Analysis of the β-Tubulin Gene from *Vittaforma corneae* Suggests Benzimidazole Resistance

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We amplified, cloned, and sequenced the β-tubulin gene of *Vittaforma corneae*, a microsporidium causing human infections. The β-tubulin gene sequence has a substitution at Glu198 (with glutamine), which is one of six amino acids reported to be associated with benzimidazole sensitivity. Benzimidazoles were assayed for antemicrosporidial activity and showed poor parasite inhibition.

Microsporidia are obligate intracellular protozoan parasites that have emerged as major opportunistic human pathogens since the onset of the AIDS pandemic (6, 11). *Vittaforma corneae* is responsible for keratitis in otherwise healthy persons (2, 9, 10, 18), but disseminated infections have occurred in human immunodeficiency virus-infected patients as well (3). *V. corneae* was first described as *Nosema corneum* in a stromal biopsy from a non-human immunodeficiency virus-infected man who suffered from central disciform keratitis for 18 months (2). The parasite was cultivated in cell culture and identified as a new species, *N. corneum* (19). Infection with this organism was subsequently established in athymic mice (20), and new taxonomically significant features found warranted placing this organism within a new genus, *Vittaforma*, as *V. corneae* (21). In the second reported case of *V. corneae* infection, dual microsporidial infection was detected in a patient with AIDS—*Encephalitozoon hellem* in the sinonasal aspirate and *V. corneae* in the urine (3), indicating that *V. corneae* is capable of dissemination and survival in deep tissues, at least in an immunocompromised host. Three further cases involving *V. corneae* infection of the corneal stroma of immunocompetent patients were published (9, 10, 18). In addition to these confirmed cases, there is molecular evidence (obtained by PCR) for *Vittaforma* infections as a cause of keratitis in India (15 cases) (12) and of enteritis in Portugal (25 cases) (23).

Benzimidazoles are widely used as anthelmintic drugs in veterinary and human medicine and have been used as antifungal agents in agriculture. Albendazole is one of the most commonly used drugs for treating microsporidiosis in humans (5). Infections with *Encephalitozoon* spp. respond especially well to therapy with albendazole, whereas infections with *Enteroctyzoa bienusi* do not respond to albendazole, and the sequence of the β-tubulin gene of *E. bienusi* suggests that this species is resistant to albendazole (1, 5). No clinical data about the response of *Vittaforma* infections to albendazole are available, and molecular data on the β-tubulin gene are not available for this species either. We therefore tested the susceptibility of *V. corneae* to different benzimidazoles in vitro and amplified, cloned, and sequenced the β-tubulin gene of *V. corneae*.

MRC-5 cells were seeded on 24-well culture plates at a concentration of 5 × 10^5/ml in minimal essential medium and incubated overnight to confluence. Confluent monolayers of cells were inoculated with 2 × 10^8 microsporidian spores (*V. corneae* and *E. cuniculi* as a control). Three hours later, non-internalized spores were washed off and fresh medium with or without drugs was added. Albendazole and fenbendazole were purchased from Sigma (St. Louis, MO), and stock solutions were dissolved in dimethyl sulfoxide at a final concentration of 10 mg/ml and thereafter diluted in culture medium for use in the assay. Culture medium was replaced every 3 days, and on day 10, 100 μl of 10% (wt/vol) sodium dodecyl sulfate was added to each well to release newly developed spores from infected host cells. The spores were counted in a hemocytometer. Each treatment was done in triplicate, and the percent inhibition of microsporidian replication was calculated as 100 – [(number of spores counted in treated culture/number of spores in nontreated cultures) × 100]. Comparison of antimicrosporidial activities was done by Student’s *t* test.

DNA was isolated from *V. corneae*-infected cell cultures, and the β-tubulin sequences were amplified with the degenerate primers btubf (5’-GCC TGC AGG NCA RTG YGG NAA YCA-3’) and btubr (5’-GCC CTC AGT RAA YTC CAT YTC RTC CAT-3’) (15). PCR fragments were ligated into the vector pCR4-TOPO (Invitrogen, Carlsbad, CA) and cloned into TOP10 competent cells (Invitrogen). Plasmids were isolated from white colonies and sequenced on an automated DNA sequencer (ABI PRISM, Applied Biosystems, Foster City, CA) with vector-directed primers T7 and M13. Sequencing of both strands of each PCR fragment was done twice, and several plasmids were sequenced. The resulting DNA sequences were assembled with GeneTool Lite, version 1.0, and a consensus sequence was generated.

Results in Fig. 1 demonstrated that albendazole displayed significantly lower (*P < 0.001*) antimicrosporidial in vitro activities against *V. corneae* compared to *E. cuniculi*. Significant antimicrosporidial activities were observed in *E. cuniculi* cultures treated with albendazole or fenbendazole at concentrations of 10 ng/ml and above, whereas in *V. corneae* cultures no antimicrosporidial activities could be detected up to 1,000 ng/ml. At concentrations of 1,000 ng/ml, clear signs of host cell

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toxicity were observed after treatment of host cells with albendazole or fenbendazole for 10 days.

PCR amplification revealed a DNA fragment that was 1,000 bp long. Sequencing of the cloned DNA fragment provided a 1,024-bp gene sequence with a CG content of 46%.

The predicted \(-\)tubulin amino acid sequence has only five of the six sites that have been reported to be associated with benzimidazole activity (His6 is not included, as the fragment is truncated at the 5’ end) and has a substitution at Glu 198 (with glutamine) (numbering is based on the \textit{Saccharomyces cerevisiae} sequence in Fig. 2).

Some microsporidia (especially the \textit{Encephalitozoon} spp.) and other protists (e.g., \textit{Giardia lamblia} and \textit{Cryptococcus neoformans}) are very sensitive to several derivatives of a group of drugs called the benzimidazoles, which are widely used to treat helminth infections in humans and animals and as systemic fungicides in agriculture (13, 17). Benzimidazoles have been shown to disrupt mitosis in sensitive organisms through binding to the \(-\)tubulin subunit of microtubules. The benzimidazole binding site and the basis for the selective toxicity of these compounds remain incompletely defined (17). Six amino acids of \(-\)tubulin are reported to be associated with benzimidazole sensitivity (His1, Ala165, Phe167, Glu198, Phe200, and Arg241; numbering is based on the \textit{S. cerevisiae} sequence in Fig. 2).

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\textit{V. corneae} and \textit{E. bieneusi} evolved simultaneously and are closely related microsporidia. The \(-\)tubulin data presented here support this relationship, and \textit{V. corneae} may be a useful surrogate organism for studies on \textit{Enterocytozoon} drug resistance; such studies have so far been hampered as \textit{E. bieneusi} could not be cultivated in long-term culture.

Organisms resistant to benzimidazoles lack one or more of the six amino acids mentioned above. Besides \textit{E. bieneusi}, \textit{Entamoeba histolytica} also has changes at Glu198 and is relatively resistant to albendazole, and \textit{Cryptosporidium parvum} and \textit{Acanthamoeba polyphaga}, which have an additional change at Phe200 (in addition to Glu198), are resistant to benzimidazoles as well. \textit{V. corneae} has Ala165, whereas \textit{E. cuniculi}, \textit{E. hellem}, and \textit{E. intestinalis} have a change from Ala165 to Cys165. Nevertheless, \textit{V. cuniculi} is resistant to benzimidazoles, whereas the \textit{Encephalitozoon} spp. are highly susceptible. Cys165 is also present in \textit{G. lamblia} and \textit{C. neoformans}, which are sensitive to albendazole (7, 16, 17). So changes in Glu198 and/or Phe200 seem to be associated with resistance to benzimidazoles, whereas changes in Ala165 seem not to be highly predictive of benzimidazole sensitivity.

The suspected resistance against albendazole was further evaluated in vitro. Previous studies have shown inconsistent results. In one study, albendazole at 2.1 or 4.2 \(\mu\)g/ml in minimal medium was tested against \textit{V. corneae} in MDCK cell monolayers and showed some antimicrosporidal activity (22). The percentage of infected cells was reduced in the presence of the drug, and there were ultrastructural abnormalities at all stages of the life cycle. The drug prevented parasite division (22). However, such high concentrations cannot be

\begin{figure}
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\includegraphics[width=\textwidth]{figure1.png}
\caption{Dose-response curves for albendazole and fenbendazole. Values are means and standard deviations of three replications (\textit{V. corneae}) or from one experiment (\textit{E. cuniculi}).}
\end{figure}
reached under therapy. In another study, albendazole was less effective against *V. corneae* than against *E. intestinalis*, based on an approximately sevenfold higher minimal inhibitory concentration for 50% of the isolates tested (4). The in vitro data presented here also show that benzimidazoles are not effective against *V. corneae* whereas *E. cuniculi* parasite growth was inhibited very effectively. There are limited clinical data about the application of albendazole in *Vittaforma* infections. One patient with keratitis due to *V. corneae* was treated with topical steroids and broad-spectrum antibiotics but ultimately required a corneal transplant (2). Another patient was treated initially with topical acyclovir and steroid, but penetrating keratoplasty was performed later and he was given two courses of oral albendazole (400 mg daily), each for 14 days, and no microsporidia were detected in a biopsy of the rejected graft 6 months later (18). A third patient underwent penetrating keratoplasty as well but was not treated with albendazole (10), and another patient was treated with both topical fumagillin bicyclohexylammonium salt and oral albendazole but failed to improve or control the progression of the infection after lamellar keratoplasty (9). Thus, there is no clinical evidence for or against the use of albendazole in *Vittaforma* infections. Our experimental data show resistance of *V. corneae* to benzimidazoles. The molecular data obtained suggest that *V. corneae* should be resistant to benzimidazoles, and this was shown in vitro. Infections with *V. corneae* should not be treated with benzimidazoles, and other treatment options, such as fumagillin or TNP-470, should be chosen.

**Nucleotide sequence accession number.** The consensus sequence generated from several sequencing reactions in this study was submitted to the GenBank database under accession no. EU031749.

**REFERENCES**


