Characterisation of the benzimidazole-binding site on the cytoskeletal protein tubulin

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Year of submission

2003
Declaration

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

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

( Louisa Mary MacDonald )
Acknowledgements

I would like to take this opportunity to acknowledge and thank my three supervisors, Jim Reynolds, Andy Thompson, and Anthony Armson. The support and encouragement they provided during this project was pivotal towards its completion. They afforded me a degree of freedom in approach and thinking which I have come to appreciate all the more since leaving Murdoch University. They were excellent mentors who provided me with valuable opportunities to realise my potential in a productive and encouraging environment. In many ways this thesis is dedicated to them as well for giving me a break.

I would also like to thank the Murdoch University Parasitology Group for many years of friendship and support. The long, sometimes rambling, conversations I had with different members of this group gave me the necessary humour and enthusiasm to successfully complete the project.

I would finally like to thank the two people who helped me to stay interested in proteins that refuse to crystallise and that disintegrate, rapidly and at every opportunity: Drs Jackie and Matthew Wilce. It was a pleasure meeting and working with them, and they were also excellent role models of how to succeed at what you are passionate about: the science ….
This thesis is dedicated to my parents

Dr Mary MacDonald

and the memory of

Dr Robert Ewen Munn MacDonald

1930 – 2001
Abstract

The binding kinetics of several benzimidazole compounds were determined with recombinant tubulin monomers and heterodimers from benzimidazole-sensitive and -insensitive organisms. This study utilised the naturally occurring high efficacy of the benzimidazoles for the parasitic protozoa *Giardia duodenalis* and *Encephalitozoon intestinalis*. The benzimidazoles are not active against the protozoan *Cryptosporidium parvum* or mammalian hosts, including humans. The affinity of several benzimidazole derivatives for monomeric and heterodimeric β-tubulin was clearly demonstrated, thus supporting previous studies of drug-resistant nematode and fungal populations. A homology model of protozoan αβ-tubulin, produced using the three-dimensional structure of mammalian αβ-tubulin, identified a strongly hydrophobic domain only on the β-tubulin protein of sensitive protozoa. This domain is proposed to be the benzimidazole-binding domain and the amino acid residues within it include three key residues which are substituted between benzimidazole-sensitive and -insensitive organisms. These residues are Ile-189, Val-199, and Phe-200 that all have non-polar, hydrophobic side groups and are proposed to bind with the Rs side chain of several benzimidazole derivatives. In addition to this, the benzimidazole derivatives were able to bind irreversibly with assembling microtubules from sensitive parasites. The incorporation of benzimidazole-bound αβ-heterodimers into assembling microtubules was shown to arrest polymerisation *in vitro* although the addition of benzimidazole compounds to assembled microtubules did not result in depolymerisation. Taken together, these results suggest that the mechanism of action of these compounds is through disruption of the dynamic equilibrium that balances the cycle of microtubule polymerisation and disintegration within these protozoa. Further, this effect is brought about by preferential binding of the benzimidazoles to a hydrophobic region on the β-tubulin protein.
Publications

Journal Articles (chronological order)


**Book Chapters**


**Published Abstracts (chronological order)**


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<tr>
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<th>Full Form</th>
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<td>two-dimensional</td>
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<td>three-dimensional</td>
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<td>A</td>
<td>adenine</td>
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<td>AGE</td>
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<td>BIA</td>
<td>biomolecular interaction assay</td>
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<td>C</td>
<td>cytosine</td>
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<td>CCT</td>
<td>chaperonin containing T-complex polypeptide-1</td>
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<td>c-cpn</td>
<td>cytosolic chaperonin</td>
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<tr>
<td>CMD</td>
<td>carboxymethyl/dextran</td>
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<tr>
<td>CTAB</td>
<td>cetyl trimethyl ammonium bromide</td>
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<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>E-site</td>
<td>exchangeable site (α-tubulin)</td>
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<td>EDC</td>
<td>N-ethyl-N’-(3-diethyl-aminopropyl)-carbodiimide</td>
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<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
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<td>ethylene glycol-bis-(β-aminoethyl ether)-N, N', N'-tetraacetic acid</td>
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<td>FBZ</td>
<td>fenbendazole</td>
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<td>FC</td>
<td>flow cell</td>
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<td>Fts</td>
<td>filament temperature sensitivity</td>
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<td>G</td>
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<td>GDP</td>
<td>guanosine 5′-diphosphate</td>
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<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
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<tr>
<td>GTP</td>
<td>guanosine 5′-triphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>------------</td>
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<tr>
<td>HBS</td>
<td>HEPES buffered saline</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid</td>
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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
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<td>kDa</td>
<td>kiloDalton</td>
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<td>$k_m$</td>
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<td>$k_{on}$</td>
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<td>$k_{off}$</td>
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<tr>
<td>$K_d$</td>
<td>equilibrium dissociation constant</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani (broth)</td>
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<tr>
<td>M-loop</td>
<td>microtubule loop</td>
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<tr>
<td>MAP</td>
<td>microtubule-associated protein</td>
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<td>MBP</td>
<td>maltose-binding protein</td>
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<td>MBS</td>
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<td>MBZ</td>
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<tr>
<td>MES</td>
<td>2-(N-morpholino) ethanesulphonic acid</td>
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<tr>
<td>MTOC</td>
<td>microtubule-organising centre</td>
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<td>N-site</td>
<td>nonexchangeable site (β-tubulin)</td>
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<td>NH</td>
<td>nonpolar hydrophobic (amino acid)</td>
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<td>nickel nitriloacetic acid</td>
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<td>OBZ</td>
<td>oxibendazole; methyl 5-N-propoxy-2-benzimidazocarbamate</td>
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<td>OD</td>
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<td>PBZ</td>
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<td>PCR</td>
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<td>PDB</td>
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<td>PEG</td>
<td>polyethylene glycol</td>
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<td>Term</td>
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<tr>
<td>pI</td>
<td>isoelectric point</td>
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<td>Pipes</td>
<td>piperazine-$N,N'$-bis(2-ethanesulphonic acid)</td>
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Chapter 1

The levels of control of microtubule dynamics and the influence of benzimidazole compounds
1.1. The cytoskeletal microtubules

The cytoskeleton is a dynamic framework which plays a central role in a wide variety of cellular processes including organelle transport, maintenance of cellular integrity, mitosis, motility, and cell division (reviewed by Desai and Mitchison, 1997). The main components of the cytoskeleton are the microtubules, formed from tubulin, microfilaments, composed of actin, and intermediate filaments composed of individual types of different proteins. The main focus of this project is tubulin which form $\alpha\beta$-tubulin heterodimers that polymerise to form the major component of microtubules (Fig. 1.1). Microtubules are regulated at several levels within cells, including assembly and disassembly through dynamic instability and treadmilling, posttranslational modifications, and stabilisation by several different microtubule-associated proteins (MAPs).

1.1.1. Structure and assembly of microtubules

Microtubules are cylindrical cytoskeletal polymers which perform a wide variety of functions in eukaryotic cells ranging from cell motility to cellular transport and mitosis (Desai and Mitchison, 1997). Some of these functions are achieved through interaction with specific motor proteins, such as dynein and kinesin, which are responsible for ciliary and flagellar motion (Bloom and Endow, 1995; Mandelkow and Johnson, 1998). The wide variety of functions performed by microtubules is partly explained by the existence of several $\alpha$- and $\beta$-tubulin isoforms or isotypes, which are the main components of microtubules. The expression of the different isotypes is controlled transcriptionally and is directly related to the cell type or developmental stage of the organism (Lewis et al., 1985; Panda et al., 1994; Schwarz et al., 1998).
The first stage in microtubule formation is the dimerisation of α- and β-tubulin monomers by a noncovalent association (Fig. 1.1). The αβ-tubulin heterodimers then polymerise to form the microtubule which is stabilised by additional proteins such as MAPs and tau (mammalian cells). The incorporation of the αβ-subunits into microtubules requires guanosine 5'-triphosphate (GTP) hydrolysis in the presence of magnesium ions (Fontalba et al., 1993; Menéndez et al., 1998).

The αβ-tubulin dimers are arranged in a head-to-tail orientation which is believed to expose particular residues at the ends of the microtubule and this creates its polarity. The exposure of the β-tubulin carboxy-terminal creates a positive or fast-growing end (Mitchison, 1993) and the α-tubulin amino-terminal is exposed at the minus or slow-growing end (Fan et al., 1996). Understanding microtubule polarity is particularly important as it is believed to form the basis of the interaction with motor proteins (Song and Endow, 1996). The two ends of the microtubule also attach to different cellular components depending on the function of the microtubules (Wolf et al., 1996).
1.1.2. Dynamic behaviour of microtubules

Microtubules are highly dynamic and can switch stochastically between growing and shortening phases both in vivo and in vitro (Margolis and Wilson, 1998). This nonequilibrium behaviour is termed dynamic instability (i.e. rapid switching between growth and rapid shortening) and treadmilling (i.e. net assembly at one end and disassembly at the opposite end). The mechanisms which underlie this switching or dynamic instability have been most widely studied using mammalian forms of the protein and are believed to be based on the binding and hydrolysis of GTP on the α- and β-tubulin subunits (Caplow and Fee, 2002; Caudron et al., 2000). Each monomer binds one GTP molecule at the nonexchangeable or N-site on α-tubulin (Bai et al., 1998) and the exchangeable or E-site on β-tubulin (Shivanna et al., 1993). After the αβ-tubulin dimers are incorporated into microtubules the E-site GTP is hydrolysed to GDP and becomes nonexchangeable (Carlier et al., 1987). There are two models which describe the dynamic instability of microtubules in vivo and in vitro: the GTP-cap model and the treadmilling hypothesis.

1.1.2.i. Dynamic instability: GTP-capping model

The more favoured of the two dynamic instability models is the GTP-cap model which is based on the gain and loss of a stabilising GTP- or GDP-P_i-liganded tubulin (Dougherty et al., 1998) at the E-site of the β-subunit at the plus or growing end of a microtubule. The GTP-monomer stabilises the microtubule and when they are stochastically lost the microtubule rapidly depolymerises (Drechsel and Kirschner, 1994; Tran et al., 1997). The most recent high-resolution models of the microtubule (Löwe et al., 2001; Nogales et al., 1999) place a β-tubulin subunit at the plus end of the microtubule which exposes its nucleotide to solution while the minus or shortening end contains an α-subunit exposing its catalytic end (Fig. 1.1). Therefore the nature of the
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subunit at either end of a microtubule is the critical factor in the GTP-cap model (Janosi et al., 2002).

![Diagram of the GTP-capping model of microtubule dynamic instability](image)

**Fig. 1.2.** Diagram of the GTP-capping model of microtubule dynamic instability. The GTP-cap is represented by a stabilising single layer of GDP-P\(_i\) molecules (Panda et al., 2002).

For the GTP-cap model to be correct when a dimer is added to the plus end of a microtubule its catalytic sites will come into contact with the E-site nucleotide (Fig. 1.2) of the polymerised subunits and the creation of this interface is what is believed to drive GTP hydrolysis (Mitchison, 1993). The GTP on the newly polymerised GTP-tubulin dimer therefore will not be hydrolysed until another dimer is added (Margolis and Wilson, 1981). This process results in the microtubule plus end having a GTP-cap on the uppermost tubulin dimers. At the minus end, as dimers are removed the catalytic portions of microtubular dimers are exposed though without any GTP or GDP involvement (reviewed by Carlier, 1982). The rapid assembly and disassembly of cytoskeletal microtubules favoured with this model allows the cytoskeleton to respond rapidly to a wide variety of stimuli which results in its role in the wide variety of processes detailed above.
1.1.2.ii. Dynamic instability: treadmillling model

The second model, which may exist simultaneously or during particular stages of the cell cycle with the GTP-cap model (Caplow et al., 1985; Caplow and Shanks, 1996), is termed treadmillling. It has been demonstrated in vitro and in vivo (Margolis and Wilson, 1998) and is described as the constant flux or recycling of αβ-dimers such that the net gain from the plus end and the net loss from the minus end is constant and a constant microtubule length is therefore maintained (Margolis and Wilson, 1981). It is still unknown what determines or controls the length of a microtubule in this model, although it is possibly functional constraints as a result of specific cellular requirements (Desai and Mitchison, 1997).

1.1.3. Microtubule nucleation

The nucleation of microtubules in cells at microtubule-organising centres (MTOCs) is also essential for their dynamic behaviour and functions (reviewed by Archer and Solomon, 1994; Bornens, 2002). MTOCs include the centrosome (animal cells), flagellar basal bodies (protozoa), and spindle pole bodies (fungi, yeast). Depending upon their function they are either stable, long-term assemblies involved in the formation of cilia and flagellar or unstable, transient assemblies required for cytoplasmic microtubules or mitotic spindle formation (Tucker et al., 1995).

The centrosome of animal cells has been widely studied, particularly for the role its interaction with microtubules plays in their dynamic instability (Rodionov et al., 1999). A typical animal cell centrosome consists of two centrioles surrounded by an amorphous cloud of pericentriolar material. While the protein composition and molecular organisation of the pericentriolar material is still largely unknown (Archer and Solomon, 1994) it has been demonstrated that one essential component is γ-tubulin (Moritz and Agard, 2001; Wiese and Zheng, 1999). γ-Tubulin has high sequence
homology with $\alpha$- and $\beta$-tubulin and is the point of attachment between the microtubule minus end ($\alpha$-tubulin) and the MTOC (Li and Joshi, 1995; Rodionov et al., 1999). More recently $\gamma$-tubulin has been shown to be part of a ring complex, termed $\gamma$-TuRC, which is thought to be the template for microtubule assembly on centrosomes (Job et al., 2003; Moritz et al., 2000; Wiese and Zheng, 2000).

A newer member of the tubulin family, $\varepsilon$-tubulin, has also been identified as being essential for the assembly and cellular positioning of centriolar and basal bodies microtubules (Dupuis-Williams et al., 2002). Further roles of $\varepsilon$-tubulin in these microtubules are being investigated and will help to identify the mechanisms underlying their development as will the roles of other recently identified tubulins such as $\delta$-tubulin (Smrzka et al., 2000; Vaughan et al., 2000) and $\eta$-tubulin (Chang and Stearns, 2000; Dutcher, 2001; McKean et al., 2001).

Several investigators have also demonstrated noncentrosomal nucleation of microtubules (Mogensen et al., 2000) particularly in a wide range of mammalian cell types such as neurons (Baas et al., 1991; Lunn et al., 1997), skeletal muscle cells (Tassin et al., 1985), kidney cells (Bré et al., 1987), and the cochlea (Tucker et al., 1998). These points of nucleation are believed to perform a pivotal role in the specialised functions of these cells and may also create a noncentrosomal population of microtubules for rapid nucleation and release (Keating et al., 1997).
1.1.4. Promotion of microtubule stability or instability

The dynamic behaviour of microtubules is further regulated at the cellular level through interaction with factors which promote either stability or instability of microtubules.

1.1.4.i. Factors which promote microtubule stability

Several MAPs have been identified and they may be divided into two groups as either structural or dynamic MAPs (reviewed by Desai and Mitchison, 1997). Structural MAPs have been demonstrated to stimulate tubulin assembly, enhance microtubule stability, and influence the spatial distribution of microtubules within cells (Gamblin et al., 1996; Mandelkow and Mandelkow, 1995). Proteins in this group include the structurally and biochemically related proteins MAP1-4 and tau, which interact with the carboxy-terminus of the α- or β-tubulin portion of the heterodimer (Al-Bassam et al., 2002; Chau et al., 1998; Saoudi et al., 1995). Dynamic MAPs, including kinesin and dynein, are molecular motors which interact with microtubules to bring about adenosine triphosphate (ATP)-dependent uni- or bi-directional movement within cells such as chromosome segregation during mitosis and meiosis (Hunter and Wordeman, 2000; Mandelkow and Johnson, 1998; Vorobjev et al., 2001). The role of MAPs has not been studied in protozoan organisms but functionally similar mammalian equivalents can be used to gain valuable information regarding microtubule stability and dynamics.
1.1.4.ii. Factors which destabilise microtubules

There are two well characterised factors which are involved in the regulation of microtubule dynamics \textit{in vivo}. One of these is the Oncoprotein18/stathmin (Op18) (Cassimeris, 2002) which is believed to promote GTP hydrolysis resulting in the loss of the stabilising GTP-cap from microtubules and subsequent depolymerisation (Amayed \textit{et al.}, 2000; Holmfeldt \textit{et al.}, 2001; Howell \textit{et al.}, 1999). Another group of microtubule depolymerisers is the kinesin-like proteins, XKCM1 and XKIF2, which are required in the formation and maintenance of the mitotic spindle (Kline-Smith and Walczak, 2002; Anderson and Wittmann, 2002). These are particularly interesting microtubule disruptors as they bind to both the plus and minus ends of the microtubule in a way which destabilises the microtubule lattice (Desai \textit{et al.}, 1999; Niederstrasser \textit{et al.}, 2002; Walczak \textit{et al.}, 2002).

1.1.5. Post-translational modifications of tubulin

In addition to the regulation of microtubules at the genetic and macromolecular level, microtubules also undergo tissue and cell-cycle specific post-translational modifications. These modifications have been identified in a wide range of organisms from the ancient protists, such as \textit{Giardia} and \textit{Trypanosoma}, through to mammalian cells. Several types of post-translational modifications occurring in tubulin are unique to it (reviewed by MacRae, 1997). These modifications have been found to be enzymatically driven, reversible, and except for acetylation they all occur at the highly variable and acidic carboxy-terminus. Some of the modifications occur on both the \(\alpha\)- and \(\beta\)-tubulin monomers and include polyglycylation and polyglutamylation.
Polyglycylation involves the addition of glycine residues onto carboxy-terminal glutamate residues and can involve the addition of up to thirty-four glycine residues (Bré et al., 1998; Weber et al., 1996). Polyglycylation may be functionally significant as it has been identified only in cells with either cilia or flagella (Bré et al., 1998; Million et al., 1999) including several species of protozoa such as Giardia duodenalis (Campanati et al., 1999, 2003; Weber et al., 1997).

Further analysis of Paramecium cilia has determined that higher polyglycylation levels are associated with inactive or stable microtubules than with active or dynamic ones (Bré et al., 1998; Vinh et al., 1999). It has been proposed that polyglycylation results in a bulkier and more negatively charged carboxy-terminal domain. Mutational analysis has also demonstrated that the amount of polyglycylation is more important than whether it occurs on either the α- or β-tubulin monomer (Xia et al., 2000) although whether or not this is functionally significant is unknown.

Polyglutamylation is the successive addition of glutamyl residues to carboxy-terminal glutamic acid residues on α- and β-tubulin (Eddé et al., 1990; Redeker et al., 1994). It is also commonly found in ciliated or flagellated cells (Gagnon et al., 1996) and neurons across a wide species range from protozoa, including G. duodenalis (Boggild et al., 2002; Weber et al., 1997), Trichomonads (Schneider et al., 1998) and Trypanosoma (Schneider et al., 1997) to mammalian cells (Regnard et al., 1999). A greater amount of this modification has been observed in proliferating cells than resting or non-dividing ones. Combined with polyglutamylation's association with mammalian centrioles and protozoan basal bodies it has been suggested it may perform a microtubule regulatory function (Bobinnec et al., 1998; Lechtreck and Geimer, 2000; Regnard et al., 1999).
Chapter 1. General Introduction

Palmitoylation and tyrosination are unique to α-tubulin. Palmitoylation involves the addition of palmitate to cysteine residues with the main site for this being cysteine-376 (Ozols and Caron, 1997). The functional significance of this modification has been investigated in platelets and palmitoylation has been found to decrease with platelet activation (Caron, 1997). The amount of palmitoylation \textit{in vitro} has also been found to be decreased by antimicrotubule drugs, including the benzimidazole compound nocodazole and the \textit{Vinca} alkaloid vinblastine, although whether this is related to their function is unknown (Caron, 1997). Palmitoylation may also contribute to the attachment of tubulin to particular cell membranes in a regulatory manner (Caron \textit{et al.}, 2001; Zambito and Wolff, 1997).

The α-tubulin specific tyrosination/detyrosination cycle involves the removal of a genetically encoded tyrosine residue from the carboxy-terminus and re-addition of another tyrosine in a dual enzyme process (reviewed by Idriss, 2000). Stable microtubules are more frequently found to be detyrosinated although it is not essential for their stability or function \textit{in vivo} (Webster \textit{et al.}, 1990) or \textit{in vitro} (Skoufias and Wilson, 1998). It has also been observed that mammalian brain microtubules are more frequently found to have the carboxy-terminal tyrosine and glutamic acid residues removed, which produces a non-tyrosinatable form of tubulin which is very stable when incorporated into microtubules (Alonso \textit{et al.}, 1993; Lafanechère and Job, 2000; Paturle-Lafanechère \textit{et al.}, 1994). This observation has also led to the connection between tissue specific modifications and the role they may perform in regulating microtubule assembly and stability \textit{in vivo} (Banerjee, 2002; Multigner \textit{et al.}, 1996). It has been demonstrated in diplomonads, such as \textit{G. duodenalis}, that tubulin does not undergo the tyrosination/detyrosination cycle but instead retains its genetically encoded terminal tyrosine residue (Boggild \textit{et al.}, 2002; Weber \textit{et al.}, 1997).
1.1.6. Folding of tubulin monomers

Analysis of the tubulin folding pathway has revealed that the \( \alpha \)- and \( \beta \)-tubulin polypeptides interact with a series of chaperone proteins in order to attain their native structure (reviewed by Lopez-Fanarraga et al., 2001). Chaperonins are ubiquitous multisubunit complexes that aid protein folding in an ATP-dependent manner. The first step in tubulin's folding pathway involves interaction between the nascent \( \alpha \)- and \( \beta \)-tubulin amino acid chains with prefoldin (Geissler et al., 1998; Vainberg et al., 1998) which then transfers them to the cytosolic chaperonin CCT (\textit{i.e.} chaperonin containing T-complex polypeptide-1; also termed TRiC, TCP-1, or c-cpn) (Fig. 1.3) (Llorca et al., 2001; Melki et al., 1997; Roobol et al., 1999; Tian et al., 1995).

Subsequent folding steps by CCT involve binding and full release (Farr et al., 1997) which consumes ATP (Melki et al., 1997; Tian et al., 1995). The resulting quasi-native tubulin intermediates then interact with a series of tubulin-specific chaperones termed cofactors A – E which bind sequentially to the \( \alpha \)- and \( \beta \)-tubulin monomers. These cofactors are essential for the formation of normal \( \alpha \beta \)-tubulin heterodimers (Tian et al., 1997, 1999).

Briefly, the folding intermediates released from CCT are bound and stabilised by cofactors B and E for \( \alpha \)-tubulin and cofactors A and D for \( \beta \)-tubulin (Fig. 1.3). The \( \alpha E \) and \( \beta D \) complexes then interact with cofactor C to form a transient pentameric complex (Tian et al., 1997). Hydrolysis of GTP bound to the \( \beta \)-tubulin component of the complex is then believed to be the trigger for the release of the native tubulin dimers (Fontalba et al., 1993; Tian et al., 1999).
Fig. 1.3. Schematic diagram of the α- and β-tubulin folding pathway. These chaperonins and cofactors are required for the formation of functional heterodimers and regulation of the monomer to dimer ratio in cells (Nogales, 2000).

The folding cofactors are also important in regulating the ratio of α- and β-tubulin within cells and normal microtubule assembly is highly dependant upon this regulation. In yeast, even a small excess of β-tubulin is lethal (Abruzzi et al., 2002) and the cofactors A and B form αB and βA complexes which remove its lethality and provide a reservoir of tubulin folding intermediates (Tian et al., 1997). Whether these complexes can be directly converted from αB and βA to αE and βD, respectively, seems unlikely as an extensive study has demonstrated that αB and βA only convert with CCT which then form αE and βD, respectively (Tian et al., 1997).
The relative levels of $\alpha$- and $\beta$-tubulin in yeast have been most extensively studied recently using native gel electrophoresis of protein mixtures. This has facilitated the identification of several additional regulatory or monomer-binding proteins. Thus, proteins such as Pac10p have been demonstrated to enhance binding of $\alpha$- to $\beta$-tubulin which suppresses $\beta$-tubulin's lethality (Alvarez et al., 1998) while others such as Rbl2p bind to $\beta$-tubulin in equilibrium with $\alpha$-tubulin and in a manner similar to cofactor A (Abruzzi et al., 2002). Other yeast cofactors, termed Alp11 (Feierbach et al., 1999), Alp1 (Hirata et al., 1998), and Alp21 (Radcliffe et al., 1999), which are homologs of cofactors B, D, and E, respectively, have all been demonstrated to interact directly with microtubules which indicates a wider role for these cofactors in different organisms (Radcliffe et al., 2000).

It has been demonstrated that tubulin undergoes a facilitated folding pathway via interaction with CCT and other cofactors, and that the mechanism of action of CCT parallels that of other chaperonins in that it generates folding intermediates that bind with several chaperonins in an ATP-dependent reaction in a process termed cycling (Tian et al., 1995). A similar cycling of intermediates occurs in the prokaryotic GroEL/GroES chaperonin pathway and produces the filament temperature sensitivity proteins (i.e. FtsA, FtsZ), which are structural homologs of eukaryotic tubulin (Löwe and Amos, 1998). It should therefore be possible to produce assembly-competent tubulin monomers in Escherichia coli-based in vitro protein expression systems.
1.1.7. Expression of α- and β-tubulin *in vitro*

As the initial parasitic organism extensively studied for benzimidazole activity and resistance was *Haemonchus contortus* it is not surprising that the first attempts to produce recombinant β-tubulin in *E. coli* were with the genes from this species (Lubega *et al.*, 1993). This and subsequent studies demonstrated that under particular conditions the α- and β-tubulin protein from several protozoa and amoebae, when expressed in *E. coli*, tended to form insoluble inclusion bodies (Hollomon *et al.*, 1998; Oxberry *et al.*, 2001a). However, when these conditions were varied, for example by altering the fusion protein partner, *E. coli* strain, or rate of recombinant protein synthesis, the α- and β-tubulin proteins were expressed in a soluble form (Hollomon *et al.*, 1998; Linder *et al.*, 1998; MacDonald *et al.*, 2001, 2003a).

When a recombinant protein is expressed in an insoluble form it requires refolding after being lysed from intracellular inclusion bodies. As it has been demonstrated that the microtubule assembly and drug-binding characteristics of refolded tubulin are significantly different to the native protein (Guha and Bhattacharyya, 1997; Sackett *et al.*, 1994; Shah *et al.*, 2001) it is preferable to produce soluble, correctly folded tubulin if it is to be used for characterisation studies.
1.1.8. Determination of the three-dimensional structure of microtubules

The resolution of the atomic structure of the $\alpha\beta$-tubulin dimer has gone through several stages of improvement which have progressively allowed the construction of maps with successively higher resolution and detail.

In order to understand the key discoveries which have led to the determination of the three-dimensional (3-D) structure of the $\alpha\beta$-tubulin dimer some terminology must initially be clarified. The two techniques which have been combined to describe the structure of tubulin are two-dimensional (2-D) projection maps and 3-D reconstructions. The projection maps of tubulin take electron microscopy to the limit of its resolution of approximately 15Å (Wolf et al., 1993). The most valuable information obtained from the optical diffraction patterns generated in this way has related to the organisation and polarity of $\alpha$- and $\beta$-tubulin monomers in zinc-induced crystalline sheets and macrotubes. 3-D reconstructions became possible after improvements in resolution resulting from the application of electron crystallography, as it is better suited to identifying $\alpha$-helices and $\beta$-sheets as well as some of the loops connecting them.

The first high-resolution model of the structure of tubulin was produced by Downing and Jontes (1992) using 2-D zinc-induced crystalline sheets (Larsson et al., 1976) which were analysed using electron crystallography. The resulting electron diffraction patterns began to provide valuable information, albeit with limited resolution, using the 2-D crystalline sheets. This model was improved the following year by Wolf et al., (1993) who continued to work at a 4Å resolution with electron crystallography. They were able to produce three polymerised forms of bovine tubulin dependent upon the pH of the crystallisation solution. These forms were zinc-sheets, microtubules, and macrotubes and the model describing them was able to assign the alternating polarity
of adjacent protofilaments. This work also provided a 2-D projection map at 15Å resolution of the zinc-sheet and macrotube crystal forms. One of the main limitations of these early projection maps is that they were not able to assign which monomer was $\alpha$- and which was $\beta$-tubulin.

This problem was addressed by the Nogales group (1995a) which produced a 6.5Å resolution 3-D reconstruction of bovine tubulin using electron crystallography of the same 2-D crystalline sheets (Fig. 1.4). They utilised the preferential binding of the antimitotic drug paclitaxel (Taxol™) to $\beta$-tubulin (Rao et al., 1992, 1995) as a marker to distinguish between the monomers. Using this method they were able to identify that the paclitaxel binding site was located near the interprotofilament contacts which agreed with observations that it bound preferentially to polymerised tubulin (Manfredi et al., 1982; Parness and Horwitz, 1981). Nogales study also supported the hypothesis that paclitaxel acts by stabilising or strengthening the lateral interactions between protofilaments (Díaz et al., 1993; Howard and Timasheff, 1988). The resolution of many regions of secondary structure was made possible with the 6.5Å level of detail and in particular a long $\alpha$-helix between the lateral and longitudinal contacts between the subunits (helix-A) was identified. The location of several internal $\beta$-sheets in both subunits was also achieved and confirmed their previously predicted perpendicular orientation to the central axis of the protofilaments (Downing and Jontes, 1992).
At this level of resolution it was not possible to identify the GTP-binding site of either monomer, that was to come later in 1998 (Nogales et al.) when the resolution was significantly improved to 3.7Å. (Fig. 1.4). Subsequent work with this model was able to relate the 3-D structure to some microtubule functions (Downing and Nogales, 1998a) such as longitudinal stability and interaction with accessory proteins.
The most recent and thorough explanation of the current atomic level information available on the $\alpha\beta$-tubulin dimer is provided by Löwe et al., (2001). This paper, written in collaboration with Nogales, extended the electron crystallography model to a resolution of 3.5Å. This allowed previously unresolved secondary structures to be identified as well as other postulated binding regions to be defined (e.g. magnesium and zinc ions). The most significant improvement to previous models was in defining the side-chains of the amino acids involved in the formation of the intra- and inter-dimer interfaces and the binding sites of the guanine nucleotides (i.e. GTP and GDP) and taxol. The 3.5Å model is a significant milestone in the determination of the structure of $\alpha\beta$-tubulin and also provides important information towards understanding the dynamic instability of microtubules (Downing and Nogales, 1998b; Meurer-Grob et al., 2001).
Table 1.1. Summary of the atomic resolution of the αβ-tubulin dimer in chronological order. Structures were determined using two-dimensional zinc-induced crystalline sheets (Larsson et al., 1976) with the exception of the study by Amos and Klug (1974).

<table>
<thead>
<tr>
<th>Investigators</th>
<th>Year</th>
<th>Resolution</th>
<th>Technique</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amos and Klug</td>
<td>1974</td>
<td>Low (&gt;20Å)</td>
<td>Optical diffraction studies</td>
<td>Identified arrangement of heterodimers in microtubules</td>
</tr>
<tr>
<td>Mandelkow et al.</td>
<td>1977</td>
<td>Low (&gt;20Å)</td>
<td>X-ray fibre diffraction</td>
<td>First structure using zinc-induced crystalline sheets</td>
</tr>
<tr>
<td>Amos and Baker</td>
<td>1979</td>
<td>20Å</td>
<td>Electron microscopy</td>
<td>20Å 3-D projection map</td>
</tr>
<tr>
<td>Ceska and Edelstein</td>
<td>1984</td>
<td>20Å</td>
<td>Electron microscopy</td>
<td>First structure of MAP-free microtubules</td>
</tr>
<tr>
<td>Beese et al.</td>
<td>1987</td>
<td>18Å</td>
<td>X-ray diffraction with electron microscopy</td>
<td>Improved resolution of microtubule internal structure</td>
</tr>
<tr>
<td>Andreu et al.</td>
<td>1992</td>
<td>30Å</td>
<td>X-ray scattering with cryo-electron microscopy</td>
<td>First model using taxol-stabilised microtubules</td>
</tr>
<tr>
<td>Downing and Jontes</td>
<td>1992</td>
<td>4Å</td>
<td>Cryo-electron crystallography</td>
<td>4Å 3-D projection map</td>
</tr>
<tr>
<td>Nogales et al.</td>
<td>1995</td>
<td>6.5Å</td>
<td></td>
<td>Resolved secondary structures and visualised taxol binding site</td>
</tr>
<tr>
<td>Wolf et al.</td>
<td>1996</td>
<td></td>
<td></td>
<td>Improved the 6.5Å 3-D model</td>
</tr>
<tr>
<td>Nogales et al.</td>
<td>1997</td>
<td>4Å</td>
<td></td>
<td>Resolved most secondary structures</td>
</tr>
<tr>
<td>Nogales et al.</td>
<td>1998</td>
<td>3.7Å</td>
<td></td>
<td>3.7Å 3-D reconstruction</td>
</tr>
<tr>
<td>Nogales et al.</td>
<td>1999</td>
<td></td>
<td></td>
<td>Docked 3.7Å tubulin model onto 20Å microtubule map</td>
</tr>
<tr>
<td>Meurer-Grob et al.</td>
<td>2001</td>
<td></td>
<td></td>
<td>Docked 3.7Å tubulin model onto 14Å microtubule map</td>
</tr>
<tr>
<td>Löwe et al.</td>
<td>2001</td>
<td>3.5Å</td>
<td></td>
<td>3.5Å 3-D reconstruction</td>
</tr>
</tbody>
</table>
1.2. Antimitotic compounds

There are several classes of compounds which bind to tubulin (reviewed by Downing, 2000) to inhibit polymerisation (colchicine: Chakrabarti et al., 1996), prevent depolymerisation by stabilising the microtubule (taxanes: Snyder et al., 2001), or prevent dimerisation (benzimidazoles: Lacey, 1988). The molecular basis of the mechanism of action of these compounds has been extensively studied and has generally focussed on sensitivity versus resistance as a basis for understanding their mechanism of action. The techniques which have been used to understand their mechanism of action have resulted in progressive improvements to the drug-binding model for each class of drugs.

1.2.1. Compounds that prevent microtubule dimerisation: Benzimidazoles

The hypothesis that tubulin is the target of the benzimidazoles is based on the ultrastructural studies of cells. These observations included the degenerative changes caused by benzimidazoles which coincided with the disappearance of cytoplasmic microtubules (Borgers et al., 1975). The broad range of activity of the benzimidazoles has been demonstrated against a wide variety of organisms including nematodes (Enos and Coles, 1990; Roos et al., 1990), fungi (Cruz et al., 1994; Del Poeta et al., 1998), and parasitic protozoa (Katiyar and Edlind, 1997; Morgan et al., 1993).
1.2.2. Benzimidazoles as anthelmintics

Since the introduction of the benzimidazoles in the 1960s as anthelmintics there has been increasing incidence and reporting of resistance amongst a range of agriculturally significant nematodes or strongylids, including *H. contortus*. The original studies of the mechanism of action of benzimidazoles in parasitic nematodes demonstrated some relatively generalised biochemical observations such as an inhibitory effect on glucose uptake and glycogen storage (Borgers *et al.*, 1975). When ultrastructural studies revealed that mebendazole caused disintegration of the microtubule network in nematodes (Borgers *et al.*, 1975) a link between benzimidazoles and tubulin was made which also provided an explanation for the biochemical observations. Subsequent studies have primarily focussed on determining the molecular basis of resistance to benzimidazole compounds. The development of benzimidazole-resistant strains in previously susceptible nematode populations assisted these studies and it was hoped that through understanding the mechanism of resistance the mode of action of the benzimidazoles would become clearer.

The mode of action of the benzimidazoles in helminths is believed to involve direct binding to the β-tubulin monomer and thus inhibition of dimerisation and polymerisation into microtubules (Gill and Lacey, 1992; Lubega and Prichard, 1990, 1991a). The mechanism of benzimidazole resistance has been studied in detail for the nematodes *H. contortus* (Kwa *et al.*, 1995; Roos *et al.*, 1990) and *Caenorhabditis elegans* (Driscoll *et al.*, 1989; Enos and Coles, 1990). In both organisms the expression of high affinity benzimidazole-binding sites has been linked to susceptibility (Lubega and Prichard, 1991a, 1991b) whereas the expression of predominantly low-affinity benzimidazole-binding sites has been linked with resistance (Beech *et al.*, 1994). The switching between the expression of predominantly high- or low-affinity binding sites has been suggested to be the result of gene deletions (Driscoll *et al.*, 1989; Roos *et al.*, 1990) or point mutations in the β-tubulin genes (Kwa *et al.*, 1995).
There is a substantial amount of evidence to suggest that the benzimidazoles generally bind to tubulin and that the amount of binding is lower for tubulin extracted from benzimidazole-resistant than -susceptible *H. contortus* while the rate of association \( (k_{on}) \) is unaltered (Lacey and Prichard, 1986). This indicated that the benzimidazoles were affecting the amount of microtubules being formed but not their rate of formation. This hypothesis was reinforced by the observation that resistance relates to the amount of benzimidazole-tubulin complex formed and that this is partially dependent on the nature of the benzimidazole compound.

Further studies by Lubega and Prichard (1990) using competitive ligand displacement studies with tritiated-benzimidazoles demonstrated that the reduction in the amount of drug bound by susceptible and resistant tubulin pools was due to a loss of high-affinity benzimidazole receptors or binding sites. They cited a limitation in the previous studies whereby there was no differentiation between high-affinity and low-affinity binding but instead only the total amount of binding was noted. They distinguished high-affinity binding as a specific type of binding that is inhibited by an excess amount of ligand as it is comparatively irreversible. Low-affinity binding is then easily inhibited, as it is more reversible and also more difficult to saturate. For this reason it is difficult to distinguish low-affinity binding from non-specific binding.

The methods which had been employed using tubulin extracted from *H. contortus* have one significant flaw and that is that they failed to take into account the heterogeneity of the extracted tubulin pools. The heterogeneity resulted from tubulin biotypes from different worm sections, which perform different functions, being extracted and purified simultaneously. This is potentially significant in drug binding studies as it is believed that at least some of the functional diversity of microtubules is due to functionally specific post translational modifications of the tubulin monomers or dimers, as discussed above. These modifications, therefore, may mask or interfere with
benzimidazole binding. The early studies also failed to differentiate between either \( \alpha \)- or \( \beta \)-tubulin as being involved in benzimidazole binding but treated binding as a heterodimeric interaction with these drugs.

Therefore, significant improvements were made to the quality of the benzimidazole binding site information once DNA-based techniques were applied. A preliminary study by Roos et al., (1990) was able to identify the existence of mutant \( \beta \)-tubulin, and not \( \alpha \)-tubulin, genes in benzimidazole-resistant \textit{H. contortus} and these were later found in other benzimidazole-resistant helminths (Beech et al., 1994; Kwa et al., 1995; Lubega and Prichard, 1991c).

### 1.2.3. Benzimidazoles as fungicides

The genetic basis of resistance to the benzimidazoles has also been widely studied in several fungi including \textit{Aspergillus nidulans} and \textit{Neurospora crassa}. The fungicidal activity of the benzimidazole compound thiabendazole has been most widely studied and its mode of action was initially described as an ability to inhibit mitosis but not DNA or RNA synthesis (Davidse and Flach, 1978). As with \textit{H. contortus} it was with the development of resistance that the opportunity to study the genetic mechanisms underlying their mode of action presented itself. The benzimidazoles mode of action had already been proposed to be the same as for several other species and to involve preferential binding by the benzimidazoles to the \( \beta \)-tubulin monomer (Jung et al., 1992; Jung and Oakley, 1990). Several studies have identified mutant \( \beta \)-tubulin genes in benzimidazole-resistant fungi (Table 1.2).
**Table 1.2.** Summary of the mutations which have been detected in benzimidazole-resistant biotypes of several fungi.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Type of BZ-resistance</th>
<th>Amino acid position</th>
<th>Type of substitution</th>
<th>Investigator</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus nidulans</em></td>
<td>Thiabendazole</td>
<td>165</td>
<td>Ala → Val</td>
<td>Jung &amp; Oakley, 1990</td>
</tr>
<tr>
<td></td>
<td>Nocodazole</td>
<td>6</td>
<td>His → Tyr</td>
<td>Jung <em>et al.</em>, 1992</td>
</tr>
<tr>
<td></td>
<td>Carbendazim</td>
<td>198</td>
<td>Glu → Lys/Gln</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Benomyl</td>
<td>200</td>
<td>Phe → Tyr</td>
<td></td>
</tr>
<tr>
<td><em>Neurospora crassa</em></td>
<td>Carbendazim – Mildly resistant</td>
<td>167</td>
<td>Phe → Tyr</td>
<td>Fujimura <em>et al.</em>, 1992a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>198</td>
<td>None detected</td>
<td>Fujimura <em>et al.</em>, 1992b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>241</td>
<td>None detected</td>
<td></td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em></td>
<td>Albendazole</td>
<td>6</td>
<td>His → Gln</td>
<td>Cruz &amp; Edlind, 1997</td>
</tr>
</tbody>
</table>

From these studies, an inconsistent model of the key amino acids involved in benzimidazole resistance has emerged. Resistance appears to relate to the nature of the organism and the structure of the benzimidazole studied. As with the resistance studies of *H. contortus* it should again be noted that many assumptions were made regarding the target of the benzimidazoles in fungi and studies were restricted to this target. These studies also focussed on deducing amino acid sequences from complete or partial β-tubulin DNA sequences to identify mutated amino acids without a clear understanding of the background or basal mutation rate in the β-tubulin gene in cultured fungi.
1.2.4. Benzimidazoles as antiprotozoan compounds

Due largely to the selectivity of benzimidazole action in mammalian hosts these compounds have been examined for antiprotozoan activity against a wide range of parasites. Two protozoa which cause significant pathological sequelae and which have been demonstrated to be sensitive to a number of benzimidazoles are *G. duodenalis* and *Encephalitozoon intestinalis*.

1.2.4.i. Significance of *Giardia duodenalis*

*G. duodenalis* is a flagellated parasitic protozoan that inhabits the upper small intestine of several hosts causing giardiasis. It is the most common cause of diarrhoeal illness worldwide (reviewed by Thompson *et al.*, 1993) and is a major cause of waterborne enteric disease (Furness *et al.*, 2000) and failure-to-thrive syndrome in children (Farthing, 1996). *Giardia* is thought to belong to the earliest lineage to diverge from the major eukaryotic line (Hashimoto *et al.*, 1994; Sogin *et al.*, 1989) and it lacks several common higher eukaryotic organelles such as mitochondria, endoplasmic reticulum, Golgi apparatus, and peroxisomes (reviewed by Adam, 2001; Gillin *et al.*, 1996). One of the most striking features of *Giardia* is its unique cytoskeleton especially the flagellar and adhesive or ventral disc. The infective *G. duodenalis* trophozoite stage is believed to attach to the intestinal cells using the ventral disc although the mechanism for attachment has yet to be clearly defined. It is also difficult to verify the attachment mechanism experimentally due to the lack of a suitable *in vitro* model. Recently, attachment to human intestinal cells has been studied *in vitro* and was demonstrated to be reduced by temperature, colchicine, mebendazole, and glucose (Céu Sousa *et al.*, 2001) which suggests that a combination of mechanical and hydrodynamic forces are involved in attachment. These authors also described how surface lectins may mediate adherence involving specific recognition by host cells, such as intestinal cells.
1.2.4.ii. Benzimidazoles in the treatment of giardiasis

The increasing emergence of resistance to existing treatments of giardiasis, whether it is a result of low compliance with therapy regimes, reinfestation, or resistance to widely used treatments such as metronidazole, has resulted in increased application of the benzimidazole, albendazole. This is a highly effective anti-giardial compound in vitro (Katiyar et al., 1994; Meloni et al., 1990) and in vivo (Reynoldson et al., 1998) although treatment failures (Brasseur and Favennec, 1995) and resistance to it have been demonstrated (Abboud et al., 2001). While there is a low reportage of treatment failures due to resistance, in vitro resistance has been propagated (Lindquist, 1996; Upcroft et al., 1996). The successful treatment of giardiasis with benzimidazoles (reviewed by Gardner and Hill, 2001; Upcroft and Upcroft, 2001a) has been demonstrated with albendazole (Hall and Nahar, 1993; Lemée et al., 2000; Reynoldson et al., 1998) and mebendazole (Al-Waili et al., 1988; Al-Waili and Hasan, 1992).

Initial ultrastructural observations of the morphological changes to G. duodenalis following treatment with albendazole and mebendazole noted a loss in their ability to adhere to solid supports and diminished viability. Fragmentation of the ventral disc was also observed (Chavez et al., 1992) and supported the hypothesis that the benzimidazoles were disrupting normal cytoskeletal microtubule functions, although whether they are interferring with microtubules in particular cellular locations (i.e. flagellar, ventral disc) has not been clearly identified by ultrastructural studies (Chavez et al., 1992; Oxberry et al., 1994).

In vitro assessment of the activity of the benzimidazoles against G. duodenalis has also demonstrated their therapeutic value. However, due to the lack of standardised methods to assess efficacy the variability in efficacy imparted by drug exposure time and drug effect has meant that results of separate drug efficacy studies are not concordant. To determine which benzimidazoles are most efficacious against
G. duodenalis several in vitro trials have been conducted utilising a variety of techniques to evaluate drug efficacy. These have included the measurement of an inhibitory concentration (Edlind et al., 1990; Katiyar et al., 1994; Morgan et al., 1993), inhibitory dose (Lemée et al., 2000), minimum lethal concentration (Cedillo-Rivera and Munoz, 1992), and minimum inhibitory concentration (Upcroft and Upcroft, 2001b). Unfortunately, this makes comparison of benzimidazole efficacy difficult and also complicates the process of relating the structure of different benzimidazoles to their efficacy.

1.2.4.iii. Significance of the Encephalitozoon species

‘Microsporidia’ is a nontaxonomic term used to describe protozoan parasites belonging to the phylum Microspora (reviewed by Franzen and Müller, 1999). Several species of microsporidia have been identified as the cause of disease in humans, especially immunocompromised (Fournier et al., 2000; Leder et al., 1998) and immunosuppressed (Gumbo et al., 1999) individuals but also in immunocompetent people (Raynaud et al., 1998). The two microsporidia species most commonly detected in human immunodeficiency virus (HIV)-positive patients are Enterocytozoon biennis and Encephalitozoon intestinalis (Sobottka et al., 1998), formerly Septata intestinalis (Hartskeerl et al., 1995). E. intestinalis is the most prevalent of the three significant Encephalitozoons which also include E. hellum and E. cuniculi.

The Encephalitozoon species are characterised as spore forming, obligate intracellular eukaryotes which parasitise other eukaryotic cells. Like Giardia, this group is considered to be an ancient eukaryote that multiplies by binary fission, has a membrane-bound nucleus, and lacks mitochondria and Golgi membranes (Franzen and Müller, 1999). The Encephalitozoons are also an emerging water-borne microsporidian species (Dowd et al., 1998) which is capable of zoonotic transfer (Deplazes et al., 1996; Didier et al., 1996; Snowden et al., 1999). In immunocompetent
individuals *E. intestinalis* causes chronic diarrhoea (Raynaud *et al*., 1998; van Gool *et al*., 1997) which combines with enteritis and systemic infections in HIV-infected individuals (Asmuth *et al*., 1994; Coyle *et al*., 1996; Franzen *et al*., 1996).

In host cells *Encephalitozoon* replicates in a unique parasitophorous vacuole and releases vegetative and spore stages into the host cell, resulting in the production of infective spores. These spores have a unique coiled polar tubule (Fig. 1.6) and it is through this tubule that they transfer their infective sporoplasm into host cells. The mechanism by which the polar tube interacts with the host cell membrane is not clearly understood, although it may involve host cell proteins such as actin (Foucault and Drancourt, 2000). The polar tubule has been investigated as a target for chemotherapy, especially as the extrusion of the polar tubule is accompanied by an influx of calcium into the spore (He *et al*., 1996) and calcium channel blockers (*e.g.* metronidazole) have been shown to inhibit extrusion and therefore infection of host cells *in vitro*.

![Fig. 1.6. Diagram of a microsporidian spore. The spore consists of a dense outer membrane (exospore, endospore, and plasma membrane), a single nucleus, posterior vacuole, coiled polar filament, and anterior anchoring disc (Franzen and Müller, 1999).](image-url)
1.2.4.iv. Activity of the benzimidazoles against Encephalitozoon species

The efficacy, but not the mechanism of action, of the benzimidazoles has been demonstrated mainly with the most prevalent Encephalitozoon species, E. intestinalis and E. hellum. In studies which have considered the benzimidazole mechanism of action it has been assumed to be the same as their action in helminths and fungi. Therefore, benzimidazoles are considered to be microtubule disruptors through selective binding to the β-tubulin monomer which inhibits polymerisation. From a therapeutic point of view the most widely used treatment is albendazole which has been demonstrated to be rapid and highly effective in the treatment of E. intestinalis (Leder et al., 1998; Molina et al., 1998). Albendazole has been reported to completely eradicate this microsporidian (Dore et al., 1995; Joste et al., 1996) although infections with E. intestinalis are reported to recur following cessation of albendazole therapy (Molina et al., 1995, 1998).

The investigation of therapeutic agents to treat microsporidiosis has been hampered by the time taken both to realise the identity of the infective agent and to culture it in sufficient amounts for in vitro drug trials, due in part to its unique host cell location. It is also due to the fact that the infection levels of Encephalitozoon species have only increased relatively recently as a result of the increasing number of HIV-positive individuals, particularly since the early 1980s (Coyle et al., 1996; Del Aguila et al., 1998). In recent years though, several in vitro methods have been developed specifically for drug assays, including utilising kidney cells (Franssen et al., 1995), fibroblasts (Beauvais et al., 1994), and a fluorescent probe to detect drug-induced morphological changes (Leitch et al., 1997).
With the development of reliable in vitro models for assessing drug efficacy several studies have evaluated fumagillin (Beauvais et al., 1994; Leitch et al., 1997), metronidazole (Franssen et al., 1995), and nifedipine (calcium-channel blocker) (He et al., 1996). When these studies have compared the efficacy of these compounds against *E. intestinalis* in vitro with different benzimidazoles the superior efficacy of the benzimidazoles to any of the other compounds tested has been clearly demonstrated (Beauvais et al., 1994; Didier et al., 1997; Leitch et al., 1997) and it is therefore the benzimidazole class of drugs which has been most widely studied.

Several in vitro trials have investigated the activity of various benzimidazoles against *Encephalitozoon* species. These studies represent a relatively thorough examination of some benzimidazole compounds for anti-*Encephalitozoon* activity, particularly albendazole and its sulphoxide and sulphone metabolites (Didier et al., 1998; Katiyar and Edlind, 1997; Ridoux and Drancourt, 1998). There is a large amount of variability in the inhibitory concentrations reported and this is attributable to the method by which the in vitro activity was assessed. Several attempts have been made recently to develop standard methods for the assessment of anti-microsporidial activity of drugs and this would greatly facilitate the comparison and screening of a large number of compounds.

As a class of drugs the benzimidazoles have been demonstrated both in vivo and in vitro to be the most promising. In addition, neither in vivo nor in vitro resistance has been observed, although they have been used therapeutically for less than ten years (De Groote et al., 1995; Lecuit et al., 1994; Molina et al., 1995). It is therefore worthwhile exploring the molecular basis of action of the highly effective benzimidazoles against *E. intestinalis* as these studies may lead to the identification of a binding site, possibly on a stably expressed protein in one or more *E. intestinalis* life stage.
1.2.5. Molecular basis of benzimidazole activity

Based largely on the studies which have been carried out with benzimidazole-sensitive and -resistant nematodes and fungi, the in vitro trials examining the molecular basis of benzimidazole activity in protozoa have focussed on the $\beta$-tubulin nucleotide and amino acid sequence. Several in vitro studies have taken a comparative approach using the $\beta$-tubulin gene from benzimidazole-sensitive and -insensitive organisms. These studies have identified phenylalanine-200 and glutamic acid-198 as being key determinants of susceptibility in G. duodenalis and E. intestinalis to benzimidazoles (Edlind et al., 1994; Katiyar et al., 1994). In addition to this, studies of the molecular basis of benzimidazole resistance in the A. nidulans fungi proposed that the benzimidazoles bind to amino acid residue 165 (Jung and Oakley, 1990). These studies of fungal resistance identified an alanine to valine mutation between sensitive and resistant A. nidulans populations. Subsequent work by Jung et al., (1992) identified several additional $\beta$-tubulin amino acid alterations in benzimidazole-resistant A. nidulans. From their analysis they proposed that amino acid residues 6, 165, 198, 199, and 200 were involved in benzimidazole binding and the subsequent development of resistance.
1.3. Rationale of this project

There is a significant lack of information and understanding of the mechanism of action of the benzimidazoles. This is partly due to the lack of direct drug binding data and is particularly true of the parasitic protozoa including *G. duodenalis* and *E. intestinalis*. Additionally, the parasitic protozoan *Cryptosporidium parvum* is insensitive to several benzimidazole compounds. Although several studies have investigated the molecular basis of benzimidazole resistance, a similar amount of information is not available to understand the basis of sensitivity or insensitivity to these compounds. This is partly due to a lack of techniques to either purify tubulin from protozoa or to express it in host cells lacking endogenous tubulin. To identify valid drug-binding sites it is also necessary that the tubulin protein is correctly folded and biochemically active in both a monomeric and heterodimeric form. These problems are compounded by the requirement for large amounts of α- and β-tubulin capable of assembling into heterodimers and microtubules for drug-binding studies.

To address this problem, this project aims to combine benzimidazole-β-tubulin binding kinetics with homology models of β-tubulin from sensitive and insensitive organisms. This approach is based upon the rationale that if β-tubulin is the target protein of the benzimidazoles in sensitive protozoa then by studying the β-tubulin protein from sensitive and insensitive protozoa the benzimidazole-binding region should be identifiable. Further to this, the identification of the benzimidazole-binding region should be possible using recombinant α-, β-, and αβ-tubulin proteins from benzimidazole-sensitive organisms.
1.4. Aims of this project

The aims of this study were:

1. To produce recombinant α- and β-tubulin from *G. duodenalis*, *E. intestinalis*, and *C. parvum* using bacterial expression systems

2. To produce three-dimensional crystals of protozoan αβ-tubulin with albendazole *in situ* utilising standard crystallisation conditions and a hanging-drop vapour diffusion method

3. To examine the validity of the proposed benzimidazole binding site on β-tubulin by homology modelling of β-tubulin from benzimidazole-sensitive and -insensitive organisms

4. To determine the binding rates and kinetics of several benzimidazoles previously demonstrated to have a range of efficacies *in vitro* against *G. duodenalis* and *E. intestinalis*
Chapter 2

Expression of recombinant protozoan tubulin in

_Escherichia coli_
Chapter 2. Expression of protozoan tubulin

2.1. Introduction

One of the barriers to high-level expression of recombinant proteins in *E. coli* is their tendency to aggregate or accumulate in inclusion bodies. This can potentially lead to problems refolding the recombinant protein *in vitro* with the increased possibility of mis-folding or incomplete folding intermediates being produced (Georgiou and Valax, 1996). Since the mid-1990s several protein expression fusion partners have been developed which favour the production of soluble recombinant proteins particularly for use in *E. coli* (Davis *et al.*, 1999; LaVallie and McCoy, 1995; Nilsson *et al.*, 1997). These include fusions with the maltose-binding protein, MBP (di Guan *et al.*, 1988; Hennig and Schäfer, 1998; Sachdev and Chirgwin, 1998b), glutathione S-transferase, GST (Smith and Johnson, 1988), and thioredoxin, TRX (LaVallie *et al.*, 1993; Yasukawa *et al.*, 1995).

There are several factors that may affect the solubility or rate of accumulation of a recombinant protein including the intrinsic characteristics of the fusion partner and the recombinant protein, and the rate of protein synthesis. Further, whether inclusion body formation is the result of the accumulation of incompletely synthesised or incorrectly folded proteins (Dobson and Ellis, 1998) or whether it is simply due to the abnormally high concentration of non-native polypeptides within *E. coli* (Lilie *et al.*, 1998) is unclear. It has also been observed that hydrophobic proteins are commonly expressed in inclusion bodies and that by fusing these proteins to a hydrophilic partner their solubility can be greatly enhanced (Lilie *et al.*, 1998). In addition to this, the solubility of a fusion protein may be based upon whether the first portion of the protein which emerges from the ribosome is soluble or insoluble and this will determine the solubility of the remaining portion of the fusion protein (Sachdev and Chirgwin, 1998a).
In order to provide further understanding of the factors which contribute to the sequestering of a recombinant protein in either a soluble or insoluble state the present study of the solubility properties of recombinant \( \alpha \)- and \( \beta \)-tubulin monomers from several protozoa was undertaken. The parasites selected for this analysis are clinically significant and so are also important for drug targeting studies. More importantly, the extraction of sufficient amounts of native tubulin from these parasites for drug binding studies has never been successfully achieved. The parasites are \( G. \ \text{duodenalis} \), \( E. \ \text{intestinalis} \), and \( C. \ \text{parvum} \) which are all enteric pathogens that cause different severities of gastrointestinal symptoms. The course of infection is normally self-limiting but can become life-threatening in immunocompromised individuals (Furness et al., 2000). Further, and of interest within the context of the overall project, the three species exhibit a range of sensitivity to the benzimidazoles.

The protozoan \( \alpha \)- and \( \beta \)-tubulin proteins are primarily cytoskeletal proteins of approximately 50kDa in size which naturally dimerise to form \( \alpha \beta \)-tubulin heterodimers that polymerise to form microtubules. They are acidic proteins with theoretical isoelectric points (pI) ranging from 4.83 to 4.98, compared with 5.17 to 6.20 for the ubiquitous mammalian forms. This overall protein charge difference is due to the high number of acidic amino acids, especially glutamic acid, in the carboxy-terminal region of all the protozoan forms of the monomers. When eukaryotic tubulin monomers have been expressed in \( E. \ \text{coli} \) previously they have been found to accumulate in inclusion bodies (Hollomon et al., 1998; Oxberry et al., 2001a) and also in soluble form (Hollomon et al., 1998; Linder et al., 1998) though without any clear reasons why a particular state is favoured (i.e. fusion partner, \( E. \ \text{coli} \) strain, intrinsic nature of tubulin).
The *G. duodenalis* β-tubulin was initially expressed as a soluble protein in *E. coli* using a poly-histidine (poly-His) fusion partner (MacDonald *et al*., 2001) and it was noted that with the same expression method recombinant tubulin from other protozoa exhibited aggregation or inclusion body formation. Hence, a fuller analysis of the solubility characteristics of protozoan tubulin was undertaken using the tubulin genes from *G. duodenalis, E. intestinalis*, and *C. parvum*. Particular attention was paid to the effect of the fusion partner, effects of the *E. coli* BL21 strains used for protein expression, and the rate of protein synthesis as determined by the temperature at which protein expression was performed (MacDonald *et al*., 2003a).

The aims of this study were:

1. To produce sufficient amounts of soluble α- and β-tubulin from several protozoa for further characterisation studies

2. To purify these proteins with minimal loses and without affecting the activity or three-dimensional structure of the recombinant tubulin
2.2. Materials and Methods

2.2.1. DNA extraction and PCR amplification of protozoan tubulin genes

The genomic DNA from *G. duodenalis* Portland strain (P1c10) and *E. intestinalis* (strain CDC:V297) was extracted using a modified cetyl trimethyl ammonium bromide (CTAB) method (Hopkins *et al.*, 1997). The *G. duodenalis* α-tubulin gene (*GD*α-1) was amplified using the polymerase chain reaction (PCR) using primers designed to the GenBank accession number AF331826 (Elmendorf *et al.*, 2001) and the β-tubulin gene (*GD*β-1) to accession number XO6748 (Kirk-Mason *et al.*, 1988) (Table 2.1).

The *E. intestinalis* α-tubulin gene (*EI*α-1) sequence was PCR amplified using degenerate primers designed to the conserved 5’ amino acid motif MRE(I/L)VH(I/V) and the 3’ motif DYEEVG(V/A)(D/E). The *E. intestinalis* β-tubulin gene (*EI*β-1) was PCR amplified using primers based on GenBank AF297876 (Table 2.1).

The PCR mixture (50 µl) contained 5 µl *Tth* Plus 10X reaction buffer (containing 670mM Tris-hydrochloride (pH 8.8), 166mM ammonium sulphate, 4.5% Triton X-100, and 2mg/ml gelatin), 1U *Tth* Plus DNA polymerase, 200 µM each dNTP, 1.5mM magnesium chloride (all from Biotech International, Bentley, Western Australia), 1U *Taq* Extender DNA polymerase, (Stratagene, La Jolla, U.S.A.), 2% dimethyl sulphoxide (DMSO) (Merck, Kilsyth, Victoria), 25pmol of each primer (Gibco BRL, Rockville, U.S.A.), 5ng genomic DNA, and sterile distilled water (to 50 µl). Amplifications were performed in a Perkin-Elmer PE 2400 thermal cycler (Foster City, U.S.A.) under the following conditions: 95°C for 5 min; 28 cycles of 95°C for 1 min, 55°C for 1 min, 74°C for 1 min; and a final 74°C elongation step for 8 min.
C. parvum oocysts (bovine assemblage) were initially suspended in 300 µl lysis buffer (10mM Tris-hydrochloride (pH 6.8), 50mM ethylene diamine tetraacetic acid (EDTA), 250mM sucrose, 8% Triton-X 100) and freeze-thawed four times to lyse the oocysts. They were then incubated at 56°C for 3 h with 50 µl proteinase K (10mg/ml) as initially described by Morgan et al., (1995). Total RNA was extracted using the SV Total RNA Isolation system (Promega, U.S.A.) and reverse transcriptase (RT)-PCR was performed as described in the Omniscript RT-PCR kit (QIAGEN, Hilden, Germany). The C. parvum α-tubulin (CPα-1) and β-tubulin (CPβ-1) genes were RT-PCR amplified using primers designed to GenBank AFO82877 (Bonafonte et al., 1999) and Y12615 (Cacciò et al., 1997), respectively, (Table 2.1).

<table>
<thead>
<tr>
<th>Gene and primer</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GDα-1</strong></td>
<td>Forward primer  5’- GCGGAAGAATTCTCACGTGAGTGCATCTCG-3’&lt;br&gt;Reverse primer 5’- GAACAAGCTTGCTTAGTAGGCGTCGTCCTC-3’</td>
</tr>
<tr>
<td><strong>GDβ-1</strong></td>
<td>Forward primer  5’- ATCAGAATTCCGTCGTGAGATTTGTCCACAT-3’&lt;br&gt;Reverse primer 5’- CAAGCTTTTGCTACTCGTCGCCGAAGTCCCTC-3’</td>
</tr>
<tr>
<td><strong>Elα-1</strong></td>
<td>Forward primer  5’- GCGGAAGAATTCTCACGTGAGGTCATCTCG-3’&lt;br&gt;Reverse primer 5’- GAACAAGCTTGCTTAGAGGCGTCGTCCTC-3’</td>
</tr>
<tr>
<td><strong>Elβ-1</strong></td>
<td>Forward primer  5’- ATCGAATTCGCAAGAGAAATCATACACTTG-3’&lt;br&gt;Reverse primer 5’- GACGTCTGCAGCAGTTAATTAACTAGAAA-3’</td>
</tr>
<tr>
<td><strong>CPα-1</strong></td>
<td>Forward primer  5’- ATATTCCTGCAGGACAGAGAAGTTATTTCA-3’&lt;br&gt;Reverse primer 5’- TTCAACCTGCAGGGATAACTGGAATCGC-3’</td>
</tr>
<tr>
<td><strong>CPβ-1</strong></td>
<td>Forward primer  5’- AAGAACCTGCAGGACAGGGAAATTGTTTCAT-3’&lt;br&gt;Reverse primer 5’- TTCAACCTGCAGGGATAAGTTAAGGCCTCAAT-3’</td>
</tr>
</tbody>
</table>

Table 2.1. Primer sequences used to amplify protozoan tubulin genes.

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.).
All the tubulin genes were amplified with unique 5' and 3' restriction sites for unidirectional cloning into pRSET-B (Invitrogen, Carlsbad, U.S.A.), pMAL-c2X (New England Biolabs, Beverly, U.S.A.), and pGEX-2T (Amersham Pharmacia Biotech, Buckinghamshire, England) and were maintained in *E. coli* strain TOP 10F' (Invitrogen). All the plasmid-tubulin constructs used for protein expression were sequenced using the ABI PRISM dye terminator cycle sequencing kit (PerkinElmer) according to the manufacturer's recommended method. Sequencing primers were designed to the vector sequence 50 basepairs upstream (forward primer) and downstream (reverse primer) of the cloning site where the tubulin genes were inserted (Table 2.2). The forward and reverse sequences were analysed using the SeqEd version 1.0.3 (PerkinElmer) programme and aligned using the Clustal W (version 1.81) sequence alignment programme (Metrowerks).

<table>
<thead>
<tr>
<th>Vector and primer</th>
<th>Primer sequence</th>
</tr>
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<tbody>
<tr>
<td>pRSET-B</td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>5'- TAATACGACTCACTATAGGG-3'</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5'- TAGTTATTGCTCAGCGGTGG-3'</td>
</tr>
<tr>
<td>pMAL-c2X</td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>5'- CGATGAAGCCCTGAAAGACGCGCA-3'</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5'- GTAACGCCAGGGTTTTCCCAGTCA-3'</td>
</tr>
<tr>
<td>pGEX-2T</td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>5'- GGGCTTGCAAGCCACGTGGTGGA-3'</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5'- TTACAGACAAGCTGTGACCGG-3'</td>
</tr>
</tbody>
</table>

Table 2.2. Primer sequences used to sequence protozoan tubulin genes.
Chapter 2. Expression of protozoan tubulin

2.2.2. Expression of protozoan tubulin in *E. coli*

The plasmid-tubulin constructs were transformed into the *E. coli* strains BL21(DE3), BL21(DE3)pLysS (Invitrogen), and BL21(DE3)RIL (Stratagene, Cedar Creek, U.S.A.) and single colonies were grown for 15 h at 37°C in 3ml Luria-Bertani (LB) broth containing 50µg/ml ampicillin and 35µg/ml chloramphenicol. The mid-log phase for each strain was determined using optical density (OD) measurements (wavelength 600nm). The *E. coli* culture volumes were increased to 50ml and grown at 15°C, 25°C, 30°C, or 37°C. Protein expression was induced with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the OD$_{600}$ was between 0.4 and 0.6. Soluble and insoluble protein expression levels were monitored by the removal of 1ml samples at 30 min intervals for a 6 h period. Uninduced bacterial cultures were also included to identify the basal bacterial protein expression levels. Negative controls for tubulin expression were the unligated plasmids pRSET-B, pMAL-c2X, and pGEX-2T which were also transformed into the *E. coli* strains and treated as described above.

2.2.3. Separation and analysis of recombinant tubulin solubility

The 50ml cultures were pelleted by centrifugation at 4°C then resuspended in 0.1M MES (pH 6.5), 0.2M sodium chloride, 0.05% sodium azide, with protease inhibitors 1mM phenylmethylsulphonyl fluoride (PMSF), 0.7mg/ml leupeptin, and 0.5mg/ml pepstatin A. The 1ml samples collected during expression were pelleted at 4°C then resuspended in 100µl sample buffer, lysed by freeze-thawing, then centrifuged at 2,500 x g to separate the soluble (supernatant) and insoluble (pellet) expression fractions.
All the samples were analysed by gel electrophoresis and immunoblotting using Bio-
Rad Mini-Protean apparatus (Hercules, U.S.A.). The protein expression samples were
analysed in a native form by agarose gel electrophoresis (AGE) and in a denatured
form by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE:
Laemmli, 1970). The native AGE sample buffer contained 62.5mM Tris-hydrochloride
(pH 6.8), 7.5% Ficoll 400, and 0.01% bromophenol blue. The denaturing SDS-PAGE
sample buffer contained 25% glycerol, 2.5% SDS, 62.5mM Tris-hydrochloride (pH 6.8),
0.01% bromophenol blue, and 12.5% deionised water. The 4% agarose gels were
prepared in buffer containing 90mM Tris-hydrochloride (pH 8.5), 90mM boric acid and
electrophoresis was performed at 80V (constant voltage) for 60 min at 4°C. The
12% Tris-glycine polyacrylamide pre-cast gels (Gradipore, Frenchs Forest, Australia)
were electrophoresed at 80V (constant voltage) for 60 min in electrophoresis buffer
containing 3.75% Tris-hydrochloride (pH 6.8), 1.25% SDS, and 18% glycine in distilled
water. The AGE and SDS-PAGE gels were then stained for 12 h in Coomassie staining
solution containing 0.1% Coomassie Brilliant Blue R-250, 50% distilled water,
40% methanol, and 10% acetic acid. The gels were de-stained in 50% distilled water,
40% methanol, and 10% acetic acid for 24 h.

Immunoblotting (i.e. Western and dot: Towbin et al., 1979) was performed using
polyvinylidene difluoride (PVDF) transfer membranes (MSI, Westboro, U.S.A.) with
polyclonal anti-poly-His (Invitrogen), anti-MBP (New England Biolabs), anti-GST
(Amersham Pharmacia Biotech), or monoclonal anti-\(\alpha\)-tubulin, and anti-\(\beta\)-tubulin
primary antibodies (ICN Biomedicals, Aurora, U.S.A.). An alkaline phosphatase-
conjugated immunoglobulin G (IgG, whole molecule) secondary antibody (Sigma
Chemical Company, St Louis, U.S.A.) was used for colorimetric detection using an
alkaline phosphatase assay (Bio-Rad) performed according to the manufacturer’s
recommended method.
2.2.4. Purification of recombinant tubulin by affinity and ion-exchange chromatography

Affinity chromatography of the tubulin fusion proteins was performed by binding the MBP-tubulin fusions to an amylose resin (New England Biolabs), GST-tubulin fusions to glutathione-agarose beads (Amersham Pharmacia Biotech), and poly-His-tubulin fusions to a nickel-nitriloacetic acid (Ni-NTA) resin (Invitrogen) at 4°C. The recombinant tubulin monomers were cleaved from the MBP partner using Factor Xa, from the GST partner using thrombin, and from the poly-His partner using an enterokinase enzyme. The tubulin was eluted in 0.1M MES (pH 6.5), 0.2M sodium chloride, and 0.05% sodium azide and was then separated from any MBP, GST, or poly-His contamination using anion-exchange chromatography with Uno-Q anion-exchange columns (Bio-Rad) at 4°C. The eluants were analysed by AGE and SDS-PAGE and immunoblots as described above and quantified using a Bio-Rad protein assay (Bradford, 1976).

2.2.5. Dimerisation of recombinant tubulin monomers

The purified recombinant α- and β-tubulin monomers (5mg/ml) were dimerised in buffer containing 0.1M MES (pH 6.5), 1mM magnesium chloride, 1mM ethylene glycol-bis-(β-aminoethyl ether)-N, N', N'-tetraacetic acid (EGTA), and 0.5mM GTP at 4°C for 30 min. The progress of dimer formation was monitored by AGE and the inclusion of both the α- and β-tubulin components in the dimers was analysed by Western blotting as described above.


2.3. Results

The results of the expression of protozoan tubulin genes in *E. coli* demonstrated that they can be expressed at relatively high concentrations, of up to 50mg/l of purified protein, under a variety of conditions (Table 2.3). The choice of fusion partner and the temperature used for protein expression produced the most significant changes in soluble fusion protein concentrations. The results also demonstrated there was no detectable difference between the expression levels for the α- and β-tubulin genes from each protozoan while there were minor differences in α- and β-tubulin expression levels between the different protozoa.

The effect of amino-terminal fusion partners on expression levels in the *E. coli* strain BL21(DE3)pLysS was tested. It was demonstrated that the two previously identified high-solubility partners, MBP and GST, produced a five-fold increase in soluble protein yields compared with the lower solubility poly-His partner (Table 2.3). This is likely to be due to an increased ability of the high-solubility proteins to compensate for and overcome the lower cytoplasmic solubility of tubulin and so maintain it in a soluble form, thus circumventing inclusion body formation. The highest expression levels prior to purification for the MBP fusion proteins of approximately 40% of the total cell protein agrees with the expected expression levels for a non-toxic MBP fusion protein in the *E. coli* cytoplasm (Hennig and Schäfer, 1998; Sachdev and Chirgwin, 1998b).
The effect of *E. coli* culturing temperature on the amount of soluble tubulin expressed was initially identified using a poly-His fusion partner (Fig. 2.1; MacDonald *et al.*, 2001). As other protein expression systems (discussed below) have given optimal yields of soluble recombinant proteins at lower temperatures this was more fully examined using a high-solvability MBP fusion partner. Of the three parameters tested, expression temperature had the greatest effect on protein yield. At 37°C, expression of all three protozoan α- and β-tubulin genes was maximal and decreased by only 20% when the temperature was decreased to 30°C. Temperatures of 25°C and below resulted in a 50% reduction in average yield of soluble tubulins.

![Fig. 2.1. Expression of *G. duodenalis* β-tubulin at 37°C, 30°C, and 25°C.](image)

The variation in expression levels of soluble *G. duodenalis* MBP-β-tubulin at 37°C (lane 1), 30°C (lane 2), and 25°C (lane 3) is illustrated in this SDS-PAGE gel.
### Table 2.3. Comparative expression of recombinant protozoan tubulin.

Average yield (mg/l) of purified soluble recombinant α- and β-tubulin from *G. duodenalis, C. parvum, and E. intestinalis* in several *E. coli* strains, expression temperatures, and fusion partners. The yields represent an average of quadruplicate samples.

<table>
<thead>
<tr>
<th>Parameter tested</th>
<th><em>G. duodenalis</em></th>
<th><em>C. parvum</em></th>
<th><em>E. intestinalis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fusion partner</strong>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>MBP</td>
<td>40 – 50</td>
<td>40 – 45</td>
</tr>
<tr>
<td></td>
<td>GST</td>
<td>35 – 40</td>
<td>40 – 45</td>
</tr>
<tr>
<td></td>
<td>poly-His</td>
<td>10 – 15</td>
<td>5 – 15</td>
</tr>
<tr>
<td><strong>E. coli strain</strong>&lt;sup&gt;2&lt;/sup&gt;</td>
<td>BL21(DE3)</td>
<td>10 – 20</td>
<td>15 – 20</td>
</tr>
<tr>
<td></td>
<td>BL21(DE3)pLysS</td>
<td>30 – 40</td>
<td>30 – 40</td>
</tr>
<tr>
<td></td>
<td>BL21(DE3)RIL</td>
<td>25 – 35</td>
<td>25 – 35</td>
</tr>
<tr>
<td><strong>Temperature</strong>&lt;sup&gt;3&lt;/sup&gt;</td>
<td>15°C</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td>15 – 20</td>
<td>15 – 20</td>
</tr>
<tr>
<td></td>
<td>37°C</td>
<td>30 – 40</td>
<td>35 – 40</td>
</tr>
</tbody>
</table>

<sup>1</sup> Expression in *E. coli* strain BL21(DE3)pLysS at 37°C

<sup>2</sup> Expression as MBP-tubulin fusion proteins at 37°C

<sup>3</sup> Expression of MBP-tubulin fusion protein in *E. coli* strain BL21(DE3)pLysS
The recombinant $\alpha$- and $\beta$-tubulin monomers were demonstrated to form $\alpha\beta$-tubulin heterodimers under standard conditions in the presence of GTP and magnesium when analysed by Western (Fig. 2.2) and dot-blotting (Fig. 2.3). These results indicated that the band corresponding to the dimeric tubulin reacted with both the anti-$\alpha$- and anti-$\beta$-tubulin antibodies. They were therefore, found to not form $\alpha\alpha$- or $\beta\beta$-homodimers which has been previously reported with recombinant nematode tubulin (Oxberry et al., 2001a). The recombinant monomers also retained their antigenicity with monoclonal antibodies and Western (Fig. 2.2) and dot-blotting (Fig. 2.3). These results are a strong indication that the structural integrity of the tubulin monomers has been preserved throughout the expression and purification processes.

Fig. 2.2. Western-blot of monomerised $\alpha\beta$-tubulin from *G. duodenalis*, *E. intestinalis*, and *C. parvum*. *G. duodenalis*, *E. intestinalis*, and *C. parvum* $\alpha\beta$-tubulin heterodimers were dissociated and separated by SDS-PAGE. This Western-blot with anti-$\alpha$-tubulin (lanes 1-3) or anti-$\beta$-tubulin (lanes 4-6) antibodies indicates that each heterodimer was composed of an $\alpha$- and $\beta$-tubulin monomer. The *G. duodenalis* (lane 1), *E. intestinalis* (lane 2), and *C. parvum* (lane 3) $\alpha$-tubulin monomers had a molecular weight of approximately 50kDa. The *G. duodenalis* (lane 4), *E. intestinalis* (lane 5), and *C. parvum* (lane 6) $\beta$-tubulin monomers had a molecular weight of approximately 48kDa.
**Fig. 2.3.** Dot-blot of monomeric recombinant α- and β-tubulin. The α- and β-tubulin from *G. duodenalis* (column 1), *E. intestinalis* (column 2), and *C. parvum* (column 3) were detected with monoclonal anti-α-tubulin (row 1) or anti-β-tubulin (row 2) antibodies.

The protozoan α- and β-tubulin genes used for protein expression were sequenced in the expression vectors (*i.e.* pMAL-c2X, pRSET-B, pGEX-2T) using a standard dye-terminator sequencing method. The sequencing primers were designed to match the vector sequences 50-basepairs upstream (forward primer) and downstream (reverse primer) from the tubulin gene insertion site. The sequences of all the tubulin-vector constructs indicated the correct codon reading frame had been maintained throughout the PCR, restriction enzyme digest, and ligation processes (Appendices I and II). The α- and β-tubulin sequences were analysed using the SeqEd programme and aligned using Clustal W with a ubiquitous *Homo sapiens* tubulin sequence for comparison. The sequences were deposited in the GenBank DNA database and have the following accession numbers: *E. intestinalis* α-tubulin AY161322; *E. intestinalis* β-tubulin AY161323; *C. parvum* α-tubulin AY161324; *C. parvum* β-tubulin AY161325; *G. duodenalis* α-tubulin AY161326; and *G. duodenalis* β-tubulin AY161327 and AF162281.
2.4. Discussion

An analysis of tubulin expression conditions as a means of maximising the amount of soluble recombinant protein through preventing or minimising inclusion body formation are presented. One of the strengths of this study was that the same protein was produced using several methods so that a comparison of the factors affecting its expression levels could be made, that is, the intrinsic effects due to tubulin were minimised. The cumulative effects of \textit{E. coli} strain, fusion partner, and expression temperature have been previously demonstrated to have a significant effect on the level of expression of soluble proteins (Hollomon et al., 1998; Sheffield et al., 1999). However, it has also been suggested that there is no correlation between the propensity to inclusion body formation by a particular protein and its intrinsic properties such as molecular weight, hydrophobicity, and folding pathways (Lilie et al., 1998).

To clearly identify the influence of the fusion partner on protein solubility a comparison was made between a relatively low solubility poly-His system (Kroll et al., 1993) and two higher solubility partners, MBP (Kapust and Waugh, 1999; Pryor and Leiting, 1997) and GST (Frangioni and Neel, 1993). These fusion partners were also chosen as they have the additional advantage of being purified using affinity chromatography and also have a fusion partner cleavage site within a few amino acids of the start of the protein of interest. These three fusion systems also use the T7 RNA promoter as indicated by the DE3 designation in the strain names signifying they contain the \( \lambda \text{DE3} \) lysogen which carries the gene for T7 RNA polymerase under the control of the \( \text{lacUV5} \) promoter. Therefore all the fusion systems used chemical induction with IPTG to initiate expression (Studier et al., 1990). Using the same promoter for all the expression systems tested removes any variability in soluble protein levels resulting from weaker or stronger promoters of protein expression.
It has previously been observed that recombinant protein expression levels can be reduced because of a limit in the amount of rare codons in *E. coli* which are present in lower amounts when compared with other organisms, particularly eukaryotes (Kleber-Janke and Becker, 2000; McNulty *et al.*, 2003), including protozoa. This has also been termed a codon bias amongst amino acid usage in *E. coli* compared with eukaryotic genes and it can reduce both the quality and quantity of recombinant proteins (Kane, 1995) as a result of premature termination of translation, bypassing of codons, and misincorporation of amino acids (Calderone *et al.*, 1996; Forman *et al.*, 1998; McNulty *et al.*, 2003).

The effects of the codon bias are believed to be particularly significant when there are multiple consecutive rare codons near the amino-terminus of the coding sequence (Kurland and Gallant, 1996). It was therefore important to understand whether codon bias also resulted in low expression levels of protozoan tubulin genes, especially as they share high amino acid sequence and structural homology (80%) with the *E. coli* FtsZ cell-division protein (Erickson, 1998; Löwe and Amos, 1998; van den Ent *et al.*, 2001). To determine the amount of codon bias between *E. coli* and protozoan genes the Kazusa database was used (Nakamura *et al.*, 2000). This contains the codon usage frequencies for the complete protein coding sequences compiled in the GenBank DNA database. The *E. coli* strains chosen for this study were BL21(DE3), BL21(DE3)pLysS, and BL21(DE3)RIL. The BL21(DE3)pLysS strain contains the pLysS plasmid which encodes the T7 lysozyme that inhibits the basal expression of the T7 RNA polymerase and consequently the recombinant protein (Studier *et al.*, 1990).
The BL21(DE3)RIL strain contains extra copies of the arginine (\textit{argU}), isoleucine (\textit{ileY}), and leucine (\textit{leuW}) transfer RNA (tRNA) genes in order to produce high expression levels of proteins affected by this type of codon bias (Carstens and Waesche, 2001). These tRNAs recognise the AGA/AGG, AUA, and CUA codons, respectively, with different comparative codon usage between \textit{E. coli} and particular protozoa (Table 2.4). Despite some codon bias differences the use of a strain providing extra rare tRNA genes, BL21(DE3)RIL, did not provide any additional benefit for the synthesis of protozoan tubulin when compared with the strain containing the pLysS vector (\textit{i.e.} BL21(DE3)pLysS). There was however, an approximately two-fold increase between the strains containing the extra tRNAs and the basic BL21(DE3) strain (Table 2.3).

<table>
<thead>
<tr>
<th>Codon</th>
<th>tRNA product</th>
<th>\textit{E. coli}</th>
<th>\textit{G. duodenalis}</th>
<th>\textit{C. parvum}</th>
<th>\textit{E. intestinalis}</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGA</td>
<td>\textit{argU}</td>
<td>3.5</td>
<td>6.1</td>
<td>24.9</td>
<td>17.7</td>
</tr>
<tr>
<td>AGG</td>
<td>\textit{argU}</td>
<td>2.0</td>
<td>9.6</td>
<td>6.3</td>
<td>10.5</td>
</tr>
<tr>
<td>AUA</td>
<td>\textit{ileY}</td>
<td>6.6</td>
<td>11.9</td>
<td>19.6</td>
<td>14.4</td>
</tr>
<tr>
<td>CUA</td>
<td>\textit{leuW}</td>
<td>4.2</td>
<td>7.1</td>
<td>7.8</td>
<td>13.4</td>
</tr>
</tbody>
</table>

\textbf{Table 2.4.} Comparative codon usage for particular codons by \textit{E. coli} (strain BL21(DE3)), \textit{G. duodenalis}, \textit{C. parvum}, and \textit{E. intestinalis}. Codon usage is indicated as frequency per thousand codons for codons thought to be in particularly low supply in \textit{E. coli} (Nakamura \textit{et al.}, 2000).

It has been demonstrated that the rate of protein synthesis can also have an effect on whether a recombinant protein is expressed in a soluble or insoluble state (Vasina and Baneyx, 1997). This has mainly been demonstrated by reducing the tendency of
certain proteins to aggregate when expressed at 37°C by lowering the rate of protein synthesis through decreasing the temperature, in turn increasing the amount of soluble protein expressed (Schein and Noteborn, 1988). The present study also demonstrated the effect of temperature upon soluble tubulin expression levels over a 37°C to 15°C range. Soluble protein expression levels were found to be significantly reduced below 25°C when compared with 30°C and 37°C, although total protein expression levels of soluble and insoluble tubulin were relatively similar (data not shown). These results indicate there is an optimal temperature for expression of proteins with different fusion partners and these should be empirically investigated when optimising a protein expression system (MacDonald et al., 2003a).

Taken together with previous studies on the expression of tubulin in a variety of host cells (Hollomon et al., 1998; Linder et al., 1997, 1998; Lubega et al., 1993; Oxberry et al., 2001a), the present results would indicate that the interplay between the intrinsic nature of tubulin, strength of the expression promoter, and choice of E. coli strain may have a greater effect on expression levels than other parameters, such as expression temperature or rate of protein synthesis. The pursuit of soluble protein expression is not a trivial one and until a more detailed structural analysis has been made of a protein in its native form, in a recombinant soluble form, and recombinant protein re-folded from inclusion bodies (Chaudhuri et al., 1999) it will not be known if incorrect conclusions are being made between in vivo and in vitro biological characteristics. These characteristics include protein-protein interactions (e.g. dimerisation) and antigenicity (e.g. monoclonal antibody reactivity). In addition to this, it has also been demonstrated that tubulin heterodimers lose some of their biological activities in a stepwise process during unfolding, activities which are not regained with refolding (Guha et al., 1997; Sackett et al., 1994). Significantly, drug-binding activities and the ability to form microtubules are functions which are lost in this manner.
Chapter 3

Crystallisation studies of recombinant protozoan tubulin
3.1. Introduction

The most recently solved atomic models of tubulin, with resolutions of 3.5Å (Löwe et al., 2001) and 3.7Å (Nogales et al., 1998), were obtained by electron crystallography of 2-D zinc-induced crystalline sheets. While both these models represent significant milestones in tubulin’s structural determination the pursuit of an X-ray derived structural model is still essential. This is because several important regions, particularly of inter-dimeric interactions and nucleotide binding, cannot be clearly resolved with 2-D crystalline sheets (Löwe et al., 2001; Nogales et al., 1998). Recently, two groups have utilised similar crystallisation techniques to produce tubulin crystals stabilised with an anti-mitotic drug, podophyllotoxin (Schönbrunn et al., 1999), and a neuron-specific protein, SGC10 (Fleury et al., 2000). Unfortunately, the resolution of both these X-ray diffraction models was relatively poor, at 12Å and 6.1Å, respectively, compared with the most recent 3.5Å electron diffraction model (Löwe et al., 2001).

There have been several unsuccessful attempts to produce X-ray quality 3-D crystals of tubulin (Amos et al., 1984; Lobert and Correia, 1991; Voter and Erickson, 1979). From these studies it has been proposed that the failure of tubulin to form 3-D crystals is primarily due to microheterogeneity in the tubulin sample as a result of it being extracted from brain tissue and therefore consisting of several different biotypes with a range of post-translational modifications. Other possible reasons for the failure of tubulin to form 3-D crystals include a high degree of thermal instability (Nogales et al., 1995b) and a tendency to polymerise into pleomorphic structures, such as rings, plates, tubes, and sheets, even in the presence of microtubule stabilising compounds, such as paclitaxel (Nogales et al., 1995c; Schönbrunn et al., 1999; Wolf et al., 1993).
Significantly, the structurally unstable carboxy-terminal of tubulin is proposed to result in it having a large amount of conformational flexibility in solution (Jimenez et al., 1999). To reduce this flexibility attempts have been made to crystallise subtilisin-treated tubulin (i.e. tubulin-S) which removes the carboxy-terminus of the β-tubulin component of the heterodimer (Lobert and Correia, 1991; Redeker et al., 1992). The carboxy-terminus of the α-tubulin portion of the heterodimer is located within the inter-monomeric region and is believed to be inaccessible to solvents (Nogales et al., 1995a). Earlier crystallisation attempts also predicted that at a neutral pH this highly acidic carboxy-terminus would form a stable though unstructured conformation (Reed et al., 1992) which has since been confirmed by electron microscopy (Wolf et al., 1996). In addition to this, the unstable carboxy-terminus has also been identified as a key site of proteolysis of tubulin which may also contribute to its thermal instability (Nogales et al., 1995b).

This study of tubulin’s crystallisation properties was therefore designed to remove as many of the historical crystallisation problems as possible. The source of the tubulin was protozoan, rather than mammalian, and it was recombinant rather than extracted from the organisms. The recombinant protozoan tubulin was purified to greater than 98% purity using affinity and anion-exchange chromatography and was maintained at 4°C or -70°C prior to crystallisation trials. Monomeric and heterodimeric tubulin was utilised along with subtilisin-treated αβ-tubulin.

The aims of this study were:

1. To investigate standard protein crystallisation techniques suitable for producing three-dimensional crystals of protozoan tubulin
2. To produce three-dimensional crystals of protozoan αβ-tubulin suitable for X-ray diffraction studies
3.2. Materials and Methods

3.2.1. Preparation and purification of recombinant tubulin

Recombinant tubulin from *G. duodenalis*, *E. intestinalis*, and *C. parvum* were prepared as previously described (Chapter 2; MacDonald et al., 2003a). Briefly, the entire α- and β-tubulin genes were cloned into the pMAL-c2X vector (New England Biolabs) and MBP fusion proteins were expressed in the *E. coli* BL21(DE3)pLysS strain (Invitrogen). The tubulin fusion proteins were purified by affinity chromatography followed by cleavage of the MBP fusion partner with Factor Xa (New England Biolabs). The resulting tubulin monomers were further purified by anion-exchange chromatography using Uno-Q ion-exchange columns (Bio-Rad). The recombinant tubulin was concentrated using Centricon P30 ultrafiltration membranes (Millipore, Bedford, U.S.A.) at 4°C to final concentrations of 10, 20, or 30mg/ml. The recombinant α- and β-tubulin monomers, αβ-tubulin heterodimers, and MBP-α- and MBP-β-tubulin fusion proteins were maintained in MBS buffer containing 0.1 M MES (pH 6.5) and 0.2 M sodium chloride (i.e. no protease inhibitors or preservatives were included) at -70°C.

Subtilisin-treated *G. duodenalis* αβ-tubulin dimers (GDαβ-tub) were prepared by incubating *G. duodenalis* αβ-tubulin heterodimers in MBS buffer with 1% subtilisin (w/w) at 25°C for 45 min (Knipling et al., 1999; Redeker et al., 1992; Wolf et al., 1996). The reaction was stopped with 1% PMSF (w/v) in 100% DMSO. The progress of carboxy-termini digestion was monitored by AGE (native) and SDS-PAGE (denatured) as described in Chapter 2.
3.2.2. Vapour-diffusion method for screening crystallisation conditions

The recombinant $\alpha$- and $\beta$-tubulin monomers, $\alpha\beta$-tubulin heterodimers, and MBP-$\alpha$- and MBP-$\beta$-tubulin fusion proteins were stored in MBS buffer at 4°C. The pH of the MBS buffer was tested from 5.4 to 6.5 at 0.1 pH unit intervals. The Hampton Research Crystal Screen reagents (Laguna Niguel, U.S.A.) were utilised to evaluate 104 unique combinations of pH, buffers, precipitants, and salt additives for their ability to promote crystal formation and growth. The details of the Crystal Screen I and 2 formulations are provided in Appendices III and IV, respectively.

The hanging-drop vapour-diffusion method was used with 200 $\mu$l Crystal Screen reagent in the reservoir and 2 $\mu$l tubulin solution mixed with 2 $\mu$l reagent in the hanging drop on a siliconised circular coverslip. Crystallisation plates were incubated at 4°C or 25°C (room temperature) and the progress of crystal development was monitored at 24 h, 48 h, weekly for several months, and at bi-monthly intervals over a 2y period using stereo microscopy (10 – 100 x magnification).

3.2.3. Crystallisation trials with albendazole

The effect of albendazole on the crystallisation of recombinant G. duodenalis $\alpha$, $\beta$, and $\alpha\beta$-tubulin was assessed by adding 5% 0.1M albendazole in 100% DMSO to the tubulin solutions and applying them to the vapour-diffusion method described above.
3.3. Results and Discussion

Recombinant tubulin from *G. duodenalis*, *E. intestinalis*, and *C. parvum* were expressed in *E. coli* to produce MAP- and post-translational modification-free tubulin monomers and dimers which were purified to greater than 98% purity as indicated by AGE and SDS-PAGE results (Chapter 2; MacDonald *et al.*, 2003a). This highly homogenous form of monomeric and dimeric tubulin was investigated for its ability to form 3-D crystals suitable for X-ray crystallography.

The combinatorial screening method tested 104 conditions for crystal formation and included a wide range of buffers, pHs, additives, and precipitants to promote crystal formation (Appendices III and IV). Initially, *G. duodenalis* α-, β-, and αβ-tubulin were tested with this screening method at 10, 20, or 30mg/ml concentrations in MBS buffer. Using this method, two sets of conditions were found to produce small *(i.e. <1.0mm)* 3-D crystals. These conditions comprised: (1) 30% PEG-5000, 0.1M MES pH 6.5, and 0.01M zinc sulphate or (2) 20% PEG-2000, 0.1M Tris, and 0.01M nickel chloride. The tubulin was heterodimeric and the crystals formed at 25°C after one week.

The formation of 2-D microtubule crystals has been achieved previously using buffer containing MES, Pipes, sodium chloride, magnesium sulphate, and zinc sulphate (Wolf *et al.*, 1996) to form zinc-induced crystalline sheets, as first described by Larsson *et al.*, (1976). It is therefore interesting that conditions containing MES, sodium chloride, and zinc sulphate were also suitable for producing small 3-D crystals of recombinant tubulin. Further investigations of these conditions included testing the pH of the solution in which the tubulin was maintained in from pH 5.4 to 6.5 (0.1 pH unit increments), addition of the β-tubulin binding-drug, albendazole, de-carboxylation of the β-tubulin dimer portion with subtilisin (Wolf *et al.*, 1996), and incubating the
crystallisation plates for over two years. These steps, however, did not improve the size or number of 3-D crystals of tubulin or produce 2-D microtubule crystals.

The addition of microtubule stabilising drugs, such as paclitaxel (Nogales et al., 1995c) and podophyllotoxin (Schönbrunn et al., 1999), have been previously shown to promote the formation of 2-D and 3-D tubulin crystal forms, respectively. As albendazole has been demonstrated to bind with a high affinity to recombinant G. duodenalis and E. intestinalis β-tubulin (discussed in Chapter 5) its tubulin stabilising properties were investigated. The albendazole, unfortunately, did not promote crystallisation with αβ- or β-tubulin from either parasite under the conditions tested in this study.

As it has been proposed that a significant amount of tubulin’s structural heterogeneity is due to its highly acidic and unstructured carboxy-terminus (Löwe et al., 2001) it was predicted that removal of this region by subtilisin digest would produce a more stable protein structure (Lobert and Correia, 1991; Wolf et al., 1996). The subtilisin digest also reduced the overall charge of the protein (Table 3.1), an effect predicted to assist in the formation of stable crystalline structures at neutral pH values (Wolf et al., 1996). Subtilisin digestion of αβ-tubulin removed a small (approximate molecular weight 2kDa) fragment of approximately twenty amino acids from the carboxy-terminal of the β-tubulin subunit (Fig. 3.1). The resulting αβs-tubulin had a greatly reduced negative charge as demonstrated by native AGE which separates the components of a mixed protein sample on the basis of their charge (Knipling et al., 1999; Redeker et al., 1992; Sackett et al., 1985).
### Table 3.1. Theoretical pI values of protozoan tubulin dimers. The decrease in acidity following removal of the carboxy-terminus of heterodimeric $\alpha\beta$-tubulin ($\alpha\beta_s$-tubulin) is compared with the native form ($\alpha\beta$-tubulin). Theoretical pI values were calculated using the ExPASy internet-based tools (http://au.expasy.org/tools/pi_tool.html) utilising the nucleotide sequences of the $\alpha\beta$-tubulin genes determined in Chapter 2 (Appendices I and II). These were used to produce the recombinant tubulin used in this study, with the exception of the *Homo sapiens* sequences which were determined using GenBank accession numbers CAA25855 ($\alpha$-tubulin) and ABO88100 ($\beta$-tubulin) for the ubiquitous forms of tubulin.

<table>
<thead>
<tr>
<th>Organism</th>
<th>$\alpha\beta$-tubulin</th>
<th>$\alpha\beta_s$-tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. duodenalis</em></td>
<td>4.90</td>
<td>5.15</td>
</tr>
<tr>
<td><em>E. intestinalis</em></td>
<td>4.94</td>
<td>5.11</td>
</tr>
<tr>
<td><em>C. parvum</em></td>
<td>5.02</td>
<td>5.30</td>
</tr>
<tr>
<td><em>H. sapiens</em></td>
<td>5.17</td>
<td>5.47</td>
</tr>
</tbody>
</table>
Fig. 3.1. Native gel of subtilisin digested *G. duodenalis* α- and β-tubulin. The carboxy-terminus of β-tubulin was removed and dimerised with α-tubulin to form αβs-tubulin. When these are separated by non-denaturing AGE on the basis of charge the difference in charge between tubulin and subtilisin-treated tubulin is clearly illustrated.

Interestingly, some investigators have examined the improvement in crystallisation properties of proteins which are difficult to crystallise brought about by the inclusion of carrier proteins such as the high-solubility fusion partners MBP (Center *et al*., 1998) and GST (Lally *et al*., 1998; Zhan *et al*., 2001). The main requirements for carrier protein-driven crystallisation are that the carrier protein must be highly soluble and form well-ordered crystals suitable for high-resolution diffraction studies (Rhodes, 2000). As MBP satisfies these criteria, this approach was also investigated in this study using the *G. duodenalis* MBP-α- and MBP-β-tubulin fusion proteins. While the MBP-tubulin fusion proteins did not form 3-D crystals this approach could be modified and potentially be successful. In this study, the MBP fusion proteins placed the MBP on the amino-terminal of the tubulin proteins. If the MBP protein expression vectors were modified to place the MBP on the carboxy-terminal of the tubulin proteins it may remove some of the tubulin’s structural heterogeneity as well as attaching a highly soluble and well characterised carrier protein to them. This may also improve the crystallisation of the tubulin monomers or heterodimers.
3.4. Conclusions

In the determination of the 3-D structure of heterodimeric tubulin one of the primary limitations of the techniques employed to date has been that only 2-D crystals have been produced and as a result of this it has not been possible to use high-resolution X-ray crystallography. This limitation has meant that only electron crystallography has been used and this has limited resolution, providing less detail of secondary structures when compared with X-ray crystallography.

In order to address this problem a 3-D crystal of $\alpha\beta$-tubulin suitable for X-ray diffraction studies is required. The present study was undertaken to investigate techniques available for the crystallisation of highly purified and highly homogeneous recombinant protozoan tubulin. As the production of recombinant tubulin results in a protein which contains only one $\alpha$- or $\beta$-tubulin isotype, and which is also free of post-translational modifications and MAPs, it is potentially suitable for the production of 3-D crystals. This approach would also address the reasons which have been proposed for extracted brain tubulin failing to form 3-D crystals, despite numerous trials and technical improvisations (Jan Löwe and Eva Nogales, personal communication). Recombinant heterodimeric tubulin however, is still conformationally unstable due primarily to the presence of a highly acidic and unstructured carboxy-terminus, and very high thermal instability. As the crystallisation trials of monomeric or dimeric tubulin, MBP-tubulin fusion proteins, and $\alpha\beta_{\varepsilon}$-tubulin produced only small 3-D crystals it is possible that other intrinsic characteristics of tubulin prevented it from successfully forming 3-D crystals.
Chapter 4

Molecular modelling of the benzimidazole binding site on protozoan tubulin
4.1. Introduction

As the production of an X-ray crystallography structure of protozoan $\alpha\beta$-tubulin was not successful (Chapter 3) an alternative approach that can be utilised to determine the structural differences between two proteins is based on comparative or homology modelling. Homology models are commonly utilised to compare the structure of different protein biotypes when a high-resolution model of one biotype is available.

To date, there is no comparative modelling data available for the benzimidazoles, either for understanding the basis of drug action or resistance. Therefore, this type of comparative modelling can provide a significant amount of structural information that cannot be produced by other techniques. This is particularly true as the formation of 3-D $\alpha\beta$-tubulin crystals (with or without benzimidazole compounds) which are suitable for X-ray crystallography has not been achieved. However, comparative modelling can be utilised to develop a model of the benzimidazole-binding site as the 3-D structure of two benzimidazole-insensitive organisms has been determined. These are the 3.7Å model of porcine $\alpha\beta$-tubulin (Nogales et al., 1999) and the 3.5Å model of bovine $\alpha\beta$-tubulin (Löwe et al., 2001). The detailed electron-density map which has been produced in this way is suitable for comparative molecular modelling. Essentially, this type of homology modelling technique is crucial to understanding the molecular basis of benzimidazole binding as it will assist in identifying which amino acids are potentially involved in the formation of the benzimidazole binding site of $\beta$-tubulin.
This study was undertaken to provide additional structural information regarding the location of the benzimidazole-binding site on protozoan $\alpha\beta$-tubulin through utilisation of the crystal structure of bovine $\alpha\beta$-tubulin as a model to predict the structure of the protozoan form of the protein. The rationale behind this approach is that the 3-D map of the electron density, hydrophobicity, and solvent accessibility of $\beta$-tubulin will illustrate significant areas of difference on $\beta$-tubulin from either benzimidazole-insensitive or -sensitive organisms. In turn, by combining the location of these differences with the amino acid residues which have been proposed to be involved in the formation of the benzimidazole-binding site, that a 3-D map of the binding site on protozoan $\alpha\beta$-tubulin will be produced.

By using this approach it was possible to develop a model of the benzimidazole-binding domain on $G.\ duodenalis$ and $E.\ intestinalis$ $\beta$-tubulin and identify an area of increased hydrophobicity, which is absent from the $\beta$-tubulin of insensitive organisms such as $Bos\ taurus$, $C.\ parvum$, and $H.\ sapiens$. The homology model-derived benzimidazole-binding site involves a distinctive hydrophobic binding domain from residues Ala-186 to Phe-213 on the $\beta$-tubulin monomer of sensitive protozoa. The interactions which are likely to occur between several benzimidazoles and the comparative side groups of the amino acids in this binding domain are described with respect to benzimidazole-sensitive and -insensitive organisms.
4.2. Materials and Methods

4.2.1. Homology modelling of protozoan tubulin

The structural co-ordinates for the 3.7Å model of porcine αβ-tubulin (1TUB.pdb: Nogales et al., 1999) and the 3.5Å model of bovine αβ-tubulin (1JFF.pdb: Löwe et al., 2001) were retrieved from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB; http://www.rcsb.org/pdb/; Berman et al., 2000; Westbrook et al., 2003). Homology models comparing the 3-D structure of the mammalian and protozoan tubulin monomers were created using the Swiss-MODEL server (http://www.expasy.ch/swissmod/). Homology models were received via e-mail and visualised using the Swiss-PDB Viewer (http://www.expasy.org/spdbv/mainpage; Schwede et al., 2003) and RasMol (http://www.openrasmol.org/) programmes. The deduced amino acid sequences of the *G. duodenalis*, *E. intestinalis*, and *C. parvum* α- and β-tubulin genes, which were expressed in *E. coli* (Chapter 2; MacDonald et al., 2001, 2003a) and were used for benzimidazole binding kinetic studies (Chapter 5; MacDonald et al., 2003b), were used to produce the homology model with the bovine α-tubulin (1JFFA.pdb) and β-tubulin (1JFFB.pdb) structural co-ordinates.

4.2.2. Modelling of benzimidazole compounds

The structure of each benzimidazole used for β-tubulin binding kinetic studies (Chapter 5; MacDonald et al., 2003b) was produced using ISIS Draw version 2.3. Additionally, the structural co-ordinates for the central benzimidine ring structure were obtained from the PDB entry for 1L5F.pdb (Cheong et al., 2002). The structural co-ordinates for albendazole and mebendazole were obtained from the HIC-Up database (http://xray.bmc.uu.se/hicup/: Kleywegt and Jones, 1998). These 3-D structures were utilised to obtain the size of each benzimidazole using the RasMol or Swiss-PDB Viewer programme.
4.3. Results

4.3.1. Amino acid sequence comparison

4.3.1.i. Comparison of β-tubulin sequences from mammalian and protozoan organisms

The β-tubulin deduced amino acid sequences from several benzimidazole-insensitive and -sensitive organisms were aligned (Appendix II) to facilitate the identification of amino acids potentially involved in the formation of the benzimidazole-binding site. The β-tubulin alignment also illustrates the differences between mammalian and protozoan forms of tubulin and the selectivity of the benzimidazoles efficacy may be reflected in these differences. The alignment of the β-tubulin deduced amino acid sequences of *B. taurus*, *H. sapiens*, *C. parvum*, *G. duodenalis*, and *E. intestinalis* (Appendix II) represents a comparison of mammalian and protozoan tubulin. There are 23 amino acid substitutions in the 439 amino acid sequence (Table 4.1) which represents a 5% difference between mammalian and protozoan β-tubulin at the amino acid level. While this type of sequence alignment has been used previously to identify potential benzimidazole-binding sites (Edlind et al., 1994; Jung et al., 1992; Kwa et al., 1995) it does not indicate the differences between benzimidazole-sensitive and -insensitive organisms. It should be noted that at the positions where two possible amino acid substitutions were observed (*i.e.* 35, 44, 56, 153, 165, 196, 200, 231, 232, 238, 268, 349, 351) the differences were always between *C. parvum* and both *G. duodenalis*, and *E. intestinalis*. These divergent substitutions represent 13 of the 23 substitutions (56%). This means that most of the amino acid substitutions observed by performing this type of primary sequence-based alignment are unlikely to play a central role in benzimidazole binding although they may be involved in the formation of the benzimidazole-binding site. This idea is further supported by the fact that these substitutions are present in the benzimidazole-insensitive *C. parvum* sequence.
4.3.1.ii. Comparison of β-tubulin sequences from benzimidazole-sensitive or -insensitive organisms

When comparison is made between the β-tubulin deduced amino acid sequences of benzimidazole-sensitive and -insensitive organisms there are 14 amino acid differences which represents 2.5% of the total amino acid sequence (Table 4.1). The notable substitutions change five amino acids from polar uncharged residues (benzimidazole-insensitive organisms) to nonpolar hydrophobic residues (benzimidazole-sensitive organisms). These polar to hydrophobic substitutions occur at amino acid residues at positions 145, 196, 200, 232, and 280 in the β-tubulin protein. In addition to this, two substitutions between benzimidazole-insensitive and -sensitive organisms alter the side-chain polarity of the amino acids involved. This occurs at positions 296 and 373 in the β-tubulin protein where the nonpolar hydrophobic alanine residue (benzimidazole-insensitive organisms) is substituted with either asparagine, serine, or cysteine (benzimidazole-sensitive organisms), which all have polar uncharged amino acid side-groups.
<table>
<thead>
<tr>
<th>Position</th>
<th>Benzimidazole-insensitive</th>
<th>Amino acid charge</th>
<th>Benzimidazole-sensitive</th>
<th>Amino acid charge</th>
<th>Effect of substitution</th>
</tr>
</thead>
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<tr>
<td>30</td>
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<td>Valine</td>
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<td>None</td>
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<tr>
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<td>PU</td>
<td>Serine</td>
<td>PU</td>
<td>None</td>
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<tr>
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<td>Histidine</td>
<td>Positive</td>
<td>Arginine</td>
<td>Positive</td>
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</tr>
<tr>
<td>145</td>
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<td>PU</td>
<td>Alanine</td>
<td>NH</td>
<td>Increased hydrophobicity</td>
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<tr>
<td>163</td>
<td>Isoleucine</td>
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<td>Methionine</td>
<td>NH</td>
<td>None</td>
</tr>
<tr>
<td>165</td>
<td>Asparagine / Glutamine</td>
<td>PU</td>
<td>Cysteine</td>
<td>PU</td>
<td>None</td>
</tr>
<tr>
<td>196</td>
<td>Threonine / Serine</td>
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<td>Alanine</td>
<td>NH</td>
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</tr>
<tr>
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<td>Tyrosine / Glutamine</td>
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<td>Phenylalanine</td>
<td>NH</td>
<td>Increased hydrophobicity</td>
</tr>
<tr>
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<td>Leucine</td>
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</tr>
<tr>
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<td>PU</td>
<td>Valine</td>
<td>NH</td>
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</tr>
<tr>
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<td>Isoleucine</td>
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</tr>
<tr>
<td>280</td>
<td>Glutamine</td>
<td>PU</td>
<td>Isoleucine or Lysine</td>
<td>NH</td>
<td>Increased hydrophobicity</td>
</tr>
<tr>
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<td>Asparagine or Serine</td>
<td>PU</td>
<td>Increased polarity</td>
</tr>
<tr>
<td>373</td>
<td>Alanine</td>
<td>NH</td>
<td>Cysteine or Serine</td>
<td>PU</td>
<td>Increased polarity</td>
</tr>
</tbody>
</table>

Table 4.1. Comparison of \(\beta\)-tubulin amino acid differences between benzimidazole-sensitive and -insensitive organisms. The charge of the amino acids shown in this table is described as nonpolar hydrophobic (NH), polar uncharged (PU), or positive.
4.3.2. Tubulin homology model validation

The quality of the homology models created using the Swiss-MODEL server was assessed by modelling the two published αβ-tubulin 3-D models against each other. In this way the α- or β-tubulin amino acid sequences used to obtain the porcine αβ-tubulin model were modelled against the bovine α- or β-tubulin models (1JFFA.pdb and 1JFFB.pdb, respectively) (Fig. 4.1 and 4.2). Conversely, the bovine α- or β-tubulin amino acid sequences were also modelled with the porcine α- or β-tubulin structures (1TUBA.pdb and 1TUBB.pdb, respectively). It should be noted that the porcine and bovine αβ-tubulin models had different resolutions; the porcine structure had a 3.7Å resolution (Nogales et al., 1998) while the bovine model had a resolution of 3.5Å (Löwe et al., 2001). The difference in the resolution in these two models resulted in the bovine 3.5Å model having fewer amino acids whose side-chain location within the 3-D structure of the protein could not be unambiguously resolved (Löwe et al., 2001). Using this approach it was possible to determine that this method of creating a homology model based on a protein amino acid sequence and the Swiss-MODEL programme was a valid means of obtaining a robust 3-D model.
Fig. 4.1. Homology model of porcine and bovine α-tubulin. This homology model shows the amino acid sequence of porcine α-tubulin (green) which was threaded onto the bovine α-tubulin (blue) 3-D structure. GDP is shown in white and the monomer is viewed from the inter-monomeric region of the α-tubulin monomer.
Fig. 4.2. Homology model of porcine and bovine β-tubulin. This homology model shows the amino acid sequence of porcine β-tubulin (green) which was threaded onto the bovine β-tubulin (blue) 3-D structure. GTP is shown in white and the monomer is viewed from the plus- or assembling-end of the microtubule.
4.3.3. Homology modelling of protozoan αβ-tubulin

The deduced amino acid sequences of *G. duodenalis*, *E. intestinalis*, and *C. parvum* β-tubulin which were expressed in *E. coli* (Chapter 2; MacDonald *et al.*, 2003a) and used for benzimidazole binding kinetic studies (Chapter 5; MacDonald *et al.*, 2003b) were utilised in creating the homology models. The structural models of protozoan β-tubulin were created using the structural co-ordinates of the 3.5Å bovine β-tubulin model (1JFFB.pdb) and Swiss-MODEL. The homology models were visualised using Swiss-PDB Viewer and RasMol. As the α-tubulin monomer has not been proposed to be involved in benzimidazole binding it was not included in the homology modelling procedure. It should also be noted that as the carboxy-terminus of bovine β-tubulin is not present in the 3.5Å model, and so would not be included in the homology model, it was deleted from the protozoan β-tubulin amino acid sequence prior to construction of the bovine-protozoan homology models.

The carboxy-terminus of the β-tubulin monomers include amino acids from Arg-280 to Gln- or Asn-439 and has been proposed to form an unstructured and highly disordered region of the protein (Nogales *et al.*, 1998; Wolf *et al.*, 1996). In addition, for the purposes of modelling potential benzimidazole-binding sites, the β-tubulin carboxy-terminus is not located within 10Å of the postulated key benzimidazole-binding residue, Phe-200 (Fig. 4.6). From this it was assumed that the carboxy-terminus region of β-tubulin is not involved in the formation of the benzimidazole-binding site.
The overall structure of the \( \beta \)-tubulin monomer was found to be highly conserved between the protozoan and mammalian forms of the protein. This high degree of homology was also predicted from the primary amino acid sequences that are 85-90% homologous. However, the location of residues that are substituted between benzimidazole-sensitive and -insensitive organisms can be visualised on this model (Fig. 4.3). From the mammalian-protozoan \( \beta \)-tubulin homology model it was observed that the substituted residues are located throughout the 3-D structure of \( \beta \)-tubulin.

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**Fig. 4.3.** The location of substituted amino acids between mammalian and protozoan \( \beta \)-tubulin. The amino acid residues that are altered between a benzimidazole-insensitive (left, red) and -sensitive (right, green) organism are shown on this \( \beta \)-tubulin homology model.
As several amino acid substitutions were identified between the benzimidazole-sensitive and -insensitive organisms that alter the hydrophobicity of the amino acids involved (Table 4.1) it was considered whether or not these substitutions would affect the overall hydrophobicity of particular regions of the $\beta$-tubulin protein. This is also an important consideration regarding benzimidazole-binding as it has been proposed to be dependent on covalent interactions (Lacey, 1988; Prichard, 2001). When the electron-density of a comparative model of $\beta$-tubulin from the benzimidazole-insensitive bovine and -sensitive *G. duodenalis* or *E. intestinalis* was visualised a substantial difference in hydrophobicity was observed (Fig. 4.4). This model was orientated so that the plus-end or assembling portion of the $\beta$-tubulin monomer was visible to mirror the manner in which the microtubule would be exposed during heterodimer polymerisation or assembly *in vivo*. Interestingly, the large hydrophobic region of *G. duodenalis* and *E. intestinalis* was located in this region of the $\beta$-tubulin protein.

![Electron density map (red) of bovine and *E. intestinalis* $\beta$-tubulin.](image)

**Fig. 4.4.** Electron density map (red) of bovine and *E. intestinalis* $\beta$-tubulin.

The homology model of *B. taurus* (left) and *E. intestinalis* (right) $\beta$-tubulin is viewed from the plus or assembling end. The yellow bars indicate the location of the M-loop on the $\beta$-tubulin monomers.
When the $\beta$-tubulin homology models were visualised from the plus-end of the microtubule the location of the structurally important M-loop was observable (Fig. 4.4). The M-loop region is composed of amino acid residues from position 279 to 287 in the $\beta$-tubulin protein (Nogales et al., 1998; Nogales, 2000). The M-loop was incorporated into the large hydrophobic region of $\beta$-tubulin from benzimidazole-sensitive organisms (i.e. G. duodenalis or E. intestinalis) but was absent from the model of $\beta$-tubulin from benzimidazole-insensitive organisms (i.e. mammalian or C. parvum). This is significant as the $\beta$-tubulin M-loop is structurally important in the formation of stable inter-dimer contacts between adjacent assembling microtubule heterodimers (Nogales et al., 1999). In particular, this region of the $\beta$-tubulin component of the microtubule-bound $\alpha\beta$-tubulin heterodimer forms lateral contacts with the H1 and H3 regions of the $\alpha$-tubulin component of $\alpha\beta$-heterodimers as they are incorporated into the microtubule (Nogales et al., 1999). This is a significant region of $\alpha$-tubulin as it is also believed to be the site of GTP binding and subsequent hydrolysis when the $\alpha\beta$-heterodimer is polymerised to form a stable component of the microtubule (Nogales, 2000).

This model provides two key pieces of evidence that the benzimidazole-binding site is located in this region of the $\beta$-tubulin protein. Firstly, it is a highly hydrophobic region of the protein found only in this region of the $\beta$-tubulin monomer. Secondly, this region of the $\beta$-tubulin monomer is exposed to the cellular environment, or ligands therein, during microtubule assembly. Benzimidazoles have been proposed to arrest microtubule assembly (Gill and Lacey, 1992; Lubega and Prichard, 1991a) and this location of the drug-binding site within this region would support this drug-binding model.
4.3.4. The \( \beta \)-tubulin–benzimidazole hydrophobic binding region

The electron density map of hydrophobicity of \( \beta \)-tubulin from benzimidazole-sensitive and -insensitive organisms has identified a structurally important region of increased hydrophobicity in the sensitive organisms that is highly accessible during microtubule assembly (Fig. 4.4). Within this region are several residues that are substituted between benzimidazole-sensitive and -insensitive organisms. These residues include amino acids Cys-165, Ala-196, Phe-200, Leu-231, and Val-232. These residues are also located within three structurally important loop regions named T5 (Ala-196), T6 (Phe-200), and T7 (Leu-231 and Val-232). In particular, the T6-loop region encompasses amino acids Ala-186 to Asp-205 (Fig. 4.5) and has been identified as being structurally important as it is located adjacent to the M-loop in the 3.5Å model of \( \beta \)-tubulin (Löwe et al., 2001; Nogales et al., 1999). The T6-loop region also includes several amino acids that have been proposed to result in the benzimidazole-resistant phenotype in the parasitic nematode, \( H. \ contortus \) (Beech et al., 1994; Kwa et al., 1995; Nare et al., 1996). More particularly, the amino acids located at position 198, 199, and 200 in the \( \beta \)-tubulin protein have been proposed to confer benzimidazole-resistance in \( H. \ contortus \) by utilising comparative nucleotide sequence data.
**Fig. 4.5.** The T6-loop region of protozoan β-tubulin. The amino acid residues spanning the region Ala-186 to Phe-213 are indicated with GTP at the top of the loop region. The T6-loop is viewed in cross-section through the β-tubulin monomer with the microtubule interior on the left and exterior on the right.
The T6-loop region creates a 3-D structure with a cone-like shape with a depth of 17.20Å and an upper distance between residues Ala-187 and Asp-205 of 13.30Å (Fig. 4.6). The benzimidazoles utilised in the drug-binding kinetics component of this study (Chapter 5) were all R₅-carbamate derivatives with the exception of thiabendazole (Fig. 5.1; Table 5.1). The benzimidazoles used in this modelling study were albendazole, albendazole-sulphoxide, fenbendazole, mebendazole, oxibendazole, parbendazole, and thiabendazole. The central benzimidine ring structure of these benzimidazoles had an average size of 4.55Å (Fig. 4.7). The benzimidazole R₅ analogues, including the R₂-carbamate side-chain, have a minimal length of 9.25Å which increased depending upon the length of the R₅ side-chain.

The models of each benzimidazole within the proposed hydrophobic binding domain (Fig. 4.5) provides some information in relation to the interaction of the different benzimidazole derivatives with the amino acids of this binding region (Fig. 4.8 to 4.21). The benzimidazole interaction plots were determined for benzimidazole-sensitive and -insensitive organisms. Within this region, the key amino acid interactions are proposed to involve Ile-189, Val-199, and Phe-200 which are all located at the base of the proposed binding region (Fig. 4.5). These three residues in benzimidazole-sensitive organisms also increased the hydrophobicity of this region of the β-tubulin monomer (Fig. 4.4).
Fig. 4.6. Distances between amino acids of the hydrophobic benzimidazole-binding domain of protozoan β-tubulin. The secondary structure of this region is also indicated with α-helix (red), β-sheet (yellow), and loop region (grey). The amino acid residues spanning the region Asn-186 to Ala-208 are indicated. The T6-loop is viewed in cross-section through the β-tubulin monomer with the microtubule interior on the left and exterior on the right.
Fig. 4.7. Distances within the R₅-carbamate benzimidazole derivatives.

The length of the central benzimidine ring structure was determined to be 4.55Å. The length of the central benzimidine rings with the R₂-carbamate group was found to be 9.25Å.

The proposed benzimidazole-binding site places the R₅ side-chain in close proximity to the side-chains of residues Ile-189, Leu-192, Val-199, Phe-200, and Cys-201 of β-tubulin from sensitive organisms. For each benzimidazole utilised in this study the chemical identity of the R₅ side-chain was shown to determine the nature of the interaction particularly with the Ile-189 to Cys-201 region of the binding domain.
4.3.5. Interaction models of benzimidazole compounds

with β-tubulin

4.3.5.i. Interaction models of albendazole and albendazole-sulphoxide with β-tubulin

In considering the structure of the benzimidazole compounds utilised in this study, albendazole and albendazole-sulphoxide differ only by the presence of an oxide group between the R₅ sulphide moiety and the propyl side-chain shared by both albendazole and albendazole-sulphoxide. The location of this oxide group is significant as this model identifies a potential interaction between this group and the aliphatic side-group of Ile-189 (Fig. 4.9). As the β-tubulin amino acid at position 189 is altered from Ile-189 to Val-189 in benzimidazole-insensitive organisms this side-group is likely to be involved in the formation of significant benzimidazole-binding interactions (Fig. 4.8).

The sulphoxide group of albendazole-sulphoxide extends the distance that the propyl side-chain extends into the hydrophobic binding region. Specifically, instead of creating one potential interaction between the R₅ side-chain of albendazole with Phe-200 (Fig. 4.8) the propyl carbon chain of albendazole-sulphoxide places both the first and third carbon groups of the propyl side-chain in contact with the aromatic side-group of Phe-200 and the aliphatic side-group of Val-199 (Fig. 4.9). This is in contrast to the comparable region of the β-tubulin model of albendazole-insensitive organisms where a relatively chemically inert oxide group (Tyr-200) and hydroxyl side-group (Thr-199) are found (Fig. 4.8).
Fig. 4.8. Structure of albendazole in proposed binding domain of benzimidazole-insensitive organisms.

Fig. 4.9. Structure of albendazole in proposed binding domain of benzimidazole-sensitive organisms.
Fig. 4.10. Structure of albendazole-sulphoxide in proposed binding domain of benzimidazole-insensitive organisms.

Fig. 4.11. Structure of albendazole-sulphoxide in proposed binding domain of benzimidazole-sensitive organisms.
4.3.5.ii. Interaction models of fenbendazole and mebendazole with β-tubulin

Fenbendazole and mebendazole are structurally distinct from the other benzimidazoles used in this study due to the presence of a phenyl ring linked to the R₅ position of the central benzimididine ring structure by either a sulphyl (fenbendazole) or carbonyl (mebendazole) group. The proposed binding model places the phenyl-ring structure in close proximity to the aliphatic side-group of Ile-189 (sensitive) (Fig. 4.13 and 4.15) which has a stronger electron-withdrawing capacity than Val-189 (insensitive) for both fenbendazole and mebendazole (Fig. 4.12 and 4.14). The sulphyl (fenbendazole) or carbonyl (mebendazole) group of both these drugs is proposed to form an electrophilic interaction with the oxide component of the sulphydryl side-group of Cys-201 (Fig. 4.13 and 4.15). The presence of a carbonyl group in the mebendazole R₅ side-chain introduces an additional interaction to those seen for fenbendazole (Fig. 4.14). This involves the hydrophobic phenyl ring of Phe-200 which is absent from the uncharged side-group of Tyr-200 of insensitive organisms (Fig. 4.14). In addition to this, the higher hydrophobicity of Val-199 (Fig. 4.15) compared with the uncharged Thr-199 of insensitive organisms (Fig. 4.14) is likely to further stabilise the carbonyl-phenyl-based interaction of mebendazole with residue Phe-200.
Fig. 4.12. Structure of fenbendazole in proposed binding domain of benzimidazole-insensitive organisms.

Fig. 4.13. Structure of fenbendazole in proposed binding domain of benzimidazole-sensitive organisms.
Fig. 4.14. Structure of mebendazole in proposed binding domain of benzimidazole-insensitive organisms.

Fig. 4.15. Structure of mebendazole in proposed binding domain of benzimidazole-sensitive organisms.
4.3.5.iii. Interaction models of oxibendazole and parbendazole with β-tubulin

Oxibendazole and parbendazole differ from albendazole and albendazole-sulphoxide only in the loss of the R₅-sulphyl group which is proposed to interact with the oxide component of the sulphhydril side-group of Cys-201 forming a relatively stable electrophilic interaction (Fig. 4.9 and 4.11). The main difference between oxibendazole and parbendazole is the extent to which the R₅ carbon side-chain extends into the binding domain (Fig. 4.16 and 4.19). When the spatial location of the side-chain is considered, the butyl side-chain of parbendazole interacts with two substituted amino acid side-groups, the aromatic phenyl group of Phe-200 and the hydrophobic aliphatic group of Val-199 (Fig. 4.19). This could suggest the formation of a more stable complex between parbendazole and β-tubulin compared with the stability proportioned by the shorter propyl-group of oxibendazole. With respect to the propyl side-chain of oxibendazole, the proposed benzimidazole-binding site amino acid which may interact with the propyl side-chain is Phe-200 as this carbon chain is too short to bind with Val-199 (Fig. 4.17). The basis of the interaction with Phe-200 may involve hydrophilic binding between the oxibendazole R₅ carbon chain with the aromatic phenyl side-group of Phe-200.
Fig. 4.16. Structure of oxibendazole in proposed binding domain of benzimidazole-insensitive organisms.

Fig. 4.17. Structure of oxibendazole in proposed binding domain of benzimidazole-sensitive organisms.
Fig. 4.18. Structure of parbendazole in proposed binding domain of benzimidazole-insensitive organisms.

Fig. 4.19. Structure of parbendazole in proposed binding domain of benzimidazole-sensitive organisms.
4.3.5.iv. Interaction model of thiabendazole with β-tubulin

To contrast with the other benzimidazoles, thiabendazole was selected as it lacks an R₅ side-chain, instead having an R₂-thiazole group. In terms of how this compound would interact with key amino acids in the proposed hydrophobic binding domain it is unlikely that there would be any significant interactions. Thiabendazole is also unlikely to form any significant interactions with the residues in the upper region of the binding domain including residues Ala-185, Thr-186, Ser-188, Ile-202, and Asp-203 (Fig. 4.20 and 4.21). These amino acids are also conserved between benzimidazole-sensitive (Fig. 4.21) and -insensitive (Fig. 4.20) organisms and so would be unlikely to form the basis of the selective binding between the benzimidazoles and protozoan β-tubulin. Additionally, it is likely that the R₂-thiazole group may prevent this compound from binding or interacting with any of the residues near the base of the hydrophobic binding domain including residues Ile-189, Leu-192, Val-199, and Phe-200 (Fig. 4.21). From this model it is predicted that thiabendazole may have a low affinity for the proposed binding domain. This is because thiabendazole lacks the necessary R₅ side-chain which would interact with amino acid residues unique to the benzimidazole-sensitive protozoan form of the β-tubulin monomer.
Fig. 4.20. Structure of thiabendazole in proposed binding domain of benzimidazole-insensitive organisms.

Fig. 4.21. Structure of thiabendazole in proposed binding domain of benzimidazole-sensitive organisms.
4.4. Discussion

In this study, comparative molecular modelling of \(\beta\)-tubulin from benzimidazole-sensitive and -insensitive organisms was utilised to visualise potential benzimidazole binding sites based on the location of substituted amino acids in the 3-D structure of \(\beta\)-tubulin. Initial analysis of the deduced amino acid sequence of \(\beta\)-tubulin from benzimidazole-sensitive and -insensitive organisms identified several amino acid substitutions. However, most of the amino acid substitutions identified by this method were between \textit{C. parvum} and \textit{G. duodenalis} or \textit{E. intestinalis}. That is, they were not amino acid differences between benzimidazole-sensitive protozoa and -insensitive mammalian or protozoan (\textit{i.e.} \textit{C. parvum}) sequences. It is therefore unlikely these residues would form a unique benzimidazole-binding site in \textit{G. duodenalis} or \textit{E. intestinalis} as the residues comprising this site are expected to be divergent between sensitive and insensitive organisms and also conserved between all benzimidazole-insensitive organisms (mammalian and protozoan).

Using the homology models of \(\beta\)-tubulin from benzimidazole-sensitive and -insensitive organisms a highly hydrophobic binding domain, which was unique to benzimidazole-sensitive protozoa, was identified. The amino acids within this domain include the residues from Ala-186 to Phe-213 on the \(\beta\)-tubulin monomer of sensitive protozoa. The involvement of residues 198, 199, and 200 in the formation of this benzimidazole-binding domain also corroborates previously identified results from investigations of the molecular basis of benzimidazole resistance in nematodes (Kwa \textit{et al.}, 1995; Nare \textit{et al.}, 1996) and fungi (Jung and Oakley, 1990; Jung \textit{et al.}, 1992). The interactions which are likely to occur between several benzimidazoles and the various side-groups of the amino acids in this binding domain were described with respect to benzimidazole-sensitive and -insensitive organisms. The principal interactions are proposed to occur
between the $R_5$ side-chain of the benzimidazole compounds and Ile-189, Val-199, and Phe-200 which all have nonpolar, hydrophobic side-groups.

In addition to this, the protozoan model places the potential benzimidazole-binding site closer to the GTP-binding site on the $\beta$-tubulin monomer or heterodimer than has been previously suggested. The proposed benzimidazole-binding domain places the drug binding site on the outer surface of the $\beta$-tubulin protein whether it is in a monomeric or heterodimeric state. Through the use of a homology model it was possible to identify that the proposed benzimidazole-binding domain is directly adjacent to the site of hydrolysis of $\beta$-tubulin-bound-GTP. As this GTP hydrolysis occurs when a new $\alpha\beta$-tubulin heterodimer binds with a microtubule bound $\alpha\beta$-heterodimer it is proposed that any changes in this region of the heterodimer may affect this process. This presents an interesting model whereby the presence of a benzimidazole compound at this location may prevent or inhibit the rate of the conformational change that is believed to accompany the hydrolysis of GTP to GDP (Muller-Reichert et al., 1998; Vulevic et al., 1997) and which is essential for microtubule assembly (Fontalba et al., 1993; Menéndez et al., 1998). The consequences of this may be that microtubules can no longer polymerise and grow or alternatively it may decrease the internal stability of the microtubule lattice thereby promoting depolymerisation. If microtubule depolymerisation occurred at a higher rate than polymerisation there would be a net loss of microtubules from the cell. This would also support microscopic observations of the apparent effect of benzimidazole compounds on several sensitive organisms (Borgers et al., 1975; Chavez et al., 1992; Oxberry et al., 1994).
By using this approach it was possible to develop a model of the benzimidazole-binding domain on *G. duodenalis* and *E. intestinalis* β-tubulin which is absent from the β-tubulin of insensitive organisms such as *B. taurus*, *C. parvum*, and *H. sapiens*. A similar approach has been utilised with the anti-tumour compound, paclitaxel (Gupta *et al.*, 2003) to identify the amino acids involved in the formation of the drug-binding site. The validity of this benzimidazole-binding site on the β-tubulin protein, however, requires further evidence that it is a key determinant of benzimidazole binding. This requires the analysis of direct-binding kinetics between the benzimidazole compounds utilised in the formation of this model and α-, β-, and αβ-tubulin heterodimers and microtubules from sensitive and insensitive organisms.
Chapter 5

Characterisation of benzimidazole binding with protozoan $\alpha$- and $\beta$-tubulin
5.1. Introduction

The in vivo and in vitro efficacy of several benzimidazole compounds against G. duodenalis (Morgan et al., 1993; Reynoldson et al., 1998) and E. intestinalis (De Groote et al., 1995; Ridoux and Drancourt, 1998; Katiyar and Edlind, 1997) has previously been demonstrated. In addition to this, several benzimidazoles have also been demonstrated to be inactive against other parasitic protozoa such as C. parvum (Fayer and Fetterer, 1995). This chapter presents an analysis of the binding kinetics involved when different benzimidazoles bind with tubulin from benzimidazole-sensitive and -insensitive organisms. The benzimidazole-sensitive organisms used in this analysis were G. duodenalis and E. intestinalis while the benzimidazole-insensitive organism was C. parvum.

The kinetic analysis involved determining the association ($K_a$) and dissociation ($K_d$) equilibrium constants for a number of benzimidazoles with recombinant $\alpha$-, $\beta$-, and $\alpha\beta$-tubulin to investigate whether their differential efficacy in vitro relates to binding to monomeric or dimeric tubulin. This analysis was performed by comparing two techniques: the first technique was based on using optical biosensors and the second technique utilised fluorescence quenching, in order to identify the optimal analytical method. The first method, a biosensor chip-based technique, measured changes in resonance brought about by altering the refractive index of materials bound to the biosensor chip. The second method utilised the quenching of fluorescence from particular amino acid residues when a ligand binds to them. These two methods were utilised to determine the apparent association rate ($k_{on}$) and dissociation rate ($k_{off}$) from which the equilibrium constants were calculated.
The benzimidazoles used in this study were all R₂-carbamate analogues with the same basic structure of 1,2-diaminobenzene (Fig. 5.1). These compounds were albendazole, albendazole-sulphoxide, fenbendazole, mebendazole, oxibendazole, and parbendazole. Thiabendazole was also studied as a non-R₂-carbamate analogue (Table 5.1). All these benzimidazoles have previously been demonstrated to have a wide range of activity against *G. duodenalis* and *E. intestinalis* and are therefore expected to display selectivity of benzimidazole binding.

![General structure of a benzimidazole compound.](image)

**Fig. 5.1.** General structure of a benzimidazole compound.

<table>
<thead>
<tr>
<th>Benzimidazole derivative</th>
<th>Side-chain composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albendazole</td>
<td>-NHCOOCH₃</td>
</tr>
<tr>
<td>Albendazole-sulphoxide</td>
<td>-NHCOOCH₃, -SCH₂CH₂CH₃</td>
</tr>
<tr>
<td>Fenbendazole</td>
<td>-NHCOOCH₃, -S-phenyl</td>
</tr>
<tr>
<td>Mebendazole</td>
<td>-NHCOOCH₃, -CO-phenyl</td>
</tr>
<tr>
<td>Oxibendazole</td>
<td>-NHCOOCH₃, -OCH₂CH₂CH₃</td>
</tr>
<tr>
<td>Parbendazole</td>
<td>-NHCOOCH₃, -CH₂CH₂CH₂CH₃</td>
</tr>
<tr>
<td>Thiabendazole</td>
<td>-4-Thiazole, -H</td>
</tr>
</tbody>
</table>

**Table 5.1.** Structure of benzimidazole derivatives tested for binding affinity with protozoan tubulin from sensitive and insensitive organisms.
The aims of this study were:

1. To determine the binding affinities and kinetics of several benzimidazole derivatives with recombinant protozoan tubulin

2. To identify whether the benzimidazoles have a differential affinity for recombinant tubulin from benzimidazole-sensitive or -insensitive organisms

3. To determine if the benzimidazoles have a differential affinity for α- or β-tubulin monomers, or αβ-tubulin dimers

4. To relate the in vitro binding kinetics of the benzimidazoles tested to their in vivo and in vitro efficacy against G. duodenalis, E. intestinalis, and C. parvum
5.2. Characterisation of benzimidazole binding using surface plasmon resonance
5.2.1. Introduction

The technique of measuring surface plasmon resonance (SPR) using an optical biosensor (reviewed by Nice and Catimel, 1999; Schuck, 1997) has been developed by BIAcore (Fågerstam et al., 1992; Jönsson et al., 1991) and relies upon measuring changes in resonance between a solid support and a liquid medium (Fig. 5.2). SPR is an optical phenomenon caused by the oscillation of electrons in a thin metallic film, usually gold, off which polarised light is reflected. The amount of resonance measured is therefore directly proportional to any changes in the refractive index brought about by an increase in the mass of molecules (e.g. proteins) attached to the surface (Stenberg et al., 1991).

The sensor chip developed by BIAcore illustrates the application of this phenomenon (Fig. 5.2) (Malmqvist and Karlsson, 1997). In this system, the target ligand (e.g. protein) is immobilised onto the sensor chip and the analyte (e.g. drug, DNA) flows over it using continuous flow conditions. The SPR signal, expressed as resonance units (RU), is measured continuously to detect changes in the refractive index caused by mass changes on the surface of the chip. These changes are brought about by binding of the mobile analyte to the immobilised ligand (Jönsson et al., 1991; Malmqvist, 1993) and are therefore proportional to the amount of binding that occurs. The continuous monitoring of the SPR signal allows the kinetics of binding to be followed in real time as they are displayed graphically as a sensorgram (i.e. RU versus time) (Fig. 5.3). From these sensorgrams the affinity and kinetic aspects of the interaction can be determined, particularly the association ($k_{on}$) and dissociation ($k_{off}$) rates, from which the equilibrium association ($K_a$) and dissociation ($K_d$) constants are calculated (Ben Khalifa et al., 2001; Quinn and O'Kennedy, 2001).
Fig. 5.2. Surface plasmon resonance detection system within BIACore instruments. The polarised light is focussed onto the sensor chip surface where the binding reaction occurs and is reflected to an optical detection unit. The angle of the reflected light varies with the refractive index of the sensor chip surface such that an increase in mass with binding to the surface results in a change in the refractive index (i.e. from I to II). The changes in reflectance with time are presented in a sensorgram.

For these interactions to be accurately described kinetically, several potential sources of error must be eliminated or minimised. These include instrument artefacts such as instrument and baseline drift, non-specific binding, and refractive index changes that are not the result of macromolecular interactions (Myszka, 1997). These problems are all eliminated through careful experimental design (i.e. randomised, duplicate samples) and particularly by using a reference binding surface. The reference surface does not contain any ligand but is treated identically to the sample surfaces within a four channel sensor chip. The changes in resonance occurring on the reference surface due to artefacts are contained within its sensorogram which is then subtracted from all the binding reaction or test surface sensorograms to eliminate them as a source of error (Roden and Myszka, 1996; Karlsson and Fält, 1997).
Fig. 5.3. Typical phases of association and dissociation described by a sensorgram. Following injection of an analyte onto a stable binding surface association binding data is collected. After sample injection, the complex dissociates in running buffer followed by regeneration of the binding surface using a low pH solution. Kinetic rate constants are derived from the association and dissociation phases of the sensorgram.

Another significant error may occur when an unstable or decaying binding surface is created (Joss et al., 1998). BIAcore has developed several different ligand immobilisation techniques to avoid this problem. These may be based on random immobilisations via amine-, thiol-, aldehyde, or streptavidin-biotin coupling (Fig. 5.4) (Johnsson et al., 1995; O'Shannessy et al., 1992) although these approaches create a degree of heterogeneity in the binding surface. There are also more specific methods which orientate the ligand via specific antibodies (Karlsson and Fält, 1997; Myszka et al., 1997) or antibodies to commonly used protein expression fusion partners (Nilsson et al., 1996). The most commonly used sensor chip (Rich and Myszka, 2001) is coated with a carboxymethyl-dextran (CMD) matrix (Löfás and Johnsson, 1990) which provides a flexible anchor for ligand immobilisation (Fig. 5.4) and is proposed to allow interactions to occur as they would in solution (Johnsson et al., 1991).
Perhaps the most significant and documented problem in producing high quality binding data is caused by mass transfer (also termed mass transport) (Fig. 5.5) (Myszka, 1997; Schuck and Minton, 1996). Many approaches have been taken to describe and so eliminate it from binding data (Myszka et al., 1997, 1998a). Mass transfer is principally due to the intrinsic design of the sensor chip and continuous flow system whereby the mobile analyte in the flowing portion of the channel diffuses through an unstirred solvent layer to bind with the immobilised ligand (Fig. 5.5) (Myszka, 1997). The rate of diffusion to the binding surface (i.e. mass transfer) may be sufficiently slow to affect the rate of binding, especially for high-affinity or rapidly associating macromolecules (Glaser, 1993; Myszka et al., 1998b).

To minimise these effects, it has been recommended that kinetic studies are performed at high flow rates (i.e. >20 µl/min) with a low amount of ligand immobilised (i.e. <100RU) (Edwards et al., 1997). The high flow rate reduces the height of the unstirred solvent layer, which increases the transfer rate of the analyte to and from the sensor chip surface (Fisher et al., 1994; Karlsson et al., 1994). Lowering the amount of surface ligand present also decreases the rate at which the analyte is depleted from the unstirred solvent layer such that diffusion does not control the association process. Similarly, if unbound analyte is not removed from the surface rapidly it may rebind producing a slower apparent dissociation rate. Rebinding is also minimised by having a low amount of ligand present (Myszka, 1997).
Fig. 5.5. Diagram of the interaction occurring at the surface of a biosensor chip. The ligand (B) is immobilised to the sensor chip via a flexible linker and the analyte (A₀) flows over the surface. The initial association phase involves the diffusion of A₀ through the unstirred solvent layer at the rate of mass transfer ($k_m$). The association ($k_a$) and dissociation ($k_d$) rates are produced when the complex AB is formed (Myszka, 1997).

The determination of binding and equilibrium kinetics from SPR data were significantly improved with the introduction of global data fitting into the BIAevaluation software (version 3.0) in 2000. Prior to this it was not possible to analyse binding curves using several different interaction models in order to identify which one best described the data. Kinetic information was previously extracted from binding curves using what has been proven, in some instances, to be an overly simplistic bimolecular model [Eqn 5.1].

$$A + B \xrightleftharpoons[\koff]{\kon} AB$$  \hspace{1cm} [Eqn 5.1]
This equation is based on A being the mobile analyte, B the immobilised ligand, and AB the complex they form with particular association \((k_{on})\) and dissociation \((k_{off})\) rates. With this model, data fitting initially involved utilising linear regression (Karlsson et al., 1991), non-linear curve fitting (O’Shannessy et al., 1993; Roden and Myszka, 1996), and later numerical integration of the equations describing the interaction kinetics (Karlsson and Fält, 1997; Morton et al., 1995; Myszka et al., 1997). Some of the problems encountered with this model were due to the fact that interactions occurring on the biosensor surface did not obey the pseudo-first-order rate equation that these data fitting models were based on (Bowles et al., 1997; O’Shannessy and Winzor, 1996). This equation assumes that, under ideal conditions, the concentration of mobile analyte is constant and that mass transfer is not affecting the rate of diffusion to or binding with the ligand (Glaser, 1993; Karlsson et al., 1994).

Significantly, it has been demonstrated previously that for these conditions to be met a relatively high concentration of ligand must be immobilised particularly to produce a significant change in refractive index upon binding (Lipschultz et al., 2000; Schuck, 1996; Strandh et al., 1998). This has also been found to affect the accuracy of the binding data through increased mass transfer, steric hindrance, crowding, and aggregation on the surface of the sensor chip (Edwards et al., 1997; Schuck, 1996; Morton and Myszka, 1998).

Many investigators have analysed approaches which could be taken to successfully describe equilibrium binding constants and in particular these have focussed on describing binding as a two-step process (Edwards et al., 1997). The first step is the transfer of the analyte \((A_0)\) to the sensor chip surface and the second step is binding of the analyte \((A)\) to the ligand \((B)\) to form the complex \(AB\) (Fig. 5.5) (Fisher et al., 1994; Morton et al., 1995; Myszka et al., 1997). The equation describing this binding model includes diffusion or transfer of the analyte to and from the binding surface [Eqn 5.2].
Chapter 5. Benzimidazole binding kinetics

\[
A_0 \xrightarrow{k_{m}} A + B \xrightarrow{k_{on}} AB
\]

[Eqn 5.2]

The mass transfer coefficient \(k_m\) is dependent on the diffusion coefficient of the protein, dimensions of the flow cell, and the flow rate (Karlsson et al., 1994). This model has been used to describe several different experimental systems (Markgren et al., 2001; Morton and Myszka, 1998; Myszka et al., 1998a; Oddie et al., 1997). The progression of this approach was made when it was identified that by fitting association and dissociation curve data for a series of ligand and analyte concentrations and flow rates simultaneously, referred to as global data fitting or analysis, discrimination could then be made between different reaction mechanisms (Morton et al., 1995) while also removing the effects of mass transfer (Myszka, 1997). With global data fitting it was possible to describe kinetic parameters from several sensorgrams simultaneously, which significantly improved the quality of the parameters (Karlsson and Fält, 1997; Roden and Myszka, 1996). This also allowed more complex interaction models, such as heterogenous ligands, mass transfer limited, and multivalent ligands, to be described (Ben Khalifa et al., 2001; Markgren et al., 2001).

SPR biosensor technology offers several advantages for characterising macromolecular interactions over traditional analysis techniques (reviewed by Rich and Myszka, 2000; Schuck, 1997). The principal advantages of SPR biosensors are that neither the ligand nor the analyte requires labelling, whilst also being highly sensitive, specific, and versatile (Myszka, 1997; O'Shannessy, 1994). For these reasons it has been applied to the description of several types of interaction including antigen-antibody (Myszka et al., 1997; Oddie et al., 1997), protein-drug or receptor-ligand (Karlsson et al., 2000; Markgren et al., 2001), protein-DNA (Bondeson et al., 1993; Fisher et al., 1994), and DNA-DNA (Bates et al., 2002; Persson et al., 1997).
While the most widely described interactions are high-affinity (Markgren et al., 2001; Myszka et al., 1998b) methods for evaluating low-affinity ($K_a < 10^4 \text{M}^{-1}$) and low-molecular-weight (<1,000Da) interactions have been described (Karlsson et al., 2000; Ohlson et al., 1997; Strandh et al., 1998). More recently some analysis has been presented whereby the interaction between high-molecular-weight ligands with very low-molecular-weight analytes, <100Da, especially drug-receptor interactions, has been described (Davis and Wilson, 2000; Karlsson et al., 2000; Rich et al., 2001). The application of SPR as a drug-screening tool is therefore a relatively recent development in the field of optical biosensors (Markgren et al., 1998; Rich et al., 2001) and one for which experimental liposome-based sensor chips have recently been developed (Baird et al., 2002; Erb et al., 2000).

As optical biosensors have now been developed for the detection of binding between high-molecular-weight targets and low-molecular-weight ligands the present study was undertaken. The aim of this study was to specifically develop a method for determining the binding kinetics ($K_a$, $K_d$) occurring between the low-molecular-weight benzimidazoles with their postulated target protein, the high-molecular-weight protozoan $\alpha$- or $\beta$-tubulin, using an SPR-based technique.
The activity of the benzimidazoles, which were initially developed as anthelmintics, has been most widely studied in the parasitic nematode *H. contortus*, although more recently their *in vitro* activity against several intestinal parasites has been demonstrated. This includes differential activity of various benzimidazoles against *G. duodenalis* (Katiyar *et al.*, 1994; Meloni *et al.*, 1990; Morgan *et al.*, 1993) and *E. intestinalis* (Franssen *et al.*, 1995; Katiyar and Edlind, 1997; Ridoux and Drancourt, 1998). Previous kinetic studies with tritiated-benzimidazoles have established that they bind with a high affinity (>10^7 M^-1) to the cytoskeletal protein β-tubulin in the benzimidazole-sensitive *H. contortus* (Gill and Lacey, 1992; Lubega and Prichard, 1991b; Prichard, 2001) although the kinetics of this binding have not been investigated. In addition to this it has also been previously shown that refolded recombinant tubulin from several organisms is capable of binding tubulin antagonists, including benzimidazole compounds (Hollomon *et al.*, 1998; Lubega *et al.*, 1993; Oxberry *et al.*, 2001b).

The aims of this study were:

1. To immobilise *G. duodenalis* α- and β-tubulin monomers onto biosensor chips using amine coupling and affinity capturing techniques

2. To allow binding to occur between several benzimidazole compounds with the immobilised tubulin monomers to determine their binding affinities
5.2.2. Materials and Methods

5.2.2.i. Immobilisation of recombinant protozoan tubulin

The BIAcore 2000 system, research grade carboxymethylated dextran CM5 sensor chips, and standard BIA-certified HBS running buffer (0.01M HEPES, pH 7.4, 0.2M sodium chloride, 3.4mM EDTA), and the Amine Coupling Kit containing N-hydroxysuccinimide (NHS), N-ethyl-N’-(3-diethyl-aminopropyl)-carbodiimide (EDC), and ethanolamine-hydrochloride were obtained from BIAcore AB (Uppsala, Sweden). Recombinant *G. duodenalis* α- (rGDα) and β-tubulin (rGDβ) were produced in *E. coli* as MBP fusion proteins as previously described (Chapter 2; MacDonald *et al.*, 2003a). The sensor chips (SC-1, SC-2) were immobilised with four binding surfaces each (Table 5.2); FC-1: unbound, blocked CMD matrix; FC-2: anti-MBP monoclonal antibody; FC-3: anti-α-tubulin (SC-1) or anti-β-tubulin (SC-2) monoclonal antibody; FC-4: rGDα (SC-1) or rGDβ (SC-2). Standard amine coupling chemistry was used to attach the anti-MBP monoclonal antibody, anti-α- or anti-β-tubulin monoclonal antibodies, or rGDα or rGDβ onto the CM5 sensor chip.

The immobilisation steps were carried out using HBS buffer at 25°C with a 20µl/min flow rate. All four channels were simultaneously activated for 7 min with 0.05M NHS and 0.2M EDC. The respective monoclonal antibodies, rGDα, or rGDβ were injected at a concentration of approximately 20µg/ml in 10mM sodium acetate, pH 4.4, for 7 min. Unreacted N-hydroxysuccinimide esters were then blocked with 1M ethanolamine-hydrochloride, pH 8.5, for 7 min. The MBP-rGDα (SC-1, FC-2) or MBP-rGDβ (SC-2, FC-2) fusion proteins and rGDα (SC-1, FC-3) or rGDβ (SC-2, FC-3) were immobilised onto their respective antibodies using a standard affinity capturing method. Subsequent analysis was performed in MBS running buffer containing 0.1M MES (pH 6.5), 0.2M sodium chloride, and 10% DMSO.
Table 5.2. Binding surfaces created on each flow channel (FC) of sensor chip-1 (SC-1) and sensor chip-2 (SC-2).

<table>
<thead>
<tr>
<th>Flow channel</th>
<th>Sensor chip</th>
<th>Approximate MW proteins (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SC-1</td>
<td>SC-2</td>
</tr>
<tr>
<td>FC-1</td>
<td>Reference surface</td>
<td>Reference surface</td>
</tr>
<tr>
<td>FC-2</td>
<td>Anti-MBP antibody + MBP-rGDα-tub</td>
<td>Anti-MBP antibody + MBP-rGDβ-tub</td>
</tr>
<tr>
<td>FC-3</td>
<td>Anti-α-tubulin antibody + rGDα-tub</td>
<td>Anti-β-tubulin antibody + rGDβ-tub</td>
</tr>
<tr>
<td>FC-4</td>
<td>rGDα-tub</td>
<td>rGDβ-tub</td>
</tr>
</tbody>
</table>

5.2.2.ii. Benzimidazole binding experiments

Stock solutions of albendazole and mebendazole (SmithKline Beecham Pharmaceuticals) were prepared in 100% DMSO and diluted immediately prior to use in MBS running buffer to minimise refractive index changes following sample injections onto the sensor chip. The solubility of albendazole and mebendazole in MBS running buffer over a 1 to 1,000nM range was assessed by light scattering at OD 320nm using a Shimadzu UV-1201 spectrophotometer at 25°C. The interaction between albendazole and the immobilised tubulin monomers was optimised by monitoring binding at flow rates of 10, 20, or 30µl/min over a 0 to 1,000nM range (200nM increments) injected for 30, 60, 90, 120, or 150 sec, randomly and in quadruplicate. A blank sample of 25µl of MBS running buffer was injected between each drug sample. Suitable regeneration conditions were determined using either 0.01M glycine (pH 2.7), 0.01M sodium hydroxide injected for 30 sec, or MBS running buffer (5 min) at 30µl/min.
5.2.2.iii. Analysis of binding reaction sensorgrams and global data fitting

The response from the four binding surfaces were initially corrected for baseline drift, instrument noise, refractive index changes, and non-specific binding using graphical tools in the BIAevaluation software (version 3.0, 2000). This was done by subtracting the reference surface (FC-1) sensorgram from each test surface (FC-2, -3, -4) for each sensor chip individually. The association ($k_{on}$) and dissociation ($k_{off}$) rates and equilibrium constants ($K_a$, $K_d$), respectively, were calculated using the global data fitting component of the BIAevaluation software. The response value at equilibrium ($RU_{eq}$) was determined for each albendazole concentration ([ABZ]) under stabilised conditions with respect to flow-rate, pH, temperature, and surface integrity. $RU_{eq}$ represents the amount of albendazole complexed with the surface, either CMD matrix alone or with the tubulin monomers. Comparison was made between the tubulin monomers immobilised directly to the CMD matrix with those anchored via a direct antibody or antibody to the MBP fusion partner.
5.2.3. Results and Discussion

The SPR-based technique was designed such that the changes in resonance occurring during benzimidazole-binding exclusively reflected the kinetic parameters of the interaction. This was achieved by attempting to minimise the occurrence of a heterogenous binding surface, non-specific binding, and the effects of mass transfer in order to produce accurate and high quality binding data suitable for kinetic analysis (Ben Khalifa et al., 2001; Roden and Myszka, 1996).

5.2.3.i. Immobilisation of recombinant G. duodenalis tubulin monomers

To minimise or eliminate the potential occurrence of non-specific and non-uniform binding of the target protein several different immobilisation methods were investigated including direct amine coupling and affinity capturing with specific antibodies. The antibodies used were either to the tubulin protein or the fusion partner of an MBP-tubulin fusion protein. There are several advantages to this approach, most of which stem from the creation of a uniform and flexible binding surface. The uniformity is brought about by immobilising the target protein by virtue of its fusion partner (MBP) which produces a highly consistent and reproducible means of exposing the target protein to compounds in the flow channel.
In addition to this, by distancing the site of immobilisation of the tubulin from potential benzimidazole binding sites there is no loss of these sites or steric hindrance resulting from non-specific binding to other sites. This is particularly relevant if the synergistic activity of several compounds is to be studied simultaneously. The flexibility of this system arises from using an antibody specific to a commonly used fusion partner and also, in this case, to the α- or β-tubulin monomers. This reduces the amount of time required to optimise the immobilisation and binding conditions and greatly increases the number of target proteins which can be studied using the same method of immobilisation.

These different binding surfaces were created to allow comparison of their suitability for the determination of benzimidazole binding kinetics. In attempting to create a uniform binding surface there are several potential problems with each approach that could result in heterogeneity in the binding surface. When tubulin was immobilised via amine coupling it involved esterification of amino groups that are found randomly over the surface of the tubulin monomers. It is therefore difficult to create a uniform orientation of the tubulin to the analytes (i.e. albendazole, mebendazole) in the flow channel using amine coupling. This may result in a variable reduction in the number of potential drug binding sites. The main advantage of direct amine coupling is that the final amount of tubulin immobilised was much higher when compared with the affinity capture based approach (Table 5.3).
The affinity capture immobilisation approach was utilised to specifically address the problem of heterogeneity of tubulin immobilised directly to the sensor chip surface. This approach involved immobilising monoclonal antibodies to α- or β-tubulin, or to the MBP fusion proteins (MBP-rGDα or MBP-rGDβ) using amine coupling. For the same reasons discussed above, some heterogeneity would therefore exist due to the random orientation of the antibodies onto the sensor chip. One way of addressing this problem, therefore, was to saturate the chip surface so that a large number of correctly orientated antibodies would be produced as a proportion of the total number of antibody molecules immobilised. The main disadvantage of this approach is that the greater the amount of antibody which is immobilised, the greater the amount of mass attached to the chip surface and so the higher the baseline resonance from the surface. Given that the maximum resonance value that can be reliably detected with the BIAcore 2000 is 70kRU, if 15kRU is occupied by the antibody, without the tubulin attached, then it will significantly reduce the working range for detection of benzimidazole binding. In addition to this, binding will occur close to the detection limit of the system and some of the conditions to minimise the effects of mass transfer will no longer be addressed.

Following immobilisation the anti-α- and anti-β-tubulin antibodies produced a baseline resonance of 9,835 and 9,725RU, respectively, while the anti-MBP monoclonal antibody produced a baseline of 10,100RU (Table 5.3). The rGDα, rGDβ, or MBP-rGDα, MBP-rGDβ were then introduced to the appropriate channels on the sensor chip and immobilised by affinity capture. As predicted, the final amount of resonance from these surfaces was significantly higher than from the directly immobilised recombinant tubulin monomers (Table 5.3). However, the final amount of tubulin molecules orientated correctly for drug binding by anchoring them via an antibody is predicted to be higher than via direct amine coupling.
## Chapter 5. Benzimidazole binding kinetics

### Table 5.3. Comparative amounts of recombinant *G. duodenalis* tubulin (rGD\(\alpha\)-tub or rGD\(\beta\)-tub) immobilised to the CMD matrix using amine coupling or affinity capturing.

<table>
<thead>
<tr>
<th>Immobilisation method</th>
<th>Ligand</th>
<th>Corrected RU</th>
<th>Amount protein (ng/mm(^2))</th>
<th>Amount tubulin (ng/mm(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amine coupling:</td>
<td>rGD(\alpha)-tub</td>
<td>5,060</td>
<td>5.1</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>rGD(\beta)-tub</td>
<td>5,171</td>
<td>5.2</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>anti-(\alpha)-tub mAb</td>
<td>9,835</td>
<td>9.8</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>anti-(\beta)-tub mAb</td>
<td>9,725</td>
<td>9.7</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>anti-MBP mAb</td>
<td>10,100</td>
<td>10.1</td>
<td>--</td>
</tr>
<tr>
<td>Affinity capturing:</td>
<td>anti-(\alpha)-tub mAb + rGD(\alpha)-tub</td>
<td>14,315</td>
<td>14.3</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>anti-(\beta)-tub mAb + rGD(\beta)-tub</td>
<td>13,510</td>
<td>13.5</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>anti-MBP mAb   + MBP-GD(\alpha)-tub</td>
<td>13,290</td>
<td>13.3</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>anti-MBP mAb   + MBP-GD(\beta)-tub</td>
<td>13,505</td>
<td>13.5</td>
<td>3.4</td>
</tr>
</tbody>
</table>
Another potential problem that was identified with this method of immobilising tubulin as a fusion protein with MBP is the additional mass that the MBP produces on the sensor chip. The $\alpha$- and $\beta$-tubulin proteins and MBP all have approximate molecular weights of 50kDa with fusions between them being approximately 100kDa. While this mass difference did not affect the amount of tubulin immobilised (Table 5.3) it did result in a much greater amount of protein being attached to the sensor chip as indicated by the higher baseline resonance on the affinity capturing surfaces. The potential problem this creates is that it may increase the effects of mass transfer by limiting the rate of diffusion of the analyte (i.e. albendazole or mebendazole) to its target ligand (tubulin). To identify whether this was occurring a binding surface of recombinant *G. duodenalis* $\alpha$- and $\beta$-tubulin alone was created (i.e. FC-4 on SC-1 and SC-2, respectively). Binding data from this surface was subtracted from the channels containing the MBP-tubulin fusion proteins to identify any MBP-related binding events.

5.2.3.ii. Determination of benzimidazoles affinity for protozoan tubulin

To minimise the effects of mass transfer on the binding results the compounds were diluted in MBS running buffer (0.1M MES (pH 6.5), 0.15M sodium chloride, 10% DMSO) and blank injections of running buffer were made between each injection of drug. The immobilised tubulin was found to be stable in this buffer, with and without the inclusion of benzimidazoles, as no decrease in resonance was observed during the course of the drug-binding assays. The injection of MBS running buffer did not produce any change in resonance which indicated that the effects of mass transfer due to refractive index differences between the solution the analyte and ligand were in had been completely eliminated. Additionally, the effects of mass transfer due to changes in volume within the flow channel accompanying sample injection had also been removed.
The benzimidazoles, albendazole and mebendazole, were tested over a 0 to 1,000nM range, with 200nM increments, and the different drug concentrations were injected randomly and in quadruplicate during each binding trial; the results of four separate trials were utilised to construct the final equilibrium binding plots (Fig. 5.6). The reference surface was treated identically to the test surfaces with respect to running buffer, addition of ligands, regeneration conditions, and blocking of unbound amine coupling sites. The amount of resonance from this surface also indicated that it did not have any antibodies or recombinant tubulin attached to its surface.

**Fig. 5.6.** Overlay plots of uncorrected binding curves showing the interaction between albendazole and *G. duodenalis* β-tubulin. The uncorrected sensorgrams from averaged quadruplicate injections of albendazole (0 - 1,000nM) over immobilised *G. duodenalis* β-tubulin are shown.
The uncorrected sensorgrams (Fig. 5.6), (i.e. reference surface not subtracted) illustrate the concentration-dependent change in resonance resulting from albendazole binding with the surface of the sensor chip. Unfortunately, when the sensorgram from the unbound, blocked CMD matrix reference surface, (i.e. FC-1 on both sensor chips) was subtracted from the test surfaces (i.e. FC-2, -3, -4, both sensor chips) a straight line at 0RU ± <0.1% was obtained (results not shown). These results indicate that the majority of the binding that is being measured involves non-specific interaction with the CMD matrix. When the binding conditions were tested at flow rates of 10, 20, or 30µl/min there was no significant change or reduction in the amount of non-specific binding (Fig. 5.7). Similarly, when binding was monitored with drug injections for 30 to 150 seconds (30 sec increments) there was no reduction in non-specific binding (data not shown). There was also no significant difference in the amount of non-specific binding between albendazole and mebendazole over the same concentration range and under identical flow rate and binding conditions.
5.2.3.iii. Global fitting of benzimidazole equilibrium binding data

In this study the careful design of experimental controls was utilised to minimise possible binding artefacts which may result from using a 1:1 binding model with data which deviates from the pseudo-first-order kinetic model (O’Shannessy and Winzor, 1996) to determine the binding kinetics [Eqn 5.2]. The binding data obtained with the range of albendazole and mebendazole concentrations tested was analysed using the global data fitting component of the BIAevaluatiion software (v. 3.0). This allowed the association ($k_{on}$) and dissociation ($k_{off}$) components of the binding curves to be analysed simultaneously at several ligand and analyte concentrations, flow rates, pH values, and injection times (Ben Khalifa et al., 2001; Roden and Myszka, 1996). From
this data the equilibrium constants ($K_a$, $K_d$) were determined with immobilised recombinant G. *duodenalis* $\beta$-tubulin (Table 5.4).

Using global data fitting several different binding models were interrogated to identify which one best described the binding data. The various models which could be demonstrated include 1:1 interactions with and without mass transfer limited, ligand multivalency, and 2:1 interactions. With the albendazole and mebendazole binding results the 1:1 interaction with mass transfer limited was found to best describe the binding curves with rapid-on and rapid-off *(i.e.* high affinity, rapid dissociation, reversible) binding profiles (Fig. 5.6). This model was confirmed by the rapid dissociation of the albendazole and mebendazole binding complexes in running buffer, without the requirement for specific additives, such as glycine, for regeneration of the binding surface or to dissociate the benzimidazoles. This rapid dissociation is also typical of non-specific interactions, as was occurring on the sensor chip with the carboxy-methylated dextran.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Albendazole</th>
<th>Mebendazole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rGD$\beta$-tubulin</td>
<td>CMD matrix</td>
</tr>
<tr>
<td>$K_a$ (M$^{-1}$)</td>
<td>$5.46 \times 10^6$</td>
<td>$1.37 \times 10^7$</td>
</tr>
<tr>
<td>$K_d$ (M)</td>
<td>$1.83 \times 10^{-7}$</td>
<td>$7.31 \times 10^{-8}$</td>
</tr>
<tr>
<td>$k_{on}$ (Ms$^{-1}$)</td>
<td>$6.54 \times 10^5$</td>
<td>$7.99 \times 10^5$</td>
</tr>
<tr>
<td>$k_{off}$ (s$^{-1}$)</td>
<td>0.12</td>
<td>0.0584</td>
</tr>
<tr>
<td>$R_{max}$ (RU)</td>
<td>217</td>
<td>65.2</td>
</tr>
</tbody>
</table>

**Table 5.4.** Affinity rates and constants determined for albendazole and mebendazole with recombinant G. *duodenalis* $\beta$-tubulin (rGD$\beta$-tubulin) or a CMD matrix.
5.2.3.iv. Non-specific binding with the CMD matrix

A possible reason for the non-specific binding is that the immobilised tubulin has been lost from the sensor chip. However, this is not believed to be the reason as the amount of resonance from the surfaces containing tubulin remained relatively constant throughout the drug binding assays. This also includes changes in baseline resonance following storage of the sensor chip at 4°C. This indicates, therefore that the amount of mass, or tubulin, attached to the surface remained constant.

More recently, several investigators have proposed that immobilised proteins can undergo 3-D conformational changes (Mannen et al., 2001; Paynter and Russell, 2002) which can result in a loss of specific activities, such as protein-protein interactions (Ober and Ward, 2002) or drug-binding capacity. It is possible that the conformation of the immobilised tubulin was altered as a result of the continuous flow system, conditions used for immobilisation, or storage of the sensor chip at 4°C. If this has occurred it could result in the loss of the benzimidazole binding site or a significant reduction in the benzimidazoles affinity for it.

The methods which could be employed to eliminate the non-specific binding with the CMD matrix depend on the cause of the binding. If it is due to the amine coupling immobilisation technique (Kortt et al., 1997) then it may be removed by using an alternative technique (e.g. thiol coupling) although this would still involve a CMD matrix. It may be more appropriate to use a different type of sensor chip and the BIAcore lipophilic membrane chips (L1 Pioneer chips) have recently been utilised by several investigators studying drug binding kinetics (Baird et al., 2002). Some researchers have also created specialised experimental surfaces, such as polylysine-heparin-polylysine (Benítez and Jiménez, 2002), which may also be applicable to this binding assay.
Alternatively, if the non-specific binding is considered from the point of view of the benzimidazole then it may be possible to attach it to a carrier peptide which could prevent it from binding with the CMD matrix. This approach may be difficult to perform successfully as it would require a peptide which does not interfere with tubulin-binding while also preventing non-specific binding. If this approach could however, be developed it would also result in a larger change in resonance following binding with immobilised tubulin. A carrier peptide could also be used to attach the benzimidazoles to the CMD matrix irreversibly, although this would be difficult to do without also interfering with the tubulin-binding properties of the drug.

It is also possible that the benzimidazoles were not able to bind with tubulin in a continuous-flow system, such as the one used in the BIAcore instruments, but instead require a static environment to interact with their binding site. This could be tested by analysing binding using a spectrophotometric method, such as fluorescence quenching (Werbovetz et al., 1999).

5.2.4. Conclusions

The development of optical biosensors has greatly facilitated measurements of the rate of formation and dissociation of complexes in real-time and without the requirement for drug or target labelling, which may interfere with drug-binding assays (Schuck, 1997). The technique developed by BIAcore is ideally suited to the quantitative characterisation of macromolecular interactions as it can be used to determine the functional activity of a complex described by its kinetic rate constants and equilibrium constants.
It has been demonstrated that different benzimidazole compounds exhibit various efficacies for *G. duodenalis* and *E. intestinalis* *in vitro*. In order to investigate whether or not this efficacy is related to binding affinity for their proposed target protein, β-tubulin, an SPR-based system was designed to characterise this interaction. This technique was based upon the initial immobilisation of recombinant *G. duodenalis* α- or β-tubulin onto a biosensor chip followed by the measurement of refractive index changes (*i.e.* resonance) resulting from benzimidazole binding. The application of this method to the determination of benzimidazole binding kinetics would be a significant improvement on existing methods involving tritiated or photo-affinity labelled benzimidazoles and heterogenous tubulin extracts (Gill and Lacey, 1992; Lacey and Prichard, 1986; Lubega and Prichard, 1991a).

In this preliminary study a CMD-matrix was utilised as an immobilisation support which utilises a hydrophilic binding environment. Despite the development of a mass transfer limited binding environment and the testing of different protein immobilisation techniques, flow rates, immobilised protein concentrations, drug concentrations, pH of interaction, and addition of buffering agents during binding trials this technique was not successful. Under the conditions utilised in this study the benzimidazole compounds, albendazole and mebendazole, were shown to bind rapidly, reversibly, and with a high-affinity (10^7 M⁻¹) with the CMD matrix to an extent which masked the detection of any binding which was occurring with the protozoan tubulin. The approach described in this study has however, clearly demonstrated the absolute importance of using a reference binding surface in SPR-based techniques.
Chapter 5

5.3. Characterisation of benzimidazole binding using fluorescence quenching
5.3.1. Introduction

The anti-protozoal activity of the benzimidazoles, which were initially developed as anthelmintics, has been demonstrated in vitro against a number of protozoa including G. duodenalis (Cedillo-Rivera and Munoz, 1992; Katiyar et al., 1994; Meloni et al., 1990; Morgan et al., 1993) and E. intestinalis (Didier et al., 1998; Katiyar and Edlind, 1997; Ridoux and Drancourt, 1998). This activity has also been demonstrated in vivo for the treatment of giardiasis (Lemée et al., 2000; Reynoldson et al., 1998) and microsporidiosis (Dore et al., 1995; Molina et al., 1995, 1998). G. duodenalis and E. intestinalis are both significant opportunistic parasites which are also recognised as significant waterborne pathogens (Dowd et al., 1998; Furness et al., 2000). They cause different severities of gastrointestinal symptoms (Furness et al., 2000; Leder et al., 1998) which are generally self-limiting but can become life-threatening in immunocompromised individuals (e.g. transplantation, HIV-positive) (Gumbo et al., 1999; Raynaud et al., 1998). At present, the most effective therapy for both these parasites is the benzimidazole group of compounds, particularly albendazole (Hall and Nahar, 1993; Meloni et al., 1990; Molina et al., 1998; Reynoldson et al., 1998).

The mechanism underlying the mode of action of the benzimidazoles has been most extensively studied with the parasitic nematode H. contortus (Lacey and Prichard, 1986; Lubega and Prichard, 1990; Nare et al., 1996). These studies have resulted in the proposal that the benzimidazoles bind to the β-tubulin monomer prior to dimerisation with α-tubulin which prevents subsequent microtubule formation (Kwa et al., 1995). More specifically, it has been proposed that the benzimidazoles bind to a high-affinity binding site on the β-tubulin monomer (Lubega et al., 1993). One of the most important limiting factors in further characterising the binding kinetics of the benzimidazoles has been the lack of techniques to either extract homogenous tubulin from protozoan parasites or produce correctly folded and biologically active recombinant tubulin.
In order to address these problems and to further investigate the molecular basis of the mode of action of benzimidazoles in protozoa an *E. coli*-based protein expression system was utilised to produce biochemically active and assembly-competent α- and β-tubulin from *G. duodenalis*, *E. intestinalis*, and *C. parvum* (Chapter 2; MacDonald *et al.*, 2003a). The recombinant tubulin produced by this method had a purity of greater than 95% and was free of post-translational modifications. When assembled into microtubules *in vitro* they were free of accessory proteins (MAPs) which made them ideal for benzimidazole-binding assays.

In this study the binding affinities of several benzimidazoles has been determined for recombinant tubulin in both a monomeric and dimeric state. This analysis was performed using a fluorescence quenching method based on measuring the amount of intrinsic fluorescence from tryptophan residues. This fluorescence is blocked when drugs bind directly with or close to these amino acids. This technique has been successfully used by several investigators to determine the binding kinetics of the anti-mitotic compounds colchicine (Banerjee and Ludueña, 1992; Banerjee *et al.*, 1997; Chakrabarti *et al.*, 1996) and taxol (Díaz *et al.*, 2000; Han *et al.*, 1996) although more recently it has been applied to *Leishmania* tubulin to assay dinitroaniline binding (Werbowitz *et al.*, 1999).
5.3.2. Materials and Methods

5.3.2.i. Expression of protozoan tubulin in E. coli

The expression of α- and β-tubulin genes from G. duodenalis, E. intestinalis, and C. parvum has been previously described (Chapter 2; MacDonald et al., 2003a). The recombinant tubulin was maintained in MBS buffer containing 0.1M MES (pH 6.5), 0.2M sodium chloride, 0.05% sodium azide, with protease inhibitors 1mM PMSF, 0.7mg/ml leupeptin, and 0.5mg/ml pepstatin A. The protein samples were quantified using a Bio-Rad Protein assay (Bradford, 1976) and analysed by SDS-PAGE and immunoblotting as previously described (Chapter 2; MacDonald et al., 2003a).

5.3.2.ii. Dimerisation and polymerisation of recombinant tubulin monomers

The recombinant tubulin monomers (5mg/ml) were dimerised and polymerised in buffer containing 0.1M MES (pH 6.5), 1mM magnesium chloride, 1mM EGTA, and 0.5mM GTP. Dimerisation was performed at 4°C for 30 min and the progress and incorporation of both α- and β-tubulin into the heterodimers was monitored by native AGE and denaturing SDS-PAGE and Western blotting as described previously (Chapter 2; MacDonald et al., 2003a). GDP- or GTP-bound dimeric tubulin was polymerised into microtubules using a previously described method (Díaz et al., 2000; Lubega et al., 1993) by incubation at 37°C for 30 min and included a control sample with 5% DMSO and test samples with the benzimidazoles (0-200mM) tested in quadruplicate. The addition of the compounds to the polymerisation samples produced a final DMSO concentration of ≤5%. Following the addition of 3.4M glycerol the samples were clarified by centrifugation at 2,500 x g at 4°C for 15 min using a Beckman Avanti J-30I centrifuge. The pelleted microtubules were rinsed in MBS buffer and analysed by SDS-PAGE and Western blotting as described (Chapter 2; MacDonald et al., 2003a).
To monitor the effect of the benzimidazoles on polymerisation, microtubule assembly was performed with increasing drug concentrations (0-200mM, 10mM increments) and aliquots were removed at 5 min intervals. The inhibition of assembly was monitored by change in turbidity measured spectrophotometrically at 351nm (Shimadzu UV-1201 spectrophotometer) and compared to control samples of recombinant αβ-tubulin ± 5% DMSO. All measurements were made in triplicate and each drug concentration was tested in quadruplicate. To determine if the benzimidazoles de-polymerise assembled microtubules they were also added at high molar concentrations (1M) to microtubules assembled as described above. Any change in turbidity accompanying microtubule disassembly was monitored spectrophotometrically at 351nm.

5.3.2.iii. Determination of benzimidazole binding kinetics

Stock solutions of the benzimidazoles, albendazole, albendazole-sulphoxide, fenbendazole, mebendazole, oxibendazole, parbendazole, and thiabendazole (SmithKline Beecham Pharmaceuticals) were prepared in 100% DMSO. The amount of binding by the benzimidazoles to α-, β-, and αβ-tubulin (2µM) from *G. duodenalis*, *E. intestinalis*, and *C. parvum* was measured following incubation at 37°C for 30 min at 5 min intervals. To determine the association rates tubulin monomers (2µM) in MBS buffer were incubated with different benzimidazoles (0-1,000nM) or 10% DMSO (negative control) at 37°C for 30 min in quadruplicate. Aliquots were removed at 5 min intervals for fluorescence analysis. The dissociation constants were determined by monitoring the time-dependent change in fluorescence from the tubulin-benzimidazole complexes as the different benzimidazoles were released from their binding sites following a 200-fold dilution of the complex. Dissociation curves were plotted and analysed using non-linear regression to determine the dissociation constant ($k_{off}$). This analysis also accounted for the proportion of tubulin-benzimidazole complexes which did not dissociate after 15 min.
All the fluorescence measurements were performed using a Perkin Elmer luminescence spectrometer LS50 with an excitation wavelength of 280nm and emission wavelength of 340nm (initially tested over 300-400nm range). The fluorescence measurements were corrected for inner-filter effects according to Mertens and Kagi (1979) [Eqn 5.3].

\[ F_{\text{corr}} = F_{\text{obs}} \cdot \text{antilog}(A_{280} + A_{320})/2 \]  

[Eqn 5.3]

where \( F_{\text{obs}} \) and \( F_{\text{corr}} \) are the observed and corrected fluorescence values and \( A_{280}, A_{320} \) are the absorbances at the excitation and emission wavelength, respectively. The association and dissociation data was analysed using a non-linear curve fitting program (Prism, GraphPad Software) and a one-site binding equation. The association rate (\( k_{\text{on}} \)) was calculated from equations 5.4 and 5.5 to determine the rate of change in fluorescence following addition of the benzimidazoles and these results were graphed semi-logarithmically against time.

\[ (F_{\text{max}} - F_t) = A \cdot e^{-k_{\text{on}}t} \]  

[Eqn 5.4]

where \( F_{\text{max}} \) is the maximum protein fluorescence, \( F_t \) is the protein fluorescence at time \( t \), \( A \) is the amplitude of the binding phase, \( k_{\text{on}} \) is the apparent association constant at time \( t \). The apparent association constant (\( k_{\text{on}} \)) for each benzimidazole tested was calculated (Eqn 5.5) as:

\[ k_{\text{on}} = \frac{\alpha}{[\text{BZ}]} \]  

[Eqn 5.5]

where \( \alpha \) is the slope of the semi-logarithmic binding plot and [BZ] is the benzimidazole concentration. The affinity constant (\( K_a \)) was obtained from the ratio of the association (\( k_{\text{on}} \)) and dissociation (\( k_{\text{off}} \)) rates.
5.3.3. Results

5.3.3.i. Effect of benzimidazole compounds on tubulin fluorescence

All the benzimidazole compounds tested (Fig. 5.1; Table 5.1) quenched the tryptophan fluorescence intensity of *G. duodenalis* β-tubulin in a concentration-dependent manner (Fig. 5.8). These initial fluorescence results also indicate that the amount of quenching by each of the benzimidazoles ranged from maximal quenching (albendazole) to minimal quenching (thiabendazole) compared with unbound tubulin. Similar results were obtained with *E. intestinalis* β-tubulin (results not shown). The relative difference in fluorescence quenching between *Giardia* and *Cryptosporidium* β-tubulin reflects the low affinity or amount of binding by the benzimidazoles tested with tubulin from the benzimidazole-insensitive *C. parvum* (Table 5.5).

---

**Fig. 5.8.** Relative amount of change in fluorescence by seven benzimidazoles (10µM) with *G. duodenalis* β-tubulin (2 µM) over a 30 min period.
The semi-log arithmetic plot (Fig. 5.9) demonstrates that each benzimidazole binds with recombinant *G. duodenalis* β-tubulin in a monophasic manner although with different apparent association rates ($k_{on}$) which were calculated from the slope of these plots. The high-affinity benzimidazoles, albendazole, fenbendazole, and mebendazole, had the highest average $k_{on}$ of $1.95 \pm 0.45 \times 10^7 \text{M}^{-1}\text{min}^{-1}$. The medium-affinity benzimidazoles, albendazole-sulphoxide, oxibendazole, and parbendazole, produced an average $k_{on}$ of $7.14 \pm 0.69 \times 10^5 \text{M}^{-1}\text{min}^{-1}$. The lowest-affinity benzimidazole tested, thiabendazole, had an average association rate of $7.05 \pm 0.55 \times 10^2 \text{M}^{-1}\text{min}^{-1}$. These results indicate there was a significant difference in the association rates between the high- and low-affinity benzimidazoles ($\alpha=0.05$, $t_{\text{value}}$ 7.5 > $t_{0.05,3}$ 2.353). Similar results were obtained with recombinant *E. intestinalis* β-tubulin (Table 5.5).

**Fig. 5.9.** Semi-log arithmetic plot of benzimidazole differential binding with *G. duodenalis* β-tubulin. The average association kinetics of high- (■), medium- (▲), and low- (▼) affinity benzimidazoles for *G. duodenalis* β-tubulin are indicated.
The average association constants ($k_{on}$) for albendazole with $\alpha$-, $\beta$-, and $\alpha\beta$-tubulin from *G. duodenalis*, *E. intestinalis*, and *C. parvum* were also determined (Table 5.5). These results clearly illustrate the high affinity of albendazole for monomeric $\beta$-tubulin and heterodimeric $\alpha\beta$-tubulin from the two benzimidazole-sensitive parasites, *G. duodenalis* and *E. intestinalis*. The association constants for *G. duodenalis* and *E. intestinalis* $\beta$-tubulin or $\alpha\beta$-tubulin were approximately $10^7\text{M}^{-1}\text{min}^{-1}$ compared with approximately $10^3\text{M}^{-1}\text{min}^{-1}$ for the benzimidazole-insensitive *C. parvum*. These results also indicate that albendazole has a very low binding affinity (*i.e.* $10^2\text{M}^{-1}\text{min}^{-1}$) for $\alpha$-tubulin from all three parasites.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Association constants ($k_{on}$) ($\text{M}^{-1}\text{min}^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha$-tubulin</td>
</tr>
<tr>
<td><em>G. duodenalis</em></td>
<td>$1.10 \times 10^2$</td>
</tr>
<tr>
<td><em>E. intestinalis</em></td>
<td>$1.50 \times 10^2$</td>
</tr>
<tr>
<td><em>C. parvum</em></td>
<td>$1.24 \times 10^2$</td>
</tr>
</tbody>
</table>

**Table 5.5.** Association constants for albendazole with $\alpha$-, $\beta$-, or $\alpha\beta$-tubulin from *G. duodenalis*, *E. intestinalis*, and *C. parvum*.

The dissociation rate of the benzimidazole-tubulin complexes was determined by incubating them in a 200-times molar excess of $\beta$-tubulin in MBS buffer which resulted in a benzimidazole specific dissociation. This was measured as an increase in relative fluorescence values as a result of a decrease in the quenching effect from the dissociating benzimidazole compounds. The resulting dissociation curves (Fig. 5.10) were used to determine the dissociation rates ($k_{off}$) with $\beta$-tubulin from *G. duodenalis* (Table 5.6), *E. intestinalis* (Table 5.7), and *C. parvum* (Table 5.8). The rate of dissociation ($k_{off}$) was 0.269min$^{-1}$ (*G. duodenalis*) and 0.261min$^{-1}$ (*E. intestinalis*) for the high-affinity benzimidazoles (*i.e.* albendazole, fenbendazole, mebendazole). The
mid-range benzimidazoles (i.e. albendazole-sulphoxide, oxibendazole, parbendazole) had dissociation rates of 0.345min\(^{-1}\) (\textit{G. duodenalis}) and 0.355min\(^{-1}\) (\textit{E. intestinalis}). The lowest affinity benzimidazole, thiabendazole, had dissociation rates of 0.542min\(^{-1}\) (\textit{G. duodenalis}) and 0.497min\(^{-1}\) (\textit{E. intestinalis}) (Fig. 5.10, Table 5.6). These results indicate that the rate of dissociation (\(k_{\text{off}}\)) for all the benzimidazole compounds was inversely proportional to the binding affinity (\(k_{\text{on}}\)) with the highest dissociation rate being observed with the lowest affinity benzimidazole, thiabendazole. Conversely, the slowest dissociating benzimidazoles were the high-affinity albendazole, fenbendazole, mebendazole. These results indicate that the differential efficacy of the benzimidazoles tested is due to a higher affinity and slower dissociation of the benzimidazole from the benzimidazole / \(\beta\)-tubulin complex.

\textbf{Fig. 5.10.} Average dissociation curves of benzimidazole derivatives from \textit{G. duodenalis} \(\beta\)-tubulin. The high- (■), medium- (▲), and low- (◆) affinity benzimidazole compounds were dissociated from \textit{G. duodenalis} \(\beta\)-tubulin. This resulted in a decrease in the amount of quenching of the fluorescence from \(\beta\)-tubulin which is indicated in these dissociation curves.
5.3.3.ii. Comparative binding affinities of benzimidazoles with protozoan tubulin

To investigate the relationship between the strength of binding to tubulin and the chemical nature of the benzimidazole compound the affinity constant ($K_a$) was calculated by the ratio of the association ($k_{on}$) and dissociation ($k_{off}$) rates. The association constant for each benzimidazole compound tested with $\beta$-tubulin from *G. duodenalis* (Table 5.6), *E. intestinalis* (Table 5.7), and *C. parvum* (Table 5.8) were determined. Using this method, there was a clearly identifiable differential affinity of the high- and medium-affinity benzimidazole compounds with $\beta$-tubulin from *G. duodenalis* and *E. intestinalis* which was not observed with *C. parvum*. In considering the affinity constant of the benzimidazoles tested with $\beta$-tubulin from the sensitive organisms, *G. duodenalis* and *E. intestinalis*, there was no significant difference between the affinity rates for any of the benzimidazoles ($\alpha=0.05, t_{value} 1.914 < t_{0.05,3} 2.353$). However, when the affinity constants of the benzimidazole-insensitive *C. parvum* was compared with those of *G. duodenalis* or *E. intestinalis*, all the benzimidazoles tested had a significantly lower affinity for *C. parvum* $\beta$-tubulin ($\alpha=0.05, t_{value} 42.9 > t_{0.05,3} 2.353$).

The differential affinity of the benzimidazole derivatives which distinguished them as having either a high-, medium, or low-affinity for $\beta$-tubulin was also observed between the two benzimidazole-sensitive organisms (Table 5.6 and 5.7). However, the difference in affinity between the high-, medium, or low-affinity benzimidazoles was not observed with the affinity constants derived from *C. parvum* $\beta$-tubulin (Table 5.8). The highest affinity constants of the benzimidazoles tested were observed with albendazole, fenbendazole, and mebendazole with an average $K_a$ of 7.25 ± 0.40 x 10$^7$M$^{-1}$ (*G. duodenalis*) and 7.13 ± 0.18 x 10$^7$M$^{-1}$ (*E. intestinalis*). Lower affinities were demonstrated with albendazole-sulphoxide, oxibendazole, and parbendazole (*G. duodenalis* $K_a$ 2.07 ± 0.58 x 10$^6$M$^{-1}$ and *E. intestinalis* $K_a$ 1.84 ± 0.48 x 10$^6$M$^{-1}$).
Thiabendazole had the lowest and most variable affinity constant (\( G. \ duodenalis \ K_a 1.30 \pm 1.1 \times 10^3 \text{M}^{-1} \) and \( E. \ intestinalis \ K_a 1.26 \pm 0.97 \times 10^4 \text{M}^{-1} \)). The trend in binding affinities was observed for monomeric \( \beta \)-tubulin from \( G. \ duodenalis \) and \( E. \ intestinalis \) but not \( C. \ parvum \) for which very low binding affinities were determined (average \( K_a 2.1 \times 10^2 \text{M}^{-1} \)) for all the benzimidazoles tested.

<table>
<thead>
<tr>
<th>Benzimidazole derivative</th>
<th>Binding parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( k_{on} (\text{M}^{-1}\text{min}^{-1}) )</td>
</tr>
<tr>
<td>Albendazole</td>
<td>( 1.95 \pm 0.45 \times 10^7 )</td>
</tr>
<tr>
<td>Fenbendazole</td>
<td>( 2.07 \pm 0.58 \times 10^6 )</td>
</tr>
<tr>
<td>Mebendazole</td>
<td>( 2.07 \pm 0.58 \times 10^6 )</td>
</tr>
<tr>
<td>Thiabendazole</td>
<td>( 7.05 \pm 0.55 \times 10^2 )</td>
</tr>
</tbody>
</table>

**Table 5.6.** Summary of binding kinetics and affinity constants for seven benzimidazoles with recombinant \( G. \ duodenalis \) \( \beta \)-tubulin.
### Table 5.7. Summary of binding kinetics and affinity constants for seven benzimidazoles with recombinant E. intestinalis $\beta$-tubulin.

<table>
<thead>
<tr>
<th>Benzimidazole derivative</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>Albendazole</td>
<td>$1.86 \pm 0.22 \times 10^7$</td>
<td>$2.61 \times 10^{-1}$</td>
<td>$7.13 \pm 0.18 \times 10^7$</td>
</tr>
<tr>
<td>Fenbendazole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mebendazole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albendazole-sulphoxide</td>
<td>$6.53 \pm 0.51 \times 10^5$</td>
<td>$3.55 \times 10^{-1}$</td>
<td>$1.84 \pm 0.48 \times 10^6$</td>
</tr>
<tr>
<td>Oxibendazole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parbendazole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiabendazole</td>
<td>$6.39 \pm 0.63 \times 10^3$</td>
<td>$4.97 \times 10^{-1}$</td>
<td>$1.29 \pm 0.97 \times 10^4$</td>
</tr>
</tbody>
</table>

### Table 5.8. Summary of binding kinetics and affinity constants for seven benzimidazoles with recombinant C. parvum $\beta$-tubulin.

<table>
<thead>
<tr>
<th>Benzimidazole derivative</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>Albendazole</td>
<td>$7.20 \pm 0.44 \times 10^2$</td>
<td>$2.88$</td>
<td>$2.50 \pm 0.20 \times 10^2$</td>
</tr>
<tr>
<td>Fenbendazole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mebendazole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albendazole-sulphoxide</td>
<td>$4.80 \pm 0.35 \times 10^2$</td>
<td>$2.41$</td>
<td>$1.99 \pm 0.35 \times 10^2$</td>
</tr>
<tr>
<td>Oxibendazole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parbendazole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiabendazole</td>
<td>$4.75 \pm 0.52 \times 10^2$</td>
<td>$2.61$</td>
<td>$1.82 \pm 0.45 \times 10^2$</td>
</tr>
</tbody>
</table>
5.3.3.iii. Inhibition of microtubule assembly by benzimidazole compounds

The recombinant $\alpha$- and $\beta$-tubulin monomers from all three parasites were dimerised and the $\alpha\beta$-tubulin heterodimers polymerised to form microtubules. The effect of the benzimidazoles on the amount of polymerisation or microtubule assembly was monitored spectrophotometrically (i.e. turbidimetry). With *G. duodenalis* and *E. intestinalis* $\alpha\beta$-tubulin, the highest affinity benzimidazoles (i.e. albendazole, fenbendazole, mebendazole), had the greatest inhibitory effect on polymerisation compared with the lower affinity benzimidazoles (i.e. albendazole-sulphoxide, oxibendazole, and parbendazole). The lowest affinity benzimidazole, thiabendazole, had the least effect on polymerisation, particularly during the initial ten minute assembly phase (Fig. 5.11).

The results for *G. duodenalis* and *E. intestinalis* microtubule assembly were not significantly different to each other while none of the benzimidazoles tested significantly reduced the rate of assembly of *C. parvum* microtubules (results not shown). With *C. parvum* $\alpha\beta$-tubulin all seven benzimidazoles tested reduced the final amount of microtubules assembled by 5 to 10% without affecting the rate at which (maximum) assembly occurred (i.e. slope of assembly phase, 3 to 10 min).

In addition to these findings, when excess molar concentrations of albendazole, fenbendazole, mebendazole were added to microtubules assembled in the absence of any benzimidazoles there was no decrease in turbidity or absorbance indicating they did not de-polymerise the microtubules under these conditions.


**Fig. 5.11.** Effect of several benzimidazoles on the *in vitro* assembly of *G. duodenalis* microtubules. The average absorbance of heterodimeric αβ-tubulin (■) mixed with a 10 times molar excess of low- (▲), medium- (◆), and high- (●) affinity benzimidazoles are indicated over a 30 min period.

### 5.3.4. Discussion

The mechanism underlying the activity of the benzimidazoles against parasitic protozoa has not been determined. This study was undertaken to identify whether the benzimidazoles bind to their proposed target protein, β-tubulin, with different affinities when this protein is derived from either benzimidazole-sensitive or -insensitive organisms. For this study, the binding affinities of a number of benzimidazole R5-derivatives were determined with monomeric and heterodimeric β-tubulin from two parasites with a clearly demonstrated high *in vitro* efficacy, *G. duodenalis* and *E. intestinalis*, and one with a low *in vitro* efficacy, *C. parvum*, to the benzimidazoles tested.
This study has clearly demonstrated two important aspects of the benzimidazoles mechanism of action in these parasites. Firstly, regardless of the chemical composition of the benzimidazole compound tested they have an indistinguishable affinity for monomeric $\beta$-tubulin and dimeric $\alpha\beta$-tubulin. Secondly, they have a demonstrably higher affinity for $\beta$-tubulin from benzimidazole-sensitive organisms when compared with a benzimidazole-insensitive organism.

Interestingly, the R$_5$-derivatives tested in this study produced distinctive binding affinities with G. duodenalis and E. intestinalis recombinant $\beta$-tubulin. These affinities, described by the constants $K_a$ and $K_d$, divided the benzimidazoles tested into high-affinity (i.e. albendazole, fenbendazole, mebendazole), medium-affinity (i.e. albendazole-sulphoxide, oxibendazole, parbendazole), or low-affinity (thiabendazole) derivatives. A similar range of affinities is reflected in their observed in vitro efficacies against these two parasites (Katiyar and Edlind, 1997; Morgan et al., 1993). These binding constant results therefore suggest that at least some part of their efficacy is due to their differential binding affinity for $\beta$-tubulin which is related to the chemical group present in the R$_5$ side-chain of the benzimidazole.

The relationship between the association ($k_{on}$) and dissociation ($k_{off}$) rates of these benzimidazole derivatives was demonstrated to be inversely proportional. This suggests that the lower in vitro efficacy of the rapidly dissociating benzimidazoles (i.e. albendazole-sulphoxide, oxibendazole, parbendazole, and thiabendazole) may be partly due to their inability to form stable benzimidazole-$\beta$-tubulin complexes with the converse being true of the higher efficacy benzimidazoles (i.e. albendazole, fenbendazole, mebendazole).
This relationship is also reflected in the effect each benzimidazole was found to have on the polymerisation of αβ-heterodimers into microtubules. One of the proposed mechanisms of action of the benzimidazoles is through binding to the microtubule which destabilises the lattice structure of the tubule, subsequently resulting in its disintegration via depolymerisation (reviewed by Lacey, 1988). The findings of this study partially support this model although via a different mechanism. The addition of the different benzimidazoles to assembling αβ-tubulin heterodimers had a benzimidazole-dependent effect on both the rate and amount of microtubules assembled for the benzimidazole-sensitive parasites. That is, the highest affinity benzimidazoles (i.e. albendazole, fenbendazole, mebendazole) decreased both the rate and amount of microtubule assembly which was observed to decrease with the medium- and low-affinity derivatives. The benzimidazoles were also demonstrated to inhibit the assembly of *G. duodenalis* and *E. intestinalis* microtubules (i.e. albendazole, fenbendazole, mebendazole) while having no demonstrable effect on the polymerisation of *C. parvum* heterodimers.

The microtubule assembly assay also demonstrated that none of the benzimidazole compounds tested were able to depolymerise assembled microtubules although these tubules were naturally depolymerised by increasing the assembly temperature above 37°C. This indicates that whilst the microtubules were capable of depolymerisation this was not promoted or favoured by the presence of the benzimidazole derivatives tested. These results may indicate that the benzimidazole-binding site on β-tubulin from sensitive protozoa is located at or near the inter-heterodimer interface as the presence of the benzimidazoles is sufficient to arrest or inhibit microtubule assembly.
Taken together, these results may indicate that a benzimidazole-β-tubulin cap is formed at the growing or assembling ends of the microtubules and this cap prevents the further elongation or polymerisation of the microtubule. Whether the inhibition of assembly is due to steric hindrance, blockage of GTP hydrolysis, or another mechanism is unclear from this study. These results also indicate that it is likely all the benzimidazoles tested interact with the same binding site on β-tubulin but the strength of this binding is partially dependent on the chemical composition of the R₅ group of the benzimidazole derivatives.
Chapter 6

General Discussion
In order to determine the binding affinity of several benzimidazoles with β-tubulin from benzimidazole-sensitive and -insensitive organisms it was initially necessary to produce protozoan α- and β-tubulin in a recombinant form using an *E. coli* expression system (Chapter 2). This method of expressing α- and β-tubulin produced high yields of the protein when it was fused to a high-solubility fusion partner, MBP or GST. The MBP and GST fusion partners, as well as providing a means of producing α- and β-tubulin as a soluble cytoplasmic protein in *E. coli*, also provided a convenient means of purifying the tubulin fusion proteins.

This resulted in the recombinant tubulin monomers being purified to a high enough level for attempts to crystallise it to be undertaken (Chapter 3). The αβ-tubulin protein has been crystallised previously but only in the form of a 2-D crystal which has meant electron, and not X-ray, crystallography was utilised to construct a 3.5Å 3-D model of the mammalian αβ-tubulin heterodimer. The differences between previously applied methods to produce 3-D αβ-tubulin crystals and those used in this project will be discussed particularly in regard to understanding why the protozoan recombinant αβ-tubulin also failed to yield X-ray quality 3-D crystals.

As a result of this, and in order to produce a 3-D model of αβ-tubulin from benzimidazole-sensitive and -insensitive organisms, a homology or comparative modelling approach was utilised (Chapter 4). The homology models produced using this method allowed a structurally significant hydrophobic benzimidazole-binding domain to be identified. The role of the individual amino acid residues which comprise this domain may perform in binding with several different benzimidazoles is discussed using this model. This discussion also takes into account previous studies of the molecular basis of benzimidazole-resistance in the nematode *H. contortus* and the fungi *A. nidulans*. 
To substantiate the role that protozoan β-tubulin is proposed to perform in binding with the benzimidazole compounds a series of biochemical experiments were conducted (Chapter 5). In this way the association and dissociation rates, and subsequently the affinity constant, for several benzimidazoles with α-, β-, or αβ-tubulin from sensitive and insensitive protozoa were determined. From these experiments it was identified that the benzimidazoles tested could be separated into three categories of having high-, medium-, or low-affinity for protozoan β-tubulin. Of the benzimidazoles used in this study, six were R₅-carbamate derivatives while the seventh benzimidazole lacked an R₅ side group (i.e. thiabendazole). The influence that the structure of each benzimidazole derivative tested had on the observed association rate, dissociation rate, and affinity constant is discussed in terms of the proposed hydrophobic binding domain. In particular, the influence of binding between the amino acid residues of the hydrophobic binding domain with the different R₅ side-groups of the benzimidazoles on the final observed affinity constants is also discussed. A discussion is also provided of the structure-activity relationships that can be identified by combining the amino acids proposed to create the hydrophobic binding domain with the observed in vitro binding affinities for each benzimidazole compound used in this study.

In order to identify the benzimidazole binding region on the β-tubulin protein it was initially necessary to produce natively folded α- and β-tubulin free of post-translational modifications and accessory proteins such as MAPs. This was necessary as post-translational modifications and MAPs may interfere with benzimidazole-binding. Additionally, the post-translational modifications may also produce a modified form of the tubulin protein which does not assemble to form microtubules. It was therefore necessary to develop a recombinant protein expression system capable of producing soluble α- and β-tubulin. It was essential that the tubulin monomers were soluble as there is considerable speculation that recombinant proteins which are encapsulated
into inclusion bodies are not correctly folded and are a sub-population of folding intermediates which the host cell either could not continue to fold into a stable 3-D structure or the cell detects as foreign and so inactivates by sequestering them in inclusion bodies.

In addition to this, and particularly when considering the tubulin folding pathway, several co-factors and chaperones are required for tubulin to obtain its correct 3-D, biologically-active structure. It is therefore reasonable to predict that tubulin which has been solubilised from inclusion bodies and completely denatured will not refold into its correct 3-D structure. This protein folding problem is commonly recognised as one of the limiting steps in producing biochemically active proteins from denatured ones.

Several studies have examined the drug-binding characteristics of re-folded recombinant tubulin monomers without considering whether a heterologous or homogenous pool of tubulin folding intermediates has been created. These studies also fail to establish the existence of other intrinsic biological functions of native tubulin in the refolded form of the protein. These essential activities include the ability of heterodimers to assemble into microtubules and also to act as a GTPase. It is reasoned that unless these essential tubulin functions are demonstrated it will not be possible to confidently examine the drug-binding characteristics of tubulin, or to relate these \textit{in vitro} drug-binding characteristics to \textit{in vivo} drug efficacy.

To avoid potential problems associated with refolding the tubulin monomers, an \textit{E. coli} host strain was utilised as it will produce recombinant tubulin without any post-translational modifications. In addition to this, \textit{E. coli} does not express endogenous tubulin such that the tubulin extracted from these host cells consisted only of the cloned protozoan tubulin.
A comparison was made between several fusion proteins which could be attached to tubulin to ensure it was expressed as a soluble protein. These fusion partners were MBP and GST which are commercially available as expression vectors and are recommended for heterologous over-expression of soluble proteins in *E. coli*. The third fusion partner, consisting of a poly-His fusion sequence, was also utilised to establish the intrinsic solubility of tubulin in the presence of this low-solubility fusion partner. The three fusion partners, MBP, GST, and poly-His, were all transcriptionally controlled by the T7 RNA promoter so that any differences in protein expression levels could be attributed to the nature of the fusion partner. In this way the low intrinsic solubility of tubulin was demonstrated and the significant improvement in solubility brought about by the MBP and GST fusion partners was clearly identifiable.

Other conditions affecting tubulin's expression level were also assessed, including the choice of *E. coli* host strain which were all based on the BL21(DE3) genotype. The BL21(DE3)pLysS strain contains an additional plasmid to minimise the basal expression of α- and β-tubulin. The third strain tested, BL21(DE3)RIL, was modified to contain high levels of codons which are normally present in prokaryotic proteins but are in limited supply in *E. coli*. The amount of soluble α- and β-tubulin expressed in each *E. coli* strain was similar for *G. duodenalis*, *C. parvum*, and *E. intestinalis* (Table 2.3). However, there was a substantial difference in soluble tubulin expression levels between the three *E. coli* strains tested. The BL21(DE3) strain yielded the lowest amount of soluble tubulin with average expression levels 25 to 35% of those observed with the BL21(DE3)pLysS and BL21(DE3)RIL strains. The highest tubulin expression levels were observed with the BL21(DE3)pLysS strain. Taken together, these results indicate that the amount of expression of soluble α- and β-tubulin in *E. coli* is influenced by the choice of host strain and strains which suppress basal expression of tubulin are preferable to ones lacking this feature.
The amount of expression of recombinant tubulin in *E. coli* was also found to be affected by the rate of protein synthesis as determined by the temperature at which the *E. coli* expressing the tubulin was cultured. Determining the effect of temperature on tubulin expression levels involved expressing the tubulin-MBP fusion proteins at 15°C, 25°C, 30°C, or 37°C in the *E. coli* strain BL21(DE3)pLysS. This parameter was found to significantly affect the amount of soluble tubulin fusion proteins produced in a temperature-dependent manner. Optimal tubulin expression levels were observed at 37°C for α- and β-tubulin from all three parasites tested (i.e. 30-40 mg/l). These total amounts of soluble protein decreased by up to 60% at 30°C (i.e. 25-35 mg/l) and a further 50% at 25°C (i.e. 15-25 mg/l). Expression of recombinant tubulin at 15°C was less than a quarter of the level observed at 37°C (i.e. <10 mg/l). These results indicated that the rate of protein synthesis at 37°C was optimal for the maximal expression of tubulin-MBP fusion proteins in the *E. coli* BL21(DE3)pLysS strain.

A further aim of producing soluble tubulin as a fusion protein was that it would provide a convenient means of purifying the tubulin monomers. This was necessary for trials to crystallise the protozoan tubulin especially to obtain a 3-D crystal suitable for X-ray crystallography. The required level of purity of protein for successful crystallisation is generally believed to be higher than 98% although this can vary depending on the nature of the protein being crystallised (Rhodes, 2000). Prior to this study, several groups have tried unsuccessfully to produce 3-D crystals of tubulin extracted from bovine or porcine brain microtubules. It should be noted that brain tissue has subsequently been found to contain α- and β-tubulin biotypes with a high degree and wide range of post-translational modifications. This would result in a highly heterogenous tubulin sample and it is believed that this has significantly hindered the formation of 3-D crystals. This has primarily been due to the high level of heterogeneity as several α- and β-tubulin biotypes were present in microtubules extracted from brain tissue.
The approach used in this study with recombinant, highly purified, and homogeneous αβ-tubulin was designed to address some of the problems that have previously been identified as potential reasons why tubulin had failed to crystallise. The tubulin biotype heterogeneity problem was addressed by producing recombinant tubulin which was double purified; initially by affinity chromatography and secondly by anion-exchange chromatography. This ensured that both the α- and β-tubulin monomers had a purity of greater than 98% as assessed by SDS-PAGE and Western blotting with specific anti-α- or anti-β-tubulin monoclonal antibodies.

A second reason suggested for why tubulin had failed to form 3-D crystals is structural instability caused by the highly acidic and unstructured carboxy-terminus of α- and β-tubulin. This is particularly significant for β-tubulin as when tubulin is in an αβ-heterodimeric form the carboxy terminus of α-tubulin is buried within the intermonomeric region while the carboxy-terminus of β-tubulin is exposed to inter-dimeric interactions (Löwe et al., 2001; Nogales et al., 1999). To address this problem the α-, β-, and αβ-tubulin proteins were treated with subtilisin, a protease enzyme which selectively cleaves each tubulin monomer so as to remove the carboxy-terminus (i.e. residue Asp-438 on α-tubulin and residue Gln-433 on β-tubulin; Redeker et al., 1992). The effect of removing the carboxy-termini on the overall charge of the tubulin monomers was clearly illustrated using a non-denaturing agarose gel which separated the tubulin monomers on the basis of their overall charge.

The final reason which has previously been cited for why tubulin has failed to form 3-D crystals was termed thermal instability which related to tubulin’s structural instability and rapid denaturation at temperatures above 4°C. As the tubulin used in crystallisation plates could therefore denature before forming crystal nuclei a solution to this problem was sought in two ways. Firstly, the tubulin was maintained at or below 4°C at all times during the protein extraction and purification process to minimise the
amount of denatured tubulin present in samples used for crystallisation trials. Crystallisation plates were also incubated at 4°C, in addition to room temperature, to minimise the denaturation of monomeric or dimeric tubulin during crystal formation. The second approach to addressing the thermal instability of tubulin was that this problem had been observed with αβ-tubulin heterodimers. It was therefore possible that monomeric α- and β-tubulin, in addition to αs- and βs-tubulin, may be significantly more stable than heterodimeric αβ-tubulin derived from extracted microtubules.

A final alternative approach which was utilised in this project was taken from the observation that all previous material used for electron crystallography was crystallised sheets of αβ-tubulin heterodimers assembled into microtubules which then aligned in an anti-parallel manner (Wolf et al., 1996). This meant that the αβ-tubulin heterodimers had been assembled into microtubules in vitro in the presence of GTP and a divalent cation such as zinc or magnesium. While this approach had successfully produced 2-D crystals it is also possible that it had prevented the formation of 3-D crystals. Therefore, in this study, the α-, β-, αs-, βs-, αβ-, and αβs-tubulin were not assembled into microtubules prior to application to crystallisation screening but were instead treated as monomers or heterodimers.

Despite testing over one-hundred separate crystallisation conditions and incubating the crystallisation plates for two-years, none of the approaches taken in this project were suitable for the formation of 3-D crystals of recombinant protozoan tubulin. Although 3-D birefringent crystals of <1.0mm were produced these were too small and fragile for X-ray analysis and without further analysis it was not possible to identify that these were αβ-tubulin crystals. Although this was a disappointing result, especially as it would have provided a conclusive model of the benzimidazole-binding site, there are alternative approaches which were utilised to obtain a lower resolution model to provide this information.
An alternative to producing a model of protozoan β-tubulin from a crystal structure was to create a homology model of protozoan β-tubulin with the mammalian 3-D structure. This homology model compared benzimidazole-sensitive organisms (G. duodenalis and E. intestinalis) and -insensitive organisms (C. parvum and B. taurus) and identified several potentially significant amino acid differences. From the homology models of β-tubulin it was possible to identify a hydrophobic binding region from amino acid residue Ala-186 to Phe-213. This hydrophobic benzimidazole-binding region is not seen on the β-tubulin from insensitive organisms (C. parvum and B. taurus).

In order to determine the binding kinetics of several benzimidazoles with recombinant α-, β-, and αβ-tubulin from benzimidazole-sensitive and -insensitive protozoa two methods were investigated. The first method involved analysing the ability of each benzimidazole compound to bind with it's target protein which had been immobilised on a biosensor chip. The basis of this method is the measurement of resonance from the sensor chip surface which is altered by the amount of mass attached to the chip surface. An initial investigation was made into whether or not this method was suitable for assessing the binding kinetics between immobilised β-tubulin from the sensitive organism G. duodenalis and the benzimidazole compounds albendazole and mebendazole. These two compounds have been previously shown to exhibit a high efficacy for G. duodenalis (Katiyar et al., 1994; Morgan et al., 1993). Following analysis of direct binding data of albendazole and mebendazole, over a 0-1000nM range, it was demonstrated that the interaction between albendazole and mebendazole with the CMD matrix was equal to or greater than the binding with the immobilised G. duodenalis α- or β-tubulin.
These results indicated that under the range of conditions tested to determine the amount of binding between albendazole and mebendazole with immobilised β-tubulin, the two compounds were instead binding with the matrix. Whilst this non-specific binding was rapid and reversible it occurred to an extent where it blocked the detection of the interaction with *G. duodenalis* tubulin. This non-specific binding was not reduced despite testing a wide range of conditions including variation in the pH of buffers, ligand surface concentration, ligand immobilisation technique, analyte (albendazole or mebendazole) concentration, and flow rate.

The second method which was investigated to analyse the binding reaction between recombinant α- and β-tubulin monomers and dimers with the benzimidazoles was a fluorescence quenching method based on measuring the amount of intrinsic fluorescence from tubulin’s tryptophan residues. The intrinsic fluorescence of αβ-tubulin monomer is primarily due to the indolic group of tryptophan residues of which α-tubulin and β-tubulin each have four residues; the fluorescence from some of these residues is blocked as a result of dimerisation which, based on the 3.5Å dimer model (Löwe *et al.*, 2001) will result in a loss of two residues into the α/β-tubulin inter-monomer interface. The amount of fluorescence emitted from monomeric or heterodimeric β-tubulin was found to be quenched when benzimidazole compounds were bound to this protein. This method has been extensively utilised to study the binding kinetics of the anti-tumour drug colchicine with tubulin (Banerjee and Ludueña, 1992; Chakrabarti *et al.*, 1996; Guha and Bhattacharyya, 1997). In addition to this, the benzimidazole derivatives tested were also shown to affect the rate and amount of polymerisation by benzimidazole-bound αβ-tubulin heterodimer as assessed by a spectrophotometric analysis of microtubule formation *in vitro*. 
The high-affinity benzimidazoles identified in this study were albendazole, fenbendazole, and mebendazole. By utilising the benzimidazole-sensitive/-insensitive homology models, these three compounds were all proposed to form significant interactions within the proposed hydrophobic binding domain. More importantly, these interactions were proposed to involve the key substituted amino acids in this binding domain, Ile-189, Val-199, and Phe-200. More specifically these interactions are proposed to be based on binding with the aliphatic side-group of Ile-189 as this has a strong electron-withdrawing potential. The hydrophobic aliphatic side-group of Val-199 is also crucial in increasing the hydrophobicity at the base of this benzimidazole-binding loop, especially when it is compared with benzimidazole-insensitive β-tubulin models. It is unlikely that Val-199 is directly involved in binding these three benzimidazoles as spatially it is predicted to be too far from the propyl side chain of albendazole or the phenyl ring of fenbendazole or mebendazole.

The most significant interaction in this binding domain was proposed to involve the highly aromatic phenyl group of Phe-200 which is located in a position where it forms an electrophilic interaction with the R5 propyl group of albendazole and the phenyl group of fenbendazole and mebendazole. The additional carbonyl group on the R5 group of mebendazole is also proposed to participate in this electron-sharing interaction with amino acid Phe-200. The amino acid-R5-side group interactions proposed in this model would therefore result in the high affinity, slow dissociation binding kinetics observed for these three benzimidazole derivatives.

The medium-affinity benzimidazoles that were identified in this study were albendazole-sulphoxide, oxibendazole, and parbendazole. As with the high-affinity benzimidazoles, the homology models identified several significant side-chain interactions between the amino acid residues of benzimidazole-sensitive protozoa and these R5 carbamates. Therefore, these interactions also involve the benzimidazole-
sensitivity defining amino acids Ile-189, Val-199, and Phe-200. Taken together, and compared with the chemical side groups of the high affinity R$_5$ derivatives, it would appear that the loss of the R$_5$-sulphyl group has had a quantifiable effect on the affinity constants of these drugs. Although this group is proposed to interact with the conserved amino acid Cys-201, this interaction is likely to be based on an electrophilic S-O bond which would appear to contribute to the overall rates of association and dissociation of the high-affinity benzimidazoles compared with the medium-affinity benzimidazoles.

Based on the proposed benzimidazole binding model the medium-affinity benzimidazoles would form an electrophilic O-O bond which would not form as stable an interaction of the S-O interaction of albendazole, fenbendazole, and mebendazole. The principal interactions identified for the medium-affinity benzimidazoles involve either a propyl- (albendazole-sulphoxide and oxibendazole) or butyl- (parbendazole) carbon chain which interacts with the aliphatic groups of Ile-189 and Val-199 and the aromatic group of Phe-200. The increased hydrophobicity of the proposed benzimidazole-binding domain as a result of the amino acid residues Ala-185, Leu-192, and Ile-202 is also proposed to have a significant effect on the formation of a relatively stable benzimidazole-β-tubulin complex as a result of the stable R$_5$ carbamates.

The final benzimidazole tested in this study was thiabendazole which lacks an R$_5$-side group, instead having an R$_2$-thiazole group. Of the seven benzimidazoles tested, thiabendazole had the lowest association rate, highest dissociation rate, and consequently the lowest affinity constant. The affinity constant was $1.4 \pm 1.1 \times 10^3 \text{M}^{-1}$ which is approximately $10^4$ times lower than the high-affinity benzimidazoles and $10^3$ times lower than the medium-affinity benzimidazoles. This represents a significant reduction in the affinity of this benzimidazole for β-tubulin which agrees with the proposed homology model. The three key amino acids within the proposed binding
site, Ile-189, Val-199, and Phe-200, are unlikely to interact with the central benzimididine ring structure as it would not be close enough to these residues for binding to occur.

With this benzimidazole-binding model the R₂-thiazole group would be situated near the amino acid residues located at the top of this binding region. Whilst some binding may occur with these residues they are conserved between benzimidazole-sensitive and -insensitive organisms. Additionally, they are also conserved between benzimidazole-sensitive and -resistant organisms, such as *H. contortus*. This means these amino acids are unlikely to form the basis of the selective activity of the benzimidazoles, including thiabendazole, for β-tubulin from particular protozoa.

Taken together, the homology model-based benzimidazole-binding domain and the *in vitro* binding kinetics provide a model of benzimidazole / β-tubulin binding which has not been clearly identified previously. This is particularly true when the molecular basis of benzimidazole-sensitivity and -insensitivity is considered. This model of benzimidazole binding proposes that the benzimidazoles bind with a hydrophobic region of β-tubulin such that the R₅ side-group of the benzimidazoles is brought into contact with three key non-conserved amino acids. These amino acid residues are Ile-189, Val-199, and Phe-200 which are located in a hydrophobic binding region which was demonstrated to only be present on the β-tubulin model of the benzimidazole-sensitive protozoa, *G. duodenalis* and *E. intestinalis*. The interaction between the benzimidazole R₅ side-group and residues Ile-189, Val-199, and Phe-200 of β-tubulin are therefore proposed to be central to the mechanism of action of these compounds.
There are several aspects of this project which could be addressed in future studies. To establish conclusively the molecular basis of this proposed benzimidazole-binding domain site-directed mutagenesis should be performed. This could initially be of each amino acid within this domain to determine which amino acid substitutions affect the benzimidazole association and dissociation rates and affinity constants. It is also technically conceivable to propose that all the key amino acids could be mutated to produce a recombinant protein with the amino acid sequence of a benzimidazole-insensitive organism but only in this benzimidazole-binding region. If there was a complete loss of benzimidazole binding then it would provide strong evidence of the location of the key amino acid(s) involved in the formation of the benzimidazole binding site.

It would also be informative to compare the benzimidazole-binding properties of β-tubulin extracted from benzimidazole-sensitive and -insensitive organisms. If the native protein was found to display a similar benzimidazole binding profile as the recombinant protein used in this study it would strengthen the argument that a valid benzimidazole-binding site has been identified. This is believed to be necessary to address the possibility that the benzimidazole-binding site which has been identified in this study using recombinant tubulin is the result of the protein being folded in *E. coli* which may have created an incorrectly folded region with benzimidazole-binding properties.
Another aspect of this study which could be addressed in future work relates to the dynamic behaviour of microtubules in organisms and the fact that the tubulin used in this study was a homogenous and purified protein interacting under controlled *in vitro* conditions with a chemically pure ligand. It would therefore be valuable to investigate the *in vivo* binding properties of the benzimidazoles with actively assembling microtubules. There are several microscope-based techniques available for studying the growth of microtubules and some have also been developed to investigate the influence of specific drugs on this process (Grego et al., 2001; Viani et al., 2000). Similar approaches could be applied to the study of benzimidazole binding with protozoan microtubules. A further advantage of this approach is that microtubules from different regions of the protozoa could be studied in this way, such as either cytoskeletal or flagellar microtubules. It should be noted that producing sufficient amounts of flagellar or ventral disc microtubules for this type of analysis would be a significant technical challenge which could potentially preclude its successful execution.
References


Downing, K. H. (2000). Structural basis for the interaction of tubulin with proteins and


Appendices
### Appendix I

**Alignment of α-tubulin deduced amino acid sequences**

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## Appendix II

### Alignment of \(\beta\)-tubulin deduced amino acid sequences

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## Appendix III

### Hampton Research Crystal Screen Solutions 1

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*Buffer pH adjusted to pH 4.75 just prior to use for better reagent compatibility with other reagent components.*
## Appendix IV

### Hampton Research Crystal Screen Solutions 2

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<td>37. 20% v/v Polyethylene glycol 200000</td>
</tr>
<tr>
<td>38. None</td>
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<td>None</td>
<td>38. 20% v/v Polyethylene glycol 200000</td>
</tr>
<tr>
<td>39. None</td>
<td>None</td>
<td>None</td>
<td>39. 3.4 M 1.6 Hexanediol</td>
</tr>
<tr>
<td>40. None</td>
<td>None</td>
<td>None</td>
<td>40. 75% v/v tert-Butanol</td>
</tr>
<tr>
<td>41. None</td>
<td>None</td>
<td>None</td>
<td>41. 1.0 M Lithium Sulfate monohydrate</td>
</tr>
<tr>
<td>42. None</td>
<td>None</td>
<td>None</td>
<td>42. 12% v/v Caprylic caprylcolnol</td>
</tr>
<tr>
<td>43. None</td>
<td>None</td>
<td>None</td>
<td>43. 5% v/v MEGU</td>
</tr>
<tr>
<td>44. None</td>
<td>None</td>
<td>None</td>
<td>44. 20% v/v Polyethylene glycol Monononyl ether 2000</td>
</tr>
<tr>
<td>45. None</td>
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<td>None</td>
<td>45. 20% v/v Polyethylene glycol Monononyl ether 2500</td>
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<tr>
<td>46. None</td>
<td>None</td>
<td>None</td>
<td>46. 2.0 M Sodium Chloride</td>
</tr>
<tr>
<td>47. None</td>
<td>None</td>
<td>None</td>
<td>47. 2.0 M Magnesium Chloride hexahydrate</td>
</tr>
<tr>
<td>48. 2% v/v Ethanol</td>
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<td>48. 10% w/w Polyethylene glycol 200000</td>
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