A molecular basis for the resistance of *Acanthamoeba* tubulins to all major
classes of anti-tubulin compounds.

*Running title: Resistance of Acanthamoeba species to anti-tubulin compounds.*

Fiona L. Henriquez1§*, Paul R. Ingram1, Stephen P. Muench2&, David W. Rice2

and Craig W. Roberts1

1 Strathclyde Institute of Pharmacy and Biomedical Sciences, University of
Strathclyde, 27 Taylor Street, Glasgow, Scotland, UK, G4 0NR§.

2 Department of Molecular Biology and Biotechnology, University of Sheffield,
Sheffield, UK, S10 2TN&.

§ present address: School of Science & Engineering, University of Paisley, High
Street, Paisley, Scotland, UK, PA1 2BE.

& present address: Institute of Molecular and Cellular Biology, Faculty of Biological
Sciences, University of Leeds, Leeds, UK, LS2 9JT.

* corresponding author: School of Science & Engineering, University of Paisley, High
Street, Paisley, Scotland, UK, PA1 2BE. Phone: 44-141-848 3119. Fax: 44-141-548
4823. Email: fiona.henriquez@paisley.ac.uk
Abstract

Tubulin is essential to eukaryotic cells and is targeted by several anti-neoplastics, herbicides and antimicrobials. We demonstrate that Acanthamoeba are resistant to five anti-microtubule compounds, unlike any other eukaryote studied so far. Resistance correlates with critical amino acid differences within the inhibitor binding sites of the tubulin heterodimers.
Tubulin is an essential structural element of the cytoskeleton of eukaryotic cells where it plays a central role in chromosomal segregation, organelle movement and cellular motility (7, 21). Tubulin has been exploited as a target for anti-neoplastics (8, 25), herbicides (18), anti-helminthic (9, 23), anti-fungal (14) and anti/protozoal (27, 28, 29) compounds. In addition, colchicine has been used for the treatment of gout in humans (2). Despite the highly conserved nature of α-tubulin and β-tubulin across the phyla, organisms present a diverse degree of susceptibility and resistance to the different groups of anti-microtubule agents. The success of benzimidazoles and dinitroanilines is due to their selectivity for helminths and plants, respectively, and their low toxicity in mammals (6, 23). However, even within these broad classifications of organisms there are many important differences. Some protozoans, including apicomplexans are susceptible to dinitroanilines (eg. *Toxoplasma gondii* IC₅₀ 0.3µM; 19), while others such as *Trypanosoma cruzi* (IC₅₀ 17.6µM) are resistant (26, 27). Similarly, there is a considerable variation in the susceptibility of protozoans to paclitaxel, as exemplified by *Leishmania* (IC₅₀ 35nM) (10) and *T. gondii* (IC₅₀ 1µM) (4). A few protozoa such as *Giardia lamblia* are susceptible to benzimidazoles, a class of drug normally used to treat helminth infections (14). Studies have demonstrated that amino acid differences that influence tertiary structure or alter inhibitor-docking regions are responsible for determining resistance to anti-tubulins. For example, site directed mutagenesis in the oryzalin-docking site on α-tubulin in *T. gondii* and *Eleusine indica* has been successful in altering the phenotype to oryzalin resistant (6, 19, 24).

Using the previously described alamar blue assay (17) we demonstrated that the two species of *Acanthamoeba* most commonly reported as causing *Acanthamoeba* keratitis in humans (15, 16, 20), *A. castellanii* and *A. polyphaga* are resistant to five
classes of tubulin inhibitor represented by oryzalin, paclitaxel, vinblastin, albendazole and colchicine (Table 1).

To explore the potential basis for these observations, both α and β-tubulin genes were cloned and sequenced from A. castellanii (neff strain) and A. polyphaga (strain 1501/18) (Accession numbers: DQ099493, DQ099491, DQ0994494 and DQ099492). The sequence identity on the amino acid level between the two species is 67% for α-tubulin and 99% for β-tubulin (supplementary Table). Using previously solved tubulin structures and their known inhibitor binding sites, it has been possible to model the tubulins from both species of Acanthamoeba and predicted inhibitor interactions.

Structure based mutagenesis studies on T. gondii α-tubulin has suggested that oryzalin binds in a pocket formed by 13 residues (19) of which 8 are identical in the Acanthamoeba family. Two of the residues, which display sequence variation lie within the N-loop (Ile42 and Asp47), implicated in inhibitor binding (Figure 1 and suppl. Figure) which is also shorter by two residues when compared to other α-tubulin homologues, contributing to the loss of potency. Val4Ile (i.e. Valine at position 4 in the oryzalin-sensitive T. gondii α-tubulin is replaced by an isoleucine at position 4 in Acanthamoeba α-tubulin), Phe24Tyr and Cys65Ala replacements are predicted to have a more subtle effect on the inhibitor pocket shape.

Paclitaxel binds to the β-tubulin subunit (13) and the structure of the mammalian (Bos taurus) β-tubulin/paclitaxel complex reveals that 22 residues form the inhibitor-binding pocket of which 7 show sequence variation relative to the Acanthamoeba proteins (Ala231Gln, Phe270Tyr, Ser275Ala, Arg276Pro, Gln279Thr, Arg359Ala and Leu361Gln replacements). Significantly Ala231, which is in the heart of the inhibitor-binding pocket is replaced by Gln, producing a severe steric clash to
the inhibitor (Figure 1). An additional steric clash may be formed by the substitution of Leu361 for Gln, with Phe270Tyr and Arg276Pro (Table 2) changing the packing interactions to the inhibitor. The remaining changes are solvent exposed and predicted to make little difference to inhibitor binding.

Vinblastin binds at the interface between α and β tubulin subunits (5). Of the 23 residues that have been implicated in inhibitor binding four show sequence variation relative to mammalian tubulin within A. castellanii and A. polyphaga proteins (Val353Cys and Asn329His replacements (castellanii) or Ser (polyphaga) in α-tubulin and Thr219Asn and Thr221Asn replacements in β-tubulin (22) (Table 2). The substitution of Thr221Asn in β-tubulin may result in a steric clash with the inhibitor. In addition Asn329 in α-tubulin makes close interactions with the inhibitor and its replacement by His in (A. castellanii) may result in a steric clash whereas its replacement by Ser (A. polyphaga) results in a loss of the packing interactions. For A. castellanii there are three additional changes not found in A. polyphaga, which can affect inhibitor binding (Ile355Val, Phe351Pro and Pro325Thr replacements; Figure 1).

Of the 13 residues, which form the putative albendazole inhibitor-binding site (12), 4 show sequence variation in the Acanthamoeba family (Table 2). Albendazole resistance is conferred when Phe167 and Phe200 are replaced by Serine and Methionine, respectively. The latter substitution is also present in Leishmania (1, 11). The replacement of Ala165 in susceptible helminth tubulin for a cysteine in Giardia duodenalis and Encephalitozoon cuniculi, has been shown to confer resistance to several members of the benzimidazole family when mutated to a larger residue (11).
Analysis of Acanthamoeba β-tubulin has shown that several key mammalian colchicine sensitive residues Val313, Ala314, Ala315 and Ile316 (Table 2) are replaced in Acanthamoeba by Ala, Ser, Ala, Val, respectively, as they are present in colchicines-resistant Leishmania spp. (28, 29). In addition there is a significant change in the environment of the hydrophobic colchicine-binding pocket due to the replacement of 4 Ala residues with bulkier Ser residues, which increases the percentage of hydrophilic residue from approximately 30% to 55%.

To verify that the sequence divergence of Acanthamoeba tubulin is responsible for the resistance of Acanthamoeba tubulin to all five compounds tested, future work should involve biochemical and structural analysis of Acanthamoeba tubulins. An important consideration is that resistance to these tubulin inhibitors may not solely be based upon changes in the inhibitor binding site alone and other factors such as drug metabolism, compartmentalization or efflux must also be potential factors. The work presented here demonstrates that the both Acanthamoeba α and β–tubulins are unusually divergent from tubulins of other organisms and offers plausible evidence for the unusual behavior of Acanthamoeba species in the presence of tubulin polymerizing and depolymerizing inhibitors.

References


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**Figure Legend**

**Figure 1.** A structural representation of the predicted *Acanthamoeba* tubulin inhibitor binding pocket for the five tubulin inhibitors; oryzalin (a), paclitaxel (b), vinblastin...
(c), albendazole (d) and colchicine (e). For all panels α and β tubulin are coloured magenta and blue, respectively, and each inhibitor is coloured grey. The residues that bind the inhibitor are represented in a stick format, with those that are divergent within the Acanthamoeba family coloured green and labelled. In panel a, the N-loop of α-tubulin is shown in yellow demonstrating its role in forming close interactions with oryzalin. All structures are based on the B. taurus tubulin structure and were produced using the graphics program PyMOL (3).

Acknowledgements

The William Ross Foundation and the University of Strathclyde Research and Development Fund funded this work.
Table 1. Relative IC$_{50}$s of *Acanthamoeba* species and Rabbit Corneal Cells (RCE) to anti-tubulin compounds$^1$

<table>
<thead>
<tr>
<th>Compounds</th>
<th><em>A. polyphaga</em></th>
<th><em>A. castellanii</em></th>
<th>RCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>oryzalin</td>
<td>&gt;100µM</td>
<td>&gt;100µM</td>
<td>&gt;500µM</td>
</tr>
<tr>
<td>paclitaxel</td>
<td>&gt;10µM</td>
<td>&gt;10µM</td>
<td>0.04µM-0.08µM</td>
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<tr>
<td>vinblastin</td>
<td>0.68µM-1.375µM</td>
<td>0.68µM-1.375µM</td>
<td>17nM</td>
</tr>
<tr>
<td>albendazole</td>
<td>&gt;47µM</td>
<td>&gt;47µM</td>
<td>0.7µM-1.469µM</td>
</tr>
<tr>
<td>colchicine</td>
<td>2.5mM-5mM</td>
<td>2.5mM</td>
<td>2.4µM</td>
</tr>
</tbody>
</table>

$^1$ Both *A. castellanii* and *A. polyphaga* were susceptible to Chlorhexidine (IC$_{50}$s were 1.5625-3.125µM and 3.125-6.25µM, respectively).
Table 2: Key amino acid residue changes in inhibitor binding sites between susceptible organisms and resistant *Acanthamoeba*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Organism</th>
<th>α-tubulin</th>
<th>β-tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α</td>
<td></td>
</tr>
<tr>
<td>oryzalin</td>
<td><em>E. indica</em></td>
<td>susceptible</td>
<td>Ile42 Asp47 Val4 Phe24 Cys65</td>
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<td></td>
<td><em>Acanthamoeba</em></td>
<td>resistant</td>
<td>- - Ile4 Tyr24 Ala65</td>
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<tr>
<td>paclitaxel</td>
<td><em>B. taurus</em></td>
<td>susceptible</td>
<td>Ala231 Gln231</td>
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<tr>
<td></td>
<td><em>Acanthamoeba</em></td>
<td>resistant</td>
<td>Phe275 Ser275</td>
</tr>
<tr>
<td>vinblastin</td>
<td><em>H. sapiens</em></td>
<td>susceptible</td>
<td>Val353 Asn329 Ile355 Pro325 Phe351</td>
</tr>
<tr>
<td></td>
<td><em>Acanthamoeba</em></td>
<td>resistant</td>
<td>Cys353 His329(c) Val355(c) Thr325(c) Pro351(c)</td>
</tr>
<tr>
<td></td>
<td><em>A. nidulans</em></td>
<td>susceptible</td>
<td>Ala165 Phe167 Glu198 Phe200</td>
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<tr>
<td></td>
<td><em>Acanthamoeba</em></td>
<td>resistant</td>
<td>Cys165 Ser167 Glu198</td>
</tr>
<tr>
<td>colchicine</td>
<td><em>H. sapiens</em></td>
<td>susceptible</td>
<td>Val313 Ala313 Ile316</td>
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<tr>
<td></td>
<td><em>Acanthamoeba</em></td>
<td>resistant</td>
<td>Ala313 Ser313 Val316</td>
</tr>
</tbody>
</table>

(c) *A. castellanii*
(p) *A. polyphaga*