td>-</td>
<td>1mL</td>
</tr>
</tbody>
</table>

2.1.2 Giardia medium composition

Table 2.2: Composition of *Giardia* medium for the *in vitro* cultivation of *Giardia duodenalis*.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Final quantity (1L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium ferric citrate (BDH)</td>
<td>0.01g</td>
</tr>
<tr>
<td>Ascorbic acid (Sigma)</td>
<td>0.2g</td>
</tr>
<tr>
<td>Biosate peptone (BBL)</td>
<td>30g</td>
</tr>
<tr>
<td>Bovine bile (Sigma)</td>
<td>0.5g</td>
</tr>
<tr>
<td>Cysteine hydrochloride (Sigma)</td>
<td>2.0g</td>
</tr>
<tr>
<td>Monopotassium dophosphate (BDH)</td>
<td>1.0g</td>
</tr>
<tr>
<td>Dipotassium phosphate (BDH)</td>
<td>0.6g</td>
</tr>
<tr>
<td>Glucose (Ajax)</td>
<td>10g</td>
</tr>
<tr>
<td>Sodium chloride (BDH)</td>
<td>2.0g</td>
</tr>
<tr>
<td>DDI H₂O</td>
<td>900mL</td>
</tr>
<tr>
<td>New Born Calf Serum</td>
<td>100mL</td>
</tr>
</tbody>
</table>

2.1.3 Haemonchus medium composition

Table 2.3: Composition of *Haemonchus* medium for larval development assays.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Final quantity (100mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>250µg/mL</td>
</tr>
<tr>
<td>0.8% Saline</td>
<td>80mL</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1g</td>
</tr>
<tr>
<td>10x Earle’s balanced salt solution</td>
<td>1mL</td>
</tr>
<tr>
<td>DDI H₂O</td>
<td>19mL</td>
</tr>
</tbody>
</table>
2.2 *Biomphalaria glabrata* maintenance

The intermediate snail host, *Biomphalaria glabrata* (*B. glabrata*) was obtained from the Queensland Institute of Medical Research (QIMR). The original Puerto Rican isolate was sourced from National Institute of Allergy and Infectious Disease (NIAID) Schistosomiasis Resource Centre. Approximately 50 breeding snails were maintained at any one time in each 60cm x 30cm x 30cm polystyrene maintenance containers at 25°C on a 12hr light and 12hr dark cycle in aquaria containing DDI H$_2$O conditioned with CaCO$_3$ chips (fortified H$_2$O). Snails were fed with fresh lettuce leaves *ad libitum* (Loker & Hertel, 1987). *Biomphalaria glabrata* infected with *S. mansoni* miracidium were kept separated from uninfected snails. Not more than 50 infected snails were maintained in each polystyrene container. To minimize exposure to light and heat, infected snails were kept in the dark.

2.3 *Biomphalaria glabrata* infection with miracidium

The livers from five mice infected with *S. mansoni* were removed and rinsed thrice in PBS. The livers were then macerated to a fine consistency, transferred to 50mL sterile conical tubes and suspended in cold PBS. The mixture was centrifuged at 2000 x rpm on an Eppendorf 5810R rotor for 10s and the supernatant removed. Subsequently, the homogenate is resuspended in calcium carbonate-fortified H$_2$O at 25°C and transferred to 50mL measuring cylinders. Measuring cylinders were blackened on the sides, leaving the top 5cm of the cylinder exposed to allow hatched miracidia to swim to the surface for observation. The liver suspension was then transferred into the cylinders and exposed to light from a 100Watt desk lamp for 30min. Twenty mature *B. glabrata* were removed from the maintenance tanks and washed thrice with H$_2$O. Snails were separated into individual wells of a 48-well culture plate (*Nunc™*, Roskilde, Denmark) filled with 1mL of fortified H$_2$O. Ten miracidia were then pipetted into each well for infection and consistently monitored overnight. Following infection with miracidia, the snails were gently transferred into a new maintenance tank. The snails were then closely monitored for 35 days post-miracidial infection.
2.4 Cercarial shedding

Cercariae was recovered from snails 35-days following infection with miracidia. All infected snails were gently removed and washed thrice in fortified H$_2$O to remove excess food and fecal matter. The snails were then transferred into a 50mL beaker and exposed to light and heat under a 100 Watt desk lamp for no more than 5min. The beaker was then immediately filled with 20mL of fortified H$_2$O at 25°C and exposed to light for approximately 30min. Cercariae were recovered and enumerated microscopically.

2.5 Infection of definitive mouse host

Six wks female ARC Swiss outbred mice were used as definitive mammalian hosts. Prior to infection, each mouse was weighed and the abdominal region shaved to allow access by S. mansoni cercariae. Each mouse was subsequently anaesthetized with a mixture of xylazine (20mg/kg), ketamine hydrochloride (100mg/kg) and DDI H$_2$O at a 1:1:10 ratio. One hundred fifty µL of the mixture was given per 10g of mouse by intraperitoneal injection using a 21G needle. Following anesthesia, each mouse was individually restrained on a heat pad using tape. A 30mm diameter brass ring was placed on top of the shaven abdominal patch to contain the infective cercariae. Exactly 120 cercariae were then pipetted into the brass ring of each anaesthetized mouse for infection. Mice were exposed and anesthetized for no more than 1hr.

2.6 In vitro cultivation of immature schistosomulum

Cercariae were shed according to 2.4 and collected in 50mL sterile conical tubes. The tubes were chilled on ice for 20min and centrifuged at 350 x g for 5min at 4°C. The top 45mL of supernatant was removed and the pellet was resuspended in 25mL of cold ELAC wash medium. The tubes were then gently shaken and chilled on ice for 10min. The tubes were then centrifuged at 350 x g for 5min at 4°C and the wash repeated twice. The cercarial pellet was resuspended in 10mL of cold E/LAC and drawn into a 50mL Luer Lock syringe for mechanical transformation using a 22G microemulsifying needle. With the microemulsifying needle firmly screwed to another 50mL Luer Lock syringe, the suspension was emulsified by pushing back
and forth for at least 25 times. The cercariae suspension was then emptied into a fresh sterile 50mL conical tube and incubated for 15min at 37°C. The top 10mL of the cercarial tail-rich supernatant was removed and the remaining supernatant made up to 10mL with fresh warmed medium 169. The mixture was then incubated for 10min at 37°C. The top 8mL of the cercarial tail-rich supernatant was then removed and the remaining supernatant made up to 10mL with fresh warmed medium 169. The resulting suspension was incubated for a further 5min at 37°C and the top 8mL of supernatant removed. The schistosomulum suspension was then made up to 10mL with warmed medium 169 and transferred into 6mL culture wells (Nunc™, Roskilde, Denmark). The schistosomule culture medium was incubated at 37°C in an atmosphere of 5% CO₂ in air and was replaced with fresh media daily. Any dead schistosomules were immediately removed from the medium.

2.7 Schistosoma egg extraction from liver

Schistosoma mansoni and S. japonicum eggs were isolated from liver tissue according to the protocol outlined by Dalton et al. (1997). Schistosoma japonicum livers were obtained from infected mice routinely maintained at the QIMR. Livers from three mice infected with S. mansoni and three mice infected with S. japonicum were removed following perfusion and macerated to a smooth consistency. The macerated liver was then resuspended in PBS and decanted into 50mL Falcon tubes. Twenty mg of collagenase B (Sigma-Aldrich, USA), 100µL of penicillin (10µg) and 100µL (20µg) of streptomycin was added and the mixture incubated overnight with shaking at 37°C. Following incubation, the tube was centrifuged at 400xg for 5min and the supernatant removed. The pellet was resuspended in 50-mL of fresh cold PBS and the wash procedure repeated twice. The pellet was then reconstituted in 25mL PBS, passed through a 250µm sieve, followed by a 150µm sieve. The filtrate was re-centrifuged at 400 x g for 5min, and the supernatant discarded. The pellet was resuspended in 3-mL of PBS and applied gently to the top of a Percoll (Sigma-Aldrich, USA) gradient prepared by mixing 8mL of Percoll with 32mL of 0.25M sucrose in a 50mL Falcon tube. The tube was centrifuged at 800xg for 10mins and the liver cells suspended at the top of the gradient removed. Pelleted eggs
were gently transferred using sterile pasteur pipettes and washed thrice with PBS. Further purification of the eggs were achieved by resuspension in 0.5mL of PBS, with subsequent application to a second Percoll gradient, prepared by mixing 2.5mL of Percoll with 7.5mL of 0.25M sucrose in a 10mL polypropylene tube. The wash procedure was repeated as before and the eggs subsequently resuspended in 1mL cold PBS. *Schistosoma* eggs were stored in a 1.5mL microfuge tube at 4°C until required.

### 2.8 *Haemonchus* egg extraction from faeces

Five hundred grams of faeces was collected from 3 sheep infected with *H. contortus*. Faeces were suspended in 600-mL of DDI H₂O and two table-spoons of kaolin and macerated to a fine consistency. The mixture was made up to 1L with DDI H₂O and centrifuged at 1,500 x rpm for 15min at 5°C using an Eppendorf 5810R centrifuge. Following centrifugation, the top layer of fecal debris was removed gently and the middle layer of material decanted into a sieve (710µm). The filtrate was passed through another sieve (150µm) and the sieve washed with DDI H₂O. The process was repeated twice more using 80µm and 25µm sieves. The final 200mL of filtrate was collected in sterile 50mL Falcon tubes and centrifuged at 3000 x rpm for 5min at room temperature using a Kokusan STAT H103-N (RF120 rotor) centrifuge. The top 25mL of the suspension was gently removed. One teaspoon of kaolin was added and the mixture made up to 50mL with 1.275SG saturated sucrose solution. The mixture was recentrifuged at 3000x rpm for 5min at room temperature using a Kokusan STAT H103-N (RF120 rotor) centrifuge. The top 20mL of suspension was gently removed and filtered using a 0.22µm filter. The filter was then washed repeatedly with DDI H₂O. Eggs were then collected reverse flushing the filter paper with DDI H₂O. Purified eggs were stored at 4°C and used the following day.

### 2.9 *In vitro* drug evaluation

#### 2.9.1 *In vitro* drug evaluation against immature schistosomulum

Immature schistosomules were transformed as described in 2.1.5. Immature schistosomules were maintained in medium 169 for four weeks post-transformation. Exactly 30 schistosomula
were transferred into duplicate wells of 48-well culture plates (Nunc™, Roskilde, Denmark). One hundred times (x) or fold stock concentrations of Pzq, Triclabendazole (Tcbz), triclabendazole sulphoxide (Tcbz-Sx) and triclabendazole sulphone (Tcbz-Sp) were diluted in DMSO to make final concentrations of 100µg/mL, 80µg/mL, 40µg/mL, 20µg/mL, 10µg/mL and 5µg/mL in medium 169. DMSO concentration in both treated and control wells were ≤ 0.1% v/v. Cultures were then exposed to individual concentrations of the drugs for 24hrs. After 24hrs of drug exposure, the drug-treated worms were then washed thrice in warmed medium 169. The schistosomulum was then transferred into new 48-well culture plates filled with warmed medium 169 and monitored by light microscopy for seven days. Morphological changes following drug exposure were monitored at two hourly intervals by light microscopy. Real-time video were captured using a Sony HandyCam™ (DCR-HC40E) connected to a Panasonic Color CCTV Camera (Model WV-CP610/G), which was subsequently attached to an Olympus CKX41 Compound Microscope.

2.9.2 In vitro drug evaluation against mature worms

Twenty-five 49 day old infected mice were injected with 100µL heparin (Sigma-Aldrich, USA) and perfused using cold PBS. Exactly 10 adult worms were distributed in duplicate 6-well culture plates (Nunc™, Roskilde, Denmark) in medium 169. Cultures were maintained at 37°C in an atmosphere of 5% CO₂ in air and acclimatized for at least a week prior to the commencement of drug screening. Duplicate cultures were then exposed to both Pzq and Tcbz for 24hrs and monitored at one hourly intervals. Cultures were then washed thrice in medium 169 following 24hrs exposure and transferred to new 6-well culture plates containing warmed drug-free medium 169. The worms were further assessed 48hrs and 72hrs post drug exposure. Worms were defined ‘dead’ if worms remained contracted and remained immotile. Morphological changes following drug exposure were monitored at two hourly intervals using OPTIMAS®. Pzq stock solutions in DMSO were made to the following concentrations: 100µM, 30µM, 10µM, 1µM and 0.3µM. Tcbz, Tcbz-Sx and Tcbz-Sp stock solutions in DMSO were
made to the following concentrations: 300µM, 100µM, 30µM, 10µM and 3µM. The final concentration of DMSO in Medium 169 was ≤ 0.1%.

2.10 Evaluation of combination drug treatment

2.10.1 Evaluation of drug combinations against Schistosoma mansoni

The synergistic action of Pzq and Tcbz-sx was assessed against both immature and mature stages of *S. mansoni*. Ten mature worms or 30 immature schistosomulum in duplicate 6-well culture plates (Nunc™, Roskilde, Denmark) filled with 1mL of medium 169 were assessed with both Tcbz and Pzq at one hour intervals following drug combination exposure for an exposure period of 24hrs. Worms were continuously monitored at 48 hours and 72hrs post-drug combination exposure. For isobologram analysis, the EC$_{50}$, EC$_{25}$ and EC$_{10}$ values were determined from the dose response curves of worms treated with Pzq and Tcbz-sx. The stock solutions of both drugs were made to give final concentrations for nine combinations of the EC$_{50}$, EC$_{25}$ and EC$_{10}$ values in medium 169. The final DMSO concentrations in medium 169 were ≤ 0.2% in both exposed and control wells. Cultures were exposed for 24hrs and enumerated. Cultures were then washed thrice in medium 169 following 24hrs exposure and transferred to new 6-well culture plates containing warmed drug-free medium 169. Further to efficacy assessment at 24hrs post-drug exposure, the worms were also enumerated at 48hrs and 72hrs post-exposure time points.

2.10.2 Evaluation of drug combinations against *Giardia duodenalis*

*Giardia duodenalis* (*G. duodenalis*) strain P1c10 was subcultured in *Giardia* media for at least seven days prior to the commencement of drug screening. A total of 25,000 trophozoites were enumerated by haemocytometer and subsequently seeded in triplicates into 10mL Nunc™ Flat-sided polystyrene tubes. One hundred x stock solutions of Pzq, Tcbz, Tcbz-sx and Tcbz-sp were made in DMSO to give final concentrations of 100mM, 10mM, 1mM, 100µM, 10µM, 1µM, 100nM and 10nM in *Giardia* media. Cultures exposed to drugs were washed with sterile warm PBS and replaced with warmed *Giardia* media and enumerated using the OPTIMAS® system of
adherence inhibition (Bong et al., in preparation). The OPTIMAS® system is a cellular and particle enumeration software adapted for the assessment of cell numbers in liquid media. To assess for synergistic drug interactions, the EC$_{50}$, EC$_{25}$ and EC$_{10}$ values were determined from the individual dose response curves. The stock solutions of both drugs were made to give final concentrations for nine combinations of the EC$_{50}$, EC$_{25}$ and EC$_{10}$ values in *Giardia* media. The final DMSO concentrations in *Giardia* Medium were ≤ 0.2% in both exposed and control wells. Cultures exposed to drugs were washed with sterile warm PBS and replaced with warmed *Giardia* media and enumerated by measuring cells that remained adhered following exposure.

2.10.3 Evaluation of drug combinations against *Haemonchus contortus*

Benzimidazole-susceptible and resistant *Haemonchus contortus* (*H. contortus*) eggs were screened using the larval development assay developed by Animal Health Laboratories, Department of Agriculture and Food, Western Australia. Eggs were extracted from *H. contortus* infected sheep routinely maintained by the Western Australia Department of Agriculture and Food. Approximately 100 eggs were transferred into individual wells of a 96-well microtitre plate made up to 100µL with 10µL *Haemonchus* nutrient media and DDI H$_2$O. The drug stock solutions were prepared to give final concentrations of 1mM, 100µM, 10µM, 1µM, 0.1µM and 0.01µM in nutrient media. Assays were performed in triplicates to ensure statistical significance. The final DMSO concentration in culture was ≤ 1%. Upon drug exposure, culture plates were incubated at 25°C and monitored daily. Upon growth of the larvae to the L3 stage, the worms were killed with iodine and enumerated using an Olympus BH2 compound microscope.

Prior to the commencement of synergistic drug studies, the EC$_{50}$, EC$_{25}$ and EC$_{10}$ values were determined from the individual dose response curves. The stock solutions of both drugs were made to give final concentrations for all nine combinations of the EC$_{50}$, EC$_{25}$ and EC$_{10}$ values in *Haemonchus* media. Cultures were established as in 2.9.1, with the exception that the final DMSO concentration in media was ≤ 2%. Cultures were then incubated at 25°C and monitored
daily. Upon growth of the larvae to the L3 stage (one wk), the worms were killed with 10% iodine and enumerated using an Olympus BH2 compound microscope.

2.11 Evaluation of drug action against Schistosoma eggs

Eggs were extracted from liver according to 2.7. Exactly 10 eggs were enumerated and transferred into triplicate wells of Falcon® Multiwell™ 24-well culture plates (Becton Dickinson, USA). Individual culture wells were made up to 1mL with PBS. Pzq, Tcbz, Tcbz-Sx and Tcbz-Sp stock solutions in DMSO were made to give final concentrations of 1mM, 100µM, 10µM, 1µM, 0.1µM and 0.01µM.

2.11.1 Assessment of praziquantel-induced hatching of eggs

Ten mature and viable S. mansoni or S. japonicum eggs were transferred into triplicate wells of Falcon® Multiwell™ 24-well culture plates (Becton Dickinson, USA). The temporal hatching pattern of S. mansoni and S. japonicum eggs after exposure to Pzq was quantified using a stopwatch according to the following criterion: ‘time to activation’ and ‘time to hatch’. The time to activation was defined as the time from which a concentration of Pzq induced the first sign of movement of ‘activation’ within the eggs. Time to hatch was defined as the total time in which the miracidium detaches/hatches completely from the egg and the vitelline membrane. Real-time video were captured using a Sony HandyCam™ (DCR-HC40E) connected to a Panasonic Color CCTV Camera (Model WV-CP610/G), which is subsequently attached to an Olympus CKX41 compound microscope.

2.11.2 Assessment of triclabendazole and its metabolites on egg viability

Ten mature and viable S. mansoni or S. japonicum eggs were transferred into triplicate wells of Falcon® Multiwell™ 24-well culture plates (Becton Dickinson, USA). Following drug inoculation, culture plates were incubated for 24hrs at room temperature. The eggs were then transferred into fresh wells filled with DDI H2O. Hatching was induced by light exposure at room temperature and subsequently enumerated under an Olympus CKX41 compound microscope.
microscope. The inhibition of hatching was measured as the proportion of eggs that remained unhatched in DDI H$_2$O after exposure to drugs for 24 hrs.

2.12 Dose response and statistical analysis

Dose response analysis was performed using GraphPad Prism 4 and the EC values calculated as follows: $\left(\text{control negative} - \text{control treated}\right) / \text{control negative} \times 100\%$. The standard error and the 95% confidence interval (C.I.) of dose response curves were determined using Prism v. 4 (GraphPad, California).
3.1 Introduction

Schistosomiasis is the most important water-borne pathogen of humans, infecting more than 207 million people annually in 74 countries (Steinmann et al., 2006). The drug of choice for the treatment of schistosomiasis is praziquantel (Pzq), a pyrazinoisoquinolone shown to be active against various cestodes and trematodes. The widespread application of Pzq is underpinned by mass chemotherapeutic programmes that have been moderately effective, but may not be sustainable in the long term. In spite of its relative success, Pzq has inherent shortcomings that limit its utility in the field. Moreover, there have been suggestions that the efficacy of some generic brands of Pzq may be less than 50% efficacy due to unregulated manufacturing practices (Guyatt et al., 1998). The indiscriminate application of suboptimal drug formulations may result in lower treatment rates and selection for Pzq resistance.

The benzimidazole class of compounds holds considerable promise as potential anti-schistosomals (el Sayed & Allam, 1997). While most benzimidazoles have broad spectrum activities, a few benzimidazole derivatives, such as triclabendazole (Tcbz), possess narrow spectrum action. Triclabendazole is currently indicated for treatment of livestock infected with Fasciola hepatica (F. hepatica) and F. gigantica (Fairweather & Boray, 1999). It is believed that Tcbz exerts its effects through the inhibition of microtubule formation (Fairweather & Boray, 1999). In trematodes, the cytoskeleton is thought to play crucial roles in maintaining tegumental architecture and the transport of tegumental granules, which are directly involved in the synthesis and turnover of the surface membrane and its outer coat (Fairweather & Boray, 1999).

There are conflicting reports on the efficacy of Tcbz and its metabolites in the literature. El Sayed & Allam (1997) reported that Tcbz has high efficacy in vitro. However, a separate study by Keiser et al. (2006) demonstrated that Tcbz and its two metabolites displayed weak and inconsistent activities in mice infected with S. mansoni. The study showed that single doses of
400mg/kg of Tcbz, Tcbz-sulphoxide and Tcbz-sulphone failed to reduce hepatic and intestinal tissue egg loads, resulting in only a 35.5% reduction in worm burden (Keiser et al. 2006). This is in contrast to a study performed by Khalil (2000), who showed a worm burden reduction of 84%. The authors suggested that host pharmacokinetics may be critical for explaining the different susceptibilities observed (Keiser et al., 2006).
3.2 Materials and Methods

3.2.1 *Schistosoma mansoni* maintenance

A Puerto Rican strain of *S. mansoni* was used for this study. Parasites were maintained in laboratory-reared *B. glabrata* snails and perpetuated in 6-wk female ARC/Swiss outbred mice. Mechanical transformation of cercariae into immature schistosomulum was performed according to established methods (Ramalho-pinto *et al*., 1974; Basch, 1981; Lazdins *et al*., 1982; Pica-Mattoccia & Cioli; 2003) and cultured for at least four wks prior to drug screening. Mice were infected with 120 cercariae precutaneously. Infected mice were subsequently sacrificed 49-days after cercarial exposure and adult worms were recovered via hepatic perfusion. Detailed methodology is available in Chapter 2: General Materials and Methods.

3.2.2 Drug treatment

Praziquantel (Pzq), triclabendazole (Tcbz), triclabendazole-sulphoxide (Tcbz-Sx) and triclabendazole-sulphone (Tcbz-Sp) were provided by Epichem Pty Ltd. Drugs were prepared in dimethyl-sulphoxide (DMSO) as 100 x stock solutions. One µL of each drug stock solutions were diluted in 1mL of medium 169 to give final concentrations of 10µM, 30µM, 50µM, 100µM, 200µM and 300µM for evaluation against immature worms. Similarly, one µL of each drug stock solutions were diluted in medium 169 to give final concentrations of 10µM, 30µM, 100µM, 150µM, 200µM and 300µM for evaluation against mature worms. The final DMSO concentration in treated and control well was ≤ 0.1% v/v.

Following drug exposure after 24hrs, worms were washed thrice with fresh medium 169, replaced in wells filled with fresh medium 169 and enumerated microscopically. For transformed immature schistosomula, drug effects were assessed daily for seven days post-exposure against 30 immature schistosomula in duplicate culture wells. For adult worms, drug effects were exposed for 24hrs and assessed at 24hrs, 48hrs and 72hrs after drug exposure against 10 mixed-sex worms in duplicate culture wells. Both studies were performed according
to the methodologies outlined by Pica-Mattoccia & Cioli (2003). Morphological changes were monitored by light microscopy and images obtained using OPTIMAS® image analysis software.

3.2.3 Drug efficacy scores

The effects of drugs against mature worms were performed according to the following scoring criteria: 4 (no contraction, movement throughout entire body), 3 (mobile in parts of body and contracted), 2 (contracted and immotile) and 1 (maximally contracted and immobile). At the end of the observation period, immature and mature worms were defined as ‘dead’ if they remain contracted, immotile and appeared opaque (Score 2 and Score 1).

3.2.4 Dose response and statistical analysis

Drug efficacy was assessed by comparing the mean number of worms in the control versus the treated group. The % worm mortality at each drug concentration assessed was calculated as follows:  
\[
\frac{\text{(control untreated worms} - \text{control treated worms)}}{\text{control untreated worms}} \times 100\%.
\]

The 95% confidence intervals and EC₅₀ values determined using Prism v. 4 (GraphPad, California).
3.3 Results

3.3.1 Efficacy of triclabendazole against immature schistosomulum

Transformed schistosomula remained viable in medium 169 for 48hrs before the first signs of mortality were observed. An estimated mortality of 20% was observed each week from Wk 1 through to Wk five. Intestinal pigments were evident within the first week in transformed schistosomula, with the stage of development (Stage 15) and length (2mm) at Wk 4 consistent with the observations of Basch (1981). Immature schistosomula were used for drug screening at Wk 4. Natural (non drug-induced) mortality was taken into consideration by enumerating only surviving schistosomula in control wells following each time assessment period.

One hundred % mortality of immature schistosomula was observed within 24hrs at the highest concentrations of Tcbz (300μM, 200μM and 100μM). Dose-dependent effects were observed in immature schistosomula exposed to Tcbz. Contraction followed by paralysis was observed within the first 30min of exposure to ≥ 100μM of Tcbz. Schistosomulum showed evidence of severe morphological damage including pronounced ‘mottling’, loss of motility and increasing opaqueness. At doses above 30μM, morphological deformities and the loss of cellular integrity was evident, with progressive shortening (or contraction) of the schistosomulum as concentration increased (see Figure 3.2). At lower concentrations (10μM), Tcbz continued to exert anti-schistosomal effects such as blebbing and contractions. The severity of morphological damage increased up to four days after drug exposure, with EC50 values remaining consistent from four to seven days of exposure.
Figure 3.1: Dose response curve of triclabendazole evaluated against immature schistomulum at Week four stage and assessed for seven days after exposure.

The concentration of Pzq required to kill 50% of immature S. mansoni (EC\textsubscript{50}) was 65.2\textmu M. Praziquantel-treated worms showed signs of mottling and widespread tegumental damage at the highest concentration assessed of 300\textmu M. All immature schistosomula exposed to 300\textmu M were contracted and dead. At lower concentrations, tegumental damage was less evident, characterized by only 50% mortality at concentrations of 100\textmu M and below. No further increases in worm mortality were observed after drug exposure and media change at 24hrs.
Table 3.1: The concentration of triclabendazole (Tcbz) and praziquantel (Pzq) required to kill 50% (EC$_{50}$) of Wk four immature schistosomula in vitro and examined for seven days after drug exposure.

<table>
<thead>
<tr>
<th></th>
<th>EC$_{50}$ concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>Tcbz</td>
<td>40.3</td>
</tr>
<tr>
<td>Pzq</td>
<td>65.2</td>
</tr>
</tbody>
</table>

There was no significant differences between the EC$_{50}$ value for Tcbz evaluated against immature schistosomula over the period of seven days (P>0.05). The EC$_{50}$ value for Tcbz were significantly lower compared to Pzq (P<0.05).
Figure 3.2: Morphological changes observed in four wk old immature schistosomulum exposed to varying concentrations of triclabendazole in vitro for seven days.

A: Untreated four wk old immature schistosomulum (100 ×)
B: Untreated four wk old immature schistosomulum (400 ×)
C: Four wk old immature schistosomulum treated with 100µM praziquantel (400 ×)
D: Four wk old immature schistosomulum treated with 1µM triclabendazole (400 ×)
E: Four wk old immature schistosomulum treated with 3µM triclabendazole (400 ×)
F: Four wk old immature schistosomulum treated with 10µM triclabendazole (400 ×)
G: Four wk old immature schistosomulum treated with 30µM triclabendazole (400 ×)
H: Four wk old immature schistosomulum treated with 100µM triclabendazole (400 ×)
I: Four wk old immature schistosomulum treated with 300µM triclabendazole (400 ×)

Key
Bb Blebs
3.3.2 Efficacy of triclabendazole and metabolites against mature worms

Dose-dependent effects were observed in immature schistosomula exposed to Tcbz and its metabolites. Morphological changes induced by Tcbz, Tcbz-Sx and Tcbz-Sp are illustrated in Figure 3.7, 3.8 and 3.9 respectively. There was evidence of precipitation at 300µM of Tcbz, Tcbz-Sx and Tcbz-Sp. Triclabendazole-sulphoxide and Tcbz-Sp were more soluble in culture media 169. Within the first hour, worms treated with Tcbz, Tcbz-Sx and Tcbz-Sp exhibited immediate contraction and assumed a tightly coiled appearance (Score 2). Worms treated with 100µM of each compound showed immediate and spasmodic contraction, but remained motile for a further three hours. After four hours of drug exposure at 300µM and 100µM of each compound, treated worms showed maximal contraction (Score 1) and were ‘mottled’ in appearance, with ‘blebbing’ and vacuole formation disseminated along the entire tegumental body. Gut movement and peristalsis were lost in treated worms, with the collapse of the ventral and oral suckers, ultimately losing structural integrity eight hours after exposure.

At lower concentrations (50µM, 30µM), contraction of worms was evident but motility was maintained (Score 3) in most treated worms. At 10µM, worms immediately contract when drugs were added but recover after four hours. No morphological damage or loss of motility was observed for worms exposed to 3µM of Tcbz and its metabolites (Score 4). The gynaecophoreal groove of all treated male worms (except those exposed to 3µM of Tcbz and its metabolites) assumed an ‘unfurled’ appearance with female worms demonstrating stronger contractions compared to male worms. All treated worms (except those exposed to 3µM of Tcbz and its metabolites) were detached from the bottom of the culture wells after drug exposure, and remained so for the entire assessed period of 24, 48 and 72hrs. A proportion of worms exposed to 10µM recovered and resumed motility (Score 3) when fresh media was added. Triclabendazole-Sx and Tcbz-Sp appeared to induce effects more rapidly compared to Tcbz,
with persistent effects noted at 48hrs and 72hrs after drug exposure. All worms in the control wells remained motile, uncontracted (Score 4) with retained structural integrity.

Dose-dependent effects were observed in worms within 30mins of exposure to Pzq. Strong contractions were observed in worms exposed to Pzq concentrations greater than 10µM (Score 2). Weak contractions (Score 3) were observed in worms at lower concentrations. Treated worms showed evidence of coiling, blebbing and vacuolization of the tegument. Worms treated at the highest Pzq concentrations of 100µM and 30µM demonstrated maximal contractions only after eight hours of exposure, with no evidence of recovery when media was changed. Some worms exposed to 3µM, 1µM and 0.3µM of Pzq regained motility after medium wash and fresh media replacement. Morphological effects induced by Pzq are illustrated in Figure 3.10. Control and untreated worms in DMSO can be observed in Figure 3.11. Table 3.2 shows the concentrations of Pzq, Tcbz, Tcbz-Sx and Tcbz-Sp required to kill 50% of adult *S. mansoni*.

**Table 3.2:** Effective concentration required to kill 50% (EC$_{50}$) of adult *Schistosoma mansoni* worms at four hour time intervals.

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Tcbz (µM)</th>
<th>Tcbz-Sx (µM)</th>
<th>Tcbz-Sp (µM)</th>
<th>Pzq (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>188.9</td>
<td>137.8</td>
<td>223.5</td>
<td>6.136</td>
</tr>
<tr>
<td>8</td>
<td>122.1</td>
<td>123.7</td>
<td>205.3</td>
<td>3.821</td>
</tr>
<tr>
<td>12</td>
<td>90.6</td>
<td>100.8</td>
<td>193.8</td>
<td>1.898</td>
</tr>
<tr>
<td>16</td>
<td>88.2</td>
<td>77.9</td>
<td>193.8</td>
<td>1.539</td>
</tr>
<tr>
<td>20</td>
<td>88.22</td>
<td>70.4</td>
<td>96.5</td>
<td>1.266</td>
</tr>
<tr>
<td>24</td>
<td>74.2</td>
<td>68.3</td>
<td>91.8</td>
<td>1.266</td>
</tr>
<tr>
<td>48</td>
<td>102.6</td>
<td>55.3</td>
<td>176.2</td>
<td>1.266</td>
</tr>
<tr>
<td>72</td>
<td>102.6</td>
<td>43.2</td>
<td>164.9</td>
<td>1.266</td>
</tr>
</tbody>
</table>
Figure 3.3: Dose response curve for praziquantel assessed against mature worms at 24, 48 and 72 hrs post exposure.

Figure 3.4: Dose response curve for triclabendazole assessed against mature worms at 24, 48 and 72 hrs post exposure.
Figure 3.5: Dose response curve for triclabendazole-sulphoxide assessed against mature worms at 24, 48 and 72 hrs post exposure.

Figure 3.6: Dose response curve for triclabendazole-sulphone assessed against mature worms at 24, 48 and 72 hrs post exposure.
Figures 3.3, 3.4, 3.5 and 3.6 shows the dose response curves for adult worms treated with Pzq, Tcbz, Tcbz-Sx and Tcbz-Sp respectively. There was no significant differences in the EC$_{50}$ value for Tcbz, Tcbz-Sx and Tcbz-Sp at 24, 48 and 72 hours after drug exposure (P>0.05). The EC$_{50}$ values for Tcbz, Tcbz-Sx and Tcbz-Sp were significantly higher compared to the EC$_{50}$ value of Pzq at all three time points (P<0.05).

### 3.3.3 Drug efficacy scores

All adult worms in the unexposed control groups remained active, characterized by whole body movement and no visible contraction (Score 4). Adult worms demonstrated progressive contraction and loss of motility when assessed every four hours after exposure to 100µM of Pzq, Tcbz, Tcbz-Sx and Tcbz-Sp (see Table 3.3). After 24hrs post exposure to Tcbz, 50% (Score 2: 30%; Score1: 20%) of treated worms were dead. For Tcbz-Sx, 60% (Score 2: 30%; Score 1: 30%) of treated worms were dead while the Tcbz-Sp treated groups demonstrated a mortality rate of 30% (Score 2: 20%; Score 1: 10%). Praziquantel-treated groups demonstrated 100% (Score 2: 30%; Score 1: 70%) mortality at 100µM over 24hrs.

Thirty percent of worms exposed to Tcbz and 20% of worms exposed to Tcbz-Sp recovered their motility 48hrs after drug exposure. Recovered worms assumed a relaxed appearance but morphological changes induced by Tcbz remained evident. A further 10% of Tcbz-Sp treated worms recovered motility 72hrs after drug exposure and media change. Triclabendazole-sulphoxide continued to exert anti-schistosomal effects for at least 48hrs after the addition of fresh media, with worms characterized by progressively stronger contractions and loss of motility. Ninety percent of worms were dead after 48 hours, with 100% dead 72hrs after exposure to 100µM of Tcbz-Sx.
Table 3.3: Percentages of worms (%) showing altered contraction and motility (Score 1 to 4) after exposure to 100µM of praziquantel (Pzq), triclabendazole (Tcbz), triclabendazole-sulphoxide (Tcbz-Sx) and triclabendazole-sulphone (Tcbz-Sp).

<table>
<thead>
<tr>
<th>Score</th>
<th>4hr</th>
<th>8hr</th>
<th>12hr</th>
<th>16hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>41</td>
<td>31</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>41</td>
<td>31</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>41</td>
<td>31</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>1</td>
<td>41</td>
<td>31</td>
<td>21</td>
<td>11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tcbz</th>
<th>40%</th>
<th>50%</th>
<th>10%</th>
<th>0%</th>
<th>40%</th>
<th>50%</th>
<th>10%</th>
<th>0%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40%</td>
<td>50%</td>
<td>10%</td>
<td>0%</td>
<td>40%</td>
<td>50%</td>
<td>10%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>40%</td>
<td>50%</td>
<td>10%</td>
<td>0%</td>
<td>40%</td>
<td>50%</td>
<td>10%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>40%</td>
<td>50%</td>
<td>10%</td>
<td>0%</td>
<td>40%</td>
<td>50%</td>
<td>10%</td>
<td>0%</td>
</tr>
</tbody>
</table>

* 4: no contraction, movement throughout body, 3: minimally contracted, motile, 2: contracted and immotile, 1: maximal contraction and immotile
Figure 3.7: Morphological changes observed in mature *Schistosoma mansoni* after exposure to triclabendazole (Tcbz) for 24 hours.

- **A)** Adult worm exposed to 10μM of Tcbz at 100 ×
- **B)** Adult worm exposed to 30μM of Tcbz at 100 ×
- **C)** Adult worm exposed to 100μM of Tcbz at 100 ×
- **D)** Adult worm exposed to 100μM of Tcbz at 100 ×, showing belbbing of the tegument
- **E)** Adult worm exposed to 100μM of Tcbz at 400 ×, showing widespread blebbing of tegument
- **F)** Adult worm exposed to 100μM of Tcbz at 400 ×, showing crumpling of ventral sucker
- **G)** Adult worm exposed to 100μM of Tcbz at 400 ×, showing blebbing and crumpled oral sucker

**Key:** *(Os)* Oral sucker; *(Vs)* Ventral sucker; *(Bb)* Blebs; *(Vc)* Vacuolization.
Figure 3.8 Morphological changes observed in mature *Schistosoma mansoni* after exposure to triclabendazole sulphoxide (Tcbz-Sx) for 24 hours.

(A) Adult worm exposed to 300µM of Tcbz-Sx at 100×, showing vacuolization of internal musculature.

(B) Adult worm exposed to 100µM of Tcbz-Sx at 100×, showing blebbing of tegument.

(C) Adult worm exposed to 100µM of Tcbz-Sx at 200×, showing vacuolized tegument.

(D) Adult worm exposed to 100µM of Tcbz-Sx at 100×, showing contracted worm.

(E) Adult worm exposed to 10µM of Tcbz-Sx at 100×, showing no observable pathology.

(F) Adult worm exposed to 100µM of Tcbz-Sx at 400×, showing widespread blebbing and sloughing of tegument.

(G) Adult worm exposed to 100µM of Tcbz-Sx at 400×, showing belbbing of tegument.

(H) Blebs induced by 100µM of Tcbz-Sx at 40x mag.

**Key:** (Os) Oral sucker; (Vs) Ventral sucker; (Bb) Blebs; (Vc) Vacuolization; (Tm) Tegument
Figure 3.9: Morphological changes observed in mature Schistosoma mansoni after exposure to triclabendazole sulphone (Tcbz-Sp) for 24 hours. 

(A) Adult worm exposed to 300µM of Tcbz-Sp at 100×, showing immediate and maximal muscle contraction upon addition of drugs. 
(B) Adult worm exposed to 100µM of Tcbz-Sx at 100×, showing strong muscle contraction upon addition of drugs. 
(C) Adult worm exposed to 30µM of Tcbz-Sx at 100×, showing moderate contraction of worm. 
(D) Adult worm exposed to 300µM of Tcbz-Sx at 400×, showing widespread blebbing and sloughing of tegument after 24 hours exposure. 
(E) Adult worm exposed to 300µM of Tcbz-Sx at 200×, showing blebbing of the tegument after 24 hours exposure. 
(F) Adult worm exposed to 100µM of Tcbz-Sx at 200×, showing blebbing of the tegument after 24 hours exposure. 
(G) Adult worm exposed to 30µM of Tcbz-Sx at 400×, showing moderate blebbing of the tegument after 24 hours exposure. 

Key: (Os) Oral sucker; (Vs) Ventral sucker; (Bb) Blebs; (Vc) Vacuolization; (Tm) Tegument; (Mc) Maximal muscular contraction
Figure 3.10: Morphological changes observed in mature Schistosoma mansoni after exposure to praziquantel (Pzq) for 24 hours.

(A) Adult worm exposed to 100µM of Pzq at 100×, showing immediate muscle contraction upon addition of drugs.
(B) Adult worm exposed to 100µM of Pzq at 100×, showing maximal muscle contraction after 24 hours of treatment.
(C) Adult worm exposed to 10µM of Pzq at 100×, showing strong contraction of worm after 24 hours of treatment.
(D) Adult worm exposed to 100µM of Pzq at 400×, showing blebbing of oral suckers after 24 hours exposure.
(E) Adult worm exposed to 100µM of Pzq at 200×, showing widespread blebbing of the tegument after 24 hours exposure.
(F) Adult worm exposed to 10µM of Pzq at 200×, showing some blebbing of the tegument after 24 hours exposure.

Key: Os: Oral sucker; Bb: Blebs, Tm: Tegument; Gc: Gynaecophoric canal
Figure 3.11: Untreated 49-day old mature worms in DMSO drug vehicle.
(A) Adult worm exposed to 0.1% v/v DMSO drug vehicle at 100×
(B) Adult worm exposed to 0.1% v/v DMSO drug vehicle at 200×
(C) Adult worm exposed to 0.1% v/v DMSO drug vehicle at 400× showing normal ventral sucker.
(D) Adult worm exposed to 0.1% v/v DMSO drug vehicle at 400× showing normal oral sucker.
(E) Adult worm exposed to 0.1% DMSO drug vehicle at 400× showing normal tegument.

Key: (Os) Oral sucker; (Vs) Ventral sucker; (Tm) Tegument.
3.4 Discussion

The objective of this study was to assess the efficacy of Tcbz and its metabolites against *S. mansoni* in comparison to Pzq. The efficacy of Tcbz and its metabolites have been determined against *Fasciola* and other trematodes (Keiser *et al.*, 2005) but there is little available data on their anti-schistosomal activities. Conflicting reports and incomplete data sets (Khalil, 2000; Keiser *et al.*, 2006) have consistently hampered the establishment of baseline values for detailed drug studies and the generation of reliable dose response analyses. To address these issues, multiple doses of Tcbz and its metabolites were assessed and their effects compared against Pzq, the positive drug control. The main finding of this study mirrored Keiser *et al.* (2006)’s *in vivo* observations; that Tcbz and its metabolites possess weak and inconsistent anti-schistosomal activity *in vitro*.

The isolate of *S. mansoni* used in this study was obtained from the Queensland Institute of Medical Research and was adapted for culture according to the protocols recommended by the National Institute of Health, *Schistosoma* Laboratory of the Biomedical Research Institute, Maryland. While every attempt was made to maintain optimal conditions for the perpetuation of the parasite, the cold temperate climate of Perth consistently reduced snail survival rates. Snail fitness appeared to be a major factor in the survivability of the miracidium. This presented considerable problems for the perpetuation of larger parasite populations for high-throughput screening. As a result, no more than 20 infected snails were ready for cercarial shedding at any one time. To circumvent these issues, breeding tanks for infected snails were maintained in absolute darkness during the incubation period (35 days). In colder months, breeding tanks were insulated with several layers of aluminum foil. To ensure maximal snail infections and consistency, only mature snails of approximately 5mm in diameter were chosen.

The methods used to transform and culture immature schistosomulum were adapted from the protocols described by Basch (1981) and Pica-Mattoccia & Cioli (2003). The high mortality rates observed in the first two weeks of culture were consistent with Basch (1981), who
observed a reduction of approximately 50% up to Wk 4. Although there was some individual variability in schistosomulum growth, asynchronous development was a prominent feature of the culture system.

Triclabendazole demonstrated irreversible dose-dependent activity against immature schistosomula. Contraction of schistosomules was observed immediately after the addition of Tcbz, resulting in worm paralysis at four hrs post exposure. Morphological effects were evident within the first six hours of drug exposure, with schistosomules assuming a knobbed and shrunken appearance at concentrations above 30µM. Blebbing of the tegument was also evident, and appeared dependent on the concentration and length of exposure. This finding agrees with studies using Fasciola species (Stitt & Fairweather, 1993), where blebbing of the tegument was the most commonly observed signs of pathology after exposure to Tcbz. Blebbing primarily results from the release of secretory bodies by microtubule-based transport processes at the surface membrane (McConville et al., 2007). The large number of blebs observed was a reflection of the reduced ability of treated worms to repair its apical membrane (McConville et al., 2007). The relatively high EC50 value observed when Pzq was used to treat immature schistosomulum suggests that the juvenile worms were largely refractory to the effects of Pzq. While the values observed in this study were significantly lower than that of Pica-Mattoccia & Cioli (2004), it is well known that different Schistosoma strains demonstrate varying susceptibilities to Pzq in vitro and in vivo (Keiser et al., 2006). It is also likely that the higher susceptibility of immature cultured worms compared to immature worms extracted from mice was a result of the culture system. Basch (1981) reported that the mean length of immature cultured worms were shorter than immature worms in vivo. When worm pairs were implanted into mesenteric veins of mice, complete maturity of the worm pairs were not attained. It appeared likely that the stimuli required for full growth were not provided by the culture conditions (Basch, 1981).

The observed susceptibility of immature schistosomulum to Tcbz is similar to the results obtained from the exposure of juvenile F. hepatica to Tcbz (Boray et al., 1983). This is
important as immature schistosomules at the pre-patent stage (Wk 4-6) are largely refractory to the action of Pzq, with gradual resumption of sensitivity upon maturation and egg laying (Gönnert & Andrews, 1977, Xiao et al., 1985, and Pica-Mattoccia & Cioli, 2004). During this developmental period, the surface membrane of the immature schistomulum is radically reorganized from the cercarial trilaminate unit to a multilaminate membrane that is connected to underlying subtegumental cells by microtubule-lined intracytoplasmic processes (Stirewalt, 1974). The extensive cytoskeletal structure is responsible for connecting the outer-surface membranes to the basal lamina in a dynamic manner that ensures sufficient flexibility of the schistosomal surface (Jones et al., 2004). Several studies have identified the role of microtubules in surface maturation. In particular, Wiest et al. (1988) demonstrated the inhibition of multilaminate membrane formation by the microtubule inhibitor colchicine; resulting in the impediment of schistosomule maturation. It is reasonable to assume that Tcbz was likely to act via similar mechanisms of action against immature worm tubulin that were more ‘exposed’ than adult worm tubulin. This may have accounted for the higher efficacy rates observed in immature worms compared to mature worms.

Praziquantel was first introduced as an anthelmintic for the treatment of cestode and trematode infections in animals. While the molecular mechanisms of action are as yet undetermined, recent studies by Kohn et al. (2001) implicated its action against voltage-gated calcium subunits. Morphologically, Pzq-induced effects were more clearly defined. The stimulation of motor activity by calcium influx causes rapid and spastic paralysis, followed by tegumental disruption characterized by the formation of vacuoles and vesicles (Pax et al., 1978). Similarly, adult worms treated with Pzq in this experiment demonstrated a dose-dependent relationship, exemplified by the gradual increase of worm contractility and loss of motility with increasing drug concentrations. Immediate contraction was observed at high concentrations with a complete cessation of spontaneous movements. Widespread disruptions were observed throughout the body of adult worms, with pronounced blebbing, vacuolization and peeling consistent with previous studies (Pax et al., 1978; Becker et al., 1980; Irie et al., 1989; Shaw & Erasmus, 1987). Egg-laying ceased in paired worms after exposure to Pzq, indicating a loss of
fecundity. Some worms recovered and regain motility after media containing lower drug concentrations (<100µM) of Pzq was removed. However, recovered worms retained tegumental damage associated with drug treatment. Morphological observations of Pzq-treated *S. mansonii* also mirrored the observations of Pzq-treated *S. mekongi* (Jiraungkorskul et al., 2006). Shaw & Eramus (1983) proposed that the erosion of teguments and the formation of vacoules could be attributed to the dilation of the basal membrane, brought about as a result of water and ionic imbalances. Praziquantel has been implicated in the impairment of tegument transport functions which can result in such imbalances.

Tegumental blebbing and contractions were more pronounced in Tcbz-treated groups compared to Pzq-treated groups, with the disruptions of blebs evident at higher concentrations. The antischistosomal activity of Tcbz and its metabolites can be ranked according to the severity of morphological changes observed in mature worms at each concentration. The results of this study showed that the order of efficacy is Tcbz-Sx > Tcbz > Tcbz-Sp. This observation is consistent with earlier studies using *Fasciola*, which showed Tcbz-Sp to be a relatively inert metabolite (Robinson et al., 2004). Adult worm pairs exposed to Tcbz and its metabolites showed reduced fecundity and became dissociated, an observation that is also a feature of Pzq treatment.

The results showed that exposure to Tcbz-Sx caused irreversible mortality in adult worms despite replacement of cell-culture media. The prolonged effects of Tcbz-Sx may be attributed to its increased availability and solubility in the media, or may be due to irreversible binding of the metabolite to target sites. While the precise molecular mechanism of action for Tcbz and its metabolites are unknown, a significant body of knowledge exists for the proposed action of Tcbz-Sx. It has been suggested that Tcbz-Sx primarily acts on microtubule-dependent secretory processes in the liver fluke (Fairweather & Boray, 1999). In the tegument, Tcbz-Sx was shown to block the transport of secretory bodies from the cell body to the apical surface of the tegument, causing severe surface damage and total disintegration of the tegument (Fairweather, 2005).
The results of this study confirmed that Pzq is a more effective drug compared with Tcbz and its metabolites. At 100µM, Pzq-treated worms demonstrated maximal contractions and 100% mortality within 24hrs compared to Tcbz, Tcbz-Sx and Tcbz-Sp, which induced a total mortality of 50%, 60% and 30% respectively. Only Tcbz-Sx and Pzq appeared to maintain persistent action and (or) irreversible binding, characterized by further increases or maintenance of mortality rates for a further 48 hours after removal of media containing 100µM of each drug. The dose response curves for Pzq showed uniform dose-dependent sigmoidal trends at all three time-points. This is in contrast to the dose response analyses for Tcbz and its metabolites, which were inconsistent and non-uniform sigmoidal.

Adult worms treated with lower concentrations (<30µM) of Pzq, Tcbz, Tcbz-Sx and Tcbz-Sp demonstrated considerable morphological damage and minimal contractions, but were not defined as ‘dead’ as slight movements were noted particularly within the ventral region of the worms. Although these treated worms were not scored as dead, further evaluation at 48 and 72hrs after exposure revealed minimal movements and no further worm recovery. This has significant implications for the current method of in vitro assessment of drug efficacies. It is evident that the evaluation of worm mortality has to be further improved to account for irreversible morphological damage and the loss of fecundity. As indicated by Andrews (1981), Xiao et al. (1985) and Pica-Mattoccia & Cioli (2004), there were conditions that produced maximal contractions, while at the same time not affecting the long term survival of the flukes. In addition, such narrowly defined efficacy measures do not take into account of the role of host immune responses in the in vivo situation. This is important because host immune responses have been implicated in further inducing worm mortality through the exposure of parasite antigens following Pzq action on the tegument (Harnett & Kusel, 1986).

This in vitro study suggested that Tcbz and its metabolites possessed weak anti-schistosomal activities, and supports the in vivo observations of Keiser et al. (2006). While its activity against adult worms remains contentious, Tcbz demonstrates good efficacy against immature
schistosomula that were refractory to Pzq. The persistent action of Tcbz and its metabolites and its activity against immature stages of *S. mansoni* suggests that the compounds can be ideal candidates for use in drug combinations with Pzq.
4.1 Introduction

The pathology induced by *S. mansoni* infections is largely attributed to the hypersensitivity of the host to soluble egg antigens, which results in a granulomatous response and subsequent fibrosis of the liver (Abouel-Nour *et al*., 2006). The current drug of choice for the treatment of schistosomiasis is praziquantel (Pzq), which principally affects mature flukes and reduces egg burdens.

The hatching of *Schistosoma* eggs is easily achieved in fresh water, although hatching can also be induced in solutions of high osmolarity. There is currently little information on the ovicidal effects of Pzq. Xu & Dresden (1986) demonstrated that the release of leucine aminopeptidase was correlated with hatching, and that Pzq caused the rapid release of leucine aminopeptidase from eggs *in vitro*. Treatment with Pzq reduces the viability of mature worm pairs also and leads to cessation in egg-laying. While the exact mode of action of Pzq on mature worms remains undefined, it has been suggested that the β-subunit of voltage-gated calcium channels are involved (Kohn *et al*., 2001).

Preliminary studies by Matsuda *et al.* (1983) partially characterized the *in vitro* and *in vivo* effects of Pzq on *S. japonicum* eggs. The study demonstrated that the number of mature eggs was reduced and the number of empty fresh egg shells was increased in the liver of mice infected with *S. japonicum* that were given four doses of 100mg/kg of Pzq over 24hrs. The number of calcified eggs observed in the liver of mice infected with *S. japonicum* continued to increase for two weeks after treatment (Matsuda *et al*., 1983). Immature eggs that were initially unaffected developed into unviable miracidia that subsequently degenerated and calcified. The experiment also demonstrated Pzq-induced hatching following the addition of at least 1ng/mL of Pzq to the eggs *in vitro*.

Katsumata *et al* (1988) showed that Pzq-induced hatching was inhibited by Ca$^{2+}$ channel blockers (lanthanum chloride, ruthenium red) and EGTA, a calcium chelator. Conversely, the
calcium ionophore A23187 stimulated the hatching of *S. mansoni* eggs in high osmolarity. Recently, Pica-Mattoccia *et al.* (2007) demonstrated that mature worms exposed to calcium channel blockers and the actin depolymerization agent, cytochalasin D, were refractory to Pzq. The results of this study supported the hypothesis that calcium channels are the primary site of action of Pzq in mature worms.

The aim of this study was to characterise morphological changes in *S. mansoni* eggs exposed to Pzq *in vitro*, and to determine if the effects are dose-dependent. To further elucidate the mechanism of Pzq action on schistosome eggs, the effects of calcium blockers ryanodine and dihydropyridine on egg hatching was assessed. The ovicidal efficacies of triclabendazole (Tcbz) and its metabolites were also assessed because previous studies have shown that Tcbz reduced *Schistosoma* egg-laying and worm fecundity (Khalil, 2000).
4.2 Materials and Methods

4.2.1 Preparation of Schistosoma eggs

_Schistosoma mansoni_ and _S. japonicum_ eggs were recovered from the livers of infected mice according to Dalton _et al._ (1997). Eggs were extracted from the livers of three mice 49 days after infection with _S. mansoni_ and _S. japonicum_ cercariae. Purified eggs were immediately stored in cold PBS at 4°C and were only used after three days to allow for maturation of the eggs in PBS (M. Jones, pers. comm.). Eggs could be stored without hatching due to the high osmolarity of PBS.

4.2.2 Evaluation of the effects of praziquantel on egg hatching

Ten viable mature _S. mansoni_ or _S. japonicum_ eggs were transferred into triplicate wells of Falcon® Multiwell™ 24-well culture plates (Becton Dickinson, USA). The viability and maturity of the eggs were determined according to Pellegrino _et al._ (1962). Individual culture wells were made up to 1mL with PBS. One hundred times stock solutions of Pzq were made in DMSO and added to triplicate wells containing schistosome eggs in PBS to a final concentration of 1 mM, 100 µM, 10 µM, 1 µM, 0.1 µM and 0.01 µM. The untreated control wells received PBS containing ≤ 1% of DMSO.

The temporal effects of Pzq on the hatching of _S. mansoni_ and _S. japonicum_ eggs was quantified by measuring the ‘time to activation’ and the ‘time to hatch’ using a stop-watch. Time to activation was defined as the length of the first sign of movement or activation within the eggs. Time to hatch was defined as the total length of time until the miracidium detached or hatched completely from the egg shell and the vitelline membrane. Real-time video recordings were made at 100 x magnification using a Sony HandyCam (DCR-HC40E) connected to a Panasonic Color CCTV Camera (Model WV-CP610/G), which was subsequently attached to an Olympus CKX41 Compound Microscope.
4.2.3 Evaluation of triclabendazole and its metabolites on egg viability

Ten mature *S. mansoni* or *S. japonicum* eggs were transferred into triplicate wells of Falcon® Multiwell™ 24-well culture plates (Becton Dickinson, USA). Individual culture wells were made up to 1mL with PBS. One hundred times stock solutions of triclabendazole (Tcbz), triclabendazole-sulphoxide (Tcbz-Sx) and triclabendazole-sulphone (Tcbz-Sp) were made in DMSO and added to triplicate wells containing schistosome eggs in PBS to a final concentration of 1mM, 100µM, 10µM, 1µM, 0.1µM and 0.01µM. The untreated control wells received PBS containing ≤ 1% DMSO.

Eggs were exposed to each treatment for 24hrs at 25°C. Eggs were then washed and the PBS replaced with fresh DDI H₂O. Hatching was induced by exposing the culture plates to a 100 Watt light source. Eggs were then examined microscopically and the number of hatched eggs recorded. The percentage of inhibition of egg hatching was corrected for natural mortality in the control wells by subtracting the number of unhatched eggs in control wells from the number of unhatched eggs in the treated wells before calculating the proportion that hatched in the treatment wells.

4.2.4 Evaluation of calcium blockers on eggs

Ten viable mature *S. mansoni* or *S. japonicum* eggs were transferred into triplicate wells of Falcon® Multiwell™ 24-well culture plates (Becton Dickinson, USA). Individual culture wells were made up to 1mL with PBS. Fluorescent-labelled BODIPY® FL-X ryanodine and fluorescent-labelled DM-BODIPY® dihydropyridine (Invitrogen, California) were added to triplicate wells at final concentrations of 25µg/mL, 2.5µg/mL, 250 ng/mL, 25ng/mL and 0.25ng/mL in 1mL of PBS. Treated culture plates were incubated for 24hrs at 25°C. The eggs were then washed by gentle pipetting and the PBS replaced with DDI H₂O. Hatching was induced by exposure of the culture plates to a 100 Watt light source. The percentage of inhibition of egg hatching was corrected for natural mortality in the control wells by subtracting
the number of unhatched eggs in control wells from the number of unhatched eggs in the treated wells before calculating the proportion that hatched in the treatment wells.

4.2.5 Dose response and statistical analysis

The percent inhibition of egg hatching caused by each compound was calculated as:

\[
\text{Number of eggs hatched (exposed)} - \frac{\text{Number of eggs hatched (exposed)}}{\text{Number of eggs hatched (unexposed)}}
\]

The EC\textsubscript{50} and 95% confidence intervals were determined using Prism 4 (GraphPad, San Diego).
Chapter 4

4.3 Results

4.3.1 Effects of praziquantel-induced hatching of *Schistosoma* eggs

Activation of unhatched miracidia was observed immediately after the addition of each concentration of Pzq greater or equal to 100nM in PBS. All *S. mansoni* and *S. japonicum* eggs exposed to 1mM of Pzq were activated within the first 30s after exposure. The mean length of time before miracidium activation in the eggs are presented in Table 4.1.

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>0.01µM</th>
<th>0.1µM</th>
<th>1µM</th>
<th>10µM</th>
<th>100µM</th>
<th>1000µM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Schistosoma mansoni</em></td>
<td>NA</td>
<td>248 (10.9)</td>
<td>136 (8.83)</td>
<td>28 (4.62)</td>
<td>22 (0.58)</td>
<td>22 (0.58)</td>
</tr>
<tr>
<td><em>Schistosoma japonicum</em></td>
<td>NA</td>
<td>281 (22.5)</td>
<td>109 (9.55)</td>
<td>91.7 (7.27)</td>
<td>88.3 (7.85)</td>
<td>22 (0.58)</td>
</tr>
</tbody>
</table>

NA = no observed activation

Complete hatching of *S. mansoni* and *S. japonicum* eggs was observed within 20 mins of exposure to Pzq concentrations above 1µM. *Schistosoma mansoni* and *S. japonicum* did not hatch when exposed to 100nM and 10nM of Pzq. The mean length of time before complete egg hatching was observed is shown in Table 4.2. Untreated eggs in PBS were not activated. When untreated eggs were exposed to fresh DDI H₂O (25ºC), miracidia activation was observed within approximately 420s (SE=30s), and complete hatching was observed in approximately 720s (SE=160s).
Table 4.2: Mean length of time required for *Schistosoma mansoni* and *Schistosoma japonicum* eggs to hatch following exposure to 0.01, 0.1, 1, 10, 100 and 1000µM of praziquantel. Standard errors are presented in parentheses.

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>0.01µM</th>
<th>0.1µM</th>
<th>1µM</th>
<th>10µM</th>
<th>100µM</th>
<th>1000µM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Schistosoma mansoni</em></td>
<td>265 (23)</td>
<td>391 (21.8)</td>
<td>752 (72.5)</td>
<td>1116 (122)</td>
<td>N/H</td>
<td>N/H</td>
</tr>
<tr>
<td><em>Schistosoma japonicum</em></td>
<td>430 (26.5)</td>
<td>329 (63.9)</td>
<td>499 (32.6)</td>
<td>1260 (34.7)</td>
<td>N/H</td>
<td>N/H</td>
</tr>
</tbody>
</table>

NA = no hatching observed

4.3.2 Morphological effects observed in eggs exposed to praziquantel

Untreated *S. mansoni* and *S. japonicum* eggs that were exposed to fresh DDI H₂O hatched within 10 to 15mins. Hatched miracidia were pyriform in shape. Complete detachment of the vitelline membrane from the egg shells was observed, with miracidium remaining viable for at least six hours.

The first signs of miracidial movement following exposure to Pzq were observed within the anterior terebratorium followed by rapid beating of the cilia. Contractions were noted throughout the entire miracidium within 30s of Pzq exposure. The rapid beating of the cilia and strong contractions of the miracidium resulted in increased turgescence of the fluid-filled vitelline membrane (Lehman’s Lacuna). The miracidium subsequently broke through the egg shell along an oblique or vertical line, with the vitelline membrane remaining intact. In most cases, the vitelline membrane remained attached to the egg shells. The miracidium remained contracted and assumed little movement within the confines of the vitelline membrane. The terebratorium eventually ruptured the expanding vitelline membrane, discharging the miracidium, lipoid bodies and granulofloccular material (Cheever bodies) into the external environment. The morphological changes of *S. mansoni* and *S. japonicum* eggs following treatment with Pzq is shown in Figure 4.1 and 4.2.
The hatched miracidium appeared deformed, strongly contracted and showed erratic swimming motions. Wave-like contractions were observed throughout the entire miracidium body. Death of the miracidium was evident within five minutes of hatching, with parasites appearing bloated and sessile. Miracidial activation was evident at lower concentrations of Pzq but no complete hatching was observed. Despite continuous activation of miracidia within the egg, there was a gradual loss of movement within four hrs after exposure to Pzq.

A video of the Pzq-induced hatching process is provided electronically (see Electronic Appendix A).

### 4.3.3 Effects of calcium blockers and inducers on eggs

*Schistosoma mansoni* and *S. japonicum* eggs did not activate or hatch in the presence of DDI H$_2$O after treatment with the calcium-channel inhibitor dihydropyridine at the highest concentration of 25µg/mL. Activation and hatching in fresh DDI H$_2$O were observed in eggs exposed to less than 25µg/mL of dihydropyridine. Miracidial activation and hatching were observed for eggs exposed to 25µg/mL of dihyropyridine followed by exposure to 10µM of Pzq. All eggs exposed to a range of ryanodine concentrations hatched when exposed to fresh DDI H$_2$O. Activation and hatching of the miracidia were observed when ryanodie-treated eggs were exposed to 10µM Pzq.
Figure 4.1: Representative images of *Schistosoma mansoni* (left column) and *Schistosoma japonicum* (right column) eggs after exposure to 10µM of praziquantel.

A: Untreated *S. mansoni* egg at 1000×

B: Untreated *S. japonicum* egg at 1000×

C: Immediate activation of *S. mansoni* eggs with detachment of vitelline membrane and premature discharge of deformed miracidium at 400×

D: Immediate activation of *S. japonicum* eggs, characterized by blisters of the vitelline membrane and premature discharge of miracidium at 400×

**Key**

(Vm) Vitelline membrane  
(M) Miracidium  
(Bs) Blisters
Figure 4.2: *Schistosoma mansoni* (left column) and *Schistosoma japonicum* (right column) eggs exposed to 10µM of praziquantel showing hatching of miracidium.

A: Detachment of *S. mansoni* vitelline membrane and miracidium from eggs shells at 1000×

B: Detachment of *S. japonicum* vitelline membrane and miracidium from eggs shells at 1000×

C: Deformed and contracted *S. mansoni* miracidium showing condensed neural mass 1000×

D: Deformed and contracted *S. japonicum* miracidium with widespread deformities at 400×

Key

(Lp) Lipoid bodies
(Ch) Cheever bodies
(C) Contraction
(T) Terebratorium
(Vm) Vitelline membrane
(Nm) Neural mass
4.3.3 Effects of triclabendazole and its metabolites on egg viability

Untreated eggs that were exposed to DDI H₂O at 25°C all hatched. The proportion of *S. mansoni* and *S. japonicum* eggs that hatched are shown in Table 4.3 below.

**Table 4.3:** Percentage of *Schistosoma mansoni* and *Schistosoma japonicum* eggs hatched after exposure to triclabendazole (Tcbz), triclabendazole (Tcbz-Sx) and triclabendazole-sulphone (Tcbz-Sp) at 10nM, 100nM, 1µM, 10µM, 100µM and 1mM. Standard errors are presented in parenthesis.

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>0.01µM</th>
<th>0.1µM</th>
<th>1µM</th>
<th>10µM</th>
<th>100µM</th>
<th>1000µM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. mansoni</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tcbz</td>
<td>51 (6.57)</td>
<td>52.7 (4.26)</td>
<td>50 (2.89)</td>
<td>29 (2.31)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tcbz-Sx</td>
<td>66.7 (1.20)</td>
<td>45 (2.52)</td>
<td>28 (2.51)</td>
<td>18.7 (2.03)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tcbz-Sp</td>
<td>54 (7.78)</td>
<td>53.7 (3.72)</td>
<td>45.7 (2.03)</td>
<td>30.7 (1.2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>S. japonicum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tcbz</td>
<td>78.6 (2.64)</td>
<td>71 (5.98)</td>
<td>61.1 (5.57)</td>
<td>31.1 (5.88)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tcbz-Sx</td>
<td>90 (10.0)</td>
<td>82.3 (9.69)</td>
<td>71.4 (8.27)</td>
<td>20.6 (8.07)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tcbz-Sp</td>
<td>69.5 (2.77)</td>
<td>69.4 (3.67)</td>
<td>54.2 (10.5)</td>
<td>42.6 (7.34)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Eggs exposed to 1mM and 100µM of Tcbz, Tcbz-Sx and Tcbz-Sp were unable to hatch following washing with PBS and exposure to DDI H₂O. There was a dose-dependent decrease in the proportion of eggs hatching exposure to Tcbz, Tcbz-Sx and Tcbz-Sp. At lower concentrations (<1µM), at least 50% eggs achieved normal hatching.

A loss of shell integrity was observed in eggs exposed to high concentrations (100µM) of Tcbz, Tcbz-Sx and Tcbz-Sp, resulting in non-viable miracidia that did not hatch. The unhatched miracidium gradually detached from the egg shells and the vitelline membrane, resulting in the leakage of lipoid bodies. The vitelline membrane appeared to separate into two layers (Reynold’s layer and von Lichtenberg’s envelope). Morphological damage was less evident in eggs exposed to Tcbz compared to Tcbz-Sx and Tcbz-Sp. The hatched miracidia exposed to lower concentrations of Tcbz, Tcbz-Sx and Tcbz-Sp appeared crumpled, with pronounced
morphological deformities such as vacuolization and blistering of the tegument. Within a few minutes the deformed miracidium assumed a bloated appearance and were sessile. In particular, the neural mass appeared condensed. Most miracidia did not detach completely from the vitelline membrane, with the anterior end remaining attached to the egg shells. The morphological effects of *S. mansoni* and *S. japonicum* eggs following Tcbz, Tcbz-Sx and Tcbz-Sp treatment are shown in Figure 4.5, 4.6, 4.7, 4.8 and 4.9.

There was a dose-dependent effect of Tcbz, Tcbz-Sx and Tcbz-Sp on the mortality of *S. mansoni* and *S. japonicum* eggs. (Figure 4.3 and Figure 4.4 respectively). There were no significant differences in the concentration of Tcbz, Tcbz-Sx and Tcbz-Sp required to kill 50% (EC₅₀) of *S. mansoni* eggs (Table 4.4). Similarly, there was no significant difference (P>0.05) in the percent mortality for *S. japonicum* eggs exposed to Tcbz, Tcbz-Sx and Tcbz-Sp (Figure 4.4).

**Table 4.4:** Concentration of triclabendazole (Tcbz), triclabendazole-sulphoxide (Tcbz-Sx) and triclabendazole-sulphone (Tcbz-Sp) required to kill 50% (EC₅₀) of *Schistosoma mansoni* and *Schistosoma japonicum* eggs.

<table>
<thead>
<tr>
<th>Drug concentration (µM)</th>
<th>Tcbz</th>
<th>Tcbz-Sx</th>
<th>Tcbz-Sp</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Schistosoma mansoni</em></td>
<td>0.096</td>
<td>0.082</td>
<td>0.276</td>
</tr>
<tr>
<td><em>Schistosoma japonicum</em></td>
<td>1.73</td>
<td>2.43</td>
<td>1.67</td>
</tr>
</tbody>
</table>
Figure 4.3: Dose response curves of *Schistosoma mansoni* eggs exposed to triclabendazole (Tcbz), triclabendazole-sulphoxide (Tcbz-Sx) and triclabendazole-sulphone (Tcbz-SP) for 24 hours.

Figure 4.4: Dose response curves of *Schistosoma japonicum* eggs exposed to triclabendazole (Tcbz), triclabendazole-sulphoxide (Tcbz-Sx) and triclabendazole-sulphone (Tcbz-SP) for 24 hours.
Figure 4.5: Microstructural effects observed in *Schistosoma japonicum* eggs exposed to triclabendazole (Tcbz), triclabendazole-sulphoxide (Tcbz-Sx) and triclabendazole-sulphone (Tcbz-Sp).

(A) Untreated *S. japonicum* eggs at 100 ×
(B) Single untreated *S. japonicum* egg at 200 ×
(C) Single untreated *S. japonicum* egg at 400 ×
(D) Single *S. japonicum* egg treated with 100µM of Tcbz at 200 × showing drug binding to egg shells.
(E) Single *S. japonicum* egg treated with 100µM Tcbz-Sx at 200 × showing drug binding to egg shells.
(F) Single *S. japonicum* egg treated with 100µM Tcbz-Sp at 200 × showing drug binding to egg shells.

**Key (Db)** Drug binding
Figure 4.6: Microstructural effects observed in *Schistosoma japonicum* eggs exposed to various concentrations of triclabendazole (Tcbz), triclabendazole-sulphoxide (Tcbz-Sx) and triclabendazole-sulphone (Tcbz-Sp).

(A) Single *S. japonicum* egg exposed to 10µM of Tcbz, with the integrity of the miracidium remaining largely intact at 200 ×

(B) Single *S. japonicum* egg exposed to 10µM Tcbz-Sx, with crumpling and miracidium deformities evident at 200 ×

(C) Single *S. japonicum* egg exposed to 10µM of Tcbz-Sp showing crumpling of the miracidium at 200 ×

(D) Single *S. japonicum* egg exposed to 100µM of Tcbz showing detachment of the vitelline membrane from the egg shell at 400 ×

(E) Single *S. japonicum* egg exposed to 100µM of Tcbz-Sx, showing detached vitelline membrane and deformed miracidium at 400 ×

(F) Single *S. japonicum* egg exposed to 100µM of Tcbz-Sp. showing detached vitelline membrane and deformed miracidium at 400 ×

**Key**

(Vm) Vitelline membrane

(S) Egg shell

(M) Miracidium
Figure 4.7: Microstructural effects of detached vitelline membrane and miracidium of *Schistosoma japonicum* eggs after exposure to triclabendazole (Tcbz), triclabendazole-sulphoxide (Tcbz-Sx) and triclabendazole-sulphone (Tcbz-SP).

(A) *S. japonicum* egg treated with Tcbz-Sx showing crumpled miracidium at 1000 ×

(B) *S. japonicum* egg treated with Tcbz-SP showing vacuolized miracidium teguments at 400 ×

(C) Hatched and deformed *S. japonicum* miracidium at 400 × after treatment with Tcbz at 10µM.

(D) Hatched *S. japonicum* miracidium after treatment with Tcbz-Sx at 400 × showing widespread vacuolization and incomplete detachment from the vitelline membrane.

**Key**
- (M) miracidium
- (Lp) lipoid bodies
- (Vm) vitelline membrane
- (Vc) vacuolization
- (F) fracture of vitelline membrane
- (Ch) Cheever bodies
Figure 4.8: Representative images of *Schistosoma mansoni* eggs after exposure to triclabendazole (tcbz), triclabendazole-sulphoxide (Tcbz-Sx) and triclabendazole-sulphone (Tcbz-Sp) for 24 hours.

(A) Control untreated *S. mansoni* egg (400 ×).
(B) *S. mansoni* egg treated with 100µM Tcbz showing drug binding (400 ×).
(C) *S. mansoni* egg treated with 10µM Tcbz showing detachment of vitelline membrane and ruptured miracidium (400 ×).
(D) *S. mansoni* egg treated with 10µM Tcbz-Sx showing leakage of lipoid bodies (400 ×).
(E) *S. mansoni* egg treated with 10µM of Tcbz-Sp showing leakage of lipoid bodies (400 ×).

Key: (M) Miracidium; (Db) Drug binding; (Sh) Shell; (Lp) Lipoid bodies; (Vm) Vitelline membrane; (C) Contraction.
Figure 4.9: Representative images of the microstructural effects of discharged *Schistosoma mansoni* miracidium after 24 hours exposure to triclabendazole (Tcbz), triclabendazole-sulphoxide (Tcbz-Sx) and triclabendazole-sulphone (Tcbz-Sc).

(A) Discharged *S. mansoni* miracidium after treatment with Tcbz at 10µM, showing bloatedness and widespread vacuolization of the tegument at 1000 ×

(B) Discharged *S. mansoni* miracidium after treatment with Tcbz-Sx, showing widespread deformities to the internal organs and tegument at 1000 ×

(C) Intact *S. mansoni* egg and discharged miracidium after treatment with Tcbz-Sc, showing bloating and some vacuolization of the tegument at 1000 ×

**Key**

(Vc) Vacuolization
4.4 Discussion

The findings of this study demonstrated the induction of schistosome egg hatching in solutions of high osmolarity in the presence of Pzq which supports the earlier observations of Matsuda et al. (1983) and Katsumata et al. (1988). Praziquantel-induced hatching of miracidia in PBS was a dose-dependent process, with hatching observed for all concentrations above 10nM. While Katsumata et al., (1998) have suggested that the hatching induced by Pzq was a Ca\textsuperscript{2+}/calmodulin dependent process there is no direct evidence to suggest that the mechanism of action is a result of calcium-channel activation. However, the strong contractions of the activated and hatched miracidia suggest that Ca\textsuperscript{2+} may play a key role in inducing hatching as the morphological changes observed are consistent with the effects of increased Ca\textsuperscript{2+} permeability (Pax et al., 1978; Blair et al., 1992). Vacuolization of the miracidial tegument was also a prominent observation, with blebs and blisters evident throughout the entire tegumental surface. The deformities observed in miracidia tegments are similar to the pathology observed in adult worms treated with Pzq (Fallon et al., 1992; Linder & Thors, 1992; Brindley, 1994), where disruption of calcium homeostasis has been implicated as a possible mode of action.

It is also likely that Pzq could have played an additional, less direct role in miracidium hatching. Xu & Dresden (1986) reported the correlation of egg hatching and leucine aminopeptidase activity in egg extracts, miracidia and adult worms of S. mansoni. The study demonstrated that the release of leucine aminopeptidase paralleled hatching, and that inhibitors of leucine aminopeptidase also inhibited hatching. Xu et al. (1988) further demonstrated that Pzq caused the rapid release of this enzyme from schistosome eggs \textit{in vitro}. More recently, Arima et al. (2005) demonstrated the modulatory roles of calcium on leucine aminopeptidase in \textit{Streptomyces griceus}. If \textit{Schistosoma} leucine aminopeptidase was both calcium-activated and stabilized as in the case of \textit{Streptomyces griceus}, it is likely that Pzq may have a supplementary role as a calcium ionophore or carrier. By mobilizing vesicular stores of calcium within the egg, Pzq may have activated calcium-modulated leucine aminopeptidase, resulting in the premature hatching of the miracidium.
The results from this study also showed that ryanodine and dihydropyridine did not inhibit Pzq-induced hatching in PBS. At 25µg/mL, dihydropyridine inhibits normal hatching in DDI H₂O but did not inhibit hatching after exposure to 10µM of Pzq. Ten µM of Pzq was the minimal concentration that induced hatching in both *S. mansoni* and *S. japonicum* eggs in the presence of dihydropyridine. However, when eggs treated with 25µg/mL of dihydropyridine were exposed to Pzq concentrations >10µM, activation and hatching were observed. The inhibition of hatching in DDI H₂O observed after treatment with 25µg/mL of dihydropyridine could be due to the compound binding on the surface of the egg shell, which inhibits normal hatching. Dihydropyridine is a blocker of the α₃-subunit of mammalian L-type channels (Knaus *et al*., 1992). Kohn *et al.* (2001) has proposed that the β-subunit of voltage-gated calcium channels was a potential target of Pzq. Therefore, it is likely that the failure to observe the effects of dihydropyridine was due to insufficient homology between mammalian and invertebrate L-type channels. In addition, Fetterer *et al.* (1980) demonstrated that mehtoxyverapamil (D-600), an inhibitor of L-type mammalian Ca⁡²⁺ channels did not block the Pzq-dependent Ca⁡²⁺ influx in adult schistosomes but blocked tonic contraction resulting from increased K⁺ concentrations.

Ryanodine is a plant alkaloid that mobilizes Ca²⁺ from intracellular stores by activating a class of inositol triphosphate-insensitive receptors (IP₃R; Rousseau *et al*., 1987; Smith *et al*., 1988). When eggs were exposed to 25µg/mL of ryanodine, there was no normal hatching and Pzq-induced hatching. Ryanodine has two distinct effects; at submicromolar concentrations, ryanodine locks calcium channels in a long-lived open state, whereas an inhibition of Ca²⁺ channel was observed at higher micromolar concentrations (Rousseau *et al*., 1987; Smith *et al*., 1988). At high concentrations, ryanodine failed to inhibit the actions of Pzq in inducing egg hatching. At concentrations below 1µM to 1nM, no activation or hatching of schistosome eggs were observed. Katsumata *et al.* (1988) demonstrated that ruthenium red at 0.1mM, 0.5mM and 1mM caused the inhibition of egg hatching under low osmotic pressure. Ruthenium red is potent blocker of ryanodine receptors, and thus, ryanodine receptors may play an inhibitory role in the normal hatching of miracidium, as shown by Katsumata *et al.* (1988). It appeared, however, that
Pzq-induced hatching is independent of this receptor, as ryanodine at high concentrations did not inhibit Pzq activation and hatching of miracidium. It is likely that Pzq-induced hatching is activated through other classes of receptors.

One interpretation of the results of this study is that Pzq-induced egg hatching is not calcium-dependent or Ca$^{2+}$ channel activated because ryanodine and dihydropyridine did not result in any observable inhibition of normal and Pzq-induced hatching. It is also likely that the concentrations used to treat the eggs were not high enough. Another interpretation is that ryanodine and dihydropyridine failed to penetrate the egg shells due to their high molecular weights. The fluorescent tags bound to the compounds may have impeded the diffusion of the compounds across the cross-linked protein shell. Neill et al. (1988) demonstrated that the cytoplasmic layer or von Lichtenberg’s envelope interposed between the host and the miracidia can effect a barrier against simple passive diffusion.

While Tcbz and its metabolites did not induce premature hatching of the miracidium, loss of egg shell and miracidia integrity was observed. This was characterized by the rupture of egg shells, and may be evidence of drug penetration into the enveloped miracidium. It has been shown that benzimidazoles (Bzs) can penetrate helminths and cestodes via passive diffusion and is the main transport mechanism for the accumulation of drugs within the parasites (Mottier et al., 2003; Mottier et al., 2006). Interestingly, Tcbz and its metabolites showed a similar ability to penetrate the tegument of trematode parasites by passive diffusion (Mottier et al., 2004).

Although the mode of action of Tcbz in schistosomes is unknown, the drug belongs to a class of microtubule-inhibiting drugs. The crumpling effect and vacuolization of the miracidia tegument is similar to the effects of Tcbz and its metabolites against another traematode parasite, Fasciola. It is likely that Tcbz and its metabolites caused miracidial pathology via the inhibition of microtubule polymerization (Stitt & Fairweather, 1993; 1994). Morphologically, the compounds can be ranked in decreasing order of efficacy: Tcbz-Sx > Tcbz-Sp > Tcbz. However, there was no statistical difference in the EC$_{50}$ values at a 5% level of confidence,
suggesting that the parent compound was as effective as its metabolites in inducing miracidial deaths. Hatching studies suggested that the drugs continued to induce effects even at 10nM, with hatched miracidia that appeared non-viable and bloated.

The efficacy of Tcbz and its metabolites against adult schistosomes have been explored recently (Keiser et al., 2006). However, their action against the egg stages has not been investigated. While the screening of drugs against the adult worms may be useful for establishing the efficacies of the compounds against systemic stages, the eggs of *S. mansoni* and *S. japonicum* are the primary causes of host pathology. The results of this study show that research to evaluate the activity of new anti-schistosomal compounds should not be limited to quantifying the effects of fecundity and integrity in adult and immature stages, but should also be extended to include potential ovicidal activities for further alleviation of disease. Given the structural robustness of the schistosome egg shell, the high efficacies of Tcbz and its metabolites against *S. mansoni* and *S. japonicum* eggs warrants further investigations.
5.1 Introduction

The aim of combination therapy is to elicit an amplified effect or treatment outcome. Drug synergism can be defined as the joint action of discrete compounds, such that the total effect generated is greater than the sum of the two effects when used alone. Combination treatments are commonly used in livestock parasite control, and are routinely used against single or co-infections of different parasites species (Abbot et al., 2004).

The use of drug combinations to treat human schistosomiasis is currently being evaluated by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (Utzinger et al., 2003). In particular, a combination of praziquantel (Pzq) and oxamniquine (Oxm) has been shown to significantly reduce adult worm and egg burden in humans infected with S. mansoni (Shaw & Brammer, 1983). Recent field trials have also demonstrated drug synergism against S. mansoni and S. japonicum infections (Pugh & Teesdale, 1983). However, a recent study has shown that limitations in the experimental models used may have resulted in overestimation of drug efficacy (Utzinger et al., 2003). Combinations of Pzq and artemisinin derivatives have also been demonstrated to reduce worm burdens compared to monotherapy regimens (Utzinger et al., 2001a; Xiao et al., 2000). However, the use of a combination treatment of Pzq and artemisinin derivatives is unlikely in endemic areas where people are also infected with Plasmodium spp. because of the risk that drug resistance will develop against artemisinin (White et al., 1999, Utzinger et al., 2001b).

Triclabendazole (Tcbz) is a benzimidazole anthelmintic that has been shown to have measurable efficacies for the treatment of S. mansoni infections (Keiser et al., 2006). Triclabendazole is currently registered for veterinary and human use for the treatment of infections with a variety of trematode species such as Fasciola, Paragonimus and Clonorchis (Keiser et al., 2005). More importantly, Tcbz is effective against juvenile Fasciola (Coles, 1986; Keiser et al., 2005).
The aim of this study was to determine the *in vitro* efficacy of a combination of Pzq and Tcbz for the treatment of *S. mansoni*. To assess the specificity of the combination, the drugs were evaluated against *Haemonchus contortus*, an intestinal nematode and *Giardia duodenalis*, an intestinal protozoan. While Tcbz has been shown to be active against certain trematode species, it is not active against nematodes (Coles, 1986; Hennessey *et al.*, 1987; Keiser *et al.*, 2005). Similarly, there are no previous studies demonstrating the efficacy of Tcbz for the treatment of *G. duodenalis*. 
5.2 Materials and Methods

5.2.1 Schistosoma maintenance

A Puerto Rican strain of *S. mansoni* was used in this study. *Schistosoma mansoni* were maintained in laboratory-reared *B. glabrata* snails and perpetuated in 6-wk female Swiss outbred mice. Mice were infected percutaneously with 120 cercariae and maintained for 49-days. Mice were subsequently sacrifices and adult schistosomes were recovered from the liver and messentery by hepatic perfusion with PBS.

5.2.2 Giardia maintenance

*Giardia duodenalis* ATCC P1c10 strain was used for this study. Twenty-five thousand *G. duodenalis* P1c10 trophozoites were seeded into 10-mL Nunc™ flat-sided tubes filled with warmed *Giardia* media (see Chapter 2; General Materials and Methods) and incubated at 37°C until monolayer growth was achieved.

5.2.3 Haemonchus maintenance

A benzimidazole-susceptible strain of *H. contortus* was maintained in mixed-sex Merino sheep, aged 12 months at the Department of Animal Health, Western Australian Department of Agriculture and Food. Feces were collected three months after infection and eggs separated from fecal matter using sucrose-floatation (Animal Health Laboratories, 2007). Feces collected overnight were immediately processed the following day. Purified eggs were transferred into Nunc™ 96-well microtitre plates filled with nutrient media (see Chapter 2; General Materials and Methods) and immediately used for drug studies.

5.2.4 Experimental Design

Individual dose response analysis for Pzq and triclabendazole-sulphoxide (Tcbz-Sx) were initially performed to establish the EC$_{50}$, EC$_{25}$ and EC$_{10}$ values required for subsequent combination isobologram analysis. The combinations of EC$_{50}$, EC$_{25}$ and EC$_{10}$ values of Pzq and Tcbz-Sx used are detailed in Table 5.1 below.
Table 5.1: Combinations of the EC\textsubscript{50}, EC\textsubscript{25} and EC\textsubscript{10} values of praziquantel (Pzq) and triclabendazole-sulphoxide (Tcbz-Sx) used in this study.

<table>
<thead>
<tr>
<th>EC combinations of Pzq and Tcbz-Sx</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC\textsubscript{10} + EC\textsubscript{10}</td>
</tr>
<tr>
<td>EC\textsubscript{10} + EC\textsubscript{25}</td>
</tr>
<tr>
<td>EC\textsubscript{10} + EC\textsubscript{50}</td>
</tr>
</tbody>
</table>

5.2.5 In vitro efficacy of single and combination treatments of praziquantel and triclabendazole–sulphoxide.

5.2.5.1 Schistosoma mansoni

Triclabendazole-sulphoxide was chosen for drug combination evaluation because it is the most active form of the drug with the highest in vivo efficacy (Robinson et al., 2004). The in vitro efficacy of each treatment was determined using methods described by Pica-Mattoccia & Cioli (2004). Individual drug efficacy experiments were initially performed using six concentrations of each drug in triplicate wells of 24-well cell-culture plates each filled with 1mL of warmed medium 169 and 10 adult *S. mansoni* worms. One hundred times stock solutions were prepared in dimethyl-sulphoxide (DMSO) to make final concentrations of 10µM, 30µM, 100µM, 150µM, 200µM and 300µM in medium 169. The final DMSO concentration in treated and control wells was ≤ 0.1% v/v. Worms were exposed for no more than 24-hrs and were washed thrice by pipetting with PBS and the media replaced with fresh media 169. Stock solutions of Pzq and Tcbz-Sx were prepared to provide final concentrations equivalent to their EC\textsubscript{50}, EC\textsubscript{25} and EC\textsubscript{10} values in DMSO (Table 5.1). Each drug combination was tested in duplicate wells containing 10 mixed-sex adult worms. The final DMSO concentration in treated and control well was ≤ 0.2% v/v. Worms were exposed to each treatment for 24 hrs and then washed thrice by pipetting with PBS and the media replaced with fresh media 169. The number of dead worms were then counted.
5.2.5.2 *Haemonchus contortus*

Approximately 100 eggs were transferred into quadruplicate wells of Nunc™ 96-well microtitre plates. The *in vitro* activity of Pzq and Tcbz-Sx was determined using methods described by the Animal Health Laboratories, Department of Agriculture and Food, Western Australia. Albendazole (Abz) was used as a positive control drug. Microtitre plates containing treated eggs were incubated at 25°C and monitored microscopically for no more than seven days. All worms were killed by adding iodine to each well when L3 stage worms were first noted in untreated controls. For the evaluation of drug combinations, the EC$_{50}$, EC$_{25}$ and EC$_{10}$ values derived from the above experiment were mixed to give the nine combinations of the EC values shown in Table 5.1 and evaluated as described above. The final DMSO concentrations for drug combination evaluation were ≤ 2% in both treated and control wells.

5.2.5.3 *Giardia duodenalis*

*Giardia duodenalis* strain P1c10 was subcultured in *Giardia* media for a week prior to the commencement of drug screening. A total of 25,000 trophozoites were enumerated and seeded into triplicate 10mL Nunc™ Flat-sided polystyrene tubes filled with warmed *Giardia* Media. The final concentration of the DMSO in each tube was ≤ 1% v/v. Stock solutions of Pzq, Tcbz-Sx and Abz (positive control) were made in DMSO to provide final concentrations of 1mM, 100µM, 10µM, 1µM, 100nM, 10nM in *Giardia* media. Culture tubes containing *Giardia* monolayers and each treatment were incubated for 24hrs at 37°C. The proportion of *Giardia* adhered to flat-sided culture tubes was determined using OPTIMAS® image analysis software. Digital images were captured from six areas of each culture tubes and the efficacy of drugs determined as percentages of adherence inhibition compared to untreated controls. For combination evaluation, Pzq and Tcbz-sx were mixed to give nine combinations of the EC$_{50}$, EC$_{25}$ and EC$_{10}$ values previously determined by singular dose response analyses. The combinations were then exposed against 25,000 *Giardia* trophozoites in 10mL Nunc™ Flat-sided polystyrene tubes. Final DMSO concentrations per tube were ≤ 2% in treated and control tubes.
5.2.6 Statistical analysis

For *S. mansoni*, the efficacy of each single and combination treatment was determined by calculating the percent worm mortality as follows:

\[
\frac{[\text{Control untreated worms} - \text{treated worms}]}{\text{Control untreated worms}} \times 100\%
\]

For *G. duodenalis*, the efficacy of single and combination treatment was determined by calculating the percent inhibition of adherence) as follows:

\[
\frac{[\text{control trophozoites adhered} - \text{treated trophozoites adhered after drug treatment}]}{\text{Control trophozoites adhered}} \times 100\%.
\]

For *H. contortus*, the efficacy of single and combination treatment was determined by calculating the inhibition of L3 stage larval development as follows:

\[
\frac{[\text{Control untreated L3 worms} - \text{treated L3 worms}]}{\text{Control untreated L3 worms}} \times 100\%
\]

The EC$_{50}$ values for each compound and each parasite species were determined using Prism V4.0 (GraphPad, San Diego).

5.2.7 Combination dose response and isobologram analysis

The level of synergy between Pzq and Tcbz-Sx was determined by isobologram analysis. A scatter plot of the percent mortality or inhibition was plotted for Pzq and Tcbz-Sx for the various EC concentrations. A trend line is then drawn between a benchmarked concentration EC$_X$ (or the effective concentration to elicit X % mortality) of both Pzq and Tcbz-Sx. The EC$_{50}$, EC$_{25}$ and EC$_{10}$ values that are plotted below the trend line shows drug synergism while values plotted above the trend line shows drug antagonism. Values that are plotted along the trend line show additive effects between drugs.
The fractional inhibitory concentration (FIC) is the interaction coefficient indicating whether the combined inhibitory effect of the drugs is synergistic, additive or antagonistic at a chosen benchmarked concentration EC$_X$.

The average FIC is derived from the average three FICs calculated using the EC$_{so}$, EC$_{25}$ and EC$_{10}$ values of the two drugs. As a rule, an FIC of <1 would indicate synergism, a value of 1 indicating additive effects while a value of >1 would indicate drug antagonism.

The FIC at EC$_X$ = (A + B), where

A= (EC$_X$ of Pzq and Tcbz-Sx combination)/ (EC$_X$ of Pzq alone)

B= (EC$_X$ of Pzq and Tcbz-Sx combination)/ (EC$_X$ of Tcbz alone)
5.3 Results

5.3.1 Efficacy of praziquantel and triclabendazole-sulphoxide against *Schistosoma mansoni*

5.3.1.1 Efficacy of individual drug treatment

The concentration of Pzq and Tcbz-Sx required to kill 50% (EC$_{50}$), 25% (EC$_{25}$) and 10% (EC$_{10}$) of *S. mansoni* in 24hrs are shown in Table 5.2. The EC$_{50}$, EC$_{25}$ and EC$_{10}$ values for Pzq are significantly lower compared to the EC$_{50}$, EC$_{25}$ and EC$_{10}$ values for Tcbz-Sx (P<0.05).

**Table 5.2:** Concentrations of praziquantel (Pzq) and triclabendazole-sulphoxide (Tcbz-Sx) required to kill 50% (EC$_{50}$), 25% (EC$_{25}$) and 10% (EC$_{10}$) of *Schistosoma mansoni* worms after 24 hours of exposure *in vitro*.

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Pzq</th>
<th>Tcbz-Sx</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC$_{50}$</td>
<td>3</td>
<td>96</td>
</tr>
<tr>
<td>EC$_{25}$</td>
<td>1.6</td>
<td>67</td>
</tr>
<tr>
<td>EC$_{10}$</td>
<td>1.1</td>
<td>55</td>
</tr>
</tbody>
</table>

5.3.1.2 Efficacy of combination drug treatment

The percent mortality of worms treated with each combination is shown in Table 5.3. Immediate and rapid contractions were observed in worms immediately after exposure of each combination treatment. Worms died within one hour after exposure as evidenced by the cessation of movement. The high efficacies observed were characterized by high mortality rates and no worm recovery, with exposed worms maximally contracted following fresh medium change and further observations for 48hrs. Mortality rates of greater than 50% (EC$_{50}$) were observed for worms exposed to all combinations of Pzq and Tcbz-Sx.
Table 5.3: Percent mortality of adult *Schistosoma mansoni* treated with combinations of praziquantel (Pzq) and triclabendazole-sulphoxide (Tcbz-Sx). Standard errors are presented in parenthesis.

<table>
<thead>
<tr>
<th>Drug combination</th>
<th>Concentration (µM)</th>
<th>% Mortality (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pzq + Tcbz-Sx</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC_{50} + EC_{50}</td>
<td>96 + 3</td>
<td>100 (0)</td>
</tr>
<tr>
<td>EC_{50} + EC_{25}</td>
<td>96 + 1.6</td>
<td>100 (0)</td>
</tr>
<tr>
<td>EC_{50} + EC_{10}</td>
<td>96 + 1.1</td>
<td>100 (0)</td>
</tr>
<tr>
<td>EC_{25} + EC_{50}</td>
<td>67 + 3</td>
<td>100 (0)</td>
</tr>
<tr>
<td>EC_{25} + EC_{25}</td>
<td>67 + 1.6</td>
<td>80 (5.78)</td>
</tr>
<tr>
<td>EC_{25} + EC_{10}</td>
<td>67 + 1.1</td>
<td>80 (5.78)</td>
</tr>
<tr>
<td>EC_{10} + EC_{50}</td>
<td>55 + 3</td>
<td>76.7 (3.34)</td>
</tr>
<tr>
<td>EC_{10} + EC_{25}</td>
<td>55 + 1.6</td>
<td>56.7 (3.34)</td>
</tr>
<tr>
<td>EC_{10} + EC_{10}</td>
<td>55 + 1.1</td>
<td>56.7 (3.34)</td>
</tr>
</tbody>
</table>

Figure 5.1: Microstructural effects of adult *Schistosoma mansoni* exposed to an EC_{50} + EC_{50} combination of praziquantel and triclabendazole-sulphoxide. Maximal contraction and vacuolization of internal worm musculature was evident. Vc: Vacuolization
Figure 5.2: Proportion of *Schistosoma mansoni* worms killed by triclabendazole-sulphoxide plotted against the praziquantel EC$_{50}$, EC$_{25}$ and EC$_{10}$ concentrations.

Figure 5.3: Proportion of *Schistosoma mansoni* worms killed by praziquantel plotted against the triclabendazole-sulphoxide EC$_{50}$, EC$_{25}$ and EC$_{10}$ concentrations.
Figure 5.2 and 5.3 showed > 50% worm mortality for all combinations assessed and suggested strong drug synergism. The EC$_{90}$ (concentration required to elicit 90% mortality) was chosen as the benchmark value (EC$_X$) and was used to derive the fractional inhibitory concentration because of the high mortality rates observed. Figure 5.4 shows the EC$_{90}$ isobologram derived by plotting the EC$_{50}$, EC$_{25}$ and EC$_{10}$ concentrations of Pzq against the concentration of Tcbz-Sx required to kill 90% of worms. The three EC values plotted below the trend line shows drug synergism between Pzq and Tcbz-Sx. The average fractional inhibitory concentration (SE) was 0.644 with a standard error of 0.055.

![EC$_{50}$ Isobologram](image)

**Figure 5.4: EC$_{90}$ isobologram of praziquantel (Pzq) and triclabendazole-sulphoxide (Tcbz-Sx) combinations assessed against *Schistosoma mansoni*.

### 5.3.2 Efficacy of praziquantel and triclabendazole-sulphoxide against *Haemonchus contortus*

#### 5.3.2.1 Efficacy of individual drug treatment

*Haemonchus contortus* in untreated wells demonstrated good development into the L3 stages, with at least 80% of eggs embryonating and hatching. The efficacies of Pzq and Tcbz-Sx were determined against *H. contortus* using the larval development assay (LDA). The EC$_{50}$ of albendazole, the positive control compound, were significantly lower compared to the EC$_{50}$ of Pzq and Tcbz-Sx (P<0.05). A significantly higher concentration of Pzq was required to inhibit 50% of L3 stage development (EC$_{50}$ of 181µM) compared to Tcbz-Sx. Praziquantel assessed
against *H. contortus* larvae demonstrated EC$_{50}$, EC$_{25}$ and EC$_{10}$ values that were significantly higher compared to the EC$_{50}$, EC$_{25}$ and EC$_{10}$ values of Tcbz-Sx. Triclabendazole-sulphoxide also caused observable changes in *H. contortus* eggs at concentrations above 100µM. Affected eggs were opaque, bloated and were unembryonated. At lower concentrations (10µM to 100µM), L1 and L2 stage larvae were sessile and displayed no visible movement, and were characterized as dead. Table 5.4 shows the concentrations of Pzq, Tcbz-Sx and Abz required to inhibit 50% of *H. contortus* larvae from developing into the L3 stage.

**Table 5.4:** Concentration of praziquantel (Pzq), triclabendazole-sulphoxide (Tcbz-Sx) and albendazole (Abz) required to inhibit 50% (EC$_{50}$), 25% (EC$_{25}$) and 10% (EC$_{10}$) of *Haemonchus contortus* L3 stage development after 24 hours of exposure *in vitro*.

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Pzq</th>
<th>Tcbz-Sx</th>
<th>Abz</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC$_{50}$</td>
<td>181</td>
<td>0.279</td>
<td>0.0054</td>
</tr>
<tr>
<td>EC$_{25}$</td>
<td>132</td>
<td>0.039</td>
<td>0.0008</td>
</tr>
<tr>
<td>EC$_{10}$</td>
<td>96</td>
<td>0.0054</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

**5.3.2.2 Efficacy of combination drug treatment**

The percent inhibition of L3 stage development for drug combination studies is shown in Table 5.5. Mortality rates of greater than 50% were observed for worms exposed to all combinations of Pzq and Tcbz except for the lowest combination of the two compounds (Pzq EC$_{10}$ and Tcbz-Sx EC$_{10}$) which showed 47.1% inhibition. Figure 5.5 and 5.6 showed > 50% inhibition of L3 larval stage for most concentrations of Pzq and Tcbz-Sx combinations. The EC$_{70}$ (concentration eliciting 70% inhibition) was chosen as the benchmark value and was used to derive the fractional inhibitory concentration.
Table 5.5: Percent inhibition of L3 development of *Haemonchus contortus* treated with combinations of praziquantel (Pzq) and triclabendazole-sulphoxide (Tcbz-Sx). Standard errors are presented in parenthesis.

<table>
<thead>
<tr>
<th>Drug combination</th>
<th>Concentration (µM)</th>
<th>% inhibition of L3 development (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pzq + Tcbz-Sx</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC50 + EC50</td>
<td>181 + 0.279</td>
<td>94.1 (2.4)</td>
</tr>
<tr>
<td>EC50 + EC25</td>
<td>181 + 0.039</td>
<td>82.4 (3.53)</td>
</tr>
<tr>
<td>EC50 + EC10</td>
<td>181 + 0.0054</td>
<td>76.5 (1.73)</td>
</tr>
<tr>
<td>EC25 + EC50</td>
<td>132 + 0.279</td>
<td>88.2 (3.53)</td>
</tr>
<tr>
<td>EC25 + EC25</td>
<td>132 + 0.039</td>
<td>82.4 (3.53)</td>
</tr>
<tr>
<td>EC25 + EC10</td>
<td>132 + 0.0054</td>
<td>64.7 (2.73)</td>
</tr>
<tr>
<td>EC10 + EC50</td>
<td>96 + 0.279</td>
<td>70.6 (3.0)</td>
</tr>
<tr>
<td>EC10 + EC25</td>
<td>96 + 0.039</td>
<td>58.8 (3.93)</td>
</tr>
<tr>
<td>EC10 + EC10</td>
<td>96 + 0.0054</td>
<td>47.1 (8.98)</td>
</tr>
</tbody>
</table>

Figure 5.7 shows the EC70 isobologram derived by plotting the EC50, EC25 and EC10 concentration of Pzq against the concentration of Tcbz-Sx required to inhibit 70% of *H. contortus* L3 larval development. The EC50, EC25 and EC10 values plotted below the trend line showed drug synergism between Pzq and Tcbz-Sx. The average fractional inhibitory concentration (SE) was calculated to be 0.68 with a standard error of 0.058.
Figure 5.5: Proportion of inhibition of L3 stage development of *Haemonchus contortus* worms by triclabendazole-sulphoxide plotted against praziquantel EC$_{50}$, EC$_{25}$ and EC$_{10}$ concentrations.

Figure 5.6: Proportion of inhibition of L3 stage development of *Haemonchus contortus* by praziquantel plotted against triclabendazole-sulphoxide EC$_{50}$, EC$_{25}$ and EC$_{10}$ concentrations.
Figure 5.7: EC\textsubscript{70} isobologram of praziquantel (Pzq) and triclabendazole-sulphoxide (Tcbz-Sx) combinations assessed against \textit{Haemonchus contortus}.

5.3.3 Efficacy of praziquantel and triclabendazole-sulphoxide against \textit{Giardia duodenalis}

5.3.3.1 Efficacy of individual drug treatment

The concentration required for Pzq and Tcbz-Sx required to inhibit 50% (EC\textsubscript{50}), 25% (EC\textsubscript{25}) and 10% (EC\textsubscript{10}) of \textit{G. duodenalis} trophozoite adherence in 24hrs are shown in Table 5.6. Albendazole and Tcbz-Sx inhibited the adherence of \textit{Giardia} trophozoites, with EC\textsubscript{50} values of 6.17\textmu M and 12.9\textmu M respectively. Both Abz and Tcbz-Sx caused crumpling of \textit{Giardia} trophozoites at concentrations above 10\textmu M, with detachment evident at lower concentrations. Loss of flagella was also observed, with some trophozoites appearing sessile and arrested at various stages of binary multiplication. Praziquantel was ineffective at inhibiting \textit{Giardia} adherence. Motility recovery and cell multiplication was observed for Pzq-treated trophozoites following drug exposure for 24 hours and replacement with fresh media. The EC\textsubscript{50} of Abz, Pzq and Tcbz-Sx were significantly different at P<0.05.
Table 5.6: Concentration of praziquantel (Pzq), triclabendazole-sulphoxide (Tcbz-Sx) and albendazole (Abz) required to inhibit 50% (EC$_{50}$), 25% (EC$_{25}$) and 10% (EC$_{10}$) of *Giardia* adherence after 24 hours of exposure *in vitro*.

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Pzq</th>
<th>Tcbz-Sx</th>
<th>Abz</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC$_{50}$</td>
<td>589</td>
<td>6.17</td>
<td>12.9</td>
</tr>
<tr>
<td>EC$_{25}$</td>
<td>295</td>
<td>2.14</td>
<td>2.95</td>
</tr>
<tr>
<td>EC$_{10}$</td>
<td>151</td>
<td>0.76</td>
<td>0.1</td>
</tr>
</tbody>
</table>

5.3.3.2 Efficacy of combination drug treatment

The percent inhibition of trophozoite adherence for combination drug treatment is shown in Table 5.7. Combinations of Pzq and Tcbz-Sx exposed to *Giardia* trophozoites showed weak inhibition of adherence.

Table 5.7: Inhibition of adherence of *Giardia duodenalis* trophozoites following exposure to combinations of praziquantel (Pzq) and triclabendazole-sulphoxide (Tcbz-Sx). Standard errors are presented in parenthesis.

<table>
<thead>
<tr>
<th>Drug combinations</th>
<th>Concentrations (µM)</th>
<th>% Inhibition of adherence (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pzq + Tcbz-Sx</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC$<em>{50}$ + EC$</em>{50}$</td>
<td>589µM + 6.17µM</td>
<td>62.3 (4.04)</td>
</tr>
<tr>
<td>EC$<em>{50}$ + EC$</em>{25}$</td>
<td>589µM + 2.14µM</td>
<td>53.3 (6.66)</td>
</tr>
<tr>
<td>EC$<em>{50}$ + EC$</em>{10}$</td>
<td>589µM + 0.76µM</td>
<td>46 (1)</td>
</tr>
<tr>
<td>EC$<em>{25}$ + EC$</em>{50}$</td>
<td>295µM + 6.17µM</td>
<td>55.5 (5.22)</td>
</tr>
<tr>
<td>EC$<em>{25}$ + EC$</em>{25}$</td>
<td>295µM + 2.14µM</td>
<td>55.7 (0.58)</td>
</tr>
<tr>
<td>EC$<em>{25}$ + EC$</em>{10}$</td>
<td>295µM + 0.76µM</td>
<td>58.7 (1.15)</td>
</tr>
<tr>
<td>EC$<em>{10}$ + EC$</em>{50}$</td>
<td>151µM + 6.17µM</td>
<td>62 (7.55)</td>
</tr>
<tr>
<td>EC$<em>{10}$ + EC$</em>{25}$</td>
<td>151µM + 2.14µM</td>
<td>50 (8)</td>
</tr>
<tr>
<td>EC$<em>{10}$ + EC$</em>{10}$</td>
<td>151µM + 0.76µM</td>
<td>17 (8.19)</td>
</tr>
</tbody>
</table>
Figure 5.8: The proportion of *Giardia* trophozoites adherence inhibited by triclabendazole-sulphoxide plotted against the praziquantel EC$_{50}$, EC$_{25}$ and EC$_{10}$ concentrations.

Figure 5.9: The proportion of *Giardia* trophozoites adherence inhibited by praziquantel plotted against the triclabendazole-sulphoxide EC$_{50}$, EC$_{25}$ and EC$_{10}$ concentrations.
Figure 5.8 and 5.9 showed the inhibition of trophozoite adherence by the EC\textsubscript{50}, EC\textsubscript{25} and EC\textsubscript{10} combinations of Pzq and Tcbz-Sx. Moderate inhibition of adherence was observed even at the highest concentration assessed. The EC\textsubscript{50} (concentration eliciting 50% inhibition) was chosen as the benchmark value and was used to derive the fractional inhibitory concentration. The best-fit of the EC\textsubscript{50}, EC\textsubscript{25} and EC\textsubscript{10} values plotted shows weak synergy or drug addition between Pzq and Tcbz-Sx.

Figure 5.10 shows the EC\textsubscript{50} isobologram derived by plotting the EC\textsubscript{50}, EC\textsubscript{25} and EC\textsubscript{10} concentration of Pzq against the concentration of Tcbz-Sx required to inhibit 50% of *Giardia* trophozoites. The average fractional inhibitory concentration (SE) was 0.89 with a standard error of 0.18. The FIC value was indicative of weak synergy or additive effects.

**Figure 5.10**: EC\textsubscript{50} isobologram of praziquantel and triclabendazole-sulphoxide combinations assessed against *Giardia duodenalis* trophozoites.
5.4 Discussion

This study was conducted to assess the level of drug synergism between Pzq and Tcbz-Sx against *S. mansoni, H. contortus* and *G. duodenalis* *in vitro*. In addition to *S. mansoni, H. contortus* and *G. duodenalis* were assessed with the drug combination for the following reasons:

1) *H. contortus* and *G. duodenalis* have well-established models for combination drug testing *in vitro* (Jiménez-Cardoso *et al*., 2004; Kotze *et al*., 2006), and is useful as benchmarks for this study, 2) To assess the specificity and relative efficacies of the Pzq and Tcbz-Sx drug combination against unrelated parasite species, and 3) to demonstrate the relative extent of morphological damages associated with drug treatment against the three parasites using light microscopy. A combination of Pzq and Tcbz-Sx was selected for two reasons; 1) there was likely to be a high correlation between the *in vitro* and *in vivo* effects because both Pzq and Tcbz-Sx have been shown to be the predominant active forms *in vivo* (Robinson *et al*., 2004) and 2) the *in vitro* efficacy of Tcbz-Sx for each parasite species was significantly higher compared to the parent Tcbz and the metabolite Tcbz-Sp. The checkerboard method of synergy assessment using a combination of EC\(_{50}\), EC\(_{25}\) and EC\(_{10}\) values showed that the Pzq-Tcbz-Sx combination was synergistic against *S. mansoni* and *H. contortus*, and was moderately synergistic/additive against *G. duodenalis*, characterized by average FIC values that were <1.

The results from experiments using *S. mansoni* showed that Pzq was more effective compared to Tcbz-Sx. In addition, *S. mansoni* worms exposed to Pzq showed more pronounced morphological effects compared to worms exposed to Tcbz-Sx. Adult worms showed maximal contraction within 24hrs drug exposure to Pzq. Triclabendazole-sulphoxide, on the other hand, demonstrated persistent effects, characterised by continued mortality up to 72hrs after drug exposure. This could have been due to irreversible binding of Tcbz-Sx to exposed worms. The use of Pzq and Tcbz-Sx in combination appeared to accelerate the effects of each compound on *S. mansoni*, with worms contracting immediately after exposure and mortality evident within the first hour of exposure. Combinations of Pzq and Tcbz-Sx at EC\(_{50}\) + EC\(_{50}\), EC\(_{50}\) + EC\(_{25}\), EC\(_{25}\) + EC\(_{50}\) and EC\(_{10}\) + EC\(_{50}\) caused 100% mortality of adult *S. mansoni*. The lowest combination
dosage of Pzq EC$_{10}$ and Tcbz-Sx EC$_{10}$ caused 55% worm mortality, demonstrating a 2.75 fold amplification of efficacy compared to the sole additive effects of the drugs when used alone. These results and the high FIC shows that significant synergism exists between Pzq and Tcbz-Sx when used against S. mansoni in vitro.

The results from the experiments using H. contortus showed that Tcbz-Sx was more effective compared to Pzq. The EC$_{50}$, EC$_{25}$ and EC$_{10}$ values of Tcbz-Sx were in the nanomolar range, while Pzq values resided within the micromolar range. At concentrations above 100µM, pronounced and observable morphological changes to H. contortus eggs were observed. Viable eggs that were exposed to Tcbz-Sx and assessed seven days later appeared ‘calcified’, bloated and unembryonated. This observation was consistent with the observations for Abz-treated Haemonchus eggs, where a bloated and opaque appearance was seen. The consistency of this observation suggested that Tcbz-Sx and Abz elicited action(s) through similar mechanisms, albeit observations that Abz was the more effective drug. Pzq, on the other hand, demonstrated weak activity and possessed no ovicidal activity, with L3 stage inhibition only evident at the higher concentrations assessed. When Pzq and Tcbz-Sx were assessed in combination, an amplification inhibition of L3 stage development was observed. These results demonstrate that drug synergism exists between Pzq and Tcbz-Sx when used against H. contortus in vitro.

The results of experiments using G. duodenalis showed that Pzq has a weak effect in vitro as evidenced by the high EC$_{50}$, EC$_{25}$ and EC$_{10}$ values. Giardia duodenalis treated with the highest concentration of 1mM of Pzq were sessile and malformed, appearing luminescent and bloated. However, at concentrations below 1mM, trophozoites appeared unaffected, even at these relatively high concentrations. In addition, G. duodenalis trophozoite recovered and multiplied after 48hrs after exposure with Pzq at all concentrations. While the in vitro activity of Pzq on Giardia have not been previously investigated, Flisser et al. (1995) reported that a single oral dose of 5mg/kg Pzq was effective in treating human giardiasis. It is possible that the parent Pzq was ineffective against Giardia in vivo and that the metabolites of Pzq were responsible for the
elimination of *Giardia in vivo*. Flisser *et al.* (1995) further postulated that Pzq may have altered internal flora, leading to the expulsion of *Giardia*.

Triclabendazole-sulphoxide demonstrated a high level of efficacy against *G. duodenalis* trophozoites. Morphological changes associated with the exposure of Tcbz-Sx included bloating, cessation of binary multiplication, loss of adherence with pronounced crumpling of the entire trophozoite body. Loss of flagella was also observed, with some trophozoites appearing luminescent. Such morphological changes were also observed in trophozoites exposed to Abz, the positive control. Surprisingly, Abz demonstrated higher EC$_{50}$ values (less effective) compared to Tcbz-Sx at 24hrs after drug exposure. However, Abz possessed pronounced persistent inhibition at 48 and 72hrs post exposure whereas trophozoites treated with Tcbz-Sx appeared to recover after 48hrs of exposure, with binary multiplication evident at concentrations below 100µM. The FIC of 0.89 for Pzq and Tcbz combinations suggested weak drug synergism or drug addition effects.

It is not possible to determine the mechanism of synergy observed for Pzq and Tcbz-Sx against *S. mansoni* and *H. contortus*. Morphological changes induced in *G. duodenalis* trophozoites after exposure to Tcbz-Sx suggest that the compound inhibited microtubule formation which agrees with observations of Abz exposure (Reynoldson *et al.*, 1992). Triclabendazole-sulphoxide had a moderate effect on *H. contortus* eggs compared to the strong ovidical activity and larvicidal activity of Abz. Lacey *et al.* (1987) showed that there is a strong correlation between the inhibition of egg hatching and the inhibition of tubulin depolymerization which leads to microtubule disruption.

The cytoskeletal elements (including microtubules) of schistosomes are not good targets of drug action due to their internal localization (Jones *et al.*, 2004). Nevertheless, the cytoplasmic bridges of tegumentary cytoplasm possess a ring of microtubules, primarily used for anterograde transport (Wiest *et al.*, 1988). Further work by Jones *et al.* (2004) showed that the stable microtubules are distributed within the interconnecting tegumental bridges of
schistosomes. The bridges represented areas rich in microtubules that extended into the distal cytoplasm and were separated from other bridges, resulting in intervening regions that were entirely devoid of microtubules. Recent studies have shown that Tcbz-Sx inhibit microtubule formation by binding to beta-tubulin subunits of *F. hepatica* (Fairweather & Boray, 1999).

However, weak and inconsistent activities of Tcbz-Sx were observed when the compound was applied against *S. mansoni* (Keiser et al., 2006). This could be due to the poor presentation of *Schistosoma* microtubules (Jones et al., 2004). In *S. mansoni*, tubulin is positioned as vertical lines stretching across the whole thickness of the syncytium, while *F. giagantica* tubulin was disseminated in tegumental cell bodies, cytoplasmic processes and the basal layer of the tegumental syncytium (Tansatit et al., 2006). It is therefore likely that the organization of *Fasciola* microtubule is more accessible to Tcbz-Sx action compared to *Schistosoma* tubulin.

If the combination of Pzq and Tcbz-Sx elicited effects through two independent mechanisms of action, it is possible that tegument sloughing (as a result of the action of Pzq) may have exposed schistosome microtubules which enhanced Tcbz-Sx induced disruption. It has also been previously demonstrated by Linder & Thors (1992) that Pzq exposes the surface spines consisting of paracrystalline actin following tegumental disruption. In a separate study, Pzq was shown to expose and disrupt underlying subtegumental/parenchymal tissues (Shaw & Erasmus, 1987). The close proximity of schistosome actin to the underlying microtubule would allow increased access to these structures following the effects of Pzq. This may have accounted for the apparent synergy of Pzq and Tcbz-Sx, resulting in worm contractions and mortality rates that were rapid and unrecoverable.
6.1 Introduction

Praziquantel (Pzq) is usually delivered in a single dose for control, or as a two dose regime for control and prevention. Praziquantel is used to treat human schistosomiasis because it has a large therapeutic index and has good efficacy against all schistosome species. Recent reports of treatment failure have prompted an evaluation of alternative treatment that may be required if extensive resistance develops against Pzq.

One alternative to Pzq is triclabendazole (Tcbz), a benzimidazole active against a range of trematodes and cestodes. Triclabendazole has previously been shown to possess good efficacy for the treatment of human Paragonimiasis and Fascioliasis (Keiser et al., 2005). Early reports by Mansoury (1997), el Sayed & Allam (1997), Schmidt (1998) and Khalil (2000) demonstrated that Tcbz significantly reduced worm burdens by up to 84% in mice infected with S. mansoni. More recently, however, Keiser et al., (2006) revealed that Tcbz and its metabolites possessed weak and inconsistent anti-schistosomal activities in vivo, characterized by worm burden reductions of only 35% at 120mg/kg. It is difficult to interpret these conflicting reports because of inconsistencies in experimental methodologies, as well as the varying inherent susceptibilities of schistosome strains (Soliman et al., 1986; el Mansoury, 1995).

Combination drug therapies are increasingly being used to treat parasitic diseases such as malaria where multi-drug resistance have developed. The aim of this study was to assess the efficacy of a combination of Pzq and Tcbz for the treatment of mice infected with S. mansoni. Drug efficacy was evaluated on the basis of adult worm burden reduction, tissue egg loads and oogram patterns (Keiser et al., 2006).
6.2 Materials and Methods

6.2.1 Schistosoma maintenance

*Schistosoma mansoni* (Puerto Rican strain) was maintained in *B. glabrata* snails for 35 days in the Queensland Institute of Medical Research. Percutaneous infection of 25 mice was performed with 120 cercariae for each mouse. Mice were maintained for 49 days before the commencement of drug trials. Mice were randomly allocated into groups of five and housed at ambient temperature on a 12hr light/dark cycle for one week prior to infection. Food and water were administered *ad libitum*.

6.2.2 Drug efficacy

Praziquantel and Tcbz (Epichem Pty Ltd., Australia) were suspended in 2.5% Cremophor EL (Sigma-Aldrich, St Louis, USA). Each group of mice were weighed to obtain the average weights. Individual groups of mice were then treated with Pzq (80mg/kg), Tcbz (120mg/kg), Pzq-Tcbz (80mg/kg+120mg/kg) and Pzq-Tcbz (250mg/kg+250mg/kg) by oral gavage. The Pzq dose chosen was based on the ED$_{50}$ value of a previous study by Pica-Matoccoia & Cioli (2004). The Tcbz dose was chosen based on the protocol described in Keiser et al., (2006). An untreated group was maintained as control. Mice were sacrificed four days after drug treatment and hepatic perfusion performed. Adult worms were recovered from the hepatic and portomesenteric vessels by perfusion according to the protocols of Smithers & Terry (1965). Worms from the mesenteric veins were removed, sexed and enumerated using a microscope. The total number of worm mortalities was calculated by subtracting the total worm burden of each treated group from the worm burden of the untreated group.

6.2.3 Oogram pattern and tissue egg load analyses

Oogram analysis was performed by excising three fragments from the middle part of the small intestines. The fragments were cut longitudinally, washed with saline and compressed between microscope slides. The slides were then examined microscopically at 100 × magnification. A total of 100 eggs per animal were enumerated and the stages of egg development assessed.
according to Pellegrino et al. (1962). The tissue egg load analysis was determined as follows: 0.3g of the liver and small intestine from each mouse were digested in 5-mL 5% KOH overnight. Three digest aliquots of 150µL were then examined microscopically and the number of eggs counted. Hepatic and intestinal tissue egg loads were expressed as eggs per gram (epg), determined by multiplying the number of eggs enumerated by the total volume of the KOH and dividing this value by the weight of the sample.

6.2.4 Statistical analysis

Statistical analysis was performed using Prism v.4 (GraphPad, San Diego). Drug efficacy was assessed by comparing the mean number of worms in treatment groups against the untreated control group. The statistical significance of any differences were determined using an unpaired two-tailed students’ test with unequal variance at a 95% confidence limit.
6.3 Results

6.3.1 Tissue egg load analysis

There was a significant decrease in the mean hepatic egg load and the mean intestinal egg load in mice treated with Pzq, Tcbz and the two drug combinations compared to untreated control mice (P<0.05; Table 6.1). Praziquantel (80mg/kg) treated mice showed significantly lower hepatic and intestinal egg loads compared to Tcbz (120mg/kg) and Pzq-Tcbz combination (80mg/kg and 120mg/kg) treated mice. The hepatic and intestinal egg loads were significantly lower (P<0.05) in mice treated with Pzq (80mg/kg) compared to mice treated with the highest Pzq and Tcbz combination dose (250mg/kg and 250mg/kg). There was also a significantly lower (P<0.05) hepatic and tissue egg load in mice treated with Tcbz (120mg/kg) compared to mice treated with Pzq (80mg/kg), Pzq-Tcbz (80mg/kg -120mg/kg) and Pzq-Tcbz (250mg/kg -250mg/kg).
Table 6.1: Mean hepatic and intestinal egg loads in eggs per gram (epg) of mice infected with *Schistosoma mansoni* and treated with Pzq (80mg/kg), Tcbz (120mg/kg), Pzq-Tcbz combination (80mg/kg -120mg/kg) and Pzq-Tcbz combination (250mg/kg - 250mg/kg). Standard deviations and standard errors are presented in parenthesis.

<table>
<thead>
<tr>
<th>Drug (dose in mg/kg)</th>
<th>Hepatic counts (SD)</th>
<th>Intestinal counts (SD)</th>
<th>Hepatic epg (SD)</th>
<th>Intestinal epg (SD)</th>
<th>Hepatic epg (SE)</th>
<th>Intestinal epg (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>49.4 (16)</td>
<td>32.2 (8.5)</td>
<td>16.5(5.3)</td>
<td>10.7(2.8)</td>
<td>16.5 (2.37)</td>
<td>10.7 (1.25)</td>
</tr>
<tr>
<td>Pzq (80)</td>
<td>24.4 (4.28)</td>
<td>1.4 (1.14)</td>
<td>8.13(1.43)</td>
<td>0.47(0.38)</td>
<td>8.13 (0.64)</td>
<td>0.47 (0.17)</td>
</tr>
<tr>
<td>Tcbz (120)</td>
<td>45.2 (10.4)</td>
<td>3.8 (2.17)</td>
<td>15.1(3.5)</td>
<td>1.27(1.2)</td>
<td>15.1 (1.56)</td>
<td>1.27 (0.54)</td>
</tr>
<tr>
<td>Pzq - Tcbz (80-120)</td>
<td>28.8 (6.6)</td>
<td>2.2 (2.68)</td>
<td>9.6(2.2)</td>
<td>0.73(0.89)</td>
<td>9.6 (0.98)</td>
<td>0.73 (0.4)</td>
</tr>
<tr>
<td>Pzq - Tcbz (250-250)</td>
<td>18 (6.12)</td>
<td>0.2 (0.45)</td>
<td>6(2.0)</td>
<td>0.067(0.022)</td>
<td>6 (0.89)</td>
<td>0.067 (0.001)</td>
</tr>
</tbody>
</table>
6.3.2 Oogram pattern analysis

There were significantly more immature eggs compared to mature eggs in the intestines of the untreated control mice group and in mice treated with Tcbz (P<0.05; Table 6.2). There were significantly more mature eggs than immature eggs in the intestines of mice treated with Pzq at 80mg/kg (P<0.05). A significantly higher (P<0.05) numbers of mature eggs compared to immature eggs was observed in the intestines of mice treated with the highest combination doses of Pzq and Tcbz (250mg/kg-250mg/kg). There was no statistical difference between the number of mature and immature eggs in the intestines of mice treated with the lower combination dose of Pzq and Tcbz (80mg/kg-120mg/kg).

Table 6.2: Oogram pattern of mice infected with Schistosoma mansoni and treated with praziquantel (Pzq; 80mg/kg), triclabendazole (Tcbz; 120mg/kg), Pzq-Tcbz combination (80mg/kg +120mg/kg) and Pzq-Tcbz combination (250mg/kg +250mg/kg). Standard errors are presented in parenthesis.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>First immature</th>
<th>Second immature</th>
<th>Third immature</th>
<th>Fourth immature</th>
<th>Total immature</th>
<th>Mature</th>
<th>Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>12.1 (2.9)</td>
<td>18.3 (12.6)</td>
<td>32.8 (5.8)</td>
<td>13.7 (2.5)</td>
<td>77 (10)</td>
<td>22.7 (10.3)</td>
<td>0.4 (0.5)</td>
</tr>
<tr>
<td>Pzq 80</td>
<td>3.42 (5.7)</td>
<td>1.64 (2.25)</td>
<td>6.06 (10.7)</td>
<td>2.04 (3.5)</td>
<td>13.16 (6.9)</td>
<td>71 (12.1)</td>
<td>15.8 (6.74)</td>
</tr>
<tr>
<td>Tcbz 120</td>
<td>1.25 (2.8)</td>
<td>6.8 (5.6)</td>
<td>35 (11.2)</td>
<td>17.8 (12)</td>
<td>60.9 (8.4)</td>
<td>8.9 (4.3)</td>
<td>30.2 (5.4)</td>
</tr>
<tr>
<td>Pzq - Tcbz (80-120)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>3.8 (6.2)</td>
<td>23.7 (28.5)</td>
<td>27.6 (24)</td>
<td>61 (24)</td>
<td>11.5 (9.15)</td>
</tr>
<tr>
<td>Pzq - Tcbz (250-250)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>6.2 (15.4)</td>
<td>6.2 (15.4)</td>
<td>68.5 (22.1)</td>
<td>25.7 (10)</td>
</tr>
</tbody>
</table>

6.3.3 Worm burden reduction

Mice treated with the four dosage regimens demonstrated mean worm burden reductions that were significantly different to each other at P<0.05 (Table 6.3). Mice treated with 80mg/kg of Pzq showed significantly higher mean worm burden reductions compared to mice treated with 120mg/kg of Tcbz. Mice treated with Pzq and Tcbz (80mg/kg-120mg/kg) significantly higher
worm burden reductions compared to mice treated with 80mg/kg of Pzq and mice treated with 120mg/kg of Tcbz. The worm burden reductions in mice treated with the highest combination dose of Pzq and Tcbz (250mg/kg-250mg/kg) were significantly higher than all other treatment groups (P<0.05). The order of efficacy according to percentage reduction of worm burden is Pzq-Tcbz (250mg/kg-250mg/kg) > Pzq-Tcbz (80mg/kg-120mg/kg) > Pzq (80mg/kg) > Tcbz (120mg/kg).

There was no significant differences in the number of male and female worms recovered from untreated mice. Praziquantel (80mg/kg), triclabendazole (120mg/kg) and Pzq-Tcbz (80mg/kg-120mg/kg) treated mice showed higher female worm burden reductions that were statistically significant (P<0.05). Infected mice treated with the highest drug combination of Pzq-Tcbz (250mg/kg-250mg/kg) showed significantly higher male worm burden reduction.

No premature deaths or adverse behavioural symptoms were noted for the treatment groups.
Table 6.3: Worm burden of mice infected with *Schistosoma mansoni* and treated with praziquantel (Pzq; 80mg/kg), triclabendazole (Tcbz; 120mg/kg), Pzq-Tcbz combination (80mg/kg +120mg/kg) and Pzq-Tcbz combination (250mg/kg +250mg/kg). Standard deviation and standard errors are presented in parenthesis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of worms</th>
<th>Mean (SD)</th>
<th>Mean males (SD)</th>
<th>Mean females (SD)</th>
<th>% Mean worm reduction (SE)</th>
<th>% Female worm reduction (SE)</th>
<th>% Male worm reduction (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td></td>
<td>47 (11.5)</td>
<td>22.4 (5.6)</td>
<td>24.6 (6.1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pzq 80</td>
<td></td>
<td>32.6 (5.68)</td>
<td>16.2 (2.95)</td>
<td>16.4 (3.21)</td>
<td>30.6 (2.54)</td>
<td>33.3 (1.32)</td>
<td>27.7 (1.43)</td>
</tr>
<tr>
<td>Tcbz 120</td>
<td></td>
<td>36.6 (4.04)</td>
<td>18.2 (3.11)</td>
<td>18.4 (1.52)</td>
<td>22.1 (1.8)</td>
<td>25.2 (1.39)</td>
<td>18.8 (0.68)</td>
</tr>
<tr>
<td>Pzq - Tcbz (80+120)</td>
<td></td>
<td>26.6 (4.83)</td>
<td>13.2 (2.39)</td>
<td>13.4 (2.7)</td>
<td>43.4 (2.16)</td>
<td>45.5 (1.07)</td>
<td>41.1 (1.21)</td>
</tr>
<tr>
<td>Pzq - Tcbz (250+250)</td>
<td></td>
<td>12.4 (4.04)</td>
<td>5.6 (2.88)</td>
<td>7.2 (1.3)</td>
<td>73.6 (1.8)</td>
<td>70.7 (1.29)</td>
<td>75 (0.58)</td>
</tr>
</tbody>
</table>
6.4 Discussion

The results of this study demonstrated that Tcbz at 120mg/kg was partially effective in treating mice infected with *S. mansoni* as evidence by a reduction in the mean worm burden. The reduction in worm burden following treatment with Tcbz is lower compared to the worm burden reductions observed by Keiser *et al.* (2006) for the Egyptian strain (35.5%) at 120mg/kg, but is considerably higher than those observed for a Liberian strain assessed at 400mg/kg (6.6%). The subcurative dose of Pzq used in this study (80mg/kg) was derived from published observations by Pica-Mattoccia & Cioli *et al.* (2004). Cioli *et al.* (2004) suggested that Pzq-susceptible isolates possessed ED$_{50}$ values that were <100mg/kg, with results suggesting a mean ED$_{50}$ of approximately 70±7 mg/kg. In a separate study by Pica-Mattoccia & Cioli (2004), an ED$_{50}$ value of 80.1mg/kg was reported using mature, bisexual worms. In this study, the worm burden was only reduced by 30.6% following treatment with 80mg/kg Pzq. The difference observed is likely to be a result of the dosing regimen employed. Pica-Mattoccia & Cioli (2004) treated mice orally for five consecutive days using different doses of Pzq. Keiser *et al.* (2006), on the other hand, only administered single doses of Pzq. This may have accounted for the mortalities of mice observed in Keiser *et al.* (2006) for both treated and untreated groups. In this study, a single dose of 80mg/kg was used. It is also important to note that different isolates of *S. mansoni* possess variable susceptibilities to drugs (Soliman *et al.*, 1986; el mansouiry & Bayoumi, 1995). Botross *et al.* (2006) further demonstrated that *S. mansoni* isolates that were less sensitive to Pzq induced lower inhibition of hepatic drug-metabolizing enzymes, resulting in higher metabolic transformation of Pzq.

In addition, treated mice were sacrificed four days after treatment compared to four weeks and three weeks used by Keiser *et al.* (2006) and Pica-Mattoccia & Cioli (2004) respectively. This time-point was used because Pzq and its metabolites are eliminated in humans within four days after oral dosing (Dayan, 2003). Mice were also perfused within four days instead of extended periods of time to minimize deaths associated with prolonged infections and incipient host morbidity. While most *in vivo* anti-schistosomal challenge trials were analyzed two to four
weeks post-drug administration (Keiser et al., 2006, Xiao et al., 2006, Pica-Mattoccia & Cioli, 2004), the results of this study reflected the immediate effects post-drug administration. Therefore, the lower than expected reduction observed in this study was likely to be a result of variations within the dosing methodologies and inherent variations in strain-drug susceptibilities, although adult worm resistance and (or) loss of drug sensitivity should not be discounted.

The worm burden reduction observed in this study suggested that the combinations have the potential to exert additive effects against *S. mansoni* worms. Individual doses of Pzq and Tcbz induced a 30.6% and 22.1% reduction in worm burden in mice infected with *S. mansoni*. The theoretical efficacy of the combination assuming an additive effect would be 45.9% (i.e. 30.6% + [22.1/100 × (100-30.6)])). Therefore, the observed efficacy of 43.4% ± 2.16% suggests a drug addition effect for a combination of Pzq and Tcbz (80mg/kg 120mg/kg) *in vivo*.

When Pzq-Tcbz was administered at a higher combination dose (250mg/kg + 250mg/kg), a mean worm burden reduction of 73.6% was observed. The purpose of this combination dose was to assess the effects of the drugs at high concentrations, as well as the potential toxicity to treated mice. No mice died as a result of the experiment, and no signs of toxicity were observed during the trials. Pica-Mattoccia & Cioli (2004) demonstrated an approximate 80% worm burden reduction when a total of 250mg/kg of Pzq was administered daily for five days. While 250mg/kg doses of Pzq and Tcbz were not assessed as single doses in this experiment, it is unlikely that the combination would have induced a reduction of worm burden above 80%. This is based on previous work by Keiser et al. (2006) who suggested that a high single dose of 500mg/kg Pzq elicited 92.9% worm burden reductions. Keiser et al. (2006) also reported that a total dose of 400mg/kg Tcbz elicited 6.6% worm burden reduction against a Liberian strain, with a worm burden reduction of 18.6% observed for two consecutive doses of 200mg/kg Tcbz. There is no currently no efficacy data in the literature for Pzq and Tcbz administered at 250mg/kg. The lack of comparable data makes it difficult to demonstrate that the high Pzq-Tcbz
combinations assessed (250mg/kg + 250mg/kg) were additive or synergistic. However, this dose regime appeared safe for administration in mice infected with *S. mansoni*.

The hepatic tissue egg load observed in untreated control mice was in agreement to the observations of Keiser *et al.*, (2006) However, lower intestinal egg loads compared to hepatic egg loads were persistently observed for all mice groups, which is in contrast to the study performed by Keiser *et al.* (2006). This observed difference is unlikely to be a result of the sampling regimen as all care was taken to ensure that samples were obtained from the middle part of the small intestine. It is possible that the worms used in this study have lower levels of fecundity compared to those used by Keiser *et al.* (2006). There was a significant reduction in egg counts in both liver and intestinal samples from mice treated with Pzq. Interestingly, mice treated with Tcbz showed significant reduction in intestinal egg load but no significant reduction in hepatic egg loads compared to untreated mice. This can be explained by the different mechanisms of drug actions against *S. mansoni* eggs. Praziquantel, in addition to its effects against adult worms, has been shown to cause pre-mature hatching of viable eggs *in vitro* (Matsuda *et al.*, 1983, Katsumata *et al.*, 1988). Praziquantel treatment also induces a reduction in the number of mature eggs and an increase in the number of empty egg shells *in vivo* (Matsuda *et al.*, 1983). Triclabendazole and its metabolites in previous *in vitro* experiments (Chapter four) have been shown to cause the premature rupturing of eggs. It is evident that Tcbz caused a reduction in egg output that was lower compared to Pzq-treated mice.

At the lower combination dose (80mg/kg-120mg/kg), a significant reduction of hepatic and intestinal egg load was observed compared to untreated mice. However, the difference in the tissue egg loads was not significant when compared to Pzq-treated groups. When a higher combination dose was administered (250mg/kg-250mg/kg), a statistically significant reduction in hepatic and intestinal egg load was observed compared to the Pzq treatment group. This demonstrates that a combination of Pzq and Tcbz at doses of 250mg/kg each can be administered to improve treatment efficacy in mice.
There is currently no data available on the pharmacokinetic profile of Tcbz in humans or mice. Alvarez-Bujidos et al. (1993) demonstrated the elimination half-life of triclabendazole-sulphoxide and triclabendazole-sulphone to be 16.86hrs and 13hrs respectively. Lecaillon et al. (1998) demonstrated an elimination half-life of 11.2hrs for the sulphoxide metabolite and 11.8hrs for the sulphone metabolite. On the other hand, Pzq elimination is rapid, with a half-life of one to two hrs in unmetabolized Pzq (Cioli & Pica-Matteocia, 2003) and three to eight hours for metabolites in most species (EMEA, 1996; Dollery, 1999). While the clearance of Pzq metabolites is limited by the rate of metabolism, most metabolites are completely cleared by four days post-oral administration (Dayan, 2003). Tcbz and its metabolites, on the other hand, bind strongly to serum albumin and can remain in plasma for extended periods of time in treated animals. The difference in the rate of elimination of both Pzq and Tcbz could explain why more dead eggs were observed in Tcbz-treated mice compared to Pzq-treated mice. Where Pzq and its metabolites are cleared rapidly, serum-bound Tcbz and its metabolites may have continued to exert ovicidal effects.

At lower combination doses, a large reduction of immature eggs was observed. At the higher combination dose, there were significantly less eggs at immature stages of development. Interestingly, both drug combinations induced large reductions in the number of first, second and third stages of immature eggs, with most eggs observed at mature stages of development. It is not possible to identify the exact cause, but it may be possible that the drugs caused a cessation or a reduced capacity in egg laying, as evidenced by the lack of immature eggs. It was also possible that the number of mature eggs observed were ‘residual’ eggs that were in transition within the mesentery or small intestines, and were laid prior to drug administration. Moreover, it has been noted in previous in vitro experiments (Chapter 3) that adult females continue to expel eggs following treatment even though the eggs were largely unviable. While the mean numbers of dead eggs for both combinatorial treatment groups were not statistically different to those of the Pzq-treated group, it is important to note that a significant egg burden reduction was observed in both intestinal and hepatic samples.
7.1 Introduction

Schistosomiasis affects more than 600 million people annually, resulting in a loss of between 1.53 million and 4.5 million disability adjusted life years worldwide (Gryseels et al., 2006; Utzinger & Keiser, 2004; WHO 2002). Current schistosomiasis control programmes are based on the provision of a single annual dose of praziquantel (Pzq) to reduce morbidity associated with the disease. The administration of a single dose rate of 40mg/kg Pzq has been well-received and its price has significantly reduced following the expiration of its patent in the early 90s. Although competitive pricing from generic producers of Pzq has enabled greater public acceptance, operational expenses such as distribution and delivery can still add considerable costs to control programmes. While care is taken to ensure that there is minimal, indiscriminate use of Pzq, such measures are difficult to enforce in developing countries, particularly when drugs are administered through mass treatment schemes.

Recent studies have shown that triclabendazole (Tcbz) may be suitable for the treatment of human schistosomiasis (Khalil, 2000; Schmidt, 1998; Mansoury, 1997; el Sayed & Allam, 1997). However, further research is required because some studies have shown that the efficacy of triclabendazole and its metabolites is variable in vivo (Keiser et al., 2006). One approach that has not been explored is the use of combinations of Pzq and other trematocides such as Tcbz.

Drug combinations are increasingly being used for the treatment of parasitic diseases such as malaria where drug resistance has limited the efficacy of existing compounds. Whilst there is no convincing evidence of Pzq resistance in schistosomes (Gryseels et al., 2001), studies have consistently shown that standard doses of 40mg/kg Pzq generally only cure 70-90% of human infections (Stelma et al., 1995; Kumar & Gryseels, 1994; Davis, 1993; WHO, 1993; Gryseels et al., 1987). In Senegal, unusually low cure rates of 18-38% have been reported by Danso-Appaiah & De Vlas (2002), raising concerns for the emergence of resistance or tolerance to Pzq.

Triclabendazole was used in this study because: (1) Tcbz and its metabolites are currently ‘off-patent’ and are available from generic manufacturers, (2) Tcbz is cheap to produce and there are
existing distribution chains in endemic regions for treating other trematode infections of animals, (3) Tcbz has regulatory approval for veterinary use with established efficacy, pharmacokinetic and mammalian toxicity profiles and may reduce the costs of obtaining regulatory approval for human use, (4) the WHO has expressed a desire to assess the use of Tcbz for treating parasite co-infections of *Schistosoma* and *Fasciola* (TDR, 2005), (5) there are no alternative drugs for the treatment of human schistosomiasis and (6) there is a need to mitigate capital risks where the funding for drug development and delivery are highly constrained.

Cost effectiveness analyses for human schistosomiasis have been performed to evaluate the efficacy of existing chemotherapeutic schemes, delivery strategies and diagnostic technologies. However, the use of cost effectiveness analyses for schistosomiasis control programmes is challenging due to the complexities of valuing the benefits arising from each programme. The aim of this chapter is to develop an economic model to evaluate the cost effectiveness of a new combination drug for the treatment of human schistosomiasis in a region of intense transmission where the efficacy of Pzq treatment is low. The cost-effectiveness of a drug combination of Pzq and Tcbz can be determined by comparing the costs to treat a target population with the benefits derived from treatment as a measure of (1) the gross national income (GNI) saved and (2) the number of days lost to disease despite treatment.
7.2 Materials and Methods

7.2.1 Data sources

Data for this cost-effectiveness analysis were primarily derived from peer-reviewed literature and published sources. Economic data projections were obtained from electronic sources such as the World Bank (http://www.worldbank.org, 2007) and the International Monetary Fund (http://www.imf.org, 2007). Epidemiological and population projections were derived primarily from the WHO (http://www.who.int, 2007) and US Census Bureau (http://www.census.gov, 2007).

7.2.2 General assumptions

Several assumptions were necessary for the development of this analysis:

- The Pzq-Tcbz combination would be available in a single dose formulation,
- The combination therapy would be administered via existing mass treatment programme frameworks,
- The combination therapy can increase the efficacy of treatment for human schistosomiasis through its additive effects,
- The combination therapy is assumed to be active against all species of schistosomes and will be available on a global scale,
- Two drug combinations using different concentrations of Pzq and Tcbz at 1-1 drug ratio (600mg-600mg) and 0.5-0.5 drug ratio (300mg-300mg) were used,
- There are negligible adverse drug interactions between Pzq and Tcbz,
- Further research and development as well as regulatory approval will be achieved at minimal expense over a period of five years.

All costs were expressed in United States dollars (US$) for the present value of money (2007). For cost projections, prices were inflated at a global core inflation rate of 3% per annum (pa.) from 2007 to 2030 (http://www.imf.org/external/pubs/ft/weo/2007/01/index.htm, 2007).

7.2.3 Drug and operational costs

Current and past median prices for one 600mg Pzq tablet was derived from the Management Sciences for Health International Drug Price Indicator Guide for the years 1996 to 2006.
(http://www.msh.org/resources/publications/IDPIG_2004.html, 2007). The International Drug Price Indicator lists pharmaceutical prices on the international market including the list of suppliers, international development agencies and government procurement agencies. The median prices depicted in the guide reflect the median prices of purchase from nine major suppliers in the international market.

On average, two 600mg Pzq tablets are required to treat each child annually, whilst an adult would require an average of three to five 600mg tablets (Gabrielli et al., 2006; Guyatt et al., 1994). The current total cost of treating a child (age 5-14) is approximately US$0.30 at a dose of 60mg/kg body weight (Fenwick et al., 2003; Kusel & Hagan, 1999; Guyatt et al., 1994).

The average prices for Tcbz were derived from the quoted average retail prices for 1kg and 100kg of generic Tcbz from five Chinese suppliers (www.chemexper.com): CstChem Ltd., Labseeker Co. Ltd., Shanghai Pi Chemicals Ltd., HalloChem Pharma Ltd. and Yick-Vic Chemicals & Pharmaceuticals (HK) Ltd. Suppliers that provided prices outside of the average price range per kg were not included in the analyses. The average market value of a 250mg tablet of Tcbz was obtained from the WHO (www.who.int, 2004).

The sale prices for a single 600mg-600mg tablet of Pzq and Tcbz in 2007 was calculated using the estimated gross margin for a 600mg tablet of Pzq in 2005. The gross margin is the ratio of gross profit to sales revenues expressed as a percentage. A gross margin of 83.5% was calculated for each 600mg Pzq tablet (Reich et al., 1998). The prices of each Pzq and Tcbz tablets were then increased using the average percent inflation (3%) of Pzq tablets from 2000 to 2005. A loss of 3% of raw materials during production was also assumed (Reich et al., 1998).

The operational cost for the delivery of the combination therapy for children and adults was based on the existing frameworks to deliver Pzq treatments. Table 7.1 shows the sources used to determine the mean operational costs and drug costs used in this analysis.
Chapter 7

Table 7.1: Sources of operational costs for the delivery of praziquantel treatments.

<table>
<thead>
<tr>
<th>% Cost</th>
<th>Operational</th>
<th>Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gabrielli et al. (2006)</td>
<td>30.6</td>
<td>69.4</td>
</tr>
<tr>
<td>Kabatereine et al. (2006)</td>
<td>11.3</td>
<td>88.7</td>
</tr>
<tr>
<td>Van der Werf et al. (2004)</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>The Partnership for Child Development (1999)</td>
<td>32</td>
<td>68</td>
</tr>
<tr>
<td>The Partnership for Child Development (1999)</td>
<td>19</td>
<td>81</td>
</tr>
<tr>
<td>Guyatt et al. (1994)</td>
<td>24</td>
<td>76</td>
</tr>
</tbody>
</table>

7.2.4 Patient number projections

The total global demand and availability of Pzq is summarised in Table 7.2.

Table 7.2: Current availability of praziquantel (600mg) and the current proportion of people with access to praziquantel (Pzq) treatment.

<table>
<thead>
<tr>
<th>Millions</th>
<th>Proportion (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global demand for Pzq (tablets)</td>
<td>424</td>
<td>Garbielli et al. (2006) Reich et al. (1998)</td>
</tr>
<tr>
<td>Current availability of Pzq (tablets)</td>
<td>89</td>
<td>Garbielli et al. (2006) Reich et al. (1998)</td>
</tr>
<tr>
<td>Current availability</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Number of people requiring Pzq</td>
<td>86.5</td>
<td>Brady et al. (2006)</td>
</tr>
<tr>
<td>Number of people targeted</td>
<td>22</td>
<td>Brady et al. (2006)</td>
</tr>
<tr>
<td>Current treated</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

The global population and its rate of increase (or decrease) by age group were based on current and projected data available on the US Census Bureau website (www.census.gov/ipc/www/idbpyr.html, 2007). The rate of increase in the total number of persons infected with schistosomiasis from 2007 through to 2030 was assumed to be proportional to this rate of increase. Approximately 200 million people are assumed to be
infected with schistosomiasis (Yosry, 2006). Eighty percent of the 200 million people currently inhabit sub-Saharan Africa, where the annual mortality is estimated to be 280,000 people (Southgate et al., 2005).

### 7.2.5 Cost-effectiveness analysis

The cost-effectiveness of the new drug combination was compared to the cost-effectiveness of Pzq treatment in Senegal. In particular, areas of intense *S. mansoni* transmission in Senegal, such as *areas in proximity to dams*, were used in this analysis. Senegal was chosen as previous studies have suggested low treatment efficacies using 40mg/kg of the Pzq. Table 7.3 shows the epidemiology and economic assumptions used to develop the analysis.

#### Table 7.3: Demographic, epidemiological and economic data for Senegal.

<table>
<thead>
<tr>
<th>Economic indicators</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNI per capita/ per day (US$)</td>
<td>2.27  <a href="http://www.doingbusiness.org">www.doingbusiness.org</a>, (2007)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Epidemiology indicators</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion at risk of schistosomiasis (%)</td>
<td>35.30  Steinmann et al., (2006)</td>
</tr>
<tr>
<td>Total at risk</td>
<td>4115335 See Electronic Appendix B</td>
</tr>
<tr>
<td>Proportion at risk near large dams (%)</td>
<td>2.20  Steinmann et al., (2006)</td>
</tr>
<tr>
<td>Total at risk near dams</td>
<td>90537 See Electronic Appendix B</td>
</tr>
<tr>
<td>Breadwinner proportion (%; male adults&gt;15yrs)</td>
<td>0.46  <a href="http://www.gvu.unu.edu/about.cfm">www.gvu.unu.edu/about.cfm</a>, (2007)</td>
</tr>
<tr>
<td>Total number of breadwinners at risk</td>
<td>41828 See Electronic Appendix B</td>
</tr>
</tbody>
</table>

The numbers of days lost due to *S. mansoni* infections and the resulting numbers of days saved due to Pzq treatment was calculated based on a study by Blas et al., (2006) for *S. japonicum* infections. The model assumes that a Pzq-Tebz drug combination has 100% efficacy while standard Pzq treatment has only 70-90% efficacy in Senegal.
7.2.6 Stochastic model development

A stochastic model (CostMod v1.0) was developed using Microsoft Excel™ (Microsoft Inc., Redmond) with the PopTools v.2.7.5 add-in (CSIRO, Canberra). PopTools is an add-in for PC versions of Microsoft Excel™ that allows for the analysis of matrix population models and simulations of stochastic processes. The input parameters are represented by distribution functions to account for the inherent variabilities of each parameter (Vose, 1998). The input distribution ensures that for each parameter value that is randomly drawn from Monte Carlo sampling, there is an associated probability that the parameter will assume this value. The re-calculation of the model is known as an iteration, where each iteration uses a value randomly drawn from the input distribution. Using PopTools, ten thousand iterations were generated for each simulation using Monte Carlo sampling. The Monte Carlo output for each sampling or simulation event is depicted according to the median, 2.5\(^{th}\) lower and 97.5\(^{th}\) upper confidence (95% confidence intervals) values. Input parameters were modelled by assigning expert (PERT) or normal confidence distributions. The PERT distribution is used exclusively for modelling expert estimates (Vose, 2004). All PERT and normal distribution estimates used in this analysis was derived from published and peer-reviewed sources. The model output is expressed as the total gross national income (GNI) and the number of days lost despite treatment (reduced illness days) as a result of drug combination treatment (Electronic Appendix B). Table 7.4 shows the distribution parameters of the variables used in CostMod. Normal distributions were parametised using the mean and standard deviations while PERT distributions were parametised using the minimum, most likely and maximum estimates with a weighting of four.

Table 7.4: Distribution parameters of variables used in CostMod for the estimation and calculation of drug production price, operational costs and days lost due to schistosomiasis. The mean and standard deviations of the distribution are presented in parenthesis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Distribution parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Production price of triclabendazole/kg (US$)</td>
<td>Normal (14.6, 0.53)</td>
</tr>
<tr>
<td>Production price of praziquantel/kg (US$)</td>
<td>Normal (35.13, 10.7)</td>
</tr>
<tr>
<td>Operational costs of drug combination (US$)</td>
<td>PERT (31.2, 16.8)</td>
</tr>
<tr>
<td>Days lost to <em>Schistosoma mansoni</em> (Days)</td>
<td>PERT (9.08, 2.76)</td>
</tr>
</tbody>
</table>
7.3 Results

7.3.1 Production costs of praziquantel and triclabendazole

The cost to purchase the raw materials for the manufacture of Pzq in 1993 was an estimated US$85 per kg (Reich et al., 1998). In 2007, the cost of raw materials has decreased to ~US$34 per kg. At a production loss of 3% (~66 tablets) from each kg of raw materials, approximately 1600 Pzq tablets (600mg) can be manufactured. The cost to produce each tablet is US$0.02.

Ciba-Geigy’s patent for triclabendazole expired on the 3rd April, 2006. Prices of Tcbz prior to the expiration of its patent (2006) were not included in this analysis. The average quoted price/kg for 100kg of Tcbz from five generic manufacturers of Tcbz was US$14.60, compared to the average quoted price of US$105.25/kg for 10kg of the compound. At US$14.60/kg for raw Tcbz, the cost to produce each 600mg tablet is US$0.009.

7.3.2 Sale price of praziquantel and triclabendazole

The median supplier price for a 600 mg Pzq tablet was compiled from the International Drug Price Indicator (www.msh.org, 2007). The median sale price derived from eight manufacturers was approximately US$0.1173 for 2005 (see Electronic Appendix B: Purchase Costs worksheet). At a production cost of US$0.02, the gross margin is approximately is 83.5% or US$0.098. The average price inflation of Pzq tablets was approximately 3% from 2000 to 2005. Assuming that all manufacturers inflate their sale prices by 3%, the new mean supplier price for 2007 will be US$0.133.

Assuming the same gross margin of 83.5%, the sale price for a 600mg tablet of Tcbz is US$0.055. Table 7.5 shows the estimated production costs and the resultant sale price of each 600mg tablet of Pzq and Tcbz. For a detailed breakdown of production costs and sale prices, please see (Electronic Appendix B: Production Costs worksheet)
Table 7.5: Production costs and sale price of one 600mg tablet of praziquantel (Pzq) and triclabendazole (Tcbz) in 2007.

<table>
<thead>
<tr>
<th></th>
<th>Cost (US$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pzq</td>
</tr>
<tr>
<td>Raw materials/kg</td>
<td>34</td>
</tr>
<tr>
<td>Number of tablets/kg*</td>
<td>1600</td>
</tr>
<tr>
<td>Cost to produce/tablet</td>
<td>0.02</td>
</tr>
<tr>
<td>Gross margin at 83.5%</td>
<td>0.098</td>
</tr>
<tr>
<td>Sale price per tablet (2007)</td>
<td>0.118</td>
</tr>
</tbody>
</table>

* Assumes 3% production loss

7.3.3 Sale price of praziquantel-triclabendazole drug combinations

Each child is currently given an average of two 600mg Pzq tablets per treatment and an adult is given five tablets per treatment (Guyatt et al., 1994; Guyatt & Chan, 1998). The Monte Carlo simulated mean costs to treat children and adults per year using the drug combinations are shown in the following Table 7.6.

Table 7.6: The mean costs to treat a child and an adult per year derived from Monte Carlo simulations. The 95% confidence intervals are presented in parenthesis.

<table>
<thead>
<tr>
<th></th>
<th>Cost in (US$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Child</td>
</tr>
<tr>
<td>Number of tablets per treatments</td>
<td>2</td>
</tr>
<tr>
<td>Number of treatments per year</td>
<td>1</td>
</tr>
<tr>
<td>Median cost of combination</td>
<td></td>
</tr>
<tr>
<td>600mg-600mg drug combination</td>
<td>0.376 (0.220, 0.534)</td>
</tr>
<tr>
<td>300mg-300mg drug combination</td>
<td>0.189 (0.108, 0.268)</td>
</tr>
</tbody>
</table>

A detailed breakdown of the Pzq-Tcbz drug combination sale price derived from Monte Carlo simulation is illustrated in Electronic Appendix B: Production Costs worksheet.
7.3.4 Patient number projections

Table 7.7 shows the total number of children (ages <15) and adults (ages >15) and the projected rates of increase in each population group in five year increments (www.census.gov/ipc/www/idbpyr.html, 2007).

Table 7.7: Global and Senegalese population distribution of children and adults from 2007 to 2030 in five year increments.

<table>
<thead>
<tr>
<th>Year</th>
<th>Total</th>
<th>Children</th>
<th>Adult</th>
<th>% children</th>
<th>% increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007</td>
<td>6600</td>
<td>1811</td>
<td>4789</td>
<td>27</td>
<td>3.6</td>
</tr>
<tr>
<td>Global</td>
<td>2010</td>
<td>6838</td>
<td>1830</td>
<td>5008</td>
<td>26.7</td>
</tr>
<tr>
<td>2015</td>
<td>7230</td>
<td>1875</td>
<td>5355</td>
<td>25.9</td>
<td>5.76</td>
</tr>
<tr>
<td>2020</td>
<td>7608</td>
<td>1916</td>
<td>5692</td>
<td>25.2</td>
<td>5.23</td>
</tr>
<tr>
<td>2025</td>
<td>7964</td>
<td>1931</td>
<td>6033</td>
<td>24.2</td>
<td>4.67</td>
</tr>
<tr>
<td>2030</td>
<td>8296</td>
<td>1926</td>
<td>6370</td>
<td>23.2</td>
<td>4.17</td>
</tr>
<tr>
<td>Senegal</td>
<td>2007</td>
<td>11.7</td>
<td>6.3</td>
<td>5.4</td>
<td>46.0</td>
</tr>
</tbody>
</table>

Approximately 42 million people are treated with Pzq each year based on the assumption that 21% of the total 200 million people infected with schistosomiasis would gain access to treatment. Table 7.8 illustrates the global distribution of children and adults that would be treated with Pzq treatment at the rate of population change shown in Table 7.6.

Figure 7.8: Projections of the number of children and adults receiving praziquantel treatment from 2007 to 2030 in all countries.

<table>
<thead>
<tr>
<th>Year</th>
<th>Total</th>
<th>Children</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007</td>
<td>42.00</td>
<td>11.3</td>
<td>30.7</td>
</tr>
<tr>
<td>2010</td>
<td>43.5</td>
<td>11.6</td>
<td>31.9</td>
</tr>
<tr>
<td>2015</td>
<td>46.0</td>
<td>11.9</td>
<td>34.1</td>
</tr>
<tr>
<td>2020</td>
<td>48.4</td>
<td>12.2</td>
<td>36.2</td>
</tr>
<tr>
<td>2025</td>
<td>50.7</td>
<td>12.3</td>
<td>30.7</td>
</tr>
<tr>
<td>2030</td>
<td>52.8</td>
<td>12.3</td>
<td>40.5</td>
</tr>
</tbody>
</table>
7.3.5 Drug costs, operational costs and total costs of programme

The drug costs for a drug combination of Pzq-Tcbz in the first year of production (2013) are illustrated in Table 7.9.

**Table 7.9:** Projected drug costs of a combination of praziquantel-triclabendazole (Pzq-Tcbz) at different drug ratios compared to the drug costs for Pzq alone in 2013.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Lower CL*</th>
<th>Upper CL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pzq-Tcbz drug costs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>600mg-600mg</td>
<td>4,253,048</td>
<td>2,738,291</td>
<td>7,019,735</td>
</tr>
<tr>
<td>300mg-300mg</td>
<td>2,124,480</td>
<td>1,501,788</td>
<td>4,139,643</td>
</tr>
<tr>
<td>600mg</td>
<td>2,993,203</td>
<td>1,187,873</td>
<td>4,749,331</td>
</tr>
</tbody>
</table>

*Lower CL denotes lower confidence limits; Upper CL denotes upper confidence limits

The average simulated operational costs were 29.6% of the total costs of treatment. The total costs (drug + operational) to deliver a Pzq-Tcbz drug combination (1-1 and 0.5-0.5 drug ratio) treatment programmes compared to Pzq treatment programmes only are summarized in Table 7.10. Detailed cost projections are shown in Electronic Appendix B: Programme Costs worksheet.

**Table 7.10:** Projected total costs to treatment programme using a combination of praziquantel-triclabendazole at different drug ratios compared to the total cost to deliver Pzq treatment alone in 2013.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Lower CL*</th>
<th>Upper CL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pzq-Tcbz drug costs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at 1-1</td>
<td>4,837,540</td>
<td>2,738,291</td>
<td>7,019,735</td>
</tr>
<tr>
<td>at 0.5-0.5</td>
<td>2,717,606</td>
<td>1,501,788</td>
<td>4,139,643</td>
</tr>
<tr>
<td><strong>Pzq drug costs</strong></td>
<td>3,622,203</td>
<td>1,534,281</td>
<td>5,848,886</td>
</tr>
</tbody>
</table>

*Lower CL denotes lower confidence limits; Upper CL denotes upper confidence limits
7.3.6 Cost-effectiveness analysis

Table 7.11 shows the mean simulated outputs for a population of breadwinners in Senegal treated with Pzq at 70, 80, 90 and 100% efficacy rates and Pzq-Tcbz drug combination treatment at a dose rate of 600mg each. Detailed cost-effectiveness data is shown in Electronic Appendix B: Cost-effectiveness analysis worksheet.

Table 7.11: The mean gross national income (GNI) saved per capita per year (US$) for every US$1 spent, the total number of days lost despite treatment and the total number of days saved with praziquantel (Pzq) treatment at 70, 80, 90 and 100% efficacy and a combination of praziquantel and triclabendazole (600mg-600mg).

<table>
<thead>
<tr>
<th>Treatment regimen</th>
<th>Efficacy</th>
<th>GNI US($)</th>
<th>Total days lost despite treatment</th>
<th>Total days saved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pzq</td>
<td>70%</td>
<td>11.72</td>
<td>73728</td>
<td>0</td>
</tr>
<tr>
<td>Pzq</td>
<td>80%</td>
<td>13.66</td>
<td>49152</td>
<td>24576</td>
</tr>
<tr>
<td>Pzq</td>
<td>90%</td>
<td>15.60</td>
<td>24576</td>
<td>49152</td>
</tr>
<tr>
<td>Pzq</td>
<td>100%</td>
<td>19.42</td>
<td>0</td>
<td>73728</td>
</tr>
<tr>
<td>Pzq-Tcbz</td>
<td>100%</td>
<td>13.40</td>
<td>0</td>
<td>73728</td>
</tr>
</tbody>
</table>
7.4 Discussion

The purpose of this study was to determine the cost-effectiveness of delivering a combination drug containing Pzq-Tcbz for the treatment of schistosomiasis. This was achieved by calculating the estimated costs of development, manufacture and supply of the Pzq-Tcbz combination therapy and Pzq therapy. The new treatment was considered cost-effective if the total cost to deliver a treatment programme to reduce morbidity was not significantly greater compared to the costs of delivering Pzq alone. The costs of the analysis are measured as the costs (US$) to treat a targeted population of breadwinners (>15 years old) at risk of infection with *S. mansoni* who live in close proximity to dams. The outputs or benefits are measured as the amount of gross national income (GNI) per capita saved in US$ as a result of treatment and the number days lost despite treatment (or illness days reduced as a result of treatment).

The use of a drug combination of Pzq-Tcbz at the drug ratio of 1-1 or 600mg-600mg was based on a standard dose of Pzq of 40mg/kg in humans and assumed that Tcbz would be administered in the drug combination at the same dose rate. Praziquantel is currently provided in a formulation containing 600mg of active ingredients per tablet. The basis for the use of a 1-1 (600mg-600mg) drug combination ratio in this analysis was to *improve the current efficacy of Pzq*. Recent studies have suggested that the efficacy of Pzq treatment is approximately 70% at the standard dose of 40mg/kg (Danso-Appiah & De Vlas, 2002). This is important because a significant number of worms would survive treatment even when Pzq is 95% effective. More importantly, it has been demonstrated that Pzq at 40mg/kg is <70% effective for treating infections in Northern Senegal and that the low cure rates observed were suggestive of resistance to Pzq treatment (Gryseels *et al.*, 1987; Davis, 1993; WHO, 1993; Kumar & Gryseels, 1994). The use of a combination of Pzq and Tcbz may improve the current efficacy of treatment against human schistosomiasis in regions with intense transmission such as Senegal. Danso-Appiah & De Vlas (2002) suggested that the low cure rates observed in Senegal were a result of the insensitivity of immature schistosomula to Pzq at the time of treatment. If Tcbz is shown to be effective against immature schistosomula in humans, a
combination of Pzq and Tcbz at 600mg each may: (1) increase efficacy rates above 70-90% against mature worms and (2) reduce treatment failure due to the insensitivity of immature schistosomula to Pzq.

The results of this analysis show that a combination of Pzq-Tcbz at a lower dose rate of 300mg-300mg would reduce the total costs of the drug combination by approximately 50%. The application of this dose rate is likely to be well-accepted in view of its lower costs. However, the lower dose rate of the combination is unlikely to significantly increase the efficacy of treatment. The higher dose rate of 600mg-600mg may be more reliable, in light of recent Pzq treatment failures at the standard dose of 40mg/kg (Dabo et al., 2000; Wolfe, 2003; Silva et al., 2005; Alonso et al., 2006). This is further reinforced by Warren et al. (1983), who suggested that the development of drug resistance can be fostered by under-dosage. It is possible that other drug combination ratios of Pzq-Tcbz may be as effective in improving treatment efficacy, such as a combination of 600mg-300mg.

At current supplier prices, the costs of a Pzq-Tcbz drug combination would not be sustainable for mass drug treatment regimens. If Tcbz were to used for the treatment of human schistosomiasis, a significant reduction in sale price is necessary to make the combination sustainable. Currently, the gross margin for the manufacture of Tcbz tablets is approximately 99% for a 600mg tablet at a supplier price of US$0.96, based on the WHO negotiated price of US$0.40 for each 250mg tablet of Tcbz. Albendazole (Abz), another benzimidazole, is currently supplied at US$0.23 for a 400mg tablet, of which US$0.20 was the drug cost (Bundy & de Silva, 1998). The low purchase prices of Abz suggested that the sale price of Tcbz can potentially be reduced to a similar level. If the gross margin for Tcbz was similar to Pzq (83.5%), the cost of each Tcbz tablet can be reduced to approximately US$0.055. Further cost reduction is also likely for Pzq. The Schistosomiasis Control Initiative currently purchases 600mg Pzq tablets at US$0.007 from Shin Poong Pharmaceutical (www.schisto.org, 2007). With improvements in peptide acetal chemistry, radical and solid phase synthesis, the cost to
synthesize Pzq is expected to decrease more in the near future (Todd et al., 2002; El-Fayoummy et al., 2006).

Census data used in this analysis projected only modest increases in the population of children <15 yrs, with a downward trend for the age group expected within the next 20 years, which has the potential to affect the cost of disease control programmes in the future. An increase in the number of adults would exponentially increase the costs of control programmes due to the higher dosage required (five tablets per adult compared to two tablets per child).

While population distribution and growth data were compiled from published sources, the economic model did not implicitly account for the effects of current control programmes, socio-political and environmental variables. It was recently suggested that an increase in ambient temperatures by 1.7°C by 2030 would result in a northward expansion of the current Schistosoma transmission regions in China (LePing et al., 2004). In addition, accurate prevalence rates of schistosomiasis in children and adults were not available. As a result, age and sex group specific prevalence data as well as projected infection rates for each group distribution were based on the projected changes in population rates. Despite such limitations, this economic model can be modified to accommodate different age and sex-specific prevalence rates of schistosomiasis.

According to DiMasi et al. (2003), the current out-of-pocket cost for the development of a new drug is approximately US$403 million (in year 2000 dollars) for approximately 10 years. The level of research funding to further develop a new drug combination is better estimated using public-private partnership models. Using this model, the cost of development of artemisinin combinational treatments for malaria was an estimated US$20 million and the cost of development of synthetic peroxides was an estimated US$35 million (Moran et al., 2005). Public-private initiatives are being increasingly encouraged to provide public goods in a cost-efficient manner (Nishtar, 2004). While it is unlikely that manufacturers or private sources of funds will be made available to progress the development of this combination, the use of
combination chemotherapy is likely to be important developments that will slow the development of resistance (Appleton & Mbaye, 2001).

The results of the cost-effectiveness analysis show that an average of 24,576 to 73,728 days will be saved by increasing the efficacy of current treatment programmes from 70% to 100%. This analysis has assumed that the efficacy of treatment can be increased to 100% by using a combination of Pzq and Tcbz. However, the higher costs of a manufacturing a combination therapy may negate any economic benefits obtained. Therefore, the development of a combination therapy is only justified if 1) it is not possible to increase the efficacy of Pzq treatment through the administration of a higher dose or 2) significant resistance to Pzq develops in schistosomes.

It is difficult to define the level of increased cost that is ‘acceptable’ for a control programme. However, there is evidence that the most expensive drugs are often the most cost-effective because they are significantly more effective than other treatments, and thus reduces the need for repeated therapy (Wiseman et al., 2006). This study has shown that a drug combination based on Pzq and Tcbz is more cost-effective when administered in regions of intense transmission, where current Pzq treatment is not 100% effective. This indicates that the increased treatment costs associated with the drug combination has been ‘compensated’ by its increased efficacy. The analysis also shows that Pzq treatments are more cost-effective if treatment efficacies are \( > 80\% \) when compared to the new combination treatment. The cost-effectiveness model has demonstrated the cost-efficacy of a combinatorial dose of Pzq and Tcbz for the treatment of schistosomiasis, provided that: (1) a dose rate of two tablets per child and five tablets per adult treated once annually for control, (2) the approximate mean cost of a single formulation tablet at a dose rate of 600mg-600mg was US$0.164 (3) the operational costs accounted for an minimal estimated 29.6% of total treatment costs, (4) the Pzq-Tcbz combinations have 100% efficacy for treatment against human mansonal schistosomiasis and (5) that existing treatment using Pzq is \( \leq 80\% \) effective.
8 General Discussion

The aims of this study were to assess the therapeutic potential of triclabendazole (Tcbz) and a combination of praziquantel (Pzq) with Tcbz for the treatment of *Schistosoma mansoni* (*S. mansoni*) infections. The efficacy of Tcbz *in vitro* was initially determined using different life-stages of *S. mansoni*. The synergistic effects of a combination of Pzq and Tcbz were then determined *in vitro* and *in vivo*. The outcomes of this study lend further support to the published observations of Keiser *et al.* (2007); that Tcbz in singular applications showed weak anti-schistosomal effects.

The Puerto Rican strain of *S. mansoni* used for this study was obtained from the Queensland Institute of Medical Research and was adapted for culture according to the protocols recommended by the National Institute of Health, *Schistosoma* Laboratory at the Biomedical Research Institute of Maryland. While all attempts were made to ensure that optimal conditions were maintained, the perpetuation of the life-cycle was consistently hampered by the temperate climate of Perth. The small scale of the culture system presented considerable challenges for the experimental design and evaluation of drugs. Consequently, some components of this research were performed in collaboration with the Queensland Institute of Medical Research.

A detailed dose response analysis of Tcbz and its metabolites against the various life-stages of *S. mansoni* has not been previously performed. Triclabendazole demonstrated considerable efficacy against 6-wk old immature worms *in vitro*. However, there was only moderate efficacy against mature worms *in vitro* as evidenced by worm mortality at high concentrations only. The efficacy of Tcbz and its metabolites were significantly inferior to Pzq. However, the metabolites of Tcbz did demonstrate some interesting effects against adult worms with evidence of irreversible and persistent drug actions against adult worms.

The effects of Tcbz and its metabolites were also analyzed against the egg stages of *S. mansoni* and *S. japonicum* (Chapter Four). This study is the first to quantitatively assess the effects of
Pzq, Tcbz and its metabolites against the egg stages of *Schistosoma* sp. *in vitro*. The result of this study supported the findings of Matsuda *et al.* (1983), who observed the premature hatching of miracidium from eggs exposed to Pzq. Praziquantel-induced hatching was demonstrated to be a dose-dependent process, with hatching observed at concentrations above 10nM. Premature rupturing of the egg shells was observed following exposure of eggs to Tcbz and its metabolites, characterized by gross ultrastructural deformities such as a loss of egg shell integrity, pronounced disruption of unhatched miracidia and premature rupturing. This is important because the premature hatching of eggs may reduce the severity of the host response to *Schistosoma* eggs, which are the primary cause of host pathology.

This is the first time that drug synergism between Tcbz-Sx and Pzq has been demonstrated *in vitro*. Synergism was observed when adult *S. mansoni* and *H. contortus* larvae were exposed to combinations of Tcbz-Sx and Pzq. A weak synergistic or additive effect was observed in *G. duodenalis*. The results of this chapter suggested that a combination of Tcbz-Sx and Pzq may increase the efficacy of treatment for human intestinal schistosomiasis. Moreover, the synergistic effects observed for *H. contortus* suggested that the combination could potentially be used against other parasitic infections in livestock.

The worm burden reductions observed when Pzq and Tcbz were used individually to treat mice infected with *S. mansoni* were lower than those of published studies (Keiser *et al.*, 2007; Pica-Mattoccia & Cioli, 2003). This was likely a consequence of variabilities in strain susceptibility or host responses to treatment. A combination of Pzq and Tcbz at 80mg/kg and 120mg/kg respectively demonstrated additive effects, characterized by reduced worm and egg burden as well as a cessation in egg laying. At higher doses of the combination, significant worm burden reductions were also observed. As this is the first study of its kind, it is difficult to assess the effects of higher combination doses in mice due to the variabilities in dosages administered in other studies. However, this study has shown that the administration of a high dose (250mg/kg each) of Pzq and Tcbz did not show any observable toxicity or side-effects. This study also showed that Tcbz possessed some ovicidal activities, albeit at lower egg burden reductions than
Further work using a range of combination doses is necessary to explore the dose-response relationship of the drug combinations using a larger mouse cohort. In particular, additional studies are required to assess the effects of the combinations against several strains of *S. mansoni*.

A cost-effectiveness model was also developed to assess the cost-effectiveness of applying a combination of Pzq and Tcbz to treat human schistosomiasis (Chapter Seven). The stochastic model served as a theoretical framework for projecting the total costs required to deliver a combination of praziquantel and triclabendazole at a dose rate of 600mg each. Senegal was chosen for this case study as there are published reports indicating that Pzq treatment efficacies were <70%. Using Monte Carlo simulations, the model assessed the cost-effectiveness of the drug combination intervention by measuring the gross national income (GNI) and reduced illness days as a result of treatment with the drug combination. The model demonstrated that a combination of Pzq and Tcbz can potentially be more cost-effective when applied within a focus of intense transmission where the efficacy of Pzq is <80%. This has implications for treatment programmes where the commitment of funds is highly constrained. If approved for use, the Pzq-Tcbz drug combination can potentially be administered as a *contingent* control measure to (1) reduce treatment failures associated with low Pzq efficacies (<80%) or (2) be used as the sole control measure in regions of high disease endemicity only.

The development of combination therapies for schistosomiasis is warranted while Pzq control is still sustainable. Abbott *et al.* (2004) for Sustainable Control of Parasite in Sheep (SCOPS) suggested that combination therapies are best applied when individual drugs are still fully effective and where resistance alleles remain at low frequencies. Ideally, compounds with independent mechanisms of action are used to target different biochemical pathways or to exploit novel mechanisms of action (s). The sequential action of drug combinations against multiple targets will circumvent the development of resistant alleles as the probability for the development of simultaneous mutations is highly unlikely, provided that such mutations are not
linked (White et al., 1999). Compared with the current policy of singular drug regimens, drug combinations have great potential in impeding resistance development.

In addition to minimizing the side-effects of drugs through the delivery of lower, effective therapeutic doses, drug combinations can also maximize and preserve the lifespan of existing compounds in the market and public health systems. Based on the observations above, a drug combination of Pzq and Tcbz-Sx can potentially:

- Demonstrate an amplification of efficacy against *S. mansoni*, leading to reduced treatment frequency,
- Demonstrate narrow spectrum of activity against *Schistosoma* species,
- Demonstrate prolonged and persistent action to compliment Pzq’s rapid onset of anti-schistosomal effects,
- Demonstrate separate and distinct mode of actions,
- Demonstrate activity against both juvenile and adult worms.

It is also to be noted that while combination chemotherapy may prove to be more effective in alleviating disease or Pzq-resistant infections, such strategies may lead to short-term increases in costs. It is, however, likely that the translated long-term benefits will outweigh immediate shortfalls by extending the useful lifespan of existing compounds. As is the case with many drug combinations for the treatment of schistosomiasis, potential limiting factors to the application of the Pzq/Tcbz-Sx drug combination include 1) limited knowledge and public awareness of combinations due to the market dominance of Pzq, 2) further costs of development and studies into appropriate formulation, efficacy and tolerability and 3) operational issues such as indiscriminate use, post-market surveillance and market acceptance (Mutabingwa, 2005).
Recent indications of resistance and (or) tolerance to Pzq necessitates the development of new anti-schistosomal drugs in the event that widespread resistance occurs. The use of a combination of Pzq and Tcbz for treatment is a possible alternative. In view of the current lack of promising candidates, Tcbz in combination with Pzq has the potential to address the shortcomings of administering Pzq alone, and may represent a new line of treatment against human intestinal schistosomiasis.
References


References


140


bancroftian filarial infection variables: assessment after 2 years. 


References


Image from figure 1.1 adapted from *Electronic Source*

<www.who.int/tdr/diseases/schisto/lifecycle.htm> Accessed on 1/05/07.
## Key terminology and definitions

<table>
<thead>
<tr>
<th>Terminology</th>
<th>Definitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Additive effects</td>
<td>A biological response to the exposure to multiple substances that equals the sum of responses of all the substances added together.</td>
</tr>
<tr>
<td>Cost of delivery</td>
<td>The total costs, including drug and operational costs, to deliver control measures.</td>
</tr>
<tr>
<td>Cost of production</td>
<td>The actual cash costs to produce or manufacture.</td>
</tr>
<tr>
<td>Global core inflation rate</td>
<td>A measure of trend movements in aggregate prices which eliminates high frequency movements such as energy and food inflation.</td>
</tr>
<tr>
<td>Gross national income</td>
<td>Previously known as the gross national product, GNI is the total value of goods and services provided by a country.</td>
</tr>
<tr>
<td>Inflation</td>
<td>An increase in the overall level of prices over a period of time.</td>
</tr>
<tr>
<td>Logistical costs</td>
<td>Planning, execution and control costs.</td>
</tr>
<tr>
<td>Operational costs</td>
<td>The costs incurred to maintain operations, eg., salaries, transport, rent, etc.</td>
</tr>
<tr>
<td>Public-private partnerships</td>
<td>Partial privatization of a service in which elements are provided through a partnership between the government and private company (s).</td>
</tr>
<tr>
<td>Revenue</td>
<td>The actual income received from total sales.</td>
</tr>
<tr>
<td>Reduced illness days</td>
<td>The reduction in the number of days afflicted with illness or disease.</td>
</tr>
<tr>
<td>Sub-optimal/therapeutic treatment</td>
<td>The concentration of drugs eliciting less than 100% efficacy.</td>
</tr>
<tr>
<td>Supplier price/ purchase price</td>
<td>The price to purchase goods from the supplier.</td>
</tr>
<tr>
<td>Synergism</td>
<td>The concentration of substances required to elicit an effect that is larger than the sole additive effects of the substances</td>
</tr>
<tr>
<td>Time value of money</td>
<td>The principle that an amount of money anticipated as income in the future is worth less that the amount at present.</td>
</tr>
</tbody>
</table>