Exploitation of the Protein Tubulin
For Controlling
African Trypanosomiasis

Natalie Lydia Giles (BSc.)

This thesis is presented for the Degree of Doctor of Philosophy of the Division of Health Sciences, Murdoch University, July 2005.
I declare that this thesis is my own account of my research and contains as its main content work which has not been submitted for a degree at any tertiary education institution.

..................................................

Natalie Lydia Giles
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To my husband, Murray. Thank you for your infinite support, encouragement and belief in me. I could never have done this without you.
This thesis presents the results of an investigation into the structural protein, tubulin, as a potential target for anti-trypanosomatid drug discovery and vaccine development. Recombinant $\alpha$- and $\beta$- tubulin proteins from *Trypanosoma brucei rhodesiense* were expressed as soluble fusion proteins in an *E. coli* expression system. The recombinant $\alpha$- and $\beta$- tubulins were used to determine the nature of binding of novel trifluralin analogues EPL-AJ 1003, 1007, 1008, 1016 and 1017. Native tubulin from rats was used to determine the extent of binding to mammalian tubulin. The results of this study clearly demonstrate two important aspects of the binding of trifluralins to tubulin. Firstly, they have specific affinity for trypanosomal tubulin compared with mammalian regardless of the chemical composition of the trifluralin analogue tested. Secondly, they have a demonstrably stronger affinity for $\alpha$-tubulin compared with $\beta$-tubulin. In addition, compounds 1007, 1008, 1016 and 1017 have strong binding affinities for $\alpha$-tubulin, with limited binding affinity for mammalian tubulin, which indicates that these compounds selectively bind to trypanosomal tubulin.

The morphology of bloodstream forms of *T. b. rhodesiense* exposed to trifluralin analogues was studied using electron microscopy and immunofluorescence to determine the ultrastructural changes these compounds induce as a result of binding to tubulin. All compounds tested induced severe irreparable damage in *T. b. rhodesiense*, including perturbation of subpellicular microtubules, extensive cytoplasmic swellings, axoneme and paraflagellar rod malformation, disconfiguration around the flagellar pocket and membrane disintegration. These results suggest that the mechanism of action of these trifluralin analogues is through the disruption of polymerization of tubulin into microtubules as a result of binding to $\alpha$-tubulin.

The potential for recombinant trypanosomal tubulins to be used as vaccine candidates was assessed by monitoring parasitaemia and length of survival of mice immunised with the proteins and challenged with a lethal infection of *T. b. rhodesiense*. Although all the mice vaccinated with recombinant tubulin developed a patent parasitaemia and did not survive, they were partially protected because their patency period and length of survival were significantly greater than the control groups. Furthermore, plasma collected from mice immunised with recombinant trypanosomal tubulin contained antibodies that recognized tubulin in a soluble extraction from *T. b. rhodesiense*. The results of this thesis confirm the potential for the structural protein, tubulin, to be used as a target for anti-trypanosomatid drug discovery and vaccine development.
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ABBREVIATIONS

< less than
> greater than
% percent
°C degrees celcius
ABZ albendazole
ATP adenosine triphosphate
b.w. body weight
BZ benzimidazole
CaCl₂ calcium chloride
CNS central nervous system
CSF cerebrospinal fluid
DAPI 4’6-diamidino-2-phenylindole
DB depolymerisation buffer
ddH₂O double deionised water
DMSO dimethyl sulphoxide
DNA deoxyribonucleic acid
dNTP deoxynucleotide triphosphates
EDTA ethylenediaminetetraacetic acid
EGTA ethylene glycol-bis(β-aminoethyl ether) N,N,N’,N’-tetraacetic acid
EM electron microscopy
et al and other people
f femto (10^{-15}x)
fmol femtomoles
g gram
g unit of gravitational field
GST glutathione s-transferase
GTP guanosine triphosphate
HCl hydrochloric acid
hr hour
IC₅₀ concentration required to cause 50% inhibition
IC₇₀ concentration required to cause 70% inhibition
IgG immunoglobulin G
i.m. intra-muscular
i.p. intra-peritoneal
IPTG isopropyl-beta-D-thiogalactopyranoside
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<th>Full Form</th>
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<td>intra-venous</td>
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<td>$K_a$</td>
<td>affinity constant</td>
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<tr>
<td>$k_{off}$</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>$k_{on}$</td>
<td>association rate constant</td>
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<tr>
<td>KCl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>potassium dihydrogen orthophosphate</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani (broth)</td>
</tr>
<tr>
<td>$\mu$(prefix)</td>
<td>micro ($10^{-6}$x)</td>
</tr>
<tr>
<td>m(prefix)</td>
<td>milli ($10^{-3}$x)</td>
</tr>
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<td>moles</td>
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<td>micromoles</td>
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<tr>
<td>mM</td>
<td>millimoles</td>
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<tr>
<td>MAP</td>
<td>microtubule-associated protein</td>
</tr>
<tr>
<td>MBP</td>
<td>maltose binding protein</td>
</tr>
<tr>
<td>MBS</td>
<td>MES buffered saline</td>
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<td>MES</td>
<td>2-[N-morpho-lino]ethanesulphonic acid</td>
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<td>MgCl$_2$</td>
<td>magnesium chloride</td>
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<td>minute</td>
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<td>molecular weight</td>
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<td>nano ($10^{-9}$x)</td>
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<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>di-sodium hydrogen orthophosphate</td>
</tr>
<tr>
<td>Na$_2$PO$_4$</td>
<td>sodium phosphate</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>p</td>
<td>pico ($10^{-12}$x)</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pH</td>
<td>minus log of the hydrogen ion concentration</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>PIPES</td>
<td>Piperazine-1,4-bis(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>pmol</td>
<td>picomoles</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>sp.</td>
<td>Species</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
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<tr>
<td>w/v</td>
<td>weight to volume ratio</td>
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<table>
<thead>
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<th>Table 5.1</th>
<th>Schedule of immunisation of mice prior to infection with <em>T. b. rhodesiense</em>.</th>
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<td>Table 5.2</td>
<td>Mean patent period (days) and length of survival (days) of mice following infection with <em>T. b. rhodesiense</em>.</td>
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PRESENTATIONS

Oral Presentations

“Exploitation of the Protein Tubulin for Controlling Human African Trypanosomiasis.”
Inaugural Australian Society for Medical Research (ASMR) Symposium for Medical Research Week.
Queen Elizabeth II Medical Centre, Western Australia
June, 2005

“Exploitation of the Protein Tubulin for Controlling Trypanosomiasis.”
Postgraduate Seminar Programme
Division of Health Sciences, Murdoch University
May, 2005

“Characterisation of Dinitroaniline Binding to Tubulin from Trypanosoma brucei.”
46th Annual Scientific Meeting of the Australian Society for Parasitology Inc.
Fremantle, Western Australia
September, 2004

“Expression of Recombinant Tubulin from Trypanosoma brucei Using Fusion Partners.”
Inaugural Australian Society for Medical Research (ASMR) Symposium for Medical Research Week.
Queen Elizabeth II Medical Centre, Western Australia
June, 2003

“Exploitation of a Novel Drug Target for the Treatment of Trypanosomiasis.”
Postgraduate Seminar Programme
Division of Health Sciences, Murdoch University
November, 2002
Poster Presentations

“The Binding of New Trifluralin Analogues to Trypanosomal Tubulin.”
Awarded “Best Poster in Drug Discovery or Diagnostics”
Postgraduate Poster Day
Division of Health Science, Murdoch University
November, 2004

“Characterisation of Dinitroaniline Binding to Tubulin from Trypanosoma brucei.”
IX European Multicolloquium of Parasitology (EMOP IX)
Valencia, Spain
July, 2004

“Expression of Trypanosoma brucei rhodesiense α-tubulin as a Soluble Protein in Escherichia coli.”
Postgraduate Poster Day
Division of Health Sciences. Murdoch University
November, 2003

and

45th Annual Scientific Meeting of the Australian Society for Parasitology Inc.
Darwin, Northern Territory
July, 2003

“Exploitation of a Novel Drug Target for the Treatment of Trypanosomiasis.”
Awarded “Best First Year Introductory Poster” and “The Dean’s Prize for Overall Best Poster”
Postgraduate Poster Day
Division of Veterinary and Biomedical Sciences. Murdoch University
November, 2002
Chapter 1

Introduction
1.1 General Introduction to African Trypanosomiasis

African trypanosomiasis is a complex and fatal neurological disease commonly called sleeping sickness in humans and nagana in domestic livestock. The causative agents for these diseases are species of the vector-borne flagellate protozoan *Trypanosoma* (Hoare, 1970b). In sub-Saharan Africa tsetse-transmitted trypanosomiasis is mainly a disease of poor, marginalized and rural populations who depend on their land and labour for their livelihood. The most productive age group (15-45 years) are the most affected, which represents a major threat to economic development (WHO/AFRO, 2005). The estimated annual economic loss is U.S.$4.5 billion in terms of the cost of treatment, losses in meat production, milk yield and manpower plus the lost potential in livestock and crop production (Hursey, 2001; WHO/TDR, 2005). Trypanosomiasis is one of the most debilitating diseases in Africa.

1.2 Classification of Trypanosomes

African trypanosomiasis is caused by haemoflagellates of the genus *Trypanosoma*, subgenus *Trypanozoon* and species *Trypanosoma brucei*, which includes three subspecies: *Trypanosoma brucei gambiense*, *T. b. rhodesiense* and *T. b. brucei*. *Trypanosoma* belong to the order Kinetoplastida, sub-order Trypanosomatina and family Trypanosomatidae (see Table 1.1) (Hoare, 1970b). The Trypanosomatidae are kinetoplastid flagellates, possessing a single motile flagella that is either free or attached to the pellicle as an undulating membrane, a second flagellum represented by a barren basal body only and a small and compact kinetoplast containing DNA fibrils (Vickerman, 1970). All members of the Trypanosomatidae family are parasites in either invertebrate or vertebrate hosts, which are in contrast to other Kinetoplastids that may be free living (Molyneux and Ashford, 1983).
Table 1.1  Classification of Trypanosomes

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<thead>
<tr>
<th>Order</th>
<th>Kinetoplastids</th>
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</tr>
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<td>T. vivax</td>
<td>T. congolense</td>
</tr>
<tr>
<td>T. equiperdum</td>
<td>T. suis</td>
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<tr>
<td>T. evansi</td>
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<tr>
<td>T. brucei</td>
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<tr>
<td>Subspecies</td>
<td>T. b. rhodesiense</td>
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<tr>
<td>T. b. gambiense</td>
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<td>T. b. brucei</td>
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1.3  Morphology and Biology of Trypanosomes

Trypanosomes typically have a spindle-shaped flattened body which is usually curved. The part of the body which is directed forward during locomotion is described as the anterior end. The main structures of the trypanosomal cell are the kinetoplast, nucleus, basal body, flagellum and undulating membrane as shown in Figure 1.1 (Hoare, 1970a).

Figure 1.1  Structure of a trypanosome a) kinetoplast, b) nucleus, c) basal body, d) e) flagellum-marginal and free portion, f) undulating membrane (Based on Hoare, 1970a).
The major diagnostic feature of the order Kinetoplastida is the possession of the kinetoplast, which is a DNA containing organelle. The kinetoplast is disc-shaped and is situated at the base of the flagellum close to the basal body (Hoare, 1970a). The central part of the kinetoplast contains DNA that is composed of fibres densely packed in coils or figures of eight. The capsule of the kinetoplast is continuous with the mitochondrial membrane. From the inner membrane of the capsule cristae typical of the mitochondrial network are found extending into the matrix (Vickerman, 1970). This intimate association between the kinetoplast and the mitochondria is characteristic of trypanosomes and together, the two organelles are often called the kinetoplast-mitochondrion complex (Molyneux and Ashford, 1983).

The plasma membrane is typical in structure and similar to that of animal cells. It is approximately 2-4nm in width and consists of outer dense osmiophilic layers separated by a clear layer (Vickerman, 1974). The plasma membrane is covered by a 10-15nm thick surface coat, which contains two stage specific glycoproteins. The variant surface glycoprotein (vsg) is present in the bloodstream stage and procyclin (parp: for procyclic acidic repetitive protein) is present in the procyclic stage (Pays and Nolan, 1998). Beneath the plasma membrane is a series of pellicular microtubules that span 10nm in width and are separated by spaces 10-25nm in width (Molyneux and Ashford, 1983).

The flagellum emerges from a basal body situated at the posterior part of the cell body (see Figure 1.1) and has a typical 9 + 2 structure of nine outer microtubule doublets with arms and two inner but separate microtubules (Molyneux and Ashford, 1983). The basal body is always found in close proximity to the kinetoplast and the distance between the two organelles remains constant throughout the life cycle (Vickerman, 1970). The flagellum runs forwards along the outer margin of a frilled fold of the pellicle, which extends along the length of the cell body and is known as the undulating membrane (see
Figure 1.1). When the flagellum reaches the anterior end it either becomes free or terminates at the end of the membrane without a free portion (Hoare, 1970a).

The nucleus is spherical or slightly ovoid in shape and is approximately 1.5-2.5μm in diameter. It is surrounded by a nuclear envelope consisting of two membranes separated by 18-25nm, which are punctured at varying intervals by nuclear pores. These nuclear pores are 65-100nm wide and physically link the cytoplasm with the nucleus (Molyneux and Ashford, 1983). Beneath the nuclear envelope, close to the inner nuclear membrane, is usually a thin layer of peripheral chromatin. The nucleolus is centrally placed and is 0.5-1.0μm in diameter (Vickerman, 1974).

The endoplasmic reticulum (ER) is continuous with the outer nuclear membrane and concentrated around the nucleus, which is similar to that of other eukaryotes. They may be rough (containing ribosomes) or smooth (without associated ribosomes). The ER is associated with subpellicular microtubules adjacent to the attachment zone linking the body of the parasite with the flagellum. The golgi apparatus is characteristically closely associated with the flagellar pocket and lies between the flagellar pocket and the nucleus (Molyneux and Ashford, 1983).

1.4 Cytoskeletal Architecture of Trypanosomes

1.4.1 Cytoskeleton

The main components making up the trypanosomal cytoskeleton are the microtubular subpellicular corset, axoneme, basal body, paraflagellar rod, flagellum attachment zone and filaments responsible for parasite attachment to insect tissues (Kohl and Gull,
1998). The highly cross-linked corset of subpellicular microtubule encircles the main cell body, determines the shape of the cell and is present throughout the complete cell cycle (Robinson et al., 1991). The single flagellum emerges from the posterior region of the cell and runs along the length of the cell body. It contains a classical 9 + 2 patterned axoneme. The paraflagellar rod is a highly ordered three-dimensional structure extending along and in close contact with most of the axoneme and is unique to trypanosomes (Hemphill et al., 1991a).

### 1.4.2 Microtubules

Microtubules are important structural proteins and are essential to all eukaryotic cells with functions extending from maintaining cell shape and cell division to cellular secretion and nutrient absorption (Lacey, 1988). There are more than 100 microtubule profiles forming the microtubular sub-pellicular corset. Microtubules are hollow, tubular organelles with an approximate lumen and outer diameter of 15nm and 24nm respectively. They are variable in length and are arranged helically along the axis of the cell with regular spacing of 18-22nm (Hemphill et al., 1991a).

Microtubules compose the most prominent part of the cytoskeleton and are precisely cross-linked to form two defined structures. The main cell body is encircled by a highly cross-linked corset of subpellicular microtubules, which determines its shape and is present throughout the complete cell cycle (Robinson et al., 1991). Microtubules are linked to each other by a series of cross-bridges called microtubule-associated proteins (MAPs). The wide diversity of functions performed by microtubules in the cell is facilitated by the dynamics of polymerisation/depolymerisation and the interaction of tubulin with MAPs and motor proteins (Hirokawa, 1994).
Microtubules are in constant dynamic equilibrium undergoing the addition and subtraction of soluble tubulin units at opposite ends of the developing tubule (Lacey, 1988). Microtubules are polar and their two ends differ in their function. The so-called ‘plus’ end is more active in assembly/disassembly whereas the opposite ‘minus’ end is less active in this process. The polarity of microtubules is essential for their function. The minus end is inserted into the centrosome, which is a microtubule-organizing centre (MTOC) and the formation and breakdown of microtubules are determined by the kinetics of assembly/disassembly that occurs at the plus end of the microtubule (MacRae, 1992).

Microtubules are also aligned in neuronal axons so that direction-specific motor proteins such as kinesin (plus-end directed) and dynein (minus-end directed) transport organelles to specific regions in the cell. The regulation of microtubule dynamics by GTP hydrolysis and MAP binding, the interactions of tubulin with cofactors and the effect of post-translational modifications and isotypic diversity on tubulin function have been studied using a wide variety of biochemical and genetic techniques (Luduena et al., 1992).

1.4.3 Tubulin

Tubulin is the structural subunit of microtubules and constitutes 10% of the total proteins of Trypanosomes (Chan, 1994). The basic building block of the microtubule is the tubulin heterodimer consisting of α- and β- tubulin subunits. The αβ heterodimer bind head to tail into protofilaments. A series of 13 protofilaments interacting laterally comprise a microtubule (see Figure 1.2) (Lacey, 1988). Alpha- and β-tubulin from T. b. rhodesiense comprise 451 and 442 amino acids respectively (Kimmel et al., 1985).
Chapter 1. Introduction

Tubulin monomers are highly acidic with pH values of between 4 and 6 because 40% of carboxy-terminal residues are acidic (Lacey, 1988).

![Figure 1.2 Structure of a microtubule (Childs, 1996)](image)

The tubulin genes of the *T. brucei* genome are arranged in a cluster of 13-18 tandem repeated alternating α/β pairs (Kimmel *et al.*, 1985; Seebeck *et al.*, 1983; Stieger *et al.*, 1984) and contain no introns (Kimmel *et al.*, 1985). This tubulin gene organization is unique in eukaryotes (MacRae and Langdon, 1989). The structural homology between α- and β-tubulin is normally >40% in all eukaryotic cells. They share 84-85% amino acid sequence homology with tubulin from mammals and 74-75% nucleotide sequence homology (Kimmel *et al.*, 1985). Steiger *et al.* (1984) found, based on peptide mapping studies, that the amino acid sequence of trypanosomal α-tubulin was more similar to mammalian tubulin compared to trypanosomal β-tubulin.

An important third member of the tubulin family is γ-tubulin. Gamma-tubulin is approximately 30% identical to α- and β-tubulin and is present in centrosomes and other MTOCs in eukaryotic cells (McKean *et al.*, 2001). It has been shown to bind microtubule minus ends and thought to be responsible for mediating the link between microtubules and MTOCs (Li and Joshi, 1995; Rodionov *et al.*, 1999). The γ-tubulin gene of *T. brucei* is present as only a single copy per haploid genome and the gene
product is estimated to be present at less than 1% the level of $\alpha$- and $\beta$-tubulin (Stearns, et al., 1991). Recent studies have suggested that $\gamma$-tubulin is involved in maintaining the strong conservation of the $9 + 2$ structure of the axoneme in *T. brucei* (McKean et al., 2003). More recently discovered members of the tubulin family present in *T. brucei* include $\delta$, $\varepsilon$ and $\zeta$. These members are not present in all eukaryotic cells and little is known about their function in *T. brucei*. However, there is a reasonable correlation between their occurrence and the presence of the motile axoneme and triplet microtubule basal body (McKean et al., 2001).

Trypanosomal tubulin is modified after synthesis and incorporated into microtubules (Figure 1.2). These post-translational modifications include acetylation/deacetylation, tyrosination/detyrosination, phosphorylation, polyglutamylation, polyglycylation and the generation of non-tyrosinatable $\alpha$-tubulin (MacRae, 1997). Post-translational modification of tubulin can influence its assembly, the ability to bind MAP and the participation of microtubules in events such as cellular differentiation and morphogenesis (MacRae and Langdon, 1989).

In contrast to eukaryotes, prokaryotes do not contain tubulin gene but instead have a similar gene called the filament temperature sensitivity Z (*ftsZ*) gene. The tubulin-like product of the *ftsZ* gene is FtsZ, a 7-amino acid tubulin motif which self-assembles into a ring structure and functions as a cytoskeletal component (Bi and Lutkenhaus, 1991). It has been suggested that proteins ancestral to tubulins may have originated in bacteria prior to the evolution of eukaryotes (Bermudes et al., 1994). Although sequence identity with tubulin is only approximately 10%, FtsZ shares a glycine-rich segment that is considered the tubulin signature motif (GGGTGS/TG) (Nogales, 2000).
1.5 **Vectors of African Trypanosomes**

African trypanosomes are transmitted via tsetse flies (Glossinids) which are Diptera of the family Glossinidae, genus *Glossina* (Pepin and Meda, 2001). In West and Central Africa, the *Palpalis* group of tsetse flies are responsible for transmitting *T. b. gambiense*. These are riverine species of tsetse and include *Glossina palpalis*, *G. tachinoides* and *G. fuscipes*. This group requires a sustained level of humidity and dense riverine habitats in order to survive. They prefer to feed on humans, who are the major reservoirs of infection, although wild and domestic animals play a minor role in particular foci of human African trypanosomiasis (Smith *et al.*, 1998).

In southern Africa the *Morsitans* group of tsetse flies transmit *T. b. rhodesiense*. These are savannah species of tsetse and include *G. morsitans*, *G. pallidipes* and *G. swynnertoni*. This group requires a drier environment in order to survive and live in open woodland areas. They prefer to feed on game animals and domestic livestock who are the main reservoirs for infection. Human infection occurs when people enter these areas, primarily poachers, hunters and gatherers and tourists (Smith *et al.*, 1998). In East Africa *T. b. rhodesiense* is transmitted by the riverine species of tsetse, in particular *G. fuscipes*. The main reservoir of infection is domestic cattle, although human infection occurs in individuals living on farms or close to natural water sources (Pepin and Meda, 2001).

1.6 **Life Cycle of Trypanosomes**

There are three developmental forms of trypanosomes that undergo cell multiplication: the non-infective procyclic, the epimastigote forms in tsetse fly and the long slender
bloodstream forms (trypomastigotes) in mammalian hosts. The two developmental forms that do not multiply are the infective metacyclic form, which is the final stage in the tsetse fly and the short stumpy form in mammalian hosts (El-Sayed et al., 2000).

When a tsetse fly feeds on an infected vertebrate the short stumpy forms that develop in the mammalian host travels with the blood meal into the crop and then into the lumen of the midgut. The developmental cycle in the tsetse fly takes 3-4 weeks depending on the external environmental conditions and the age and sex of the tsetse (Seed and Sechelski, 1989). In the midgut of the tsetse fly the parasites develop into procyclics and replicate by binary fission (see Figure 1.3). The trypomastigotes then develop their mitochondria, undergoes biochemical differentiation, change their surface coat to contain procyclin and begin dividing. From the midgut the trypomastigotes migrate into the salivary glands where they develop into epimastigotes (see Figure 1.3). Epimastigotes are not infective to mammals and they attach to the epithelial cells of the salivary gland via their flagella and continue to divide forming microcolonies (Vickerman, 1985). When the population size of the microcolony reaches a certain density some epimastigotes transform into non-dividing metacyclic trypomastigotes by ceasing division, detaching from epithelial cells and acquiring a variable surface glycoprotein (vsg) coat. Metacyclic trypomastigotes are small, highly motile short stumpy forms which are infective to mammalian hosts (Seed and Wenck, 2003).
Figure 1.3 Schematic representation of the developmental cycle of *T. brucei* in the mammal and tsetse fly vector (Da Silva and Moser, 2003)

When the metacyclic trypomastigotes are injected via the bite of a tsetse fly into the dermal connective tissue of a mammalian host they proliferate and cause a local skin reaction, called a chancre (Barry and Emergy, 1984). Trypomastigotes then enter the bloodstream via the lymphatic system and continue to divide by binary fission into long slender trypomastigotes (Figure 1.3). During high parasitaemia the trypomastigotes continue to divide by binary fission into long slender trypomastigotes (Figure 1.3). During parasitaemia remission, the short stumpy non-multiplicative form is abundant in order to conserve energy while infection continues in the mammal (Seed and Wenck, 2003).
1.7 Pathogenesis of Human African Trypanosomiasis

Human African Trypanosomiasis (HAT) is a debilitating disease and is characterised by two distinct phases; early and late (Molyneux and Ashford, 1983). The three subspecies of African trypanosomes are morphologically identical but differ in their ability to infect various hosts. *T. b. brucei* is a parasite of domestic and game animals and is not infectious to humans because it is lysed by a haptoglobin-like molecule present in human serum called human serum lytic factor (Smith et al., 1998). The subspecies *T. b. gambiense* and *T. b. rhodesiense* cause chronic and acute HAT respectively (Pepin and Meda, 2001).

Following infection through a tsetse fly bite, trypanosomes proliferate at the site of infection resulting in a localized inflammation. A painful chancre usually develops at this site lasting 3-4 weeks (Barry and Emergy, 1984). During the first stage of disease the trypanosomes spread to the draining lymph node and bloodstream where they encounter the host immune system (Vickerman, 1985). Epitopes exposed on the surface of the vsg are readily detected by the host which leads to a humoral immune response. This results in the rapid clearing of the trypanosomes from the bloodstream. However, at any given time the trypanosome changes its vsg coat, resulting in new antigenic variants called variable antigenic types (VATs). This mechanism allows some trypanosomes to escape the antibody response directed against the previous vsg. The continuous interplay between the host immune reaction and antigenic variation of the trypanosome shapes the pattern of parasitaemia that is characteristic of trypanosomal infection (Pays and Nolan, 1998).
The ability of trypanosomes to change their surface coat appears to be the principle mechanism by which they survive in the host (Vickerman, 1974). Through regular changing of the vsg, the trypanosome exhausts the host immune system which is continuously being challenged to react to new surface determinants (Vanhamme and Pays, 1998). The genetic basis of the antigenic variation involves the switching of the gene coding for the vsg (Pays and Nolan, 1998). This means that the vsg is controlled in two ways; it is regulated throughout the life cycle and once the decision to turn on vsg expression is taken, populations expressing different vsgs succeed each other during infection (Vanhamme and Pays, 1998).

As a result of the mounting immune response, the host experiences symptoms such as malaise, headache, nausea, transient fever and anaemia which are nonspecific and are easily confused with those from other diseases such as influenza and malaria (Hide, 1999). However, enlarged submandibular lymph nodes, called Winterbottom's sign, are considered to be characteristic of early sleeping sickness (Burchmore et al., 2002).

The late phase begins when the trypanosomes have invaded other organs including the central nervous system (CNS). This can occur within the first month of infection with T. b. rhodesiense or after months to years with T. b. gambiense infection (Burchmore et al., 2002). Trypanosomes can infect the CNS by crossing the blood-brain barrier. The blood-brain barrier (BBB) consists of endothelial cells separated by tight junctions and has selective permeability. The BBB separates circulating blood from the CNS and maintains the physico-chemical composition of the CNS by regulating the flow of compounds to and from the brain (Enanga et al., 2002). During the course of HAT the integrity of the BBB is compromised allowing the trypanosomes to invade the CNS. Until recently it was not clear whether trypanosomes were directly responsible for the weakening of the BBB or whether it was due to pro-inflammatory mediators responding
to the parasite infection and causing lesions in the BBB. Recent studies have shown that human endothelial cells are directly activated by *T. b. gambiense* in vitro by enhancing the expression of surface molecules involved in cell adhesion, and the synthesis of pro-inflammatory mediators (Girard *et al.*, 2005).

The invasion of the CNS by trypanosomes induces a cell-mediated immune response characterised by an infiltration of mononuclear cells with associated vasculitis and perivascular cuffing (Aloisi *et al.*, 2000). The subsequent pathological lesions in the neural tissue results in the classical symptoms associated with HAT. These include sensory, motor and psychiatric disturbances (Poltera, 1985). Alteration of sleep pattern is the most characteristic clinical sign associated with this phase. The sleep-wake cycle becomes disorganized and fragmented which results in sleep episodes during the day and waking periods during the night (Lundkvist *et al.*, 2004). Other neurological disorders such as tremors, delayed hyperaesthesia, ataxia and psychoses can also occur. Weight loss and endocrine abnormalities are also common symptoms. Brain function eventually deteriorates severely, resulting in coma and then death (Burchmore *et al.*, 2002).

### 1.8 The Re-emergence of Human African Trypanosomiasis

#### 1.8.1 Distribution of Human African Trypanosomiasis

Human African trypanosomiasis (HAT) occurs in 36 countries of sub-Saharan Africa within the “tsetse fly belt” which covers nearly 10 million square kilometres. Within the tsetse fly belt there are more than 200 active foci of HAT (WHO/TDR, 2001). Approximately 60 million people and 50 million animals are at risk from
trypanosomiasis infection, mostly in rural areas. Only 7% of people at risk are under surveillance with regular examination or access to a health centre that can provide screening (Smith et al., 1998). Infection by T. b. gambiense occurs in West and Central Africa and infection by T. b. rhodesiense occurs in East and southern Africa. T. b. brucei occurs in livestock and wild game in all areas of the tsetse fly belt (Hide, 1999).

In the early 1900's strict measures were introduced to control infection. These included the culling of wild animal reservoirs, clearing of vegetation that harboured tsetse flies and the screening of the human population for infection and treatment of those shown to be infected (Barrett, 1999). During the second half of the 20th century widespread civil disturbance and war, declining economies and reduced health financing caused the dismantling of disease control programmes. This has resulted in a dramatic resurgence of HAT in sub-Saharan Africa with T. b. gambiense infection spreading epidemically in large areas of central Africa especially in southern Sudan, the Democratic Republic of Congo (DRC), Angola, Uganda and the Central African Republic (Smith et al., 1998).

The actual number of people currently infected with African trypanosomes is unknown because of underdiagnosis, especially in countries with a high prevalence of infection. The World Health Organization estimates that the current number of cases is between 300,000 and 500,000 (WHO/TDR, 2005). The estimated number of new cases of HAT is approximately 100,000 per year (Pepin and Meda, 2001). In addition, Hursey (2001) estimated that the number of deaths caused by African trypanosomiasis is 100 people and 10,000 cattle per day. Reports from control programmes in individual countries can only give a rough estimate of the extent of this disease because the capacity to collect accurate national statistics varies between countries. This is mainly because it is often
difficult to obtain accurate reports from some remote villages due to instability, civil war, and disruption of communication systems (Pepin and Meda, 2001). In some provinces of Sudan, the DRC and Angola the prevalence of HAT is as high as 20-50% and is the greatest cause of mortality ahead of AIDS (Ekwanzala et al., 1996; WHO/TDR, 2001). Highly endemic countries where the prevalence is moderate but likely to increase include Cameroon, the Central African Republic, Chad, Côte d'Ivoire, Guinea, Mozambique, Uganda and the United Republic of Tanzania. Countries where the endemic level is low include Benin, Burkina Faso, Equatorial Guinea, Gabon, Kenya, Mali, Togo and Zambia. Countries whose present status is not clear are Botswana, Burundi, Ethiopia, Liberia, Namibia, Nigeria, Rwanda, Senegal and Sierra-Leone (Pepin and Meda, 2001; WHO/TDR, 2001).

1.8.2 Human African Trypanosomiasis in Angola

HAT was first recognized as a health problem in Angola in 1871 (Abel et al., 2004). By 1926 the Portuguese colonial government had created the first specialized unit for HAT control and in 1949 a national programme to control sleeping sickness was implemented (Stanghellini and Josenando, 2001). This involved the use of mobile health teams that visited each village at least once a year so that the entire population of Angola had access to diagnosis and treatment. This was effective in reducing the incidence of the disease and its mortality (Smith et al., 1998).

In 1976, Angola became an independent country and experienced a devastating civil war that lasted 20 years. This caused the complete collapse of all treatment programmes and the re-emergence of sleeping sickness as a major cause of mortality (Smith et al., 1998; Stich et al., 2003). In 1974 only 3 new cases were reported compared to 2001
when over 12,000 new cases were reported (Stanghellini and Josenando, 2001; Stich et al., 2003). During times of war the Catholic Church has held privilege to reach both sides of conflict in order to carry out certain operations. In 1996 the church began an HAT program named ANGOTRIP (an acronym combining the two Portuguese words for Angola and trypanosomiasis) which involved setting up treatment centres equipped with health professionals to treat infected individuals, mobile teams of professionals to actively seek infected individuals in remote villages and Lancien type traps along riverbanks, water holes, farms and other tsetse infested areas to control vector populations (Abel et al., 2004). This has helped manage HAT in some areas in Angola however, continuing civil war continues to obstruct efforts to control the problem (Abel et al., 2004; Stich et al., 2003). In 2003, 3,000 new cases of HAT were reported to the WHO from Angola (WHO/AFRO, 2005).

### 1.8.3 Human African Trypanosomiasis in the Democratic Republic of Congo

The first significant outbreak of HAT occurred in the Belgian Congo (now the Democratic Republic of Congo [DRC]) during the 1920’s and it took several decades and a large scale sustained effort to control the disease. The number of cases was reported to be 33,502 in 1930, which dropped to 11,837 in 1940 and continued to decrease over several decades (Van Nieuwenhove et al., 2001). This decrease was due to the concerted effort of mobile teams that screened the population every 6 months and administered treatment to those shown to be infected. By the 1950’s the number of mobile teams reached 250 and in 1959 the number of reported cases was only 1,000 (Ekwanzala et al., 1996).
In 1960, the Belgian Congo became Zaire, an independent country. HAT control efforts ceased because of civil unrest, which resulted in poor road conditions, widespread corruption and poorly paid unmotivated staff. Increasing instability in the area resulted in a devastating civil war that continued until 1967. Epidemics of trypanosomiasis emerged once again (Ekwanza et al., 1996). New control efforts began in 1965 supported by Belgian technical and financial assistance. The control programmes continued until 1990. During this time 30 mobile teams were operating and helped stabilise the incidence to approximately 5-10,000 new cases per year. During 1994 to 1996 the number of cases increased to more than 19,000 (Smith et al., 1998). According to Ekwanza et al (1996), the true number was most likely double that figure. The current level of disease has reached levels comparable to those seen in the early 1930’s with the number of infected individuals in 2001 exceeding 100,000 (Van Nieuwenhove et al., 2001). In 1998, approximately 2% of the total population of the DRC were affected and in some communities the prevalence was as high as 70% (Barrett, 1999). In 2003, 11,000 new cases of HAT were reported from the DRC to the WHO (WHO/AFRO, 2005).

1.8.4 Human African Trypanosomiasis in Uganda

From 1901 to 1916 there were massive epidemics of HAT in Uganda, which resulted in 250,000 deaths (Smith et al., 1998). After this time the disease became confined to a few discrete foci with the major focus being in the Busoga region. This region remains a significant focus for sleeping sickness today (Hide, 1999). During the 1940's an epidemic emerged due to civil war, insecurity and deteriorating economic circumstances (Smith et al., 1998). In addition, changes in agricultural practices led to vegetation changes in areas where cotton was produced which resulted in the overgrowth of
*Lantana camara*, that supported large populations of *G. fuscipes fuscipes* (Abaru, 1985). Between 1976 and 1990 there were 40,000 confirmed cases in the Busoga district and a further 6,000 in neighbouring areas with people dying within three months of initial infection (Smith *et al.*, 1998). Uganda is the only country to have endemic foci of both *T. b. gambiense* in the northwest and *T. b. rhodesiense* in the southeast of the country (Abaru, 1985).

In 1985 control programmes were initiated and health centres were equipped to diagnose HAT and provide treatment. These programmes helped decrease the proportion of late stage patients admitted to hospital from 80% to 25% (Pepin and Meda, 2001). Furthermore, the increased surveillance enabled epidemic areas to be more accurately defined. By 1998 the number of notifications of the disease was less than 20 per month (Smith *et al.*, 1998). A recent outbreak of *T. b. rhodesiense* infection occurred in 2001 outside the defined foci in the district of Soroti and was found to be associated with the importation of infected cattle from areas where the disease was endemic (Fevre *et al.*, 2001). As a result of this, 133,000 people have been put at risk of infection and in 2004, 428 new cases were presented at the only health facility equipped to diagnose and treat the disease in Soroti district. Of these cases, 18 people died at the facility. It was also estimated that a further 299 cases were unreported based on a deterministic model (Fevre *et al.*, 2005).
1.9 Vector Control

1.9.1 Methods of Vector Control

The control of the tsetse fly vector is an important component of programmes to reduce the incidence of HAT (Allsopp, 2001). The first method of controlling tsetse flies involved the slaughter of game animals and clearance of large areas of woodland in order to deprive tsetse flies of food and shelter. This was quickly considered to be environmentally unacceptable and it was during the 1940's that insecticides came into use. Ground spraying of persistent insecticides such as DDT, dieldrin, endosulfan or synthetic pyrethroids became the primary method for controlling tsetse fly populations for over 40 years. Ground spraying of insecticide enabled the eradication of tsetse flies from thousands of square kilometres of land including 230,000km$^2$ in Nigeria alone (Pepin and Meda, 2001). Aerial spraying of residual insecticides also began during the 1940's, which was successful in South Africa and combined with ground spraying kept trypanosomiasis under control in Cameroon for 18 years (Allsopp, 2001). During the 1940's, large scale sequential aerial applications of low-dosage, non-residual aerosols (sequential aerial technique, SAT) were evaluated in Zambia. SAT became the method of choice for trypanosomiasis control in Botswana for 20 years and was also carried out in Zimbabwe, Ivory Coast, Somalia, Nigeria and Uganda (Allsopp, 2001).

Currently, the most widely implemented method for tsetse control is the bait technique using visual or olfactory attractants to lure tsetse flies in order to trap or kill them. The most commonly used trap is the bi-conical trap which was developed in the early 1970's (Holmes, 1997). This trap when impregnated with a mixture of acetone, octenol and cow urine is very effective in trapping tsetse flies (Belete et al., 2004).
More recently, cloth screens impregnated with insecticide (targets) have replaced the
more traditional cone-shaped traps. Blue and black coloured cloth and attractants such
as carbon dioxide, acetone, and urine phenols were found to be effective in luring tsetse
to the target where the applied insecticide killed the flies on contact (Schofield and
Maudlin, 2001). Insecticide-impregnated traps and targets require only a fraction of the
insecticide compared to aerial spraying. Insecticide-impregnated targets are more
effective and cheaper than traps, although the display of dead tsetse flies in traps helps
motivate local people to participate in their use (Holmes, 1997). Traps baited with
attractants have been successfully used in Ethiopia (Belete et al., 2004) and Tanzania
(Kasilagila, 2003). A new multipurpose fly trap, the Nzi trap, consisting of cloth with
blue and black panels and netting to trap the flies has also been used effectively in
Kenya (Mihok, 2002).

Insecticide treated cattle can also be used as live bait. Pour-on insecticides such as
synthetic pyrethroids are highly effective in killing tsetse whilst exhibiting low
mammalian toxicity (Allsopp, 2001). Traps, targets and bait technology are particularly
suitable for community based control programmes with relatively cheap materials and
unsophisticated technology. However, traps and targets require regular supervision to
reduce damage and theft and their effectiveness varies between species and geographic
subspecies of Glossina (Holmes, 1997).

A recent development in tsetse control is the sterile insect technique. This method
involves sterilizing male tsetse flies by irradiation and releasing them by light aircraft in
affected areas. The males engage in sterile intercourse with females. The tsetse
population density is first suppressed, usually with insecticides, before the release of the
sterile male tsetse. A plan was announced in 2000 by the International Atomic Energy
Agency (IAEA) to implement a continent wide release of sterile tsetse in order to eradicate tsetse flies completely from Africa (Stich et al., 2003). This programme involved dispersing flies twice weekly across specific flight lines spaced by a distance of 1-2km. More than 8.5 million flies were released this way from August 1994 to December 1997 and resulted in the eradication of tsetse flies from the island of Zanzibar (Vreysen et al., 2000).

The African governments have recently developed a new initiative, titled the Pan African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC), which aims to eliminate tsetse flies from mainland Africa completely. PATTEC was derived from a decision made by the African Heads of State and Government at the 36th summit of the Organisation of African Unity (OAU), to 'act collectively and rise to the challenge of eradicating tsetse flies from the African continent in the shortest time possible' (Kabayo, 2002). The plan of action utilises all available control methods in order to eradicate tsetse flies from pockets of infestation at a time. Tsetse-free zones are aimed to be created in this way which will then be linked over larger areas. The governments of Ethiopia, Mali, Burkina Faso, Botswana, Kenya, Uganda and Tanzania have begun to implement the plan in selected areas of their respective countries (Kabayo, 2002).

A more recent development is the launch of a five-point strategy by WHO to eliminate most cases of HAT by 2015. The strategy includes the implementation of national sleeping sickness policies in 80% of African countries where the disease is widespread by 2007, and training enough staff to work on disease control by 2008. In addition, targeted vector control interventions are aimed to be in place by 2012 (WHO/AFRO, 2005).
1.9.2 Problems Associated with Vector Control

The main problem with existing tsetse control methods as with many operations in Africa is sustainability. Reinvasion of tsetse flies is inevitable unless the entire population of tsetse is eliminated or cleared areas are protected (Schofield and Maudlin, 2001). During times of civil unrest and associated lack of finance maintaining tsetse control programmes becomes a low priority. As a result of this almost all major tsetse control campaigns implemented during the 20th century have ultimately failed (Allsopp, 2001). This is primarily the reason why PATTEC’s proposal has been met with much scepticism and criticism from other scientists. It has been viewed that the proposal is overly ambitious and that it may have been more feasible to implement a control strategy rather than complete eradication. The success in eradicating tsetse flies from Zanzibar is not considered a good model for the mainland because it is a small island that is isolated from the mainland of Africa by 30km of water (Rogers and Randolph, 2002). The success of this campaign relies heavily on the concerted participation and dedication of international agencies and all African governments, which taken from the history of HAT in Africa, is highly unlikely.

1.10 Vaccines

Currently there are no available vaccines against African trypanosomiasis and prospects for the development of a vaccine are considered to be very poor (Seed, 2001). This is mainly due to the ability of trypanosomes to undergo antigenic variation, where they periodically switch their vsg coat to evade the host immune response. When designing a vaccine, proteins present on the cell surface of the target organism are usually exploited because these proteins are most accessible to the host immune cells. Since trypanosomes have the ability to switch their vsg surface coat, they can escape the
antibody response directed against the previous antigen rendering a vsg vaccine useless (Barbet and McGuire, 1978; Donelson et al., 1998). Despite this, partial protection against *T. brucei* infection has been reported in cattle immunised with viable irradiated *T. brucei* where antibodies elicited by irradiated trypanosomes were found to be specific for antigenic determinants on the vsg exposed on the surface of live trypanosomes (Morrison et al., 1982).

Invariable trypanosomal components have recently been studied as potential vaccines against trypanosomiasis. Cattle were partially protected from *T. congolense* and *T. vivax* infection when immunized with flagellar pocket antigen from *T. b. rhodesiense* (Mkunza et al., 1995) and mice were fully protected against trypanosomiasis infection when immunized with a vaccine comprising trypanosomal microtubule-associated proteins p15 and p52, with aldolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Balaban et al., 1995; Rasooly and Balaban, 2004). In addition to this, mice vaccinated with native trypanosomal tubulin were also shown to be fully protected from *T. brucei* (Lubega et al., 2002a). Mice immunized with paraflagellar rod protein conferred full protection against *T. cruzi* infection (Miller et al., 1996; Wrightsman et al., 1995).

1.11 Treatment of African Trypanosomiasis

1.11.1 Current Treatment Regimes for Human African Trypanosomiasis

Current treatment regimes for HAT rely on a limited number of drugs developed several decades ago and little progress has been made in developing new drugs in the last fifty years with the exception of Eflornithine, which was developed in 1990. There are many
problems associated with current drugs including resistance, toxicity and a lack of guaranteed supply (Barrett and Barrett, 2000). Toxicity is a major problem, with all the drugs inducing side effects of varying severity partly because they were developed prior to the introduction of modern regulatory requirements with respect to toxicity, with the exception of Eflornithine (Barrett and Barrett, 2000). These drugs would probably not have received licenses if they were introduced today (Barrett and Gilbert, 2002).

Infectious diseases are considered neglected when there is a lack of effective, affordable or easy to use drug treatments (Yamey and Torreele, 2002). People in developing countries are too poor to buy drugs and are therefore, largely ignored by the pharmaceutical industry (Veeken and Pecoul, 2000). Of 1393 new drugs marketed between 1975 and 1999, only 16 were for neglected diseases (Trouiller et al., 2002). The lack of profit and the difficulties in synthesizing current drugs have caused some manufacturers to abandon production of certain drugs. The production of Eflornithine, for example, ceased in 1995 by its original supplier Marion-Merrell-Dow, which left the WHO searching for a new supplier (Barrett, 2000). Over the past decade the WHO, with vigorous support from Médecins sans Frontières (Drugs for Neglected Diseases Initiative), have increased their efforts in campaigning for the continued production of current anti-trypanosomatid drugs (Veeken and Pecoul, 2000) and to promote research into developing new drugs for the treatment of HAT (Etchegorry et al., 2001). As a result of the campaigning the pharmaceutical company Aventis have guaranteed to produce Eflornithine, Melarsoprol and Pentamidine for at least 5 years and Bayer will continue to produce Suramin indefinitely (WHO/TDR, 2001). Moreover, the WHO and the public sector have created favourable marketing conditions persuading industry to enter public-private partnerships to tackle neglected diseases such as malaria, HIV and tuberculosis.
The best strategy for ensuring maximum efficiency in treating those infected with trypanosomes is early diagnosis. This is not always feasible, nor is it currently possible to avoid the need for a lumbar puncture to establish the diagnosis by examination of the cerebrospinal fluid (CSF). Lumbar puncture requires trained medical staff to carry out, is invasive and often painful (Bouteille et al., 2003). The criteria currently used for diagnosing HAT are a high leucocyte count in the CSF (>5 cells/μl) or an increase in CSF protein (>37mg/100ml according to the colorimetric method) with the chance detection of trypanosomes in the cell counting chamber. The problem with following these criteria is that evidence of trypanosomes is not necessary to make a diagnosis of infection. This makes the test non-specific as other CNS infections may cause high leucocyte counts in the CSF. In addition, the test is not standardized which makes them unreproducible (Bouteille et al., 2003).

Research into improving diagnosis of CNS involvement in HAT have shown promise, with the finding that trypanosomiasis related immunoglobulin patterns are of value in differential diagnosis. It was proposed that CNS involvement is diagnosed only in patients with >20 cells/μl or with intrathecal IgM synthesis (Lejon et al., 2003). However, sufficient evidence of the efficacy of the suggested diagnostic test is yet to be determined. Initial diagnosis in the field still relies on the detection of trypanosomes, most often with insensitive techniques that have been used for decades. Thick blood smears are usually adequate for initial screening for T. b. rhodesiense infection as parasitaemia is readily detected in the peripheral blood, especially in early infection. However, with T. b. gambiense infection parasitaemia is usually low and too variable for blood smears to be an accurate diagnostic technique. Lymph node aspiration is a common technique for diagnosing early T. b. gambiense infection but requires trained medical staff to perform them (Smith et al., 1998).
Other existing problems with current trypanocidal drugs involve administration and supply of the drug to infected patients. All current trypanocides, with the exception of pentamidine, must be administered by intravenous (i.v) injection and their use is associated with severe side-effects. In addition, the economic instability, deterioration in transport and the public health infrastructure in some epidemic countries, makes it virtually impossible to deliver these drugs (Barrett, 2000). Currently no trypanocide can be administered orally, which means mass administration to areas of need is problematic. In addition, the potentially life threatening side effects that occur with treatment for late-stage trypanosomiasis means that hospitalization of patients is often required for a full month (Gutteridge, 1985). Resistance is also rapidly becoming a problem in the field. The relapse rate in patients treated with melarsoprol in Angola, Southern Sudan, DRC and northern Uganda is 25-30% (Legros et al., 1999).

1.11.2 Pentamidine

Pentamidine isothionate is an aromatic diamidine (Figure 1.4) and was first introduced in 1937 under the trade name Pentacarinate. It is now manufactured under the name of Lomidine® (Pepin and Milord, 1994). It does not cross the blood-brain barrier and is the standard treatment of early *T. b. gambiense* infection or in patients with CNS involvement only to clear blood of trypanosomes prior to treatment with melarsoprol (Docampo and Moreno, 2003). The mode of action of Pentamidine has not been established, although it has been shown to bind to nucleic acids and a network of circular DNA molecules present in the kinetoplast leading to disruption of the structure of the kinetoplast. However, Wang (1995) has reported that this mechanism of action would be insufficient to kill the parasites and therefore, another undiscovered mechanism of action exists.
Treatment using Pentamidine involves 7-10 doses at 4mg/kg body weight (b.w.) by intra-muscular (i.m) injection either daily or every other day (see Table 1.2) (Legros et al., 1999). Local reactions can occur at the site of injection with pain and the formation of sterile gluteal abscesses. Other side effects include pruritus, rash, tachycardia, nausea and vomiting (Docampo and Moreno, 2003). Nephrotoxicity, hepatotoxicity and pancreatic toxicity occur in some patients (Fairlamb, 2003; Pepin and Milord, 1994) and diabetes mellitus can develop after therapy (Burchmore et al., 2002; Nok, 2003). Some resistance has been reported in the laboratory with *T. b. brucei*. Drug-resistant strains were shown to have a diminished ability to import the drug, which is believed to be the primary cause of Pentamidine resistance (Wang, 1995). Fortunately, widespread resistance has not yet emerged in the field (Docampo and Moreno, 2003; Pepin and Milord, 1994).

![Figure 1.4](image.png)

**Figure 1.4** Structures of drugs used to treat human African trypanosomiasis: a) Suramin, b) Melarsoprol, c) Pentamidine and d) Eflornithine (Fairlamb, 2003)
1.11.3 Suramin

Suramin is a polysulphonated naphthylamine polyanionic compound (Figure 1.4). Its original trade name was Bayer 205 and it was first used for the treatment for HAT in 1922 (Wang, 1995). It was developed because its two close analogues trypan blue and trypan red were shown to be effective anti-trypanosomatids in the early twentieth century (Docampo and Moreno, 2003). The main mechanism of action of suramin is still unclear but has been shown to inhibit many enzymes such as dihydrofolate reductase, thymidine kinase and glycolytic enzymes (Wang, 1995). It is thought to inhibit multiple targets which could explain why there has been no significant clinical resistance to Suramin in its 80 years of use (Fairlamb, 2003). Its selectivity for trypanosomes is due to their ability to readily take up the drug. Suramin is slowly excreted because it binds to many serum proteins and its half life of around 90 days is one of the longest ever documented for drugs given to humans (Fairlamb, 2003; Pepin and Milord, 1994).

The present protocol for treatment using Suramin is administration of 5mg/kg b.w. at day 1, 10mg/kg b.w. at day 3 and 20mg/kg b.w. at days 5, 11, 23, and 30. This is administered by slow i.v. injection (see Table 1.2) (Docampo and Moreno, 2003). Subcutaneous or intramuscular injections are not recommended because Suramin causes local inflammation and necrosis (Pepin and Milord, 1994). Suramin is only used during early stages of the disease because it is highly ionic and does not penetrate well into the CNS (~1% of serum levels) (Fairlamb, 2003). It can be used for the treatment of early stage T. b. rhodesiense and T. b. gambiense, although it is generally used only for T. b. rhodesiense most likely due to treatment failures, the duration and cost of treatment and the need for it to be administered by i.v. injection (Pepin and Milord, 1994). The most
The common toxic effect of Suramin treatment is renal toxicity, although it is usually mild (Docampo and Moreno, 2003). Collapse and shock also occurs in some treated patients (Fairlamb, 2003). Delayed reactions include optic atrophy, blindness, exfoliative dermatitis, haemolytic anaemia, jaundice, adrenal insufficiency and severe diarrhoea in some patients (see Table 1.2) (Fairlamb, 2003; Pepin and Milord, 1994).

### Table 1.2 Drug programmes currently in use against Human African Trypanosomiasis.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Eflornithine</th>
<th>Melarsoprol</th>
<th>Pentamidine</th>
<th>Suramin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemistry</td>
<td>difluoromethyl-ornithine</td>
<td>arsenical</td>
<td>diaminidene</td>
<td>sulphated naphthylamine</td>
</tr>
<tr>
<td>Application</td>
<td>intra-venous</td>
<td>intra-venous</td>
<td>intra-muscular</td>
<td>intra-venous</td>
</tr>
<tr>
<td>Indications</td>
<td>early - late stage</td>
<td>early - late stage</td>
<td>early stage</td>
<td>early stage</td>
</tr>
<tr>
<td></td>
<td><em>T. b. gambiense</em></td>
<td><em>T. b. gambiense</em></td>
<td><em>T. b. gambiense</em></td>
<td><em>T. b. gambiense</em></td>
</tr>
<tr>
<td></td>
<td><em>T. b. rhodesiense</em></td>
<td><em>T. b. rhodesiense</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dosage</td>
<td>$4 \times 100 \text{mg/kg b.w/day}$ every 6hr × 7-14 days</td>
<td>$2.2 \text{mg/kg b.w/day}$ × 10 days</td>
<td>$4 \text{mg/kg b.w/day}$ × 7 days</td>
<td>$5 \text{mg/kg b.w Day 1}$ $10 \text{mg/kg b.w Day 3}$ $20 \text{mg/kg b.w 5,11,23,30}$</td>
</tr>
<tr>
<td>Mode of Action</td>
<td>Inhibits biosynthesis of polyamines</td>
<td>unknown</td>
<td>Reduces biosynthesis polyamines</td>
<td>Inhibits enzymes eg RNA polymerase</td>
</tr>
<tr>
<td>Side Effects</td>
<td>convulsions, diarrhoea anaemia, leucopenia thrombocytopenia</td>
<td>reactive encephalopathy polynuerophathy, exfoliative dermatitis, joint pain, fever, malaise g.i. disturbances, headache, renal damage, hypertension</td>
<td>pruritis, rash, tachycardia hepato and nephrotoxicity, pancreatic toxicity nausea, vomiting</td>
<td>nephrotoxicity, blindness exfoliative dermatitis haemolytic anaemia diarrhoea, jaundice collapse, shock</td>
</tr>
</tbody>
</table>

#### 1.11.4 Melarsoprol

Melarsoprol is a trivalent organic melaminophenyl arsenical (Figure 1.4) that was first synthesised over 50 years ago by Freidheim (Docampo and Moreno, 2003). Its trade
name is Arsobal and it is used to treat late stages of \textit{T. b. gambiense} and \textit{T. b. rhodesiense} infection because it is able to cross the blood-brain barrier (Burchmore \textit{et al.}, 2002; Docampo and Moreno, 2003). Melarsoprol is poorly soluble in water, alcohol or ether and is administered intra-venously dissolved in propylene glycol, a solvent that is highly irritant to tissues (Fairlamb, 2003; Nok, 2003). Melarsoprol is very effective but the exact mechanism by which this is achieved remains elusive. The most likely mode of action is that it inhibits glycolytic enzymes leading to a blockage of glycolysis and cell lysis (Wang, 1995). Treatment of trypanosomiasis using Melarsoprol consists of 3-4 series of 3-4 daily injections of 3.6mg/kg b.w. separated by rest periods of 7-10 days (Docampo and Moreno, 2003) (see Table 1.2).

Melarsoprol is extremely toxic and often patients relapse after treatment (Burri and Brun, 2003). The worst side effect is a reactive encephalopathy, which occurs in 5-10% of patients and results in the death of 10-50% of those affected (Docampo and Moreno, 2003; Pepin \textit{et al.}, 1995). Other side effects include polyneuropathy (10%), exfoliative dermatitis (1%), abdominal and chest pain, fever, headache, malaise, joint pain, gastrointestinal disturbances, renal damage and hypertension (see Table 1.2) (Docampo and Moreno, 2003; Pepin and Milord, 1994). Administration of the drug causes pain and thrombophlebitis at the site of injection is common (Nok, 2003). The relapse rate in patients treated with Melarsoprol is 3-10% however, in epidemic areas of \textit{T. b. gambiense}, this rate has increased dramatically in recent years to 30% in northwestern Uganda, 21% in southern Sudan, 25% in northern Angola (Burri and Brun, 2003). Recent studies have shown that men have a higher risk of treatment failure than women, although the cause of this apparent difference is unknown (Pepin \textit{et al.}, 2002).
A new treatment schedule was proposed recently based on pharmacokinetic studies and computer modelling, which decreases the time required for treatment and the amount of drug administered to 10 daily doses of 2.2mg per kg body weight (Blum and Burri, 2002). This new treatment schedule was tested in an open randomised clinical trial involving 500 patients in Angola (Burri et al., 2000). A follow-up was conducted two years after this trial to determine the relapse rate compared with those patients treated using the standard Melarsoprol schedule (Schmid et al., 2004). The findings of this study showed that, although cure rates and relapse rates were the same in both groups, the amount and cost of drug was reduced by approximately 30%. In addition, the number of patients requiring hospitalization was reduced by approximately 50% and the total amount of Melarsoprol given is reduced which will decrease toxicity without reducing efficacy (Schmid et al., 2004). The shorter protocol has recently been recommended by the International Scientific Council for Trypanosomiasis Research and Control for the treatment of late-stage HAT caused by T. b. gambiense (Schmid et al., 2005).

1.11.5 Eflornithine

Eflornithine is the only new drug registered for treatment of HAT in the last fifty years (Burri and Brun, 2003). It is a difluoromethyl ornithine (see Figure 1.4) and was registered for use in 1990 under the trade name of Ornidy (Docampo and Moreno, 2003). Eflornithine is effective against T. b. gambiense infection even in the late stages of the disease (Burchmore et al., 2002; Milord et al., 1992). It inhibits trypanosomal ornithine decarboxylase (ODC), which is the key enzyme in the pathway leading to the biosynthesis of polyamines essential for cell proliferation. Following treatment the trypanosomes become non-dividing stumpy forms that are vulnerable to the host
immune system because they cannot alter their variant surface glycoproteins (Wang, 1995). Eflornithine is therefore trypanostatic rather than trypanocidal (Pepin and Milord, 1994). *T. b. rhodesiense* is resistant to Eflornithine because of its higher ODC turnover (Burri and Brun, 2003).

Large doses of Eflornithine are required in order to be effective against HAT. The treatment protocol is 400mg per kg body weight per day in 4 daily intra-venous infusions for 7-14 days (Burchmore *et al.*, 2002) (see Table 1.2). This costs approximately U.S.$350 per patient for the drug alone. Due to the high cost, Eflornithine is mainly used as a second line treatment after Melarsoprol (Burri and Brun, 2003). Attempts to reduce the treatment course of Eflornithine have not been successful and it has been shown that a 7-day course of Eflornithine treatment is less efficacious compared to a 14-day course with new cases, however, a 7-day course is recommended where infection has relapsed (Khonde *et al.*, 1997; Pepin *et al.*, 2000). Some adverse reactions accompany the use of Eflornithine, which are completely reversible once treatment ceases (Burri and Brun, 2003). These side effects include convulsions and other neurological effects (7%), gastrointestinal disturbance such as nausea, vomiting and diarrhoea (10-39%) and bone marrow toxicity leading to anaemia, leucopenia and thrombocytopenia (25-50%) (Burri and Brun, 2003; Pepin and Milord, 1994).

Eflornithine and the trivalent arsenicals (Melarsoprol) have been shown to act synergistically in animal models and promising results from preliminary trials of combination therapy for melarsoprol-resistant *T. b. gambiense* infection suggests that this approach should be explored further (Mpia and Pepin, 2002).
1.11.6 Treatment for Nagana

Drug resistance is a major problem in the chemotherapy of trypanosomiasis in domestic ruminants in Africa. There are currently three drugs available for the treatment of animal trypanosomiasis caused by *T. congolense*, *T. vivax* and *T. b. brucei*. Isometamidium has both prophylactic and curative effects and can be administered either subcutaneously or intra-muscularly (Geerts *et al.*, 1999). Homidium also has both prophylactic and curative effects whilst diminazene has only curative effects. The supply of these drugs was once strictly controlled by Government Veterinary Departments, however, the recent privatisation of veterinary services and a general deregulation of pharmaceutical markets have resulted in them becoming freely available to farmers, which has increased the risk of their misuse. Trypanocides are often the first drugs administered to cattle developing any symptom of disease because at U.S.$1 per treatment it is most affordable for farmers (Geerts *et al.*, 2001). As a result they are frequently used without an accurate diagnosis and under-dosage commonly occurs (Holmes, 1997). Moreover, it has been estimated from studies in Zambia that half the animals treated with trypanocides are not infected with trypanosomes (Geerts *et al.*, 2001).

1.11.7 Future Prospects for Chemotherapy

Current treatments for trypanosomiasis are unsatisfactory due to severe adverse reactions, high treatment cost and increasing treatment failures. New cost-effective and easy to administer drugs are urgently needed. The immediate focus is on the development of new treatment programmes using existing drugs such as Eflornithine more efficiently (Fairlamb, 2003).
Combination therapy could increase the efficacy of treatment if there is a synergistic effect, which may increase cure rates, lower dose rates or shorten duration of treatment (Keiser et al., 2001). Several combinations of drugs have been tested clinically including Suramin and Pentamidine (Pepin and Khonde, 1996), Eflornithine and Melarsoprol (Mpiia and Pepin, 2002) and Eflornithine and Suramin (Clerinx et al., 1998). Nifurtimox, a 5-nitrofuran is used to treat trypanosomiasis in South America (Pepin and Milord, 1994) and has been studied in combination with Melarsoprol and Eflornithine with promising results (Van Nieuwenhove, 1992). However, these studies were performed on a small-scale and none of the protocols have been tested in full clinical trials.

A potential new trypanocidal drug is currently undergoing clinical trials. DB289 [2,5-bis(4-amidinophenyl)furan-bis-O-methylamidoxime] is a new oral drug for the treatment of first stage HAT and has progressed through phase I trials and is currently undergoing phase II clinical trials (http://www.sti.ch/scih/africa1.htm). It is a derivative of the diamidine DB75 [2,5-bis(4-amidinophenyl)furan] which was previously tested in primates infected with T. b. rhodesiense in the 1980’s. DB75 failed to show any therapeutic advantage over Pentamidine and was not developed further (Fairlamb, 2003). DB289 has an advantage over Pentamidine in the treatment of first stage infection because it can be administered orally. However, it takes at least six years for a drug to be approved for use from Phase I (Keiser et al., 2001) and so it seems unlikely that DB289 will be registered this decade.

Compounds extracted from medicinal plants have also been evaluated as possible treatment for HAT. Proanthocyadinin is a known antioxidant and can be extracted from Kola nuts from Kola acuminata trees, which are indigenous to West and Central Africa.
This compound was shown to be more effective against *T. brucei* in vitro than pentamidine. However, it only exhibited trypanostatic effects in vivo and more research is required to elucidate the target and mode of action in trypanosomes and also to improve its physico-chemical properties (Kubata *et al.*, 2005).

In the long term, since sequencing of the *T. brucei* genome is complete (Berriman, *et al.*, 2005), the future question is how best to exploit new information about trypanosomes because a number of new potential targets have been identified. These new targets include trypanothione (Schmidt and Krauth-Siegel, 2002), proteasome (Nkemngu *et al.*, 2002) polyamine biosynthetic enzymes (Heby *et al.*, 2003), enzyme transporters involved in galactose metabolism (Roper *et al.*, 2002), (S)-adenosyl-L-methionine:Δ^{24}-sterol methyltransferase (24-SMT) (Lorente *et al.*, 2004) and tubulin (Ochola *et al.*, 2002). Studies using compounds that are known to bind to some of these targets have begun, for example, trileucine methyl vinyl sulfone, a proteasome inhibitor and azasterols, inhibitors of 24-SMT, have shown in vitro activity against *T. brucei* with promising results (Lorente *et al.*, 2004; Steverding *et al.*, 2005).

### 1.12 Tubulin as a Novel Drug Target

#### 1.12.1 Potential of Tubulin as a Drug Target

Tubulin is a promising target for the development of new trypanocidal drugs because they are highly conserved within the Trypanosomatidae family but differ from that of mammals. Disruption of the tubulin-microtubule equilibrium can lead to a cascade of direct and indirect biochemical and physiological changes resulting in the loss of
cellular homeostasis. If this state in the cell is maintained, the result is fatal (Lacey, 1988).

1.12.2 Benzimidazoles

Benzimidazoles have been widely used since 1961 as anthelmintics in veterinary and human medicine (Kohler, 2001) and as antifungal agents in agriculture (Katiyar et al., 1994). They are thought to bind to β-tubulin (Hollomon et al., 1998; Lubega et al., 1993; Nogales, 2000) which interferes with the polymerization of tubulin and subsequently inhibit mitosis (Martin, 1997). Benzimidazoles exhibit selective toxicity binding more strongly and irreversibly with helminth tubulin in comparison to mammalian tubulin (Lacey, 1988). Benzimidazoles have demonstrated activity in vitro against Giardia duodenalis (Meloni et al., 1990; Morgan et al., 1993), Plasmodium falciparum (Dieckmann-Schuppert and Franklin, 1989), Trichomonas vaginalis (Katiyar et al., 1994) and T. b. brucei (Ochola et al., 2002).

Little or no activity has been observed against Leishmania infantum (Armson et al., 1999a), L. major, Entamoeba histolytica, Acanthamoeba polyphaga (Katiyar et al., 1994) and Fasciola hepatica (Robinson et al., 2001). Most recently, the binding kinetics of benzimidazoles have been studied on β-tubulin from G. duodenalis, E. intestinalis, C. parvum (Macdonald et al., 2004) and P. falciparum (Low, 2005), further supporting the hypothesis that benzimidazoles bind to β-tubulin selectively.
1.12.3 Dinitroanilines

Dinitroanilines are tubulin-binding agents that were originally recognised for their herbicidal properties and are used commercially for the elimination of grasses and broadleaf weeds (Chan and Fong, 1994). They act by disrupting tubulin polymerization, inhibiting mitosis and causing a loss of orientation of cell wall microfibrils so that rectangular shaped cells become round (Chan et al., 1991; Chan et al., 1993).

The anti/protozoal activity of dinitroanilines has been demonstrated against a number of protozoan parasites. Trifluralin has shown activity against *C. parvum* (Armson et al., 1999b), *Leishmania sp.*, *T. brucei* (Chan and Fong, 1990; Chan et al., 1993) and *Plasmodium sp.* (Dow et al., 2002) (Kaidoh et al., 1995). Oryzalin, ethalfluralin and trifluralin have shown activity against *Toxoplasma gondii in vitro* with low toxicity to mammalian host cells (Morrissette et al., 2004; Stokkermans et al., 1996). In addition, anti/protozoal activity has been demonstrated *in vivo* by trifluralin and oryzalin against *C. parvum* (Armson et al., 1999b), *Leishmania sp.* (Chan et al., 1993). However, not all protozoan parasites are sensitive to dinitroanilines. *Entamoeba histolytica* was not sensitive to trifluralin even at high concentrations and, whilst oryzalin arrested mitosis, the effects were reversible on removal of the drug (Makioka et al., 2000).

The putative target of dinitroanilines is α-tubulin. Recent studies showed that the dinitroaniline oryzalin was binding consistently and with great affinity to α-tubulin (Morrissette et al., 2004) (Stokkermans et al., 1996). Moreover, oryzalin resistant *Toxoplasma* possessed a point mutation in the single α-tubulin gene. In the dinitroaniline resistant weed *Eleusine indica*, a point mutation in the α-tubulin gene was also found (Anthony and Hussey, 1999). Interestingly, the activity of dinitroanilines is
restricted to plants and protozoa. These compounds are ineffective against mammalian or fungal microtubules (Bajer and Mole-Bajer, 1986; Chan and Fong, 1990; Dow et al., 2002; Hess and Bayer, 1977; Hugdahl and Morejohn, 1993).

1.13 The Present Study

The hypothesis being investigated in this project is that the structural protein, tubulin, is a potential target for anti-trypanosomatid drug discovery and vaccine development. Recombinant $\alpha$- and $\beta$- tubulin proteins from T. brucei will be expressed, drug binding kinetics will be studied and potential anti-trypanosomatid drugs evaluated based on strength and affinity of binding to tubulin. In addition to this, recombinant trypanosomal tubulin will be used to explore the possibility of its ability to provide protective immunity in an animal model.

A safe and effective anti-trypanosomal agent has yet to be found. The most promising class of compounds being explored recently are the dinitroanilines, particularly trifluralins. Further evaluation of these compounds requires pure tubulin for use in drug-binding assays to determine the binding characteristics of trifluralins and to enable the development of more active analogues. In addition, drug-treated bloodstream forms of T. brucei will be studied using electron microscopy and immunofluorescence in order to determine what ultrastructural changes these compounds induce as a result of tubulin binding.

Currently there are no available vaccines against African trypanosomiasis and prospects for the development of a vaccine are considered to be very poor due to the fact that
trypanosomes regularly change their vsg surface coat. Alternatively, invariant trypanosome components are currently being explored as potential vaccines and this study will focus on whether tubulin can provide a target for vaccine development. Mice will be immunized with recombinant $\alpha$- and $\beta$-tubulin, challenged with *T. brucei* and monitored for trypanosome infection and survival. It will also be determined whether a difference exists between $\alpha$-tubulin and $\beta$-tubulin in providing immuno-protection. With inadequate treatment currently available against African trypanosomiasis, the discovery of a vaccine would be ideal as a safe and cost-effective way of controlling African trypanosomiasis.
Chapter 2

Expression and Purification of Recombinant Tubulin From *Trypanosoma brucei* Using Fusion Partners.
2.1 Introduction

Bacterial expression systems provide economic and logistic advantages in producing large amounts of recombinant protein. However, there are also many problems associated with expressing protein in bacteria because proteins tend to aggregate or accumulate in inclusion bodies when produced to high levels, become degraded by host proteases (Wilkinson and Harrison, 1991) and purifying the protein of interest can be difficult (Davis et al., 1999). This leads to problems refolding the recombinant protein in vitro with an increased possibility of mis-folding or incomplete folding intermediates being produced. Despite improvements in the procedures for refolding proteins from inclusion bodies (De Bernardez Clark, 1998; Lilie et al., 1998) the recovery yield of biologically active protein is frequently unsatisfactory (Lilie et al., 1998). To avoid this, it is desirable to maximise the expression of the protein in completely soluble form.

Over the past decade, much progress has been made in the understanding of protein structure and protein folding (Hockney, 1994). This has lead to the development of a strategy to aid in the solubilization of recombinant proteins in *E. coli*, which involves fusing the gene of interest to a second “carrier” gene to produce a fusion protein (Sassenfeld, 1990). An appropriate gene fusion can enhance solubility, decrease proteolysis of target protein and provide an affinity purification tag (Georgiou and Valax, 1996; LaVallie and McCoy, 1995; MacDonald et al., 2003; Nilsson et al., 1997). The most common carrier proteins for fusion protein expression in *E. coli* are maltose-binding protein (MBP) (New England Biolabs, Beverly, MA) (di Guan et al., 1988; Hennig and Schafer, 1998; Riggs, 2000; Sachdev and Chirgwin, 1998; Sachdev and Chirgwin, 2000) glutathione S-transferase (GST) (Amersham Biosciences) (Smith et al., 1998) thioredoxin (TrxA) (Invitrogen) (LaVallie et al., 1993; Sachdev and
Chapter 2. Expression of Recombinant Trypanosomal Tubulin

Chirgwin, 1998; Yasukawa et al., 1995) and polyhistidine (Novagen; Qiagen). Other systems include protein A and its derivative protein Z (Pharmacia Biotech), FLAG peptide (Kodak) and PinPoint (Promega) (Nilsson et al., 1997).

MBP is commercially available as a fusion protein kit (New England Biolabs, Beverly, MA) that utilises the pMAL plasmid vector (Figure 2.1) containing the malE gene encoding maltose-binding protein. The cloned gene of interest is inserted downstream from the malE gene resulting in the expression of an MBP fusion protein (di Guan et al., 1988; Maina et al., 1988). MBP, a protein found in E. coli, is exported into the periplasmic space where it binds specifically to maltose or maltodextrins for subsequent transport across the cytoplasmic membrane (Duplay et al., 1984). A high level expression of the cloned gene occurs by use of a strong chemically inducible “tac” promoter (Amann and Brosius, 1985) and malE translation initiation signals (Duplay et al., 1984). Once the fusion protein is expressed in E. coli it can be purified using affinity chromatography using a column of a cross-linked amylose matrix that binds MBP. The MBP fusion protein is subsequently eluted using MBP’s affinity for maltose (Kellermann and Ferenci, 1982). The pMAL vector also contains the sequence coding for the recognition site of the specific protease Factor Xa (Nagai and Thogersen, 1984), which is located 5′ to the polylinker insertion sites. This allows MBP to be cleaved from the protein of interest after purification. Factor Xa cleaves after its four amino acid recognition sequence so that few or no vector-derived residues are attached to the protein of interest, depending on the site used for cloning.
Figure 2.1 Map of the maltose-binding protein fusion vector (pMAL™-2) showing the reading frame and main features. Arrows indicate the direction of transcription. Unique restriction sites are indicated.

Figure 2.2 Map of glutathione S-transferase fusion vector (pGEX-6P-1) showing the reading frame and main features. Arrows indicate the direction of transcription. Unique restriction sites are indicated.
GST is also commercially available as a fusion protein kit (Amersham Biosciences, Buckinghamshire, England) and uses the pGEX plasmid vector (Figure 2.2) containing the gene encoding GST. The cloned gene of interest is inserted downstream from the GST gene resulting in the expression of a GST fusion protein. This vector also contains the strong “tac” promoter for chemically inducible high-level expression in *E. coli*. GST is a 26kDa protein naturally occurring in the parasitic helminth *Schistosoma japonicum* (Smith *et al.*, 1986). Using the pGEX vector GST can be expressed in *E. coli* with full enzymatic activity (Smith and Johnson, 1988) and a high degree of solubility (Smith *et al.*, 1988). Purification is carried out under non-denaturing conditions using affinity chromatography by immobilizing GST to cofactor glutathione and then eluting with reduced glutathione (Simons and Vander Jagt, 1981). The pGEX vector also contains the sequence coding for the recognition site of the specific proteases thrombin and Factor Xa to allow the cleavage of the GST fusion partner (Smith and Johnson, 1988).

Trypanosomal tubulin proteins are predominantly cytoskeletal proteins, comprising of up to 10% of the total cellular protein content (Chan and Fong, 1994). Each molecule of tubulin is a heterodimer consisting of α- and β- tubulin subunits. The αβ heterodimer polymerise to form microtubules (Lacey, 1988). Alpha-tubulin is composed of 451 amino acids and has a M.W of 49.7kDa and β-tubulin is composed of 442 amino acids and has a M.W. of 49.8kDa (Kimmel *et al.*, 1985). They are both acidic proteins with theoretical pI of 4.93 for α-tubulin and 4.72 for β-tubulin. The acidity is due to the high number of acidic residues, especially glutamic acid in the carboxy-terminal of the monomers (Lacey, 1988).

Wu and Yarbrough (1987) expressed the tubulin genes from *T. brucei* in *E. coli* but they were unable to solubilize them from the inclusion bodies. Both tubulin subtypes from
the parasitic protozoa *Giardia duodenalis*, *Cryptosporidium parvum*, *Encephalitozoon intestinalis* (MacDonald *et al.*, 2001; MacDonald *et al.*, 2003) and *Plasmodium falciparum* (Low, 2005) have been expressed in *E. coli* in soluble form using various expression systems.

Tubulins are highly conserved within the Trypanosomatidae but are different from that of mammalian hosts (Lacey, 1988). Past work suggests that tubulin from kinetoplastid parasites could potentially be an efficient drug target (Armson *et al.*, 1999b; Ochola *et al.*, 2002). Production of pure tubulin for use in drug-binding assays will provide significant insights into the binding characteristics of the trifluralin analogues and allow a faster and more efficient screening of the various analogues of trifluralin.

The aims of this study were:

1. To produce soluble recombinant $\alpha$-tubulin and $\beta$-tubulin protein from *Trypanosoma brucei*.
2. To purify the recombinant $\alpha$- and $\beta$-tubulin with minimal losses whilst maintaining the structural integrity of the proteins.
2.2 Materials and Methods

2.2.1 Trypanosomes

*T. b. rhodesiense* (ATCC, 30027) were purified from the blood of highly parasitaemic rats by overlaying 1ml of infected blood onto 5ml of Percoll at a specific gravity of 1.075 (Amersham Biosciences, Buckinghamshire, England) and centrifuging at 1,500 \( \times \) g for 10min at 4°C. The layer above the Percoll containing trypanosomes was removed and washed once with 5ml of PBS (20mM Na\(_2\)PO\(_4\), 150mM NaCl, pH 7.2) and centrifuged again at 1,500 \( \times \) g for 2min to collect the pellet. The pellet of trypanosomes was resuspended in 1ml of PBS and stored at –80°C until required for use.

2.2.2 DNA extraction and PCR amplification of trypanosomal tubulin genes

Whole genomic DNA from *T. b. rhodesiense* was extracted using the Ultra Clean Gel Spin™ DNA purification kit (Mo Bio, Solana Beach, CA) and Prep-a-gene matrix (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Forward and reverse primers for \( \alpha \)-tubulin and \( \beta \)-tubulin were designed using sequence data from Kimmel, *et al.* (1985) (Table 2.1). The primers were designed to introduce unique restriction sites into the tubulin genes for unidirectional ligation into their respective vectors: namely, 5’ *BamHI* and 3’ *XbaI* for \( \alpha \)-tubulin to be cloned into the pMAL-c2x vector (New England Biolabs, Beverly, MA) and 5’*BamHI* and 3’*XhoI* for \( \beta \)-tubulin to be cloned into the pGEX-6p1 vector (Amersham Biosciences, Buckinghamshire, England). No start codons were included to ensure the tubulin sequence was translated as part of the fusion protein.
The likelihood of a PCR product being generated with these primers was assessed using the PCR Amplify programme version 1.2 (Bill Engels, Madison, U.S.A.) The theoretical specificity of the primers was determined using BLAST against Genbank.

Table 2.1. Primer sequences used to amplify T.b.rhodesiense tubulin genes.

<table>
<thead>
<tr>
<th>Gene and Primer</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-tubulin</td>
<td>5'-GATTAAGGATCCCGTGAGGCTATC-3’</td>
</tr>
<tr>
<td>forward primer</td>
<td>5'-CGGTCTAGACACTTTTCTAGTACTC-3’</td>
</tr>
<tr>
<td>reverse primer</td>
<td></td>
</tr>
<tr>
<td>β-tubulin</td>
<td>5'-GATTAGGATCCCGAAATCGTCTGC-3’</td>
</tr>
<tr>
<td>forward primer</td>
<td>5'-GGCTTACTCGAGCTATGCTCCTC-3’</td>
</tr>
<tr>
<td>reverse primer</td>
<td></td>
</tr>
</tbody>
</table>

PCR amplification of α- and β-tubulin genes were prepared as follows:

- 10μl GeneAmp 10× PCR Buffer
- 4U AmpliTaq DNA Polymerase
- 200μM each dNTP (all from Applied Biosciences, Foster City, CA)
- 25pM each primer (Gibco BRL, Rockville, U.S.A)
- 5ng genomic DNA
- PCR grade ultra pure water to a final volume of 100μl.

Amplifications were performed on a Perkin-Elmer PE 2400 Thermal Cycler (Foster City, USA) under the following conditions: 95°C for 5min; 30 cycles of 95°C for 1min, 61°C for 45sec, 72°C for 1min, 30sec and a final 72°C elongation step for 7min for α-tubulin. The same PCR conditions were followed for β-tubulin with the exception of the annealing temperature, which was changed to 55°C.
2.2.3 Preparation of plasmid and tubulin genes for cloning

PCR products were cleaned using the Wizard Prep Kit (Promega, Madison, WA) following the manufacturer’s instructions and then a restriction endonuclease digest was performed to prepare the PCR products for cloning. These were performed in two steps. For the first step, the following reaction was prepared; 0.5μg of α-tubulin DNA, 10 units of XbaI restriction endonuclease, 2.0μl 10× XbaI buffer (Promega, Madison, WA) were added to a final volume of 20μl ddH2O. This was incubated for 18hr in a 37°C water bath. The restriction endonuclease was then inactivated in an 85°C water bath for 15min before the second part of the reaction was conducted. To the initial reaction 10 units of BamHI restriction endonuclease, 2.0μl of 10× BamHI buffer (Promega, Madison, WA) were added to a final 25μl volume of ddH2O and incubated for 5hr at 37°C. The same procedures were performed on the β-tubulin insert replacing XbaI with XhoI (Promega, Madison, WA).

To prepare the pMAL and pGEX plasmid vectors for cloning a restriction endonuclease digestion was performed using the method used for their respective inserts on the same amount of DNA. The cleaved vectors were then treated with 1U of alkaline phosphatase (Promega, Madison, WA) for 1hr at 37°C to remove the phosphate ends and prevent the ends re-ligating.

2.2.4 Plasmid construction and sequencing of tubulin genes

Once digestion of the α-tubulin gene and pMAL vector were complete, a ligation reaction mixture was prepared as follows: 90fmol of α-tubulin, 30fmol of pMAL, 2U of T4 DNA Ligase (Gibco BRL, Rockville U.S.A.), 2.0μl 10×T4 Ligase Buffer (Gibco
BRL, Rockville U.S.A.) in a total volume of 20μl with ddH₂O. This reaction mixture was incubated at 16°C for 24hr. The same protocol was followed for β-tubulin substituting α-tubulin for β-tubulin and the pMAL vector for the pGEX vector.

The tubulin constructs were transformed into DH5α E. coli competent cells by a standard heat shock procedure, plated onto Luria-Bertani (LB) plates containing 50 μg/ml ampicillin and incubated overnight at 37°C. Single colonies were cultured in 2ml of LB broth containing 50μg/ml ampicillin for 18hr with shaking at 37°C. The construct DNA was isolated from the culture using standard extraction procedures and then sequenced in both directions to ensure complete integrity using sequencing primers designed by the manufacturers for the pMAL and the pGEX vectors respectively (Table 2.2) and the ABI PRISM dye terminator cycle sequencing kit (Perkin-Elmer, Foster City, CA) according to the manufacturer’s recommended method.

<table>
<thead>
<tr>
<th>Vector and Primer</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMAL-c2x</td>
<td>forward primer 5’-CGATGAAGCCCTGAAAGACGCGCAGAC-3’</td>
</tr>
<tr>
<td>reverse primer</td>
<td>5’-TGGGTAACGCCAGGGTTTTCCCAGTCA-3’</td>
</tr>
<tr>
<td>pGEX-6p1</td>
<td>forward primer 5’-GGGCTGGCAAGCCACGCGCAGAC-3’</td>
</tr>
<tr>
<td>reverse primer</td>
<td>5’-CCGGGAGCTGCATGTGTCAGAGG-3’</td>
</tr>
</tbody>
</table>

### 2.2.5 Transformation of E. coli and induction of gene expression

Once the trypanosomal tubulin constructs were confirmed (pMAL-α-tubulin and pGEX-β-tubulin) they were transformed into BL21(DE3)pLysS protein expressing cells
(Invitrogen) and single colonies were cultured in 2ml of LB broth containing 50μg/ml of ampicillin and 35μg/ml of chloramphenicol and incubated at 37°C for 18hr with shaking.

To determine the optimal protein expression conditions, the volume of the *E. coli* cultures were increased to 50ml and grown at 25°C, 30°C or 37°C. The mid-log phase was determined using optical density (OD) measurements (wavelength 600nm). Once the OD$_{600}$ was between 0.4 and 0.6 protein expression was induced by adding 0.1mM, 0.5mM or 1mM total concentration of isopropyl-beta-D-thiogalactopyranoside (IPTG). Samples of 1ml were removed hourly after induction to determine the optimal incubation time, incubation temperature and IPTG concentration that resulted in the highest yield of fusion protein. The concentration of fusion protein in uninduced bacterial cultures was also determined to provide the basal bacterial protein expression levels. The unligated plasmids pMAL-c2x and pGEX-6p1 were also transformed into BL21(DE3)pLysS cells and treated as described above to provide samples of the fusion partner only.

**2.2.6 Fusion protein analysis**

The 1ml samples collected during expression were centrifuged at 14,000 × g for 1min at 4°C and the pellet resuspended in 100μl of protein sample buffer (10% SDS, 0.5M Tris-HCl pH 6.8, 50% glycerol, 0.01% bromophenol blue, 0.1ml 2β-mercaptoethanol made up to 10ml with ddH$_2$O). The cells were then lysed by freeze-thawing 3 times and sonicated using a Misonix ultrasonic sonicator (Daintree Scientific, Tasmania) for 15sec with 10sec intervals for a total of 10 cycles. The crude lysate was then centrifuged at
14,000 × g for 1 min at 4°C to separate soluble (supernatant) and insoluble (pellet) fractions. Proteins were visualised using SDS-PAGE (Laemmli, 1970) to determine the optimal temperature, time and IPTG concentration for highest yield of soluble fusion protein. Samples of 20μl were loaded onto 10% Tris-glycine polyacrylamide pre-cast gels (Gradipore, Frenchs Forest, Australia) and electrophoresed at 90V (constant voltage) for 120 min in electrophoresis buffer (containing 3.75% Tris, 1.25% SDS and 18% glycine in ddH2O) in a Bio-Rad Mini-Protean apparatus (Hercules, U.S.A.). The gels were stained in Coomassie staining solution (50% ddH2O, 40% methanol, 10% acetic acid and 0.1% Coomassie Brilliant Blue R-250) for 18 hr. The gels were then destained for 24 hr in a solution of 50% ddH2O, 40% methanol and 10% acetic acid.

Western and Dot Blots were performed using polyvinylidene difluoride (PVDF) membranes (MSI, Westboro, MA) according to the method of Towbin et al. (1979). For the putative MBP-α-tubulin fusion protein the presence of the MBP carrier protein was determined using a monoclonal anti-MBP antibody (Sigma, St Louis, MO) and confirmation that the fusion protein contained α-tubulin was performed using a monoclonal anti-α-tubulin primary antibody (Sigma, St Louis, MO). For the putative GST-β-tubulin fusion protein, the presence of the GST carrier protein was confirmed using a monoclonal anti-GST antibody (Amersham Biosciences, Buckinghamshire, England) and confirmation that the fusion protein contained β-tubulin was performed using a monoclonal β-tubulin primary antibody (Sigma, St Louis, MO). An alkaline phosphatase-conjugated anti-mouse immunoglobulin G (IgG, whole molecule) (Sigma, St Louis, U.S.A.) was used as a secondary antibody for both fusion proteins. The conjugate was detected using a colorimetric alkaline phosphatase assay (Bio-Rad, Hercules, CA), performed as recommended by the manufacturer.
2.2.7 Purification of recombinant tubulin by affinity chromatography

The MBP-α-tubulin fusion and MBP proteins were purified from the cell supernatant by affinity chromatography using amylose resin (New England Biolabs, Beverly, MA) under the manufacturer’s instructions with some modifications to attain the highest yield of soluble protein. Briefly, the recombinant protein was passed through the column twice to ensure maximal binding to the resin and the amount of NaCl in the column buffer was increased from 200mM to 500mM to prevent the protein precipitating once it was eluted from the column. In addition, during the elution step, the column was plugged after the elution buffer was loaded for at least 2hr to allow more time for the recombinant protein to separate from the amylose resin. This entire procedure was performed at 4°C.

The GST-β-tubulin fusion and GST proteins were purified using glutathione-agarose beads (Amersham Biosciences, Buckinghamshire, England) as per the manufacturer’s instructions with some modifications. Briefly, the recommended protein binding and elution times were increased to 18hr and the process was repeated twice using fresh glutathione beads to ensure the maximum yield of recombinant protein was obtained. This procedure was performed at 4°C.

SDS-PAGE, Western Blots and Dot Blots were performed (as described in 2.2.6) on the purified fusion proteins in order to determine the correct size of the proteins and whether the structural integrity of the proteins were preserved throughout the purification process.
The purified proteins were concentrated using Amicon Centripreps (Millipore, Billerica, MA) as recommended by the manufacturer and quantified using a Bio-Rad method based on the Bradford Method (1976). The concentration of the proteins was adjusted to approximately 1mg/ml with MBS buffer (0.1M MES pH 6.5, 0.2M sodium chloride, 0.05% sodium azide, with 1mM phenylmethylsulfonyl fluoride (PMSF) protease inhibitor) and stored at –80°C until required.

Where possible recombinant α-tubulin was cleaved from MBP using the specific protease Factor Xa (New England Biolabs, Beverly, MA). Recombinant β-tubulin was cleaved from GST using PreScission Protease (Amersham Biosciences, Buckinghamshire, England). The cleaved tubulins were then purified from MBP or GST by anion-exchange chromatography using Uno-Q anion-exchange columns (Bio-Rad, Hercules, CA) at 4°C. The eluents were analysed by SDS-PAGE and Western Blots as described previously and quantified using the Bio-Rad protein assay (Bradford, 1976).
2.3 Results

2.3.1 Recombinant tubulin fusion protein expression

The supernatant fractions of lysed *E. coli* samples were analysed by SDS-PAGE in order to establish the conditions required for the tubulin fusion proteins to be expressed in soluble form. The concentration of IPTG, incubation time and incubation temperature that provided the highest yield of soluble protein for MBP-α-tubulin fusion protein was 0.1mM IPTG, for 5hr at 37°C and for GST-β-tubulin fusion protein was 0.1mM IPTG, for 4hr at 37°C. The amount of soluble MBP-α-tubulin fusion protein produced increased steadily over a 5hr period and then plateaued after this time (Figure 2.3). After 5hr of expression the ratio of soluble to insoluble protein decreased markedly. This pattern also occurs for GST-β-tubulin fusion protein, however, a shorter expression time of 4hr was found to be optimal (Figure 2.4).

![Figure 2.3](image1.png)

**Figure 2.3** SDS-PAGE gel image illustrating MBP-α-tubulin fusion protein expression in soluble form over a 6hr time period. The time post-induction is shown above each lane.

![Figure 2.4](image2.png)

**Figure 2.4** SDS-PAGE gel image illustrating GST-β-tubulin fusion protein expression in soluble form over a 6hr time period. The time post-induction is shown above each lane.
2.3.2 Recombinant tubulin fusion protein purification

The presence of MBP and GST fusion protein tags has allowed the purification of α-tubulin and β-tubulin using relatively straightforward affinity chromatography methods. Figure 2.5 depicts the course of the purification of MBP-α-tubulin fusion protein using the amylose resin column and Figure 2.6 depicts the course of the purification of GST-β-tubulin using the glutathione-agarose column. In Lane 2 of Figures 2.5 and 2.6, samples of the uninduced cultures in are shown to contain no fusion protein. Lanes 3 and 4 contain samples of the expressed fusion protein and soluble fraction of the fusion protein respectively. Lanes 5 in Figure 2.5 and Lanes 5 and 6 in Figure 2.6 contain samples of the flow-through from the columns showing the proteins that did not bind.

Figure 2.5. SDS-PAGE gel image illustrating the fractions from the purification of MBP-α-tubulin fusion protein. Lane 1, protein MW standard; Lane 2, uninduced cells; Lane 3 total proteins produced in cells following 5 hours of expression; Lane 4 soluble proteins recovered from the supernatant of sonicated cells; Lane 5 flow through from the amylose column of proteins that did not bind; Lane 6, washing flow through confirming no target protein is being released from the resin at this stage and Lane 7, purified MBP-α-tubulin eluted from the amylose column of approximately 100kDa in size.
Washing the column with their respective column buffers did not elute any bound fusion protein as shown in Lane 6 in Figure 2.5 and Lanes 7 and 8 in Figure 2.6. The eluted MBP-α-tubulin fusion protein of size 100kDa is shown in Lane 7 in Figure 2.5 and the eluted GST-β-tubulin fusion protein of size 75kDa is shown in Lanes 9 and 10. The SDS-Page gel picture in Figure 2.7 contains samples of the purified and concentrated recombinant MBP-α-tubulin, GST-β-tubulin, MBP and GST proteins of the correct size.

**Figure 2.6** SDS-PAGE gel image illustrating the fractions from the purification of GST-β-tubulin fusion protein. Lane 1, protein MW standard; Lane 2, uninduced cells; Lane 3, total proteins produced in cells following 4 hours of expression; Lane 4 soluble proteins recovered from the supernatant of sonicated cells; Lanes 5 and 6, flow through from the glutathione-agarose beads of proteins that did not bind; Lanes 7 and 8, washing flow through confirming no target protein is being released from the beads at this stage and Lane 9 and 10, purified GST-β-tubulin eluted from the glutathione-agarose beads of approximately 75kDa in size.
2.3.3 Recombinant tubulin fusion protein analysis

The Western (Figure 2.8) and Dot (Figure 2.9) Blots performed on purified fusion protein detected bands of the expected sizes for each recombinant protein demonstrating recognition by their respective monoclonal antibodies. These results also indicate that the structural integrity of the tubulin fusion proteins has been preserved throughout the expression and purification processes.
Chapter 2. Expression of Recombinant Trypanosomal Tubulin

Figure 2.8 Western Blot analysis of recombinant MBP-α-tubulin and GST-β-tubulin a) MBP was detected using monoclonal anti-MBP antibody, b) α-tubulin was detected using monoclonal anti-α-tubulin antibody c) GST was detected using monoclonal anti-GST antibody and d) β-tubulin was detected using monoclonal anti-β-tubulin antibody.

Figure 2.9 Dot Blot analysis of recombinant MBP-α-tubulin and GST-β-tubulin. a) MBP was detected using monoclonal anti-MBP antibody, b) α-tubulin was detected using monoclonal anti-α-tubulin antibody c) GST was detected using monoclonal anti-GST antibody and d) β-tubulin was detected using monoclonal anti-β-tubulin antibody.
2.4 Discussion

This is the first time the α-tubulin and β-tubulin genes from *Trypanosoma brucei* have been expressed in soluble form in *E. coli*. In previous studies, α- and β-tubulin from trypanosomes were expressed in an insoluble form in *E. coli* and attempts to solubilize the proteins were unsuccessful (Wu and Yarbrough, 1987). The development of fusion protein vectors during the 1990’s enables large quantities of soluble protein to be reliably produced (Sachdev and Chirgwin, 1998) with high levels of purity, which makes them suitable for characterisation studies such as drug-binding assays (MacDonald *et al.*, 2003).

Previous studies have shown that there is an optimal temperature, time and concentration of IPTG for expression of particular proteins with different fusion partners and that these should be determined empirically when optimising a protein expression system (MacDonald *et al.*, 2003). MBP is generally more efficient than GST in increasing recovery of soluble protein (Kapust and Waugh, 1999; Sachdev and Chirgwin, 1998; Sachdev and Chirgwin, 2000; Smyth *et al.*, 2003), however, for the expression of trypanosomal β-tubulin, this was not the case. The use of the pMAL protein expression and purification system only produced a small amount of soluble recombinant GST-β-tubulin fusion protein which may not be sufficient for characterisation experiments. In order to ensure that sufficient β-tubulin was produced, the pGEX protein expression and purification system was employed to produce recombinant β-tubulin. The yield of soluble protein produced in this system was substantially increased.
Attempts to cleave the MBP and GST carrier proteins from tubulin using Factor Xa and PreScission protease respectively were only partially successful. Approximately 50% of the fusion protein was resistant to cleavage even after incubation for 24 hours with a ten-fold increase in concentration of enzymes. This could have been due to use of specific protease that was inefficient or because the fusion protein was folded in such a way that the cleavage site was inaccessible to the protease. Although it is desirable to obtain pure recombinant tubulin protein for characterisation studies it is not essential because the attachment of MBP and GST to the N terminus of most proteins does not interfere with their function. The structure and tertiary folding of these carrier proteins are well characterised and many experiments have been conducted using purified but uncleaved fusion proteins (Sachdev and Chirgwin, 2000), including crystallization studies (Smyth et al., 2003). Furthermore, fusion partners can be advantageous in vivo because potentially they can protect the target protein from intracellular proteolysis (Sorensen and Mortensen, 2005), which would aid vaccine studies.

Use of the pMAL and pGEX E. coli based protein expression and purification systems resulted in the production of soluble recombinant tubulin that is greater than 95% pure and free from post-translational modifications and microtubule-associated proteins. Tubulin generated in this manner is suitable for a number of applications, including drug-binding and vaccine studies.
Chapter 3

Investigation of the Interactions of Novel Trifluralin Analogues with Recombinant Tubulin from *Trypanosoma brucei.*
3.1 Introduction

The dinitroanilines are tubulin-binding agents that were originally recognised for their herbicidal properties. Trifluralins and other dinitroaniline analogues (Figure 3.1) are used commercially for the elimination of grasses and broadleaf weeds (Chan and Fong, 1994). They act by disrupting tubulin polymerization, inhibiting mitosis and causing a loss of orientation of cell wall microfibrils so that rectangular shaped cells become round (Chan et al., 1991; Chan et al., 1993). The anti-protozoal activity of the dinitroanilines has been demonstrated in vitro against a number of protozoan parasites including *C. parvum* (Armson et al., 1999b), *Plasmodium sp.* (Dow et al., 2002; Kaidoh et al., 1995), *T. gondii* (Morrissette et al., 2004; Stokkermans et al., 1996), *Leishmania sp.* and *T. brucei* (Chan and Fong, 1990; Chan et al., 1993), with low toxicity to mammalian cells.

![Figure 3.1](image-url) Structures of the major dinitroaniline compounds (Stokkermans et al., 1996).

The underlying mode of action of the dinitroanilines has been studied extensively in *T. gondii* (Morrissette et al., 2004; Stokkermans et al., 1996). Recent studies showed that the dinitroaniline oryzalin bound consistently and with great affinity to α-tubulin from...
T. gondii (Morrissette et al., 2004). Moreover, oryzalin resistant Toxoplasma contained a single point mutation in the α-tubulin gene. A single point mutation in the α-tubulin gene was also found in the dinitroaniline resistant weed, Eleusine indica, (Anthony and Hussey, 1999). In contrast, most compounds that have been shown to interfere with microtubule function, such as the benzimidazoles, are known to bind to and act on β-tubulin (Nogales, 2000).

Interestingly, the activity of dinitroanilines is restricted to plants and protozoa. These compounds are ineffective against mammalian or fungal microtubules (Bajer and Mole-Bajer, 1986; Chan and Fong, 1990; Dow et al., 2002; Hess and Bayer, 1977; Hugdahl and Morejohn, 1993). A novel oryzalin analogue called GB-II-5 has recently been synthesised that shows potent, selective antimitotic activity against L. donovani and T. brucei (Werbovetz et al., 2003). GB-II-5 is a proven tubulin binding compound and further supports the suggestion that analogues of dinitroanilines are strong candidates as chemotherapeutic compounds against trypanosomiasis infection (Bhattacharya et al., 2004).

The kinetics of the binding of trifluralin and its analogues to trypanosomal tubulin have not been determined to date mainly because of the difficulty in extracting homogenous tubulin from trypanosomes free from microtubule-associated proteins. The successful production of recombinant α- and β-tubulin from T. b. rhodesiense (Chapter 2) has enabled such binding studies. The fluorescence quenching technique is based on measuring the reduction of intrinsic fluorescence from tryptophan residues as a result of the binding of compounds directly with or close to tryptophan residues. Fluorescence quenching has been successfully applied to determine the binding kinetics of colchicine...
(Banerjee and Luduena, 1992; Chaudhuri et al., 2000) and taxol (Diaz et al., 2000; Han et al., 1996) to mammalian tubulin. Most recently, fluorescence quenching was successfully used to quantify the kinetics of binding of benzimidazoles to tubulin from G. duodenalis, E. intestinalis, C. parvum (Macdonald et al., 2004) and P. falciparum (Low, 2005) and of dinitroaniline compounds to tubulin from Leishmania and T. brucei (Werbovetz et al., 1999; Werbovetz et al., 2003).

The aim of this study was:

1. To quantify the kinetics of binding of novel trifluralin analogues to recombinant tubulin from T. b. rhodesiense.
3.2 Materials and Methods

3.2.1 Expression of recombinant trypanosomal tubulin

The expression and purification of α-tubulin and β-tubulin proteins from *T. b. rhodesiense* has been previously described (see Chapter 2). The recombinant α- and β-tubulin fusion proteins were maintained in MBS (0.1M MES pH 6.5, 0.2M sodium chloride, 0.05% sodium azide, 1mM PMSF) at a concentration of 1mg/ml and kept at −80°C until required for use.

3.2.2 Extraction of native rat brain tubulin

Tubulin was extracted from the brains of rats (*Rattus norvegicus*) using a modification of the method of Castoldi and Popov (2003). Eight euthanased rats were obtained from the Animal Resource Centre (Murdoch University) and their brains removed, stored in ice-cold PBS (20mM Na₂PO₄, 150mM NaCl, pH 7.2) and the tubulin extracted within 24hr. The brains were cleaned of blood clots and surrounding meningeal membranes in PBS and weighed. Cold (+4°C) depolymerisation buffer (DB) (50mM MES, 1mM CaCl₂, pH 6.6) was added at a ratio of 1ml/g brain tissue. The brains were cut into small pieces with scissors, placed into 15ml centrifuge tubes and homogenized using an Ultra Turax T25 homogenizer (Kika Works, Malaysia) twice for 30sec with a 20sec rest period. The homogenates were centrifuged at 29,000 × g for 60min at 4°C. The supernatants were harvested, pooled and an equal volume of warm (37°C) high-molarity PIPES buffer (1M PIPES, 10mM MgCl₂, 20mM EGTA, pH 6.9), ATP (1.5mM final concentration) and GTP (0.5mM final concentration) was added. An equal volume (1/3 of the final volume) of pre-warmed (37°C) anhydrous glycerol was also added to this solution. This mixture was incubated in a 37°C water bath for 1hr to allow the tubulin to
polymerise. The polymerised tubulin was then centrifuged at 100,000 \( \times \) g for 30min at 37\(^\circ\)C. The resulting microtubule pellet was resuspended in 20ml of cold (4\(^\circ\)C) DB and incubated on ice for 30min to allow the tubulin to depolymerise. The suspension of depolymerised tubulin was centrifuged at 70,000 \( \times \) g for 30min at 4\(^\circ\)C and the supernatant containing soluble tubulin was collected. A sample of the tubulin extracted from rat brain was analysed using SDS-PAGE (performed as previously described in Chapter 2) and Western and Dot Blots using monoclonal anti-\(\alpha\)-tubulin primary antibody and monoclonal anti-\(\beta\)-tubulin primary antibody (Sigma, St Louis, MO). The solution of rat tubulin was quantified using the Bio-Rad Protein assay (Bradford, 1976), concentrated using Amicon centripreps (Millipore, Billerica, MA) and maintained in MBS at a concentration of approximately 1mg/ml. It was stored at –80\(^\circ\)C until required for use.

### 3.2.3 Novel trifluralin analogues

Novel analogues of trifluralin EPL-AJ1003 (1003), EPL-AJ1007 (1007), EPL-AJ1008 (1008), EPL-AJ1016 (1016), and EPL-AJ1017 (1017) were synthesized and supplied by Epichem Pty. Ltd (Murdoch, WA). Compounds 1003, 1007, 1016 and 1017 are second generation trifluralin analogues. These analogues have in common a 2,4-dinitro-6-(trifluoromethyl)phenyl moiety and differ in the substituents at the C1 position of the phenyl ring (Figure 3.2). Compound 1008 is a third generation analogue of trifluralin and now considered to be a bi-phenyl compound because its chemical composition is considerably different from the other analogues. This compound is not patented and therefore, permission to publish the chemical composition has not been granted.
Albendazole and trifluralin were also supplied by Epichem Pty. Ltd., and colchicine was purchased from Sigma (St Louis, MO). Stock solutions of these compounds were prepared in 100% dimethyl sulphoxide (DMSO) at a concentration of 1mM and stored in 50µl aliquots at −20°C until required for use.

### 3.2.4 Fluorescence Quenching Assay

The interaction of each test compound with recombinant trypanosomal α- and β-tubulin and native rat brain tubulin was measured as the amount of reduction in the intrinsic fluorescence of tryptophan residues following incubation with each compound. An initial binding study was performed by incubating 2μM concentrations of each compound and 2μM concentrations of each tubulin subtype at 37°C for 30min. Fluorescence was immediately measured in a quartz cuvette (Starna Pty Ltd) with excitation and emission path lengths of 10mm using a Luminescence spectrometer LS50.
(Perkin Elmer) at excitation wavelength of 280nm and emission wavelengths of 350nm for MBP-α-tubulin, MBP and GST, 340nm for native rat tubulin and 330nm for GST-β-tubulin (initially optimised over a 300-400nm range for each protein).

The fluorescence measurements were corrected for inner-filter effects (caused by the addition of each compound) according to Mertens and Kagi (Mertens and Kagi, 1979) as follows:

\[ F_{corr} = F_{obs} \cdot \text{antilog} \left( \frac{A_x + A_m}{2} \right) \]

where \( F_{corr} \) is the corrected fluorescence intensity, \( F_{obs} \) is the observed intensity and \( A_x \) and \( A_m \) are the measured absorbances at the excitation and emission wavelengths, respectively. The corrected fluorescence values were then used to determine the percentage reduction of fluorescence intensity using the fluorescence value of the DMSO control as the maximum value. Each compound was tested in triplicate and controls of MBS, 5% DMSO in MBS, test compounds in MBS, MBP fusion tag protein in MBS and GST fusion tag protein in MBS were also tested.

### 3.2.5 Equilibrium Binding Assay

Equilibrium binding was determined by measuring the reduction in fluorescence intensity of recombinant trypanosomal α- and β-tubulin and native rat brain tubulin following incubation with a range of concentrations (0.1, 0.2, 0.5, 1, 2, 5 and 10μM) of each compound. Each concentration of compound was incubated with 2μM of tubulin for 30min at 37°C and fluorescence was immediately measured following the same conditions as previously described (3.2.4). Data obtained was used to generate binding
curves for comparisons between the two forms of recombinant trypanosomal tubulins and native rat tubulin. Each compound was tested in triplicate and controls of MBS alone, 5% DMSO in MBS, test compound in MBS, MBP fusion tag protein in MBS and GST fusion tag in MBS were also tested.

3.2.6 Binding Kinetics Study

The kinetics of novel analogues of trifluralin (EPL-AJ 1003, 1007, 1008, 1016 and 1017), trifluralin, albendazole and colchicine with recombinant trypanosomal tubulins and native rat brain tubulin were determined under pseudo-first order conditions. Association rates were determined by measuring the reduction in fluorescence following the incubation of 2μM concentrations of test compounds to 2μM concentrations of recombinant trypanosomal tubulin and native rat tubulin, at 37°C. The amount of fluorescence was measured every 5min for 30min. This study was performed following the same conditions as previously described (3.2.4). Each compound was tested in triplicate and controls of MBS, 5% DMSO in MBS, test compound in MBS, MBP fusion tag protein in MBS and GST fusion tag protein in MBS were also tested.

Data were analysed using a non-linear curve fitting analysis and a one-site binding equation according to (Bane et al., 1984; Chakrabarti et al., 1996; Chaudhuri et al., 2000; Lambeir and Engelborghs, 1981; Pyles and Hastie, 1993). The association rate ($k_{on}$) was calculated using the change in fluorescence ($F_{max} - F_t$) attributable to the binding of each compound as follows:

$$(F_{max} - F_t) = Ae^{-αt}$$
where $F_{\text{max}}$ is maximum fluorescence intensity, $F_t$ is the fluorescence intensity at time $t$, $A$ is the amplitude of the binding phase and $\alpha$ is the observed rate constant of the binding phase. The parameters $A$ and $\alpha$ can be determined from the logarithmic plot of $\ln(F_{\text{max}} - F_t)$ against time $t$.

The apparent association constant ($k_{\text{on}}$) for each compound was calculated as follows:

$$k_{\text{on}} = \alpha / [D]$$

where $\alpha$ is the slope of the semi-logarithmic binding plot and $D$ is the concentration of the compound tested.

Dissociation rates ($k_{\text{off}}$) were determined by measuring the change in fluorescence following a 200-fold dilution of each compound-tubulin complex by tubulin in MBS and monitoring the increase in fluorescence as the compounds were released from their binding sites. The dissociation rate constant was determined using the equation:

$$\ln(F_{\text{max}} - F_t) = -k_{\text{off}}t + C$$

where $C$ is the integration constant.

The affinity constant ($K_a$) was determined for each compound with each tubulin according to the formula:

$$K_a = k_{\text{on}} / k_{\text{off}}$$
3.2.7 Analysis of the tubulin amino acid sequences from *T. b. rhodesiense*, *L. major*, *Rattus norvegicus* and *Homo sapiens*

Amino acid sequences for α-tubulins from four different species were obtained from the following references: *T. b. rhodesiense* (Kimmel et al., 1985), *L. major* (Ivens et al., 1998), *R. norvegicus* (Strausberg et al., 2002) and *H. sapiens* (Strausberg et al., 2002). Amino acid sequences for β-tubulins from the same species were obtained from the following references: *T. b. rhodesiense* (Kimmel et al., 1985), *L. major* (Ivens et al., 1998), *R. norvegicus* (Ginzburg et al., 1985) and *H. sapiens* (Shiina et al., 2001). These sequences were manually aligned using Microsoft Word and the percent homology between the sequences were calculated manually.
3.3 Results

3.3.1 Native rat brain tubulin extraction and analysis

Soluble tubulin extracted from rat brains was approximately 50kDa in size and free from impurities as shown in Figure 3.3.

![Figure 3.3 SDS-PAGE gel image illustrating the size and relative purity of native tubulin extracted from rat brains.](image)

Western and Dot Blot analysis performed using both monoclonal anti-α-tubulin and anti-β-tubulin antibodies (Figure 3.4) indicate that the structural integrity of the rat tubulin was maintained throughout the extraction process.

![Figure 3.4 a) Western Blot and b) Dot Blot analysis of native tubulin extracted from rat brains using monoclonal anti-α-tubulin and anti-β-tubulin antibodies.](image)
3.3.2 Effect of Test Compounds on Tubulin Fluorescence

All five of the novel trifluralin compounds and commercial trifluralin significantly reduced the fluorescence intensity of recombinant trypanosomal α-tubulin (57-84%) at 2μM (Figure 3.5). In comparison, test compounds 1008, 1007 and 1017 and albendazole induced moderate reductions in the fluorescence intensity of recombinant trypanosomal β-tubulin (29-58%). Compounds 1003 and 1016 induced only low reductions in fluorescence intensity of recombinant trypanosomal β-tubulin (1-14%). In contrast, compound 1003 induced a significant reduction (63%) and colchicine induced a moderate reduction (42%) in the fluorescence intensity of native rat brain tubulin. Compounds 1016 and 1008 and 1017 did not alter the fluorescence intensity of native rat brain tubulin and compound 1007 induced a low reduction in fluorescence intensity (11%).

Figure 3.5 The proportion of tubulin fluorescence that was quenched following the addition of 2μM concentrations of test compounds; Colchicine (Colch), Trifluralin (Trif), Albendazole (ABZ), EPL-AJ1003 (1003), EPL-AJ1016 (1016), EPL-AJ1008 (1008), EPL-AJ1007 (1007) and EPL-AJ1017 (1017) to recombinant trypanosomal α-tubulin (α-tubulin), recombinant trypanosomal β-tubulin (β-tubulin) and native rat brain tubulin (rat tubulin).
3.3.3 Equilibrium Binding of Test Compounds with Tubulin

All five of the novel trifluralin compounds interacted with recombinant trypanosomal α-tubulin and quenched fluorescence in a concentration-dependent manner (Figure 3.6). The binding curves generated for the interaction of compounds 1003, 1016, 1017 and commercial trifluralin with α-tubulin show a hyperbola shape. Compounds 1007 and 1008 each exhibit distinct binding curves. Saturation of compound-tubulin complexes was achieved at concentrations between 0.5μM and 2μM as demonstrated by the plateau in the curves. A return in fluorescence occurs following saturation point due to the high concentrations of compounds exhibiting strong intrinsic fluorescence and/or conformational changes occurring in α-tubulin causing dissociation of compounds.

Figure 3.6 Equilibrium binding of test compounds Trifluralin (Trif), EPL-AJ1003, 1007, 1008, 1016 and 1017 with recombinant trypanosomal α-tubulin.
In contrast, the compounds tested on recombinant trypanosomal β-tubulin exhibited varying curve shapes for each test compound (Figure 3.7). Compounds 1003 and 1016 induced low reductions in fluorescence intensity of β-tubulin that did not change with increasing concentrations of compounds. The binding curve generated by compound 1008 did not follow a concentration-dependent trend. Compounds 1007 and 1017 interacted with recombinant trypanosomal β-tubulin and quenched fluorescence in a concentration-dependent manner with a plateau between 1μM and 5μM concentrations similar to albendazole. A return in fluorescence occurred following saturation point due to the high concentrations of compounds exhibiting strong intrinsic fluorescence and/or conformational changes occurring in β-tubulin causing dissociation of compounds.

Figure 3.7 Equilibrium binding of test compounds Albendazole (ABZ), EPL-AJ1003, 1007, 1008, 1016 and 1017 with recombinant trypanosomal β-tubulin.
Compounds 1017 and 1007 caused small percentage reductions in rat brain tubulin fluorescence that were not concentration-dependent (Figure 3.8). Compounds 1008 and 1016 did not alter the fluorescence intensity of native rat brain tubulin. Compound 1003 induced a significant reduction in fluorescence that was concentration-dependent without the development of a plateau even at the maximum concentration of 10μM. Colchicine induced a concentration-dependent reduction in the fluorescence intensity of native rat tubulin with saturation of the complex occurring between 1μM and 5μM concentrations as shown in the plateau of the hyperbolic curve, followed by a return in fluorescence.

Figure 3.8 Equilibrium binding of test compounds Colchicine (Colch), EPL-AJ1003, 1007, 1008, 1016 and 1017 with native rat brain tubulin.
3.3.4 Kinetics of Trifluralin Binding to Tubulin

The semi-logarithmic plots of the association of each compound with recombinant trypanosomal α- and β-tubulin and native rat brain tubulin are shown in Figures 3.9-3.13. Association rates could not be calculated for the binding of compounds 1008, 1016 and 1017 with native rat brain tubulin because these compounds did not reduce the fluorescence intensity of rat brain tubulin (see Figures 3.5 and 3.8). The association rates for the binding of each compound with recombinant trypanosomal α-tubulin were in the range of $1.22 \times 2.38 \times 10^4$ M$^{-1}$min$^{-1}$ (Table 3.1), which is higher than the association with recombinant trypanosomal β-tubulin and native rat brain tubulin which were in the range of $2.00 \times 10^2$ to $1.01 \times 10^4$ M$^{-1}$min$^{-1}$ (Table 3.2) and $1.65 \times 10^3$ to $1.19 \times 10^4$ M$^{-1}$min$^{-1}$ respectively (Table 3.3).

![Figure 3.9](image-url)  
**Figure 3.9** Semi-logarithmic plot of the associations of compound 1008 with recombinant trypanosomal α- and β-tubulin. (N.B. Compound 1008 did not induce a reduction of the fluorescence of native rat brain tubulin).
Figure 3.10 Semi-logarithmic plot of the associations of compound 1016 with recombinant trypanosomal α- and β-tubulin. (N.B. Compound 1016 did not induce a reduction of the fluorescence of native rat brain tubulin).

Figure 3.11 Semi-logarithmic plot of the associations of compound 1007 with recombinant trypanosomal α- and β-tubulin and native rat brain tubulin.
Figure 3.12 Semi-logarithmic plot of the associations of compound 1017 with recombinant trypanosomal α- and β-tubulin and native rat tubulin. (N.B. Compound 1017 did not induce a reduction of the fluorescence of native rat brain tubulin).

Figure 3.13 Semi-logarithmic plot of the associations of compound 1003 with recombinant trypanosomal α- and β-tubulin and native rat brain tubulin.
The semi-logarithmic plots of the dissociation of each compound with recombinant trypanosomal α- and β-tubulin and native rat tubulin are shown in Figures 3.14-3.18. The dissociation rates for the binding of each of each compound with recombinant trypanosomal α-tubulin were in the range of $1.70 \times 10^{-2} \text{ min}^{-1}$ (Table 3.1), which is lower compared to the dissociation rates for binding to recombinant trypanosomal β-tubulin, which were in the range of $6.0 \times 10^{-4}$ to $3.93 \times 10^{-2} \text{ min}^{-1}$ (Table 3.2). The dissociation rates for the binding of compounds 1003 and 1008 to recombinant trypanosomal β-tubulin could not be determined because neither compound reduced fluorescence initially (Table 3.2). Dissociation rates for colchicine and compound 1003 with native rat tubulin were $3.38 \times 10^{-2} \text{ min}^{-1}$ and $5.62 \times 10^{-2} \text{ min}^{-1}$ respectively (Table 3.3). The dissociation rate could not be determined for compound 1007 with native rat tubulin because fluorescence increased beyond the $F_{\text{max}}$ value.

![Semi-logarithmic plot of the dissociation of compound 1008 from recombinant trypanosomal α-tubulin.](image)

**Figure 3.14** Semi-logarithmic plot of the dissociation of compound 1008 from recombinant trypanosomal α-tubulin.
Figure 3.15 Semi-logarithmic plot of the dissociations of compound 1016 from recombinant trypanosomal α- and β-tubulin.

Figure 3.16 Semi-logarithmic plot of the dissociations of compound 1007 from recombinant trypanosomal α- and β-tubulin.
Figure 3.17 Semi-logarithmic plot of the dissociations of compound 1017 from recombinant trypanosomal α- and β-tubulin.

Figure 3.18 Semi-logarithmic plot of the dissociations of compound 1003 from recombinant trypanosomal α-tubulin and native rat brain tubulin.
The affinity constants ($K_a$) for the binding of the novel trifluralin analogues, trifluralin, albendazole and colchicine to recombinant trypanosomal α- and β-tubulin and native rat brain tubulin are shown in Tables 3.1 to 3.3. Affinity constants for the binding of each compound with recombinant trypanosomal α-tubulin were in the range $3.20 \times 10^5$ to $1.23 \times 10^6$ M$^{-1}$ (Table 3.1), which is significantly higher compared to the affinity constants for binding to recombinant trypanosomal β-tubulin and native rat tubulin which were in the range of $1.50$ to $3.33 \times 10^5$ M$^{-1}$ (Table 3.2) and $2.12$ to $2.90 \times 10^5$ M$^{-1}$ (Table 3.3) respectively.

**Table 3.1** The association ($k_{on}$), dissociation ($k_{off}$) and affinity ($K_a$) constants for the binding of trifluralin analogues with recombinant trypanosomal α-tubulin.

<table>
<thead>
<tr>
<th>Trifluralin analogue</th>
<th>Binding Parameter</th>
<th>$k_{on}$ (M$^{-1}$ min$^{-1}$)</th>
<th>$k_{off}$ (min$^{-1}$)</th>
<th>$K_a$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1003</td>
<td></td>
<td>$1.85 \times 10^4$</td>
<td>$1.70 \times 10^{-2}$</td>
<td>$1.09 \times 10^6$</td>
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<tr>
<td>1007</td>
<td></td>
<td>$2.38 \times 10^4$</td>
<td>$1.93 \times 10^{-2}$</td>
<td>$1.23 \times 10^6$</td>
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<td>$1.50 \times 10^4$</td>
<td>$3.16 \times 10^{-2}$</td>
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<tr>
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<tr>
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<tr>
<td>Trif.</td>
<td></td>
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<td>$7.74 \times 10^5$</td>
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</table>
Table 3.2 The association \((k_{on})\), dissociation \((k_{off})\) and affinity \((K_a)\) constants for the binding of trifluralin analogues and albendazole with recombinant trypanosomal \(\beta\)-tubulin.

<table>
<thead>
<tr>
<th>Trifluralin analogue</th>
<th>Binding Parameter</th>
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<tr>
<td></td>
<td>(k_{on} (M^{-1} \text{ min}^{-1}))</td>
<td>(k_{off} (\text{min}^{-1}))</td>
<td>(K_a (M^{-1}))</td>
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<tr>
<td>1003</td>
<td>(4.50 \times 10^2)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1007</td>
<td>(1.01 \times 10^4)</td>
<td>(5.13 \times 10^{-2})</td>
<td>(1.97 \times 10^5)</td>
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</tr>
<tr>
<td>1008</td>
<td>(3.95 \times 10^3)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1016</td>
<td>(2.00 \times 10^2)</td>
<td>(6.00 \times 10^{-4})</td>
<td>(3.33 \times 10^5)</td>
<td></td>
</tr>
<tr>
<td>1017</td>
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<td>(3.93 \times 10^{-2})</td>
<td>(1.95 \times 10^5)</td>
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</tr>
<tr>
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<td>(3.67 \times 10^{-2})</td>
<td>(1.50 \times 10^5)</td>
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Table 3.3 The association \((k_{on})\), dissociation \((k_{off})\) and affinity \((K_a)\) constants for the binding of trifluralin analogues and colchicine with native rat brain tubulin.

<table>
<thead>
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<th>Trifluralin analogue</th>
<th>Binding Parameter</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(k_{on} (M^{-1} \text{ min}^{-1}))</td>
<td>(k_{off} (\text{min}^{-1}))</td>
<td>(K_a (M^{-1}))</td>
<td></td>
</tr>
<tr>
<td>1003</td>
<td>(1.19 \times 10^4)</td>
<td>(5.62 \times 10^{-2})</td>
<td>(2.12 \times 10^5)</td>
<td></td>
</tr>
<tr>
<td>1007</td>
<td>(1.65 \times 10^3)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Colch.</td>
<td>(9.80 \times 10^3)</td>
<td>(3.38 \times 10^{-2})</td>
<td>(2.90 \times 10^5)</td>
<td></td>
</tr>
</tbody>
</table>

3.3.5 Analysis of the tubulin amino acid sequences from *T. b. rhodesiense*, *L. major*, *Rattus norvegicus* and *Homo sapiens*

There is 94.5% homology between the amino acid sequences of trypanosomal and leishmanial \(\alpha\)-tubulin and 85% homology between trypanosomal and rat \(\alpha\)-tubulin (Figure 3.19). There is 95.2% and 83.7% homology between the amino acid sequences of trypanosomal and leishmanial \(\beta\)-tubulin and trypanosomal and rat \(\beta\)-tubulin respectively (Figure 3.20). Homology between rat and human \(\alpha\)- and \(\beta\)-tubulin is 99.3% and 95% respectively.
**Figure 3.19** Comparison of amino acid sequences of α-tubulin from *T. b. rhodesiense* (Kimmel et al., 1985), *Leishmania major* (Ivens et al., 1998), *Rattus norvegicus* (Strausberg et al., 2002) and *Homo sapiens* (Strausberg et al., 2002). (N.B. * areas of variability).
### Chapter 3. Binding Kinetics of Trifluralin Analogues

<table>
<thead>
<tr>
<th>Species</th>
<th>Amino Acid Sequence</th>
</tr>
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<tbody>
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<td>MREIVCQAG QCNGNIGSKF WEVISDEHG DPTGTYQGDS DLQLERINVY</td>
</tr>
<tr>
<td>L.major</td>
<td>MREIVSCQAG QCNGNIGSKF WEVISDEHG DPTGTYQGDS DLQLERINVY</td>
</tr>
<tr>
<td>R.norv</td>
<td>MREIVHIQAG QCNGNIGPKF WEVISDEHG DPTGSYHGDS DLQLERINVY</td>
</tr>
<tr>
<td>H.sapien</td>
<td>MREIVHIQAG QCNGNIGKAF WEVISDEHG DPTGSYHGDS DLQLDRI SVY</td>
</tr>
</tbody>
</table>

** T.brucei: FDEATGGRYV PRSVLIDLEP GTMDSVRAGP YGQIFRPDGF IFGQSGAGNN
** L.major: FDEATGGRYV PRSVLIDLEP GTMDSVRAGP YGQIFRPDGF IFGQSGAGNN
** R.norv: YNESAGGNVK PRAILVDLEP GTMD SVRSGP FGQLFRPDGF VFGQSGAGNN
** H.sapien: YNESAGGNVK PRAILVDLEP GTMD SVRSGP FGQLFRPDGF VFGQSGAGNN

** T.brucei: WAKGHYTEGA ELIDSVLDCV KEAESCDCL QGFOICSHLGG TGTSSGMGTL |
** L.major: WAKGHYTEGA ELIDSVLDCV KEAESCDCL QGFOICSHLGG TGTSSGMGTL |
** R.norv: WAKGHYTEGA ELIDSVLDCV KEAESCDCL QGFOICSHLGG TGTSSGMGTL |
** H.sapien: WAKGHYTEGA ELIDSVLDCV KEAESCDCL QGFOICSHLGG TGTSSGMGTL |

** T.brucei: LISKLREQYP DRIMMTFSII PSPKVSDTVV EPYNATLSVH QLVENSDESM |
** L.major: LISKLREQYP DRIMMTFSII PSPKVSDTVV EPYNATLSVH QLVENSDESM |
** R.norv: LISKLREQYP DRIMMTFSII PSPKVSDTVV EPYNATLSVH QLVENSDESM |
** H.sapien: LISKLREQYP DRIMMTFSII PSPKVSDTVV EPYNATLSVH QLVENSDESM |

** T.brucei: CIDNEALYDI CFRTLKLTTP TFGDLNHLVS AVMSVGTCC VLRFPGQLNSDL |
** L.major: CIDNEALYDI CFRTLKLTTP TFGDLNHLVS AVMSVGTCC VLRFPGQLNSDL |
** R.norv: CIDNEALYDI CFRTLKLTTP TFGDLNHLVS AVMSVGTCC VLRFPGQLNSDL |
** H.sapien: CIDNEALYDI CFRTLKLTTP TFGDLNHLVS AVMSVGTCC VLRFPGQLNSDL |

** T.brucei: RKLAVNLVPF PRLHFFMMGF APLTSRGSQQ YRGLSVPELT QQMFDAKNMM |
** L.major: RKLAVNLVPF PRLHFFMMGF APLTSRGSQQ YRGLSVPELT QQMFDAKNMM |
** R.norv: RKLAVNLVPF PRLHFFMMGF APLTSRGSQQ YRGLSVPELT QQMFDAKNMM |
** H.sapien: RKLAVNLVPF PRLHFFMMGF APLTSRGSQQ YRGLSVPELT QQMFDAKNMM |

** T.brucei: QAADPRHGGRY LTASALFRGR MSTKEVDEQM LNVQKNNSSY FIEWIPPNIK |
** L.major: QAADPRHGGRY LTASALFRGR MSTKEVDEQM LNVQKNNSSY FIEWIPPNIK |
** R.norv: QAADPRHGGRY LTASALFRGR MSTKEVDEQM LNVQKNNSSY FIEWIPPNIK |
** H.sapien: QAADPRHGGRY LTASALFRGR MSTKEVDEQM LNVQKNNSSY FIEWIPPNIK |

** T.brucei: SSVCDIPPKG LKMAVTFIGN NTCQEFMGRR VGEOFTLMFR RKAFLHWYTG |
** L.major: SSVCDIPPKG LKMAVTFIGN NTCQEFMGRR VGEOFTLMFR RKAFLHWYTG |
** R.norv: SSVCDIPPKG LKMAVTFIGN NTCQEFMGRR VGEOFTLMFR RKAFLHWYTG |
** H.sapien: SSVCDIPPKG LKMAVTFIGN NTCQEFMGRR VGEOFTLMFR RKAFLHWYTG |

** T.brucei: EGMDEMEMEF TEAESMNDLVSV EYQQYQDATI EEEGFDEEEE QY |
** L.major: EGMDEMEMEF TEAESMNDLVSV EYQQYQDATI EEEGFDEEEE QY |
** R.norv: EGMDEMEMEF TEAESMNDLVSV EYQQYQDATI EEEGFDEEEE QY |
** H.sapien: EGMDEMEMEF TEAESMNDLVSV EYQQYQDATI EEEGFDEEEE QY |

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**Figure 3.20** Comparison of amino acid sequences of β-tubulin from *T. b. rhodesiense* (Kimmel et al., 1985), *Leishmania major* (Ivens et al., 1998), *Rattus norvegicus* (Ginzburg et al., 1985) and *Homo sapiens* (Shiina et al., 2001). (N.B. * areas of variability).
3.4 Discussion

This study was undertaken to determine if five novel analogues of trifluralin bind to the putative target, α-tubulin, in *T. b. rhodesiense* and whether the nature of the binding of each analogue differs. In addition, the extent of binding of each compound with β-tubulin and mammalian tubulin was also investigated.

The results of this study showed that five new analogues of trifluralin and commercial trifluralin strongly interacted with recombinant trypanosomal α-tubulin, inducing significant concentration dependent reductions in the intrinsic fluorescence. These compounds also produced distinctive binding affinities for recombinant trypanosomal α-tubulin. The structural differences between the five novel trifluralin analogues have led to a significant difference in the binding affinity of each compound with the three forms of tubulin. However, these differences did not alter the ability of these compounds to bind to trypanosomal α-tubulin.

These results are consistent with the results of previous studies that showed that trifluralin binds to α-tubulin from *T. gondii* (Morrissette *et al.*, 2004; Stokkermans *et al.*, 1996). The large reduction observed in intrinsic fluorescence suggests that tryptophan residues are directly involved in the binding of these compounds to trypanosomal α-tubulin. Compound 1003 was the only analogue to bind with native rat brain tubulin, inducing a significant concentration dependent reduction in intrinsic fluorescence as a result of the C1 substituent of 4-morpholino. This suggests that tryptophan residues are also directly involved in the binding of this compound to rat brain tubulin. Compound 1003 bound more strongly to trypanosomal α-tubulin compared to rat brain tubulin as evidenced by the significant difference in their affinity.
constants. This finding differs from previous reports that the dinitroaniline class of compounds do not bind to mammalian tubulin (Bajer and Mole-Bajer, 1986; Chan and Fong, 1990; Dow et al., 2002; Hess and Bayer, 1977; Hugdahl and Morejohn, 1993) and may suggest that dinitroanilines have the potential to cause toxicity in mammals. However, there appears to be no correlation between these findings and the results of an *in vitro* whole cell toxicity assay (unpublished data).

Compounds 1007, 1008 and 1017 induced moderate, concentration dependent reductions in the fluorescence of trypanosomal β-tubulin. These moderate reductions suggest that tryptophan residues are indirectly involved in the binding of these compounds to trypanosomal β-tubulin. However, the affinity of binding was low for compounds 1007 and 1017 and compound 1008 demonstrated no binding affinity, which suggests that binding of these compounds to trypanosomal β-tubulin is weak and unstable. This result also suggests that compounds 1007 and 1017 may bind to both α- and β-tubulin, which agrees with previous suggestions that the dinitroanilines act on both α- and β-tubulin in weeds (Anthony et al., 1998; Chan and Fong, 1994).

Morrissette, et al. (2004) has identified the putative binding site of common dinitroanilines on α-tubulin from *T. gondii*. This binding site was postulated to involve amino acid residues Arg2 (R), Glu3 (E), Val4 (V), Trp21 (W), Phe24 (F), His28 (H), Ile42 (I), Asp47 (D), Arg64 (R), Cys65 (C), Thr239 (T), Arg243 (R) and Phe244 (F). All but two of these residues are present in the amino acid sequence of α-tubulin of *T. brucei*. The two residues absent in trypanosomal α-tubulin are Val4 (V), which is substituted for Ala4 (A) and Cys65 (C), which is substituted for Ala65 (A). The presence of the tryptophan residue Trp21 (W) in the dinitroaniline binding site in *T.
*gondii* correlates with the strong quenching of intrinsic fluorescence of *T. brucei* α-tubulin observed in this study. In addition, the *T. brucei* β-tubulin amino acid sequence shares only four common residues which may explain the low affinity of trifluralin analogues with trypanosomal β-tubulin.

Mammalian α-tubulin also shares the common residues found in the postulated dinitroaniline binding site in protozoal α-tubulin. The same two residues differ in mammalian and trypanosomal α-tubulin. The amino acid residue Val4 (V) is substituted for Cys4 (C) and Phe24 (F) is substituted for Tyr24 (Y) in mammalian α-tubulin. This may explain why compound 1003 bound with native rat brain tubulin but does not explain the selective action of the other four compounds for trypanosomal α-tubulin. The amino acid residues Arg2 (R), Glu3 (E), Trp21 (W), Phe24 (F), His28 (H) and Ile42 (I) are located in areas of the α-tubulin amino acid sequence where strong variations exist between trypanosomal and mammalian tubulin. It is possible that the binding site of these new analogues of trifluralin involves residues that are present in trypanosomal α-tubulin but absent in mammalian α-tubulin and that compound 1003 binds to a different site on α-tubulin.

A mechanism of action for common dinitroanilines has recently been proposed using computational techniques (Morrissette *et al.*, 2004). When oryzalin was inserted into the α-tubulin binding site of *T. gondii*, it was situated beneath the N loop between protofilaments in the microtubule. It has previously been established that protofilament-protofilament contact is mediated by N loops interacting with M loops of laterally adjacent subunits (Li *et al.*, 2002). If oryzalin binds beneath the N loop, this interaction may be inhibited with the consequence of microtubule disruption. This differs from the
mechanism of action of other tubulin-binding compounds, such as colchicine and vinblastine which have been shown to interfere with tubulin assembly (Ravelli et al., 2004; Gigant et al., 2005).

Future studies should concentrate on establishing the molecular basis of the proposed trifluralin binding domain in trypanosomal \( \alpha \)-tubulin. The single point mutation Thr239Ile and double mutation Ser165Ala/Thr239Ile in the \( \alpha \)-tubulin amino acid sequence of \textit{T. gondii} conferred dinitroaniline resistance (Morrissette et al., 2004). Similar approaches could be applied to the study of trifluralin binding to \textit{T. brucei} \( \alpha \)-tubulin. All but two of the amino acid residues involved in dinitroaniline binding in \textit{T. gondii} are shared by \textit{T. brucei} and can provide a starting point for establishing their involvement in the trifluralin binding site by use of site-directed mutagenesis. Initially, single amino acids residues could be substituted followed by all relevant amino acid residues, in order to determine the effects this would have on the binding kinetics of these trifluralin analogues and also to determine the point at which a complete loss of trifluralin binding occurs.

Differences in tubulin drug binding can be the basis for selective chemotherapeutic intervention against trypanosomal infection. The results of this study and others have demonstrated that differences in drug binding exist between protozoan parasite tubulin and mammalian tubulin and specifically trypanosomal tubulin and mammalian tubulin (Bhattacharya et al., 2004; MacRae and Gull, 1990; Werbovetz et al., 1999). Selective susceptibility is thought to be due to amino acid differences in tubulin structure between lower eukaryotes and mammals (Lacey, 1988). Trypanosomal tubulins have diverged significantly from the tubulins of mammalian species (Kimmel et al., 1985). A
comparison of the amino acid sequences of α-tubulin reveal that there is a high degree of homology between trypanosomal and leishmanial tubulin and only moderate homology between trypanosomal and rat tubulin. Therefore, it may be possible that these trifluralin analogues would also bind to leishmanial tubulin with similar affinity. However, previous studies suggest that this may not be the case and that small changes in amino acid sequence can have a dramatic influence on the binding of compounds to tubulins from different species (Bhattacharya et al., 2004).

The tubulin used in this study was a homogenous and pure protein interacting with a chemically pure ligand under controlled conditions. Microtubules are in constant dynamic equilibrium undergoing the addition and substraction of soluble tubulin units at opposite ends of the developing tubule (Lacey, 1988). The formation and breakdown of microtubules are determined by the kinetics of assembly/disassembly that occurs at the plus end of the microtubule (MacRae, 1992). This dynamic behaviour of microtubules and the effect trifluralin analogues have on growing microtubules could be investigated using established microscope-based techniques (Grego et al., 2001; Viani et al., 2000). This would aid in the understanding of the mechanism of action these trifluralin have on trypanosomal microtubules.

Understanding the nature of the interaction between trifluralins and trypanosomal tubulin is fundamental for the development of tubulin-specific anti-trypanosomatid compounds with selective activity, appropriate pharmacokinetic properties, and low human toxicity. The exact mechanism by which binding occurs is unclear, but might result from protein conformational changes induced by the binding of compounds to regions where amino acids differ between trypanosomal and mammalian tubulin. Trifluralin and its analogues possess strong binding with trypanosomal tubulin, which
provides further evidence that tubulin is a target for selective chemotherapeutic agents. Most notably, the results of this study have identified four trifluralin analogues (EPL-AJ 1007, 1008, 1016 and 1017) as potential anti-trypanosomatid agents that warrant further development.
Chapter 4

Effects of Novel Trifluralin Analogues on the Ultra-Structure of *Trypanosoma brucei.*
4.1 Introduction

A major characteristic of trypanosomes is a tightly defined, spatially co-ordinated cytoskeleton (Robinson et al., 1995), which is responsible for maintaining the shape and form of the cell and the modulation of cell shape between different life cycle stages (Hemphill et al., 1991). The main cytoskeletal components are the microtubular subpellicular corset, axoneme, basal body, paraflagellar rod (PFR), flagellum attachment zone and filaments responsible for attachment to insect tissues (Kohl and Gull, 1998). Microtubule-mediated events occur throughout the cell cycle. Cell shape and form are entirely dependent on the microtubular subpellicular corset, and polymerisation of new microtubules is an essential prerequisite for cell growth within the cell cycle (Robinson et al., 1995; Sherwin and Gull, 1989). Other microtubule dependent processes involving microtubule polymerisation/depolymerisation include kinetoplast segregation and repositioning (Matthews et al., 1995), basal body duplication, flagellar axoneme growth, mitosis and cytokinesis (Ploubidou et al., 1999).

The effects of three common dinitroaniline analogues (oryzalin, ethalfluralin and trifluralin) have been shown to alter the cytoskeletal structure of Toxoplasma gondii in vitro (Stokkermans et al., 1996). These compounds blocked nuclear division by inhibiting intranuclear spindle formation. Other cytoskeletal components were differentially affected depending on the analogue tested. In oryzalin-treated T. gondii, the subpellicular microtubules were absent and in ethalfluralin-treated T. gondii, large fragments of the inner membrane complex remained intact but the endoplasmic reticulum and nuclear envelope were highly distended (Stokkermans et al., 1996). Trifluralin and oryzalin have been shown to inhibit the polymerization of subpellicular microtubules of Leishmania sp. (Chan and Fong, 1990; Chan et al., 1991) and trifluralin
has been shown to induce the disassembly of subpellicular microtubules of Plasmodium sp. (Kaidoh et al., 1995). However, trifluralin treatment of T. cruzi in vitro induced many round, multi-flagellated forms without subpellicular or flagellar microtubule disruption (Bogitsh et al., 1999).

It is hypothesised that if a compound binds to tubulin then it will inhibit the polymerization of tubulin into microtubules (Chan et al., 1991) or that microtubules will be depolymerised, which would ultimately result in microtubule disassembly and the loss of microtubules from the cell (Morrissette et al., 2004). These changes are likely to cause abnormalities in the cytoskeleton and possibly also in the nucleus and kinetoplast of trypanosomes.

The positional ordering and segregation of the kinetoplast involves direct interaction with the microtubule cytoskeleton (Robinson and Gull, 1991). The kinetoplast and nucleus can be visualised using fluorescent cytological probes allowing the identification of the cell cycle stage. Cells early in the cell cycle possess a single kinetoplast and a single nucleus producing a 1K1N (1 kinetoplast, 1 nucleus) cell. As the cell cycle progresses the kinetoplast replicates and segregates in a discrete “S” phase prior to division of the nucleus to produce a 2K1N (2 kinetoplasts, 1 nucleus) cell. Cells then enter mitosis and the cell becomes 2K2N (2 kinetoplasts, 2 nuclei) and once cytokinesis occurs, cleaving the cell into two 1K1N siblings (Robinson et al., 1995).

The effects dinitroanilines have on the ultrastructure of T. brucei are unknown. In this study, the interaction between new analogues of trifluralin and T. b. rhodesiense was investigated and an assessment of the effect of these compounds on the ultrastructure of
trypanosomes using light, fluorescence and transmission electron microscopy (TEM) was made.

The aim of this study was:

1. To investigate the ultra-structural effects of new analogues of trifluralin on *T. b. rhodesiense*.
4.2 Materials and Methods

4.2.1 Novel Trifluralin analogues

New trifluralin analogues EPL-AJ1003 (1003), EPL-AJ1007 (1007), EPL-AJ1008 (1008), EPL-AJ1016 (1016), and EPL-AJ1017 (1017) were synthesized and supplied by Epichem Pty. Ltd. Albendazole and commercial trifluralin were also supplied by Epichem Pty. Ltd., and colchicine was purchased from Sigma (St Louis, MO). Stock solutions of these compounds were prepared in 100% dimethyl sulphoxide (DMSO) at a concentration of 1mM and stored in 50μl aliquots at –20°C until required for use.

4.2.2 Trypanosome culture

Blood stream forms of *T. b. rhodesiense* were cultured from cryopreserved stocks in HMI-9 media in 25cm³ flasks according to Hirumi and Hirumi (1994) until a concentration of approximately 1 × 10⁶ cells/ml was reached.

4.2.3 Treatment of *T. b. rhodesiense* bloodstream forms with analogues of Trifluralin.

Cultured blood stream forms of *T. b. rhodesiense* were seeded in 48-well plates at a concentration of 1 × 10⁶ cells/ml before the addition of their respective IC₅₀ and IC₇₀ concentrations of compounds EPL-AJ1003, EPL-AJ1007, EPL-AJ1008, EPL-AJ1016, and EPL-AJ1017. The IC values in μM were determined from previous dose response curves (unpublished data) (Table 4.1).
### Table 4.1 Concentrations of 5 trifluralin analogues that inhibit 50% (IC$_{50}$) and 70% (IC$_{70}$) of the growth of *T. b. rhodesiense* in vitro

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (μM)</th>
<th>IC$_{70}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPL-AJ1003</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>EPL-AJ1007</td>
<td>0.35</td>
<td>1.25</td>
</tr>
<tr>
<td>EPL-AJ1008</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>EPL-AJ1016</td>
<td>0.15</td>
<td>0.3</td>
</tr>
<tr>
<td>EPL-AJ1017</td>
<td>0.1</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Control cultures of trypanosomes were incubated with only DMSO. Each culture was observed regularly using light microscopy over a 48hr period to determine the time point at which the trypanosomes were visibly affected. The cells were then processed for immunofluorescence or transmission electron microscopy.

#### 4.2.4 Immunofluorescence study

Glass slides were wiped with 70% ethanol to degrease them and then wiped with PBS (pH 7.2) to reduce their hydrophobicity. The slides were then wiped with 0.1% glutaraldehyde to enhance cell adherence. Two millilitres of culture medium containing *T. b. rhodesiense* was removed from each plate well and centrifuged at 1,000 × g for 5min, resuspended in 200μl of HMI-9 media, smeared onto treated slides and air-dried for 30-45 min at room temperature. The slides were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in PBS (pH 7.2) for 10min at room temperature. The aldehyde groups were then neutralized by washing the slides gently with 50μl of 100mM Glycine twice for 10min each time. The slides were then immersed gently into methanol and left to incubate for 2hr at -20°C. The slides were then gently removed from the methanol
and immersed gently into PBS (pH 7.2) two times for 5min each time to rehydrate the cells.

The cells were incubated with 20μl of monoclonal anti-alpha tubulin antibody (TAT1) (Professor Keith Gull, Sir William Dunn School of Pathology, University of Oxford) for 60min in a humid chamber at room temperature. The slides were washed twice for 5min each time in PBS (pH 7.2). Anti-mouse FITC-conjugated rabbit antibody (F-9137, Sigma, St Louis, MO) was added to the slides at a dilution of 1:100 in PBS (200μl total) and incubated for 60min in a humid chamber at room temperature in the dark. The slides were washed twice in PBS (pH 7.2) for 5min each time and then flooded with 1μg/ml 4,6-diamidine-2-phenylindole (DAPI) in ddH₂O for 4min to stain cellular DNA in the kinetoplast and nucleus and then washed with 200μL of 100mM HEPES (pH 7.5) twice for 5min. The slides were then treated with antifade reagents from the SlowFade® Light Antifade Kit (Molecular Probes, Invitrogen) following the manufacturer’s instructions to reduce fading of the fluorescein. The slides were covered with a cover slip and sealed with white nail varnish and immediately viewed under an Olympus BX51 Photomicroscope (Olympus, Australia).

4.2.5 Transmission electron microscopy

The remaining culture medium containing *T. b. rhodesiense* was removed from each well and centrifuged at 1,000 × g for 10min, washed with 25ml of PBS (140mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.8mM KH₂PO₄, 80mM glucose, pH 7.4) and centrifuged again at 1,000 × g for 10min to obtain a pellet of trypanosomes for each treatment. Each pellet was fixed by incubation in a solution containing 4%
glutaraldehyde, 4% paraformaldehyde, 0.1% tannic acid in 0.1M cacodylate buffer (pH 7.4) at room temperature for 2hrs. The samples were then washed three times by centrifugation at 1,000 × g for 10min in ddH₂O. They were then post-fixed in a solution containing 1% osmium tetroxide and washed again in ddH₂O as described above. The pellets were stained overnight by incubating in a solution containing 2% magnesium-uranyl-acetate at 4°C. The samples were then washed with ddH₂O as described previously and dehydrated in a graded ethanol series of 30%, 50%, 70% for 30min each and 90% overnight. They were dehydrated again by immersion three times in absolute ethanol for 60min and then once overnight. The samples were then incubated in the following ratios of spurs resin:ethanol mixtures; 30%, 50%, 70% and 90% for 3hrs each before incubations in 100% spurs resin three times for 1hr each time. A final incubation in 100% spurs resin overnight was performed before the samples were embedded and polymerised in epoxy resin overnight at 60°C. 70nm sections were cut and stained with lead citrate for 5min at room temperature and a solution containing 5% uranyl acetate in 1% acetic acid for 30min at 60°C. The processed and stained sections were examined using a Philips CM100 Biotwin transmission electron microscope (Philips Electronics NV, The Netherlands).
4.3 Results

4.3.1 Immunofluorescence Microscopy

Untreated *T. b. rhodesiense* cells appeared to be morphologically normal maintaining their typical fusiform shape. Three stages of the trypanosome cell cycle were identified in these control samples. The DAPI-stained image revealed the presence of 1K1N and 2K2N cells, with intact nuclei and kinetoplasts. The cells probed with TAT1 antibody stained positive for tubulin showing the outline of the cytoskeleton including the flagellum, which is attached along the whole length of each cell body (Figure 4.1).

Examination of trypanosomes treated with the IC$_{50}$ concentration of compound 1008 showed some normal cells with typical morphology and some distended cells with abnormal morphology. DAPI staining revealed some normal 1K1N cells and some cells with no kinetoplast. Bright fluorescence was observed in the TAT1 antibody probed cells, clearly outlining the shape of the cytoskeleton including the flagellum, which is attached along the whole of the cell body in some cells but detached in others (Figure 4.2). Trypanosomes that were treated with the IC$_{70}$ concentration of compound 1008 generally appeared distorted. DAPI staining revealed cells that were multi-nucleated and with multiple kinetoplasts. TAT1-probed cells revealed the presence of tubulin but distortion of the cytoskeleton including the flagellum, which is detached from the cell body (Figure 4.2).
Figure 4.1 Untreated *T. b. rhodesiense* processed and viewed using a) Phase contrast light microscopy, b) DAPI fluorescence, c) TAT1 probed fluorescence, d) merged images of DAPI fluorescence and TAT1 probed fluorescence.

Figure 4.2 *T. b. rhodesiense* treated with the IC$_{50}$ concentration of compound 1008, processed and viewed using a) Phase contrast light microscopy, b) DAPI fluorescence, c) TAT1 probed fluorescence, d) merged images of DAPI fluorescence and TAT1 probed fluorescence. *T. b. rhodesiense* treated with the IC$_{70}$ dose of compound 1008, processed and viewed using e) Phase contrast light microscopy, f) DAPI fluorescence, g) TAT1 probed fluorescence, h) merged images of DAPI fluorescence and TAT1 probed fluorescence.
Examination of trypanosomes treated with the IC$_{50}$ concentration of compound 1003 showed abnormal shaped cells that were often clustered together. DAPI staining revealed only 1K1N cells and TAT1 fluorescence staining showed the presence of tubulin but distortion of the cytoskeleton and the clustering of the cells (Figure 4.3). Trypanosomes that were treated with its IC$_{70}$ concentration of compound 1003 also appeared distorted and clustered together. DAPI staining displayed distorted indistinguishable kinetoplasts and nuclei. TAT1 probed cells appeared as one large cluster of undefined cells (Figure 4.3).

Trypanosomes treated with the IC$_{50}$ concentration of compound 1016 generally appeared normal with some cells demonstrating a change in morphology similar to those induced by compound 1008. DAPI staining revealed mostly 1K1N cells containing intact kinetoplast and nucleus. TAT1 staining revealed cells with typical shaped cytoskeleton and intact flagellum (Figure 4.4). Trypanosomes treated with the IC$_{70}$ concentration of compound 1016 were distorted with either detached or abnormally long flagellae. DAPI staining of these cells revealed damaged nuclei and damaged or repositioned kinetoplasts. TAT1-probed cells stained positive for tubulin which revealed the distorted shape of the cytoskeleton and flagellae that were completely detached from the cell bodies (Figure 4.4).
Figure 4.3 *T. b. rhodesiense* treated with the IC$_{50}$ concentration of compound 1003, processed and viewed using a) Phase contrast light microscopy, b) DAPI fluorescence, c) TAT1 probed fluorescence, d) merged images of DAPI fluorescence and TAT1 probed fluorescence. *T. b. rhodesiense* treated with the IC$_{70}$ dose of compound 1003, processed and viewed using e) Phase contrast light microscopy, f) DAPI fluorescence, g) TAT1 probed fluorescence, h) merged images of DAPI fluorescence and TAT1 probed fluorescence.

Figure 4.4 *T. b. rhodesiense* treated with the IC$_{50}$ concentration of compound 1016, processed and viewed using a) Phase contrast light microscopy, b) DAPI fluorescence, c) TAT1 probed fluorescence, d) merged images of DAPI fluorescence and TAT1 probed fluorescence. *T. b. rhodesiense* treated with the IC$_{70}$ dose of compound 1016, processed and viewed using e) Phase contrast light microscopy, f) DAPI fluorescence, g) TAT1 probed fluorescence, h) merged images of DAPI fluorescence and TAT1 probed fluorescence.
Trypanosomes treated with the IC$_{50}$ concentration of compound 1007 appeared morphologically similar to those treated with the IC$_{50}$ of compound 1003. Most cells generally appeared normal in shape but were clustered together. DAPI staining revealed only 1K1N cells. TAT1-probed cells appeared morphologically abnormal although most cells contained a normal cytoskeleton and flagellum (Figure 4.5). Trypanosomes treated with the IC$_{70}$ concentration of compound 1007 were also generally irregular in their morphology. DAPI staining revealed damaged nuclei and kinetoplasts. TAT1-probed cells revealed the presence of tubulin but showed that cells were swollen and distended with detached flagella (Figure 4.5).

Trypanosomes treated with the IC$_{50}$ concentration of compound 1017 generally appeared normal but some abnormal cells were detected. TAT1-probed cells appeared swollen with detached flagella. DAPI staining revealed some 1K1N and some multinucleated cells (Figure 4.6). Trypanosomes treated with the IC$_{70}$ concentration of compound 1017 generally appeared similar to those treated with the IC$_{50}$ concentration. Many TAT1-probed cells emphasised the swollen morphology and detached flagella. DAPI staining revealed some cells that were either multinucleated with damaged kinetoplasts or the positioning of the kinetoplast had been altered, appearing closer to the nucleus (Figure 4.6).
Figure 4.5 *T. b. rhodesiense* treated with the IC\textsubscript{50} concentration of compound 1007, processed and viewed using a) Phase contrast light microscopy, b) DAPI fluorescence, c) TAT1 probed fluorescence, d) merged images of DAPI fluorescence and TAT1 probed fluorescence. *T. b. rhodesiense* treated with the IC\textsubscript{70} dose of compound 1007, processed and viewed using e) Phase contrast light microscopy, f) DAPI fluorescence, g) TAT1 probed fluorescence, h) merged images of DAPI fluorescence and TAT1 probed fluorescence.

Figure 4.6 *T. b. rhodesiense* treated with the IC\textsubscript{50} concentration of compound 1017, processed and viewed using a) Phase contrast light microscopy, b) DAPI fluorescence, c) TAT1 probed fluorescence, d) merged images of DAPI fluorescence and TAT1 probed fluorescence. *T. b. rhodesiense* treated with the IC\textsubscript{70} dose of compound 1017, processed and viewed using e) Phase contrast light microscopy, f) DAPI fluorescence, g) TAT1 probed fluorescence, h) merged images of DAPI fluorescence and TAT1 probed fluorescence.
4.3.2 Transmission Electron Microscopy

Untreated trypanosomes displayed typical morphology when viewed using TEM. Although the sections stained darker than what is considered optimal, the main microtubular structures can be clearly visualised. Both the cell body and flagellum are limited by a three-ply plasma membrane which appears normal. The subpellicular microtubules appear intact with typical arrangement. The microtubules in the axoneme are also intact and appear to have the typical $9 + 2$ arrangement of nine outer microtubule doublets with arms and two inner but separate microtubules (Figure 4.7).

Trypanosomes treated with the IC$_{70}$ concentration of compound 1008 appeared to be morphologically distorted. Figure 4.8 shows a cell with two flagella with disrupted plasma membranes and a degenerate subpellicular corset (Figure 4.8). The cytoplasm of the cell appears to contain membranous material and has no distinguishable organelles. The flagellum at the top of the image has a normal shape and an axoneme of typical arrangement but lacks a PFR. The flagellum at the bottom of the image contains a deformed axoneme and PFR. The subpellicular microtubules are either absent, leaving holes where there should be microtubules, or there are abnormally large gaps between microtubules (Figure 4.8).
**Figure 4.7** a) Transmission electron micrograph of a transverse section of an untreated *T. b. rhodesiense* cell b) high power of boxed area in a) showing paraflagellar rod (PFR), axoneme (AX), subpellicular microtubules (MT), and plasma membrane (PM).

**Figure 4.8** a) Transmission electron micrograph of a transverse section of a compound 1008 treated *T. b. rhodesiense* cell b) high power of boxed area in a) showing degenerate plasma membrane (PM), flagellum with axoneme (AX) and no paraflagellar rod, holes in place of subpellicular microtubules (MT) and a flagellum (F) with deformed axoneme and paraflagellar rod.
Trypanosomes treated with the IC$_{70}$ concentration of compound 1003 have similar morphological abnormalities observed in trypanosomes treated with compound 1008 (Figure 4.8). These cells also contained unknown membranous material in their cytoplasm and had no distinguishable organelles. No microtubules could be visualised in the subpellicular array. Figure 4.9 shows a cell that has two flagella. The flagellum on the left of the image has an axoneme of typical arrangement but does not have a PFR and the flagellum on the right of the image has a malformed axoneme composition and no PFR. The plasma membrane is degenerate around the subpellicular corset and flagella.

Trypanosomes treated with the IC$_{70}$ concentration of compound 1016 appeared severely distorted. The flagella pocket is enlarged containing unknown fine tubular material with a detached flagellar pocket membrane. The flagellum has a malformed axoneme and no distinct PFR (Figure 4.10). The plasma membrane is disturbed in most areas of the cell and particularly surrounding the flagellum where it has fragmented. Microtubules are absent throughout the cell (Figure 4.10).
Figure 4.9 a) Transmission electron micrograph of a transverse section of a compound 1003 treated *T. b. rhodesiense* cell b) high power of boxed area in a) showing degenerate plasma membrane (PM) in the subpellicular corset containing no subpellicular microtubules, and flagellum with axoneme (AX) and no paraflagellar rod.

Figure 4.10 a) Transmission electron micrograph of a transverse section of a compound 1016 treated *T. b. rhodesiense* cell b) high power of boxed area in a) showing degenerate plasma membrane (PM) in the subpellicular corset with no subpellicular microtubules and flagellum (F) containing a degenerate plasma membrane, axoneme and paraflagellar rod.
Trypanosomes treated with the IC$_{70}$ concentration of compound 1007 appeared distorted in a similar manner to trypanosomes treated with compound 1016 (Figure 4.10). The flagella pocket is enlarged and contains unknown fine tubular material. The flagellar pocket membrane is detached. The flagellum has a malformed axoneme containing no microtubules and no PFR. The plasma membrane is perturbed in most areas, particularly surrounding the subpellicular corset and flagellum where it is completely disintegrated. Few microtubules can be seen in the subpellicular membrane (Figure 4.11).

Trypanosomes treated with the IC$_{70}$ concentration of compound 1017 appeared severely damaged. Cells contained large vesicles and membranous material in their cytoplasm. Most organelles have become degenerate and are indistinguishable, with the exception of the kinetoplast, which is clearly visible. The cell lacks a flagellum. The subpellicular corset is severely disfigured, containing no microtubules and the plasma membrane has disintegrated is most areas of the cell (4.12).
Figure 4.11 a) Transmission electron micrograph of a transverse section of a compound 1007 treated *T. b. rhodesiense* cell b) high power of boxed area in a) showing degenerate plasma membrane (PM) in the subpellicular corset containing no pellicular microtubules, and flagellum (F) with degenerate plasma membrane (PM) and containing no axoneme or paraflagellar rod.

Figure 4.12 a) Transmission electron micrograph of a transverse section of a compound 1017 treated *T. b. rhodesiense* cell b) high power of boxed area in a) showing degenerate plasma membrane (PM) in the subpellicular corset containing no subpellicular microtubules, degenerate organelles (O) and intact kinetoplast (K).
4.4 Discussion

The purpose of this study was to investigate the morphological and ultrastructural effects that five novel analogues of trifluralin have on T. b. rhodesiense. The effect these new compounds have on microtubules was of particular interest because they have previously been shown to bind to recombinant trypanosomal tubulin (see Chapter 3). All compounds tested induced severe irreparable damage in T. b. rhodesiense, including perturbation of subpellicular microtubules, extensive cytoplasmic swellings, axoneme and paraflagellar rod (PFR) malformation, disfiguration around the flagellar pocket and membrane disintegration.

To determine if tubulin and the cytoskeleton were affected by the test compounds, immunofluorescence was performed using monoclonal TAT1 antibody, which binds to tyrosinated trypanosomal α-tubulin. Treatment of cells with IC$_{50}$ and IC$_{70}$ concentrations of each compound did not alter the ability of the TAT1 antibody to bind to tubulin as shown by the positive staining of tubulin. This demonstrates that the trifluralin binding and TAT1 antibody binding sites are not adjacent on tubulin. In addition, tubulin monomers do not appear to be affected by the binding of these compounds to them. If microtubules were depolymerised as a result of the compounds binding to tubulin, the tubulin monomers remained in the cell and were not degraded.

The resulting fluorescence clearly outlines the cytoskeleton of the cells and emphasises the cytoskeletal abnormalities induced by treatment of the test compounds. At IC$_{70}$ concentrations of the five compounds, major cytoskeletal abnormalities were induced including the elongation and detachment of flagella. Compounds 1003, 1007 and 1017 also induced these deformities at IC$_{50}$ concentrations. These results are consistent with
reports that cell shape and form of trypanosomes are microtubule-dependent and polymerisation of new microtubules is a critical prerequisite for cell growth (Robinson et al., 1995; Sherwin and Gull, 1989).

To determine trypanosome orientation, cell cycle stage and whether the nucleus and kinetoplast were affected, cells were treated with DAPI, which stains nuclear and kinetoplast DNA. DAPI staining of cells treated with the IC₅₀ concentrations of compounds 1003, 1007, 1008 and 1016 revealed some 1K1N cells and cells with no kinetoplasts indicating that cell replication was abnormal or had ceased. Trypanosomes that were treated with the IC₇₀ concentrations of compounds 1008, 1003, and 1017 were multi-nucleated and had multiple kinetoplasts indicating that the cells underwent mitosis before damage ensued and therefore, failed to complete cytokinesis. Previous studies using anti-microtubule drugs have shown that during drug treatment nuclear DNA synthesis occurs, which is then followed by an abnormal mitosis. Mitosis in the absence of a spindle is aided probably by interaction of the nuclear DNA with the nuclear envelope (Ploubidou et al., 1999). Trypanosomes treated with IC₇₀ concentration of compounds 1008, 1016 and 1007 contained damaged nuclei and kinetoplasts. Some trypanosomes treated with compounds 1016 and 1017 contained kinetoplasts that were positioned in close proximity to the nucleus. These cells also appeared round in shape and the flagellum was detached, which agrees with the findings of previous studies that disturbing the flagellum early in the cell cycle causes the cell to become morphologically abnormal and the kinetoplast to be repositioned closer in proximity to the nucleus (Kohl et al., 2003). These results differ from similar studies in *T. brucei* treated with the anti-microtubule rhizoxin, which produced 1K0N cells, called zoids (Robinson et al., 1995). Zoids were also described in *L. donovani* treated with
novel oryzalin analogues (Werbovetz et al., 2003). No zoids were found in this study.

The most prominent effect of these compounds at IC\textsubscript{70} concentrations was on the subpellicular microtubules that were missing from trypanosomes treated with compounds 1016, 1003 and 1017. The finding that trypanosomes treated with compounds 1007 and 1008 lacked microtubules agrees with previous electron microscopy studies that showed trifluralin-treated \textit{P. falciparam} contained fragmented subpellicular microtubules (Kaidoh \textit{et al.}, 1995) and oryzalin-treated \textit{T. gondii} were completely devoid of subpellicular microtubules (Stokkermans \textit{et al.}, 1996).

Morphological events occurring early in the \textit{T. brucei} cell cycle include duplication of basal bodies, formation of the new flagellum and building a new subpellicular array of microtubules. The precise duplication and positioning of cytoskeletal elements are necessary to ensure correct segregation to the two daughter cells (Gull, 1999). The process of building a new subpellicular array of microtubules involves the insertion of new microtubules between old microtubules (Sherwin and Gull, 1989). Subpellicular microtubules are cross-linked to each other and to the plasma membrane (Hemphill \textit{et al.}, 1991). The loss of subpellicular microtubules would therefore affect the plasma membrane.

In the algae, \textit{Chlamydomonas}, oryzalin has been shown to bind specifically to tubulin causing microtubules to depolymerise due to the tubulin-oryzalin complex binding to the growing end of the microtubule and thereby inhibiting microtubule growth. With depolymerisation of the microtubule from the minus end of the microtubule, the tubules become progressively shorter, eventually resulting in a complete loss of microtubules.
(Hess and Bayer, 1977). Therefore, the most dynamic microtubules are the most affected. This would occur early in the cell cycle when new subpellicular microtubules are being formed. A number of treated trypanosomes that were observed contained holes in areas where microtubules should be or gaps between microtubules in the subpellicular array (Figure 4.8). It is a reasonable assumption that these are areas where new microtubules would ordinarily be assembled and that the binding of trifluralin analogues to tubulin have disrupted this process. The most structurally damaged cells were observed to lack subpellicular microtubules (Figures 4.9-4.11). It appears that the tubulin-binding of trifluralins prevented new microtubules from forming, causing the eventual destruction of the subpellicular array and subsequently arresting cell division.

Some multi-flagellated trypanosomes were observed that appear to be similar to those observed in trifluralin-treated *T. cruzi* (Bogitsh *et al*., 1999) and oryzalin-treated *Leishmania* (Chan *et al*., 1991). Most of these flagella were structurally abnormal containing malformed axonemes with PFRs also malformed or absent. The axoneme is a highly organized structure composed of microtubules and the PFR is a highly ordered structure extending along and in close contact with most of the axoneme (Hemphill *et al*., 1991). It appears that the malformation of the axoneme and PFR is a direct result of the disassembly of microtubules in the axoneme. Mutant *T. brucei* cell lines have been produced using RNA interference (RNAi) (Bastin *et al*., 2000; Rusconi *et al*., 2005). These cells lack the ability to generate PFRs when the expression of an inverted PFR gene is induced, resulting in cell paralysis. RNAi could be applied in future studies whereby mutant *T. brucei* could be induced to cease axoneme assembly which would determine if this has a direct effect on the PFR and subsequent flagellum construction.
Non-flagellated forms of trypanosomes were also observed in compound 1017 treated cells by TEM. Non-flagellated forms have not been observed in dinitroaniline studies undertaken on any other organisms. However, Kohl (2003) showed that if the flagellum is affected early in the cell cycle, daughter cells will lack a flagellum.

It is possible that these trifluralin analogues are affecting other targets in addition to microtubules or that microtubules are secondary targets. The drug-induced loss of subpellicular microtubules may be a secondary phenomenon that occurred as a result of perturbation of the plasma membrane and subsequent exposure of the subpellicular microtubules to environmental factors. However, trypanosomes treated with compounds 1003, 1008 and 1016 had intact plasma membranes despite the presence of areas of complete microtubule loss. In addition, damage observed in the subpellicular array was almost always coupled with absent or malformed axoneme and paraflagellar rod, and where cells contained a degenerate subpellicular corset (completely devoid of subpellicular microtubules), severe flagellum malformation (with abnormal axoneme and absent PFR) or a lack of flagellum accompanied. Therefore, it is more likely that disruption of microtubules in this study is the primary mechanism for the subsequent disintegration of the cytoskeleton which arrested cell division and caused eventual lysis.

Improvements in the visualisation of cytoskeletal structures of protozoan parasites using high-resolution field emission scanning electron microscopy (FESEM) has recently been achieved which enabled the study of the profile of subpellicular microtubules, the protein bridges cross-linking them as well as the filaments that form the PFR and its connection to the axoneme in *T. brucei*. In addition, the use of the high-resolution backscattered electrons (BSE) detector allowed immunolabelled tubulin in the *T. brucei*
cytoskeleton to be accurately mapped (Sant'anna et al., 2005). This approach could be applied to trifluralin-treated *T. b. rhodesiense* to provide further evidence that the primary target of these compounds is tubulin.

It is not possible to unequivocally conclude that the disruption to microtubule architecture observed in this study is the direct result of the binding of the new trifluralin analogues to \(\alpha\)-tubulin of trypanosomes. To achieve this further research is required to demonstrate that these inhibit polymerisation of tubulin into heterodimers and/or depolymerise heterodimers into monomers. However, the potency of these novel analogues of trifluralin against *T. b. rhodesiense* clearly illustrates their potential use as chemotherapeutic agents and merits further investigation into their effects.
Chapter 5

Recombinant Tubulin from *Trypanosoma brucei* as a Candidate for a Vaccine to prevent African Trypanosomiasis
Chapter 5. Recombinant Tubulin as a Vaccine

5.1 Introduction

There are currently no vaccines to prevent African trypanosomiasis and prospects for the development of a vaccine are considered poor (Seed, 2001). This is mainly due to the ability of trypanosomes to undergo antigenic variation to evade the host immune response and prolong their survival in the host (Barbet and McGuire, 1978; Donelson et al., 1998). The genetic basis of antigenic variation involves switching of the genes coding for the variable surface glycoproteins (vsg), which completely cover the surface of the cell (Pays and Nolan, 1998). Switching of the vsg genes occurs spontaneously at a frequency of $10^{-7}$ and $10^{-2}$ switches per cell per generation for laboratory-adapted and wild type strains, respectively (Pays and Nolan, 1998; Turner and Barry, 1989). Regular switching of the vsg eventually exhausts the host immune system, which is continuously being challenged to react to new surface determinants (Vanhamme and Pays, 1998).

A number of studies have clearly demonstrated that different trypanosome cytoskeletal constituents can induce protective immunity in mice to a lethal challenge of *T. brucei* including: the microtubule-associated protein (MAP) p52 in combination with the glycosomal enzymes aldolase and glyceraldehyde-3-phosphate dehydrogenase (Balaban et al., 1995), the MAP p15 (Rasooly and Balaban, 2004) and native trypanosomal tubulin (Lubega et al., 2002a) In addition, Mkunza et al. (1995) were able to induce partial protection in cattle to natural infection with *T. congolense* and *T. vivax* by immunising them with a native flagellar pocket antigen purified from *T. b. rhodesiense*. Furthermore, mice immunised with native paraflagellar rod protein (PFR) (Wrightsman et al., 1995) and recombinant PFR protein (Luhrs et al., 2003) were fully protected when infected with *T. cruzi*. 
This study aims to extend the results of a study conducted by Lubega, et al. (2002a), which showed that mice immunised with native tubulin purified from *T. brucei* survived a lethal infection with *T. brucei*. To confirm that tubulin alone was responsible for the observed protection, the experiments were repeated using recombinant trypanosomal tubulin.

The aims of this study were:

1. To determine if mice immunised with recombinant trypanosomal tubulin were protected from a lethal infection with *T. b. rhodesiense*.

2. To determine whether there is a significant difference in the immunity induced by α-tubulin, β-tubulin or a combination of both α- and β-tubulin in mice.
5.2 Material and Methods

5.2.1 Experimental animals and trypanosome isolates

Experimental studies were performed with permission of the Murdoch University Animal Ethics Committee (AEC MU/R999/03), which complies with the guidelines from the National Health and Medical Research Council, Australia. A total of 30 female, 6 week old, swiss ARC mice were obtained from the Animal Resource Centre, Murdoch University. The mice were provided with food and water ad libitum.

Cryopreserved stablites of \textit{T. b. rhodesiense} (ATCC 30027) were stored in liquid nitrogen and propagated in two swiss ARC mice prior to infection of experimental animals. The mice were sacrificed and their blood collected when the parasitaemia was approximately $10^9$ trypanosomes per ml of blood. Parasites were enumerated using a Neubauer haemacytometer. Blood was diluted with PBS (140mM NaCl, 10mM Na$_2$HPO$_4$, 2.7mM KCl, 1.8mM KH$_2$PO$_4$, pH 7.3) immediately prior to infection of experimental mice.

5.2.2 Recombinant trypanosomal tubulin production

The expression of $\alpha$-tubulin and $\beta$-tubulin from \textit{T. b. rhodesiense} as fusion proteins has been previously described (Chapter 2). The recombinant tubulin was maintained in PBS (pH 7.3) at a concentration of 1mg/ml and stored at -80°C until required.
5.2.3 Immunization of mice

Mice were allocated randomly into 6 groups of 5 mice. Each group was immunised by intraperitoneal (i.p.) injection with recombinant MBP-α-tubulin, GST-β-tubulin, MBP-α-tubulin and GST-β-tubulin, recombinant MBP, GST, or PBS (pH 7.3) emulsified in Titermax® adjuvant (Titermax USA, Inc., Georgia, USA) according to the schedule summarised in Table 5.1. Mice were monitored visually for any adverse reactions 30min after vaccination. Any altered behaviour in the mice, such as hunching, lethargy, reluctance to move and/or recumbency was noted.

| Table 5.1 Schedule of immunisation of mice prior to infection with *T. b. rhodesiense*. |
|-----------------------------------------------|---------------|----------|----------|
| Group            | Dose (μg) at day | 1 | 8 | 21 |
| PBS              | -              | - | - | - |
| MBP              | 40             | 20 | 20 | 20 |
| GST              | 40             | 20 | 20 | 20 |
| MBP-α           | 80             | 40 | 40 | 40 |
| GST-β           | 80             | 40 | 40 | 40 |
| MBP-α/GST-β     | 80             | 40 | 40 | 40 |

NB: PBS group were immunised with 20μl PBS emulsified with Titermax®

All mice were infected with $10^3$ *T. b. rhodesiense* (ATCC 30027) by intraperitoneal injection 29 days after the first immunisation was administered. Mice were observed visually twice daily after infection and parasitaemia monitored by microscopic examination of tail-tip blood for the presence of motile trypanosomes 3 days after infection and daily thereafter until mice died or became moribund. The level of parasitaemia was estimated using the matching method (Herbert and Lumsden, 1976).
Mice that developed high a terminal parasitaemia (i.e. $10^8$-$10^9$ trypanosomes/ml of blood) and/or showed signs of clinical illness were euthanased.

### 5.2.4 Data analysis

The mean patent period was calculated using the number of days after infection when trypanosomes were first detected in blood and the length of survival was calculated from the number of days after infection when mice died or became moribund and were euthanased. The protection rate was calculated as the percentage of mice that survived until 30 days after infection and that did not have a detectable parasitaemia. The statistical significance of any differences was calculated using a one-way analysis of variance (ANOVA) and Tukey’s highly significant difference test with a 95% confidence limit.

### 5.2.5 Evaluation of the antibody responses to trypanosomal tubulin

Approximately $10^5$ *T. b. rhodesiense* cultured according to Hirumi and Hirumi (1994) were centrifuged at $1,000 \times g$ for 10min, washed with 1ml of PBS (pH 7.3) and centrifuged again at $1,000 \times g$ for 10min. The pellet of cells was resuspended in 100μl of PBS (pH 7.3) and freeze-thawed 3 times to disrupt the cells. The total soluble protein fraction was collected from the supernatant after centrifuging at $12,000 \times g$ for 1min and stored at -80°C until required.

Blood was collected into heparinised capillary tubes from the tails of two mice from each group on days 7, 20, and 28 (i.e. after each immunization). Capillary tubes were
centrifuged at 12,000 × g and the plasma extracted. The plasma was pooled according to vaccine group and day of collection and stored at -80°C until required.

Western Blot analysis was performed (as previously described in Chapter 2) using pooled plasma diluted 1:100 and the total soluble protein extracts of *T. b. rhodesiense* to determine the presence of anti-tubulin antibodies after each vaccine boost.

5.3 Results

5.3.1 Immuno-protection study

Trypanosomes were detected in the blood of all mice 3 to 4 days following infection with *T. b. rhodesiense*. No mice survived infection with *T. b. rhodesiense* therefore, the protection rate was 0. The mean patent period was significantly higher in the group of mice immunised with GST-β-tubulin compared to all other treatment groups (*p* < 0.05) (Table 5.2). There was no significant difference between the mean patent period observed in mice immunised with PBS, MBP, GST, MBP-α-tubulin and MBP-α-tubulin/GST-β-tubulin (Table 5.2). Mice immunised with MBP-α-tubulin, GST-β-tubulin and MBP-α-tubulin/GST-β-tubulin survived significantly longer compared with mice immunised with PBS alone (*p* < 0.05) (Table 5.2). There was no significant difference in the length of survival between the groups vaccinated with the recombinant proteins.
Table 5.2  Mean patent period (days) and length of survival (days) of immunised mice following infection with *T. b. rhodesiense*.

<table>
<thead>
<tr>
<th>Vaccine Group</th>
<th>n</th>
<th>Mean patency (95% C.I.)</th>
<th>Mean survival (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>5</td>
<td>3.0</td>
<td>3.8</td>
</tr>
<tr>
<td>MBP</td>
<td>5</td>
<td>3.0</td>
<td>4.2</td>
</tr>
<tr>
<td>GST</td>
<td>5</td>
<td>3.0</td>
<td>4.4</td>
</tr>
<tr>
<td>MBP-α</td>
<td>5</td>
<td>3.0</td>
<td>5.1 *</td>
</tr>
<tr>
<td>GST-β</td>
<td>5</td>
<td>4.0 *</td>
<td>5.3 *</td>
</tr>
<tr>
<td>MBP-α/GST-β</td>
<td>5</td>
<td>3.2</td>
<td>5.0 *</td>
</tr>
</tbody>
</table>

* value significantly higher than PBS control (*p* < 0.05)

5.3.2 Evaluation of the presence of anti-tubulin antibodies in the plasma of immunised mice

SDS-PAGE analysis of a crude extract of soluble protein from *T. b. rhodesiense* revealed the presence of proteins ranging from approximately 25kDa to 250kDa in size (Figure 5.1). Plasma collected from mice immunised with recombinant α- and β-tubulin fusion proteins contained antibodies that recognized a 55kDa band that corresponds to tubulin as shown in the Western Blots (Figures 5.2, 5.3 and 5.4). The amplitude of the antibody reactions appeared to increase after each immunisation as evidenced by an increase in the intensity of the colour reaction. Plasma collected from the PBS, MBP and GST control groups did not recognize any antigen in the soluble fraction of *T. b. rhodesiense* (Figure 5.5).
Figure 5.1 SDS-PAGE gel image illustrating the total soluble protein extracted from *T. b. rhodesiense*.

Figure 5.2 Western Blot analysis of the total soluble protein extracted from *T. b. rhodesiense* using pooled plasma collected from mice a) 7, b) 20 and c) 28 days after immunisation with MBP-α-tubulin.

Figure 5.3 Western Blot analysis of the total soluble protein extracted from *T. b. rhodesiense* using pooled plasma collected from mice a) 7, b) 20 and c) 28 days after immunisation with GST-β-tubulin.
Figure 5.4 Western Blot analysis of the total soluble protein extracted from *T. b. rhodesiense* using pooled plasma collected from mice a) 7, b) 20 and c) 28 days after immunisation with MBP-α-tubulin and GST-β-tubulin.

Figure 5.5 Western Blot analysis of the total soluble protein extracted from *T. b. rhodesiense* using pooled plasma collected from mice 28 days after immunisation with a) PBS, b) MBP and c) GST.
5.4 Discussion

The aim of this study was to determine if recombinant trypanosomal tubulins were able to elicit protective immunity in mice. The efficacy of the recombinant trypanosomal tubulins as vaccine candidates was assessed by monitoring the survival and parasitaemia of immunised mice following challenge with a lethal infection of *T. b. rhodesiense*. Although all the mice vaccinated with recombinant tubulin developed patent parasitaemias and did not survive, they were partially protected by GST-β-tubulin because their patency period and length of survival were significantly longer than the controls (Table 5.2). Furthermore, the plasma taken from mice immunised with recombinant trypanosomal tubulin contained antibodies that recognized native tubulin from the *T. b. rhodesiense* total soluble extract.

Most vaccines use proteins that are present on the surface of the target organism because these proteins are most accessible to the host immune response. However, vaccines using vsg are ineffective because trypanosomes undergo antigenic variation (Barbet and McGuire, 1978; Donelson *et al.*, 1998). Despite this, cattle immunised intravenously with $10^7$ irradiated *T. brucei* were fully protected against homologous infection 14 days later and most of the antibodies elicited by the irradiated trypanosomes were specific for vsg proteins. (Morrison *et al.*, 1982). However, only 1 of 3 cattle given weekly doses of $10^9$ irradiated *T. b. rhodesiense* was immune to homologous infection 7 days after the third dose (Wellde *et al.*, 1973).

The results of this study support the findings of Lubega, *et al* (2002a), which demonstrated that mice immunised with native trypanosomal tubulin survived a lethal infection with *T. brucei*. The basis of this protection is not clear because there is no
prior evidence that host antibodies are internalised by trypanosomes. However, Lubega
\textit{et al.} (2002b) further showed that serum from mice immunised with tubulin inhibited
the growth of \textit{T. brucei in vitro}. This would suggest that there is a mechanism for
endocytosis of host immunoglobulins. It has been suggested that the flagellar pocket
may be involved in the internalisation process, since antibodies that become bound to
the trypanosome surface migrate to the region of the flagellar pocket by patching (Gull,
2002). Once internalized, antibodies against tubulin may compromise the mitotic
spindle, axoneme, or cytoskeleton, which would have trypanocidal effects. However,
recent studies have shown that surface-bound antibodies can be internalised and then
degraded within the endosomal system, providing another mechanism by which
bloodstream forms of \textit{T. brucei} can evade the host immune response. This process
involves two trypanosomal Rab proteins, TbrAB11 and TbrAB4, which are central
components of vesicle transport systems (Pal \textit{et al.}, 2003). It is likely that this
mechanism played a role in preventing the full protection of immunised mice from \textit{T.
brucei} infection.

Mice immunised with recombinant tubulin mounted strong antibody responses against
native trypanosomal tubulin, although this was not sufficient to protect the mice from
lethal infection. Lubega \textit{et al} (2002a) showed that the passive transfer of anti-tubulin
antibodies to naïve mice conferred 80% protection, suggesting that the basis for the
protection is humoral. These findings differ from studies on \textit{T. cruzi} that showed that
protective immunity in mice immunized with PFR proteins is associated with a TH1-
type response (Miller \textit{et al}., 1996). It is more likely that both humoral and cell-mediated
is required to provide full immuno-protection against trypanosomal infection.
There was no significant difference between the lengths of survival between the three vaccine groups, although the patent period was significantly longer for the GST-β-tubulin group compared to the MBP-α-tubulin and MBP-α-tubulin/GST-β-tubulin groups. The reason for this difference is unclear although it is possible that the relative concentration of each protein differed because GST is approximately half the molecular weight of MBP. It is possible that the relatively low dose of protein and short immunisation schedule used in this study was not sufficient to provide protective immunity. The quantity of recombinant protein used in this study was based on Lubega, et al (2002a) where an initial dose of 40μg of native trypanosomal tubulin was followed by two boosts of 20μg of the same protein. The amount of recombinant tubulin used in this study was twice that used by Lubega, et al (2002a) to make allowance for the presence of the fusion partner. Vaccine studies using native PFR from T. cruzi demonstrated that the optimal dose for producing 100% immuno-protection in mice was an initial dose of 40μg followed by 2 boosts of 20μg (Miller et al., 1996). Miller et al. (1996) also demonstrated that a 50% reduction in the amount of protein used resulted in failure to protect mice infected with T. cruzi because there was insufficient humoral immunity to clear infection. The presence of the fusion partner may also have masked antigenic epitopes. However, previous studies using recombinant proteins as GST or MBP fusions have shown that the fusion partner does not interfere with the development of protective immunity in animal models against infection with Schistosoma mansoni (Schechtman et al., 2001), Plasmodium sp. (Kang et al., 1998) (Oliveira-Ferreira et al., 2004) and T. gondii (Lunden et al., 1997). Furthermore, it has been reported that fusion partners can be advantageous in vivo because they may protect the target protein from intracellular proteolysis (Sorensen and Mortensen, 2005). However, there are insufficient data from this study to confirm or reject this possibility.
Future studies could be improved by using pure recombinant α- and β-tubulin without a fusion partner or by using different expression systems that utilise smaller fusion partners such as histidine.

It may be possible that other immunogenic contaminants were present in the fusion protein preparations and that they are partially responsible for the results observed. However, the recombinant tubulin fusion proteins MBP-α-tubulin and GST-β-tubulin used in this study were purified and analysed for purity (see Chapter 2). A single band was produced for each fusion protein; 100kDa for MBP-α-tubulin and a 75kDa sized band for GST-β-tubulin. Immuno-blotting confirmed the presence of both the fusion protein tag and the accompanying tubulin subtype for each fusion protein (see Chapter 2). It is possible that other more sensitive staining methods, such as silver staining, could reveal trace amounts of contaminants of different molecular weights. However, the immune sera collected from the vaccinated mice only recognized a protein approximately 55kDa in size, which is equivalent to the molecular weight of tubulin. It is unlikely that a contaminant of equal size to tubulin could be responsible for the partial immuno-protection observed in the mice. This is because the recombinant proteins used to vaccinate the mice were produced in bacterial cells and purified using affinity chromatography that is specific for the two fusion protein tags.

It has been suggested that the choice of adjuvant can have a significant effect on the level of protection provided by a vaccine (Daly and Long, 1996) and this should be empirically investigated when optimising vaccine conditions in animal models. Titermax® was chosen for this study because it was reported to be as effective as Freunds complete adjuvant (FCA) without the toxic effects that FCA can induce in mice.
(Bennett *et al.*, 1992). It has also been suggested that the route of inoculation may affect vaccine efficacy (Daly and Long, 1996). However, Lubega *et al.* (2002a) successfully immunised mice by intraperitoneal administration of native trypanosomal tubulin. Future studies to optimise vaccine efficacy should evaluate the use of alternative adjuvants, such as alum, FCA and saponin and alternative routes of administration, such as intramuscular and subcutaneous.

Suppression of the proliferative T-cell response to trypanosomal antigens has previously been reported (Alcina and Fresno, 1985; Charoenvit *et al.*, 1981; Corsini *et al.*, 1977; Masake *et al.*, 1981), although the role of the T-cell response to *T. brucei* infection remains unclear to date. In this study, the humoral response was not sufficient to provide full protection in mice infected with *T. brucei* and so future vaccine studies should concentrate on inducing both a humoral and cell-mediated immune response. The next logical step would be to investigate tubulin DNA as a vaccine as either naked plasmid DNA, tubulin DNA delivered in a viral vector or by following a DNA-priming protein-boosting immunization regime. Use of tubulin DNA as a potential vaccine could be explored in future studies. Studies using an adenoviral vector containing the gene coding for MAP p15 was found to generate strong CD8+ T-cell proliferation and provided full immuno-protection in mice challenged with a lethal infection of *T. brucei* (Rasooly and Balaban, 2004). It has previously been established that immunisation using “naked” plasmid DNA (i.e. DNA that is not associated with any delivery vehicle such as virus) provides an effective activator of both humoral and cellular immune responses and has been used in preclinical animal models for viral, bacterial and parasitic diseases (Donnelly *et al.*, 1997), although has not been trialled against *T. brucei* infection. An alternative immunisation protocol could use a DNA-prime protein-boost immunization schedule consisting of trypanosomal tubulin DNA followed by
recombinant trypanosomal tubulin protein. DNA-priming protein-boosting immunization takes advantage of the ability of DNA immunization to guide the immune responses toward a cell-mediated response. This method has successfully been applied in studies using PFR protein 2 from *L. mexicana* as a vaccine against homologous infection in hamsters (Saravia *et al.*, 2005). Similar studies in *T. cruzi*, found that although a DNA prime of the catalytic domain of trans-sialidase (TS) followed by recombinant TS protein boost generated a strong cell-mediated response, it did not provide immuno-protection against *T. cruzi* infection in mice (Vasconcelos *et al.*, 2003).

A successful recombinant vaccine based on tubulin would be a significant advancement in the control of human and animal African trypanosomiasis. This study has provided further evidence that tubulin is a potential target for the development of a vaccine to prevent African trypanosomiasis. However, further investigation is required to optimise vaccine preparations and maximise protective immune responses against recombinant tubulin.
Chapter 6

General Discussion
The purpose of this study was to explore if tubulin, in particular $\alpha$-tubulin, from *T. b. rhodesiense* are valid targets for the development of new therapeutic agents for the treatment or prevention of human African trypanosomiasis (Sleeping Sickness). The impetus for this study came from the fact that current treatment regimes for trypanosomiasis are unsafe, expensive, difficult to administer and often ineffective.

The production of recombinant trypanosomal $\alpha$- and $\beta$-tubulin proteins in bacterial expression systems enabled the purification of large quantities of soluble protein, free from impurities, post-translational modifications and microtubule-associated proteins, making them suitable for drug-binding and vaccine studies. These fusion proteins were successfully used to determine the binding kinetics of several compounds to trypanosomal and mammalian tubulins. Ideally this study would have been completed with only the protein of interest without their fusion partners but this was not possible. The MBP and GST fusion partners did not appear to have affected the drug-binding studies conducted in this project although they may have impeded access of the host immune responses to the tubulin monomers *in vivo* during the vaccine studies.

There are no published reports of studies using recombinant trypanosomal tubulins and few reports of the use of native trypanosomal tubulins to determine the characteristics of binding of the dinitroanilines. Five new analogues of trifluralin were shown to bind strongly to recombinant trypanosomal $\alpha$-tubulin and only 3 compounds bound weakly to recombinant trypanosomal $\beta$-tubulin. Only one compound was shown to bind with any affinity to native rat tubulin probably as a result of the C1 substituent of 4-morpholino. This demonstrates the dramatic effects that alterations in the chemical composition of trifluralin can have on specificity of their binding. The mechanism by
which binding occurs is unknown although it appears that the binding site of these new analogues of trifluralin directly involves tryptophan residues and other residues that are present in trypanosomal $\alpha$-tubulin but absent in mammalian $\alpha$-tubulin. Future studies will need to concentrate on determining the mechanism of binding and to determine their effects on the growth of microtubules.

The novel trifluralin analogues induced severe irreparable damage to bloodstream forms of *T. b. rhodesiense* with the most prominent effect being the disruption to the subpellicular array. This dramatic effect on the cells is strong evidence that trifluralin and its analogues target tubulin and disrupt microtubule formation. However, further work is required to prove that these compounds inhibit the polymerisation of tubulin before we can unequivocally conclude that tubulin is their primary target.

Past attempts to develop vaccines to prevent trypanosomiasis have failed mainly because of the parasites ability to evade the host immune response. Recombinant trypanosomal tubulin induced a humoral response in vaccinated mice but was insufficient to prevent infection. There is now a need to optimise the vaccine preparation used in this study and further evaluate the mechanism of the immune protection observed in this study.

Tubulin is a valid chemotherapeutic target for nematocidal and antiprotozoal drugs. This study has shown that tubulin offers a potential new target for the development of novel chemical and immunological therapeutic agents for the treatment and prevention of trypanosomiasis.
Chapter 7

References


