Antimicrobial Agents
and Chemotherapy

Molecular Basis for Resistance of *Acanthamoeba* Tubulins to All Major Classes of Antitubulin Compounds

Fiona L. Henriquez, Paul R. Ingram, Stephen P. Muench, David W. Rice and Craig W. Roberts

Antimicrob. Agents Chemother. **2008, 52(3):1133. DOI: 10.1128/AAC.00355-07.**
Published Ahead of Print 10 December 2007.

Updated information and services can be found at:
http://aac.asm.org/content/52/3/1133

These include:

SUPPLEMENTAL MATERIAL Supplemental material

REFERENCES This article cites 28 articles, 8 of which can be accessed free at: http://aac.asm.org/content/52/3/1133#ref-list-1

CONTENT ALERTS Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: **http://journals.asm.org/site/misc/reprints.xhtml**
To subscribe to to another ASM Journal go to: **http://journals.asm.org/site/subscriptions/**

Journals.ASM.org

Downloaded from http://aac.asm.org/ on April 4, 2014 by UNIVERSITY OF STRATHCLYDE

Antimicrobial Agents and Chemotherapy, Mar. 2008, p. 1133–1135 Vol. 52, No. 3
0066-4804/08/$08.00+0 doi:10.1128/AAC.00355-07
Copyright © 2008, American Society for Microbiology. All Rights Reserved.

NOTES

Molecular Basis for Resistance of *Acanthamoeba* Tubulins to All Major Classes of Antitubulin Compounds[▽]†

Fiona L. Henriquez,[1]* Paul R. Ingram,[1] Stephen P. Muench,[2]§ David W. Rice,[2] and Craig W. Roberts[1]

Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 27 Taylor Street, Glasgow G4 0NR, United Kingdom,[1] and Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, United Kingdom[2]

Received 16 March 2007/Returned for modification 22 July 2007/Accepted 27 November 2007

Tubulin is essential to eukaryotic cells and is targeted by several antineoplastics, herbicides, and antimicrobials. We demonstrate that *Acanthamoeba* spp. are resistant to five antimicrotubule 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 antineoplastics (8, 25), herbicides (18), and antihelminthic (9, 23), antifungal (14), and antiprotozoal (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 diverse degrees of susceptibility and resistance to the different groups of antimicrotubule 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 (e.g., *Toxoplasma gondii*, with a 50% inhibitory concentration [IC_{50}] of 0.3 μM [19]), while others, such as *Trypanosoma cruzi* (IC_{50} of 17.6 μM), are resistant (26, 27). Similarly, there is considerable variation in susceptibility of protozoans to paclitaxel, as exemplified by *Leishmania* spp. (IC_{50} of 35 nM) (10) and *T. gondii* (IC_{50} of 1 μM) (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 antitubulins. 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, vinblastine, 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) (GenBank 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 (see the table in the supplemental material). By 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 of *T. gondii* α-tubulin have 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

TABLE 1. Relative IC_{50}s of *Acanthamoeba* species and rabbit corneal cells (RCE) to antitubulin compounds[a]

Compound	IC_{50}		
	A. polyphaga	*A. castellanii*	RCE
Oryzalin	>100 μM	>100 μM	>500 μM
Paclitaxel	>10 μM	>10 μM	0.04–0.08 μM
Vinblastine	0.68–1.375 μM	0.68–1.375 μM	17 nM
Albendazole	>47 μM	>47 μM	0.7–1.469 μM
Colchicine	2.5–5 mM	2.5 mM	2.4 μM

[a] Both *A. castellanii* and *A. polyphaga* were susceptible to chlorhexidine (IC_{50}s were 1.5625 to 3.125 μM and 3.125 to 6.25 μM, respectively).

* Corresponding author. Present address: School of Science and Engineering, University of Paisley, High Street, Paisley PA1 2BE, United Kingdom. Phone: 44-141-848 3119. Fax: 44-141-548 4823. E-mail: fiona.henriquez@paisley.ac.uk.

§ Present address: Institute of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, United Kingdom.

† Supplemental material for this article may be found at http://aac.asm.org/.

▽ Published ahead of print on 10 December 2007.

Downloaded from http://aac.asm.org/ on April 4, 2014 by UNIVERSITY OF STRATHCLYDE

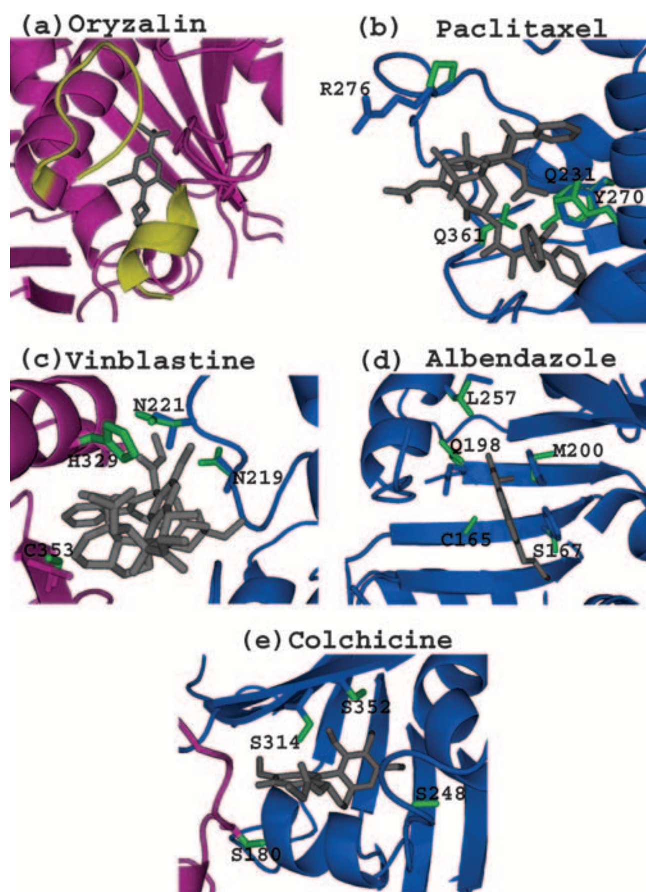

FIG. 1. Structural representation of the predicted *Acanthamoeba* tubulin inhibitor binding pocket for the five tubulin inhibitors oryzalin, paclitaxel, vinblastine, albendazole, and colchicine. For all panels, α- and β-tubulin are colored magenta and blue, respectively, and each inhibitor is colored gray. The residues that bind the inhibitor are represented in a stick format, with those that are divergent within the *Acanthamoeba* family colored green and labeled. In panel a, the N-loop of α-tubulin, which plays a role in forming close interactions with oryzalin, is shown in yellow. All structures are based on the *B. taurus* tubulin structure and were produced using the graphics program PyMOL (3).

(Ile42 and Asp47) lie within the N loop, implicated in inhibitor binding (Fig. 1; also see the figure in the supplemental material), which is also shorter than other α-tubulin homologues by 2 residues, 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; 7 of these residues 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 (Fig. 1). An additional steric clash may be formed by the replacement of Leu361 with 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.

Vinblastine binds at the interface between α- and β-tubulin subunits (5). Of the 23 residues that have been implicated in inhibitor binding, 4 show sequence variation relative to mammalian tubulin within *A. castellanii* and *A. polyphaga* proteins: Val353Cys and Asn329His (for *A. castellanii*) or Asn329Ser (for *A. polyphaga*) in α-tubulin and Thr219Asn and Thr221Asn in β-tubulin (22) (Table 2). The Thr221Asn replacement 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 (in *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) (Fig. 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 con-

TABLE 2. Key amino acid residue changes in inhibitor binding sites between susceptible organisms and resistant *Acanthamoeba* spp.

Compound	Organism	Phenotype	Residue in:												
			α-Tubulin					β-Tubulin						
Oryzalin	*E. indica*	Susceptible	Ile42	Asp47	Val4	Phe24	Cys65							
	Acanthamoeba	Resistant			Ile4	Tyr24	Ala65							
Paclitaxel	*B. taurus*	Susceptible						Ala231	Phe270	Ser275	Arg276	Gln279	Arg359	Leu361
	Acanthamoeba	Resistant						Gln231	Tyr270	Ala275	Pro276	Thr279	Ala359	Gln361
Vinblastine	*Homo sapiens*	Susceptible	Val353	Asn329	Ile355	Pro325	Phe351	Thr219	Thr221					
	Acanthamoeba	Resistant	Cys353	His329 (c)[a] Ser329 (p)	Val355 (c)	Thr325 (c)	Pro351 (c)	Asn219	Asn221					
Albendazole	*A. nidulans*	Susceptible						Ala165	Phe167	Glu198	Phe200			
	Acanthamoeba	Resistant						Cys165	Ser167	Gln198	Met200			
Colchicine	*Homo sapiens*	Susceptible						Val313	Ala314	Ile316				
	Acanthamoeba	Resistant						Ala313	Ser314	Val316				

[a] c, *A. castellanii*; p, *A. polyphaga*.

Downloaded from http://aac.asm.org/ on April 4, 2014 by UNIVERSITY OF STRATHCLYDE

ferred when Phe167 and Phe200 are replaced by serine and methionine, respectively. The latter replacement is also present in *Leishmania* spp. (1, 11). The replacement of Ala165 in susceptible helminth tubulin with 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 the key mammalian colchicine-sensitive residues Val313, Ala314, Ala315, and Ile316 (Table 2) are replaced in *Acanthamoeba* by Ala, Ser, Ala, and Val, respectively, as they are present in colchicine-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 residues 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 analyses of *Acanthamoeba* tubulins. An important consideration is that resistance to these tubulin inhibitors may not be based upon changes in the inhibitor binding site alone; other factors, such as drug metabolism, compartmentalization, or efflux, must also be potential factors. The work presented here demonstrates that the *Acanthamoeba* α- and β-tubulins are both 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.

The William Ross Foundation and the University of Strathclyde Research and Development Fund funded this work.

REFERENCES

1. **Armson, A., S. W. Kamau, F. Grimm, J. A. Reynoldson, W. M. Best, L. M. MacDonald, and R. C. Thompson.** 1999. A comparison of the effects of a benzimidazole and the dintroanilines against *Leishmania infantum*. Acta Trop. **73:**303–311.
2. **Cronstein, B. N., and R. Terkeltaub.** 2006. The inflammatory process of gout and its treatment. Arthritis Res. Ther. **8**(Suppl. 1)**:**S3.
3. **Delano, W. L.** 2002. The PyMOL molecular graphics system. Delano Scientific LLC, San Carlos, CA. http://www.pymol.org.
4. **Estes, R., N. Vogel, D. Mack, and R. McLeod.** 1998. Paclitaxel arrests growth of intracellular *Toxoplasma gondii*. Antimicrob. Agents Chemother. **42:**2036–2040.
5. **Gigant, B., C. Wang, R. B. Ravelli, F. Roussi, M. O. Steinmetz, P. A. Curmi, A. Sobel, and M. Knossow.** 2005. Structural basis for the regulation of tubulin by vinblastine. Nature **435:**519–522.
6. **Hugdahl, J. D., and L. C. Morejohn.** 1993. Rapid and reversible high-affinity binding of the dintroaniline herbicide oryzalin to tubulin from Zea mays L. Plant Physiol. **102:**725–740.
7. **Hyams, J. S., and C. W. Lloyd.** 1993. Microtubules, p. 1-439. *In* J. B. Harford (ed.), Modern cell biology, vol. 13. Wiley-Liss, New York, NY.
8. **Johnson, I. S., J. G. Armstrong, M. Gorman, and J. P. Burnett, Jr.** 1963. The vinca alkaloids: a new class of oncolytic agents. Cancer Res. **23:**1390–1427.
9. **Jung, H., M. Hurtado, M. Sanchez, M. T. Medina, and J. Sotelo.** 1992. Clinical pharmacokinetics of albendazole in patients with brain cysticercosis. J. Clin. Pharmacol. **32:**28–31.
10. **Kapoor, P., M. Sachdeva, and R. Madhubala.** 1999. Effect of the microtubule stabilising agent taxol on leishmanial protozoan parasites *in vitro*. FEMS Microbiol. Lett. **176:**429–435.
11. **Katiyar, S. K., V. R. Gordon, G. L. Mclauglin, and T. D. Edlind.** 1994. Antiprotozoal activities of benzimidazoles and correlations with beta-tubulin sequence. Antimicrob. Agents Chemother. **38:**2086–2090.
12. **Kwa, M. S., J. G. Veenstra, and M. H. Roos.** 1994. Benzimidazole resistance in Haemonchus contortus is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. Mol. Biochem. Parasitol. **63:**299–303.
13. **Lowe, J., H. Li, K. H. Downing, and E. Nogales.** 2001. Refined structure of ab-tubulin at 3.5 Å resolution. J. Mol. Biol. **313:**1045–1057.
14. **MacDonald, L. M., A. Armson, A. R. Thompson, and J. A. Reynoldson.** 2004. Characterisation of benzimidazole binding with recombinant tubulin from *Giardia duodenalis*, *Encephalitozoon intestinalis*, and *Cryptosporidium parvum*. Mol. Biochem. Parasitol. **138:**89–96.
15. **Marciano-Cabral, F., and G. Cabral.** 2003. *Acanthamoeba* spp. as agents of disease in humans. Clin. Microbiol. Rev. **16:**273–307.
16. **Martinez, A. J., and G. S. Visvesvara.** 1997. Free-living, amphizoic and opportunistic amoebas. Brain Pathol. **7:**583–598.
17. **McBride, J., P. R. Ingram, F. L. Henriquez, and C. W. Roberts.** 2005. Development of colorimetric microtiter plate assay for assessment of antimicrobials against *Acanthamoeba*. J. Clin. Microbiol. **43:**629–634.
18. **Morejohn, L. C., and D. E. Fosket.** 1991. The biochemistry of compounds with anti-microtubule activity in plant cells. Pharmacol. Ther. **51:**217–230.
19. **Morrissette, N. S., A. Mitra, D. Sept, and L. D. Sibley.** 2004. Dinitroanilines bind alpha-tubulin to disrupt microtubules. Mol. Biol. Cell **15:**1960–1968.
20. **Niederkorn, J. Y., H. Alizadeh, H. Leher, and J. P. McCulley.** 1999. The pathogenesis of *Acanthamoeba* keratitis. Microbes Infect. **1:**437–443.
21. **Nogales, E., S. G. Wolf, and K. H. Downing.** 1998. Structure of the alpha beta tubulin dimer by electron crystallography. Nature **391:**199–203.
22. **Rai, S. S., and J. Wolff.** 1996. Localization of the vinblastine-binding site on beta-tubulin. J. Biol. Chem. **271:**14707–14711.
23. **Rossignol, J. F., and H. Maisonneuve.** 1984. Albendazole: a new concept in the control of intestinal helminthiasis. Gastroenterol. Clin. Biol. **8:**569–576.
24. **Roy, D., and A. Lohia.** 2004. Sequence divergence of Entamoeba histolytica tubulin is responsible for its altered tertiary structure. Biochem. Biophys. Res. Commun. **319:**1010–1016.
25. **Schiff, P. B., J. Fant, and S. B. Horwitz.** 1979. Promotion of microtubule assembly *in vitro* by taxol. Nature **277:**665–667.
26. **Stokkermans, T. J., J. D. Schwartzman, K. Keenan, N. S. Morrissette, L. G. Tilney, and D. S. Roos.** 1996. Inhibition of *Toxoplasma gondii* replication by dinitroaniline herbicides. Exp. Parasitol. **84:**355–370.
27. **Traub-Cseko, Y. M., J. M. Ramalho-Ortigao, A. P. Dantas, S. L. de Castro, H. S. Barbosa, and K. H. Downing.** 2001. Dinitroaniline herbicides against protozoan parasites: the case of *Trypanosoma cruzi*. Trends Parasitol. **17:** 136–141.
28. **Werbovetz, K. A.** 2002. Tubulin as an antiprotozoal drug target. Mini Rev. Med. Chem. **2:**519–529.
29. **Werbovetz, K. A., J. J. Brendle, and D. L. Sackett.** 1999. Purification, characterization, and drug susceptibility of tubulin from *Leishmania*. Mol. Biochem. Parasitol. **98:**53–65.

Downloaded from http://aac.asm.org/ on April 4, 2014 by UNIVERSITY OF STRATHCLYDE