In Vitro Susceptibilities of the AIDS-Associated Microsporidian 
Encephalitozoon intestinalis to Albendazole, Its Sulfoxide Metabolite, and 12 Additional Benzimidazole Derivatives

S. K. KATIYAR* AND T. D. EDLIND

MCP*Hahnemann School of Medicine, Allegheny University of the Health Sciences, Philadelphia, Pennsylvania 19129

Received 25 July 1997/Returned for modification 10 September 1997/Accepted 25 September 1997

Recent reports have described the successful treatment of Encephalitozoon intestinalis infection in AIDS patients with albendazole. However, this compound is rapidly metabolized in vivo to albendazole sulf oxide, and furthermore it is only 1 of about 15 commercially developed benzimidazole derivatives. To compare the activities of albendazole, albendazole sulfoxide, and other benzimidazoles, an in vitro system involving infection of green monkey kidney cell (E6) monolayers with E. intestinalis spores was developed. After 14 days, the effects of benzimidazoles on spore production were determined. Ten of fourteen derivatives tested, including albendazole, were inhibitory at concentrations of 1 to 10 ng/ml. Derivatives modified at the 1 or 2 position were less active. Albendazole sulf oxide was 1.7-fold more inhibitory than albendazole but significantly less toxic to E6 cells, a finding that explains the clinical efficacy of this compound. Potential alternatives to albendazole are discussed. No albendazole-resistant E. intestinalis mutants were obtained following in vitro selection.

Microsporidia are obligate intracellular parasites of a wide variety of vertebrate and invertebrate hosts. Infection typically begins with injection of the sporoplasm from a spore into the host cell via a polar tubule. Intracellular replication (merogony) is followed by the formation of environmentally resistant spores (sporogony), which are released upon lysis of the host cell. The taxonomic status of the phylum Microspora is currently unclear; while the highly divergent ribosome components (17, 28) and minimal complement of organelles imply that they are early-branching eukaryotes, recent studies of tubulin genes from microsporidia suggest a close relationship to fungi (19, 22).

Two microsporidial species have been repeatedly associated with intestinal infections in humans with AIDS: Enterocytozoon bieneusi and Encephalitozoon intestinalis (formerly Septata intestinalis) (2, 10, 26; for a review, see reference 29). It was reported in 1992 (7) that Enterocytozoon bieneusi infections were responsive to treatment with albendazole, 1 of 15 or so benzimidazole derivatives developed for use as anthelmintics in human and veterinary medicine and as fungicides in agriculture. Subsequent reports indicated that albendazole was only partially effective against Enterocytozoon bieneusi (2, 11) but highly effective against E. intestinalis and related Encephalitozoon species (1, 2, 9, 16, 25, 24, 30, 31). Benzimidazoles act by blocking the polymerization of tubulin into microtubules (for a review, see reference 20). While the selective toxicity of this group is based largely on differences in tubulin structure (for a review, see reference 20), the effects of benzimidazoles on spore production were determined. Ten of fourteen derivatives tested, including albendazole, were inhibitory at concentrations of 1 to 10 ng/ml. Derivatives modified at the 1 or 2 position were less active. Albendazole sulfoxide was 1.7-fold more inhibitory than albendazole but significantly less toxic to E6 cells, a finding that explains the clinical efficacy of this compound. Potential alternatives to albendazole are discussed. No albendazole-resistant E. intestinalis mutants were obtained following in vitro selection.

Materials and Methods

Benzimidazoles. Parbendazole, oxibendazole, and albendazole sulfoxide were obtained from SmithKline Beecham (Philadelphia, Pa.), benomyl and carbendazim were from Du Pont (Wilmington, Del.), fenbendazole was from Hoechst-Roussel (Somerville, N.J.), oxendazole was from Syntex (Palo Alto, Calif.), cambendazole was from Merck (Rahway, N.J.), flubendazole and cyclobendazole were from Janssen (Beerse, Belgium), and thiabendazole, albendazole, mebendazole, and nocodazole were from Sigma (St. Louis, Mo.). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at −20°C.

Cell culture and preparation of spores. E. intestinalis spores (strain CDC: V297, isolated from the urine of an AIDS patient [27]) and the E6 line of African green monkey kidney (Vero) cells were provided by G. S. Visvesvara (Centers for Disease Control and Prevention, Atlanta, Ga.). Cells were grown in 25-cm2 green monkey kidney (Vero) cells were provided by G. S. Visvesvara (Centers for Disease Control and Prevention, Atlanta, Ga.). Cells were grown in 25-cm2 culture flasks at 37°C in 5% CO2 and typically subcultured every 96 h. The medium was minimum essential medium supplemented with 1-glutamine (Gibco BRL, Bethesda, Md.), 5% heat-inactivated fetal bovine serum (HyClone, Logan, Utah), 2 μg of fungizone per ml, and 50 μg of gentamicin per ml. To prepare E. intestinalis spores, actively growing cells were infected at a ratio of three to four spores/cell, and the medium was collected and changed every 72 to 96 h for up to 4 weeks. The collected medium was centrifuged at 2,000 × g for 15 min, and the pellet was resuspended in fresh medium to a concentration of 20 × 106 spores/ml and stored at 4°C.

Assay of benzimidazole activity. Confluent monolayers of E6 cells were detached with trypsin-EDTA (Gibco BRL) and centrifuged at 1,000 × g for 10 min. The cell pellet was resuspended in fresh medium to a concentration of 5 × 104 cells/ml. One milliliter was placed in each well of a 24-well tissue culture plate, and the plates were incubated 12 to 15 h. Medium was removed and replaced with 1 ml of fresh medium containing E. intestinalis spores at a ratio of three to four spores/cell. One microliter of freshly diluted benzimidazole solution (1, 3, 10, 30, and 100 μg/ml in DMSO) was added; control wells received 1 μl of DMSO alone (0.1% final concentration). After 72 h, the medium was replaced with fresh medium and drug. This was repeated after additional incubations of 72 and 96 h; preliminary experiments indicated that essentially all spores that had failed to infect cells were removed by these three medium changes. Two weeks postinfection (following a final 96-h incubation), medium was collected and progeny spores were counted in a hemocytometer. Concentrations of drug inhibiting E. intestinalis replication to 50% of the level in control cultures (IC50) were estimated from plots of spore number versus log benzimidazole concentration.

Assay of benzimidazole toxicity. Uninfected E6 cultures (1 ml) were prepared in 24-well plates as described above. One microliter of benzimidazole solution in
DMSO was added to a final concentration of 10 to 3,000 ng/ml, and incubation was continued for 72 h; controls received DMSO alone. Cultures were first examined microscopically for abnormal appearance and then detached with trypsin-EDTA and counted in a hemocytometer. IC₅₀[s] were estimated as described above.

### RESULTS

Beauvais et al. (6), Ditrich et al. (12), and Franssen et al. (13) have described in vitro methods for testing drug activity against *Encephalitozoon cuniculi* or *Encephalitozoon hellem*. In their studies, infected cells were detected by staining and counted. We found it more convenient and accurate to count released spores rather than infected cells. The convenience derives from the ability to visualize spores without staining. Also, cultures can be sampled multiple times for spore production but can be stained only once. The improvement in accuracy derives from the absence of ambiguity in the counting of infected cells: infected cells contain variable numbers of progeny parasites, and variation among microscopic fields of cell monolayers can be large.

With two exceptions (thiabendazole and cambendazole), commercially developed benzimidazoles are 2-carbamate derivatives (Fig. 1; Table 1). The 5-position-unsubstituted benomyl and carbendazim are widely used as agricultural fungicides, while the 5-substituted derivatives include a number of anthelmintics important in human and veterinary medicine (e.g., mebendazole, albendazole, and fenbendazole). Nocodazole is unique in that it was originally developed as an anti-cancer agent due to its toxicity for dividing mammalian cells.

The inhibitory activities of benzimidazoles against *E. intestinalis*, expressed as IC₅₀[s], are presented in Table 1. The most potent compound was nocodazole (IC₅₀ = 1 ng/ml), but nine additional derivatives were also highly active, with IC₅₀[s] of ≤10 ng/ml. Albendazole was typical of this group, with an IC₅₀ of 5 ng/ml. All of these are 2-position carbamate derivatives that differ widely in their 5 positions. Two compounds with 2-position thiazole, cambendazole and thiabendazole, were less active, as was benomyl, which is modified at the 1 position.

The E6 line of green monkey kidney cells was used as a host for *E. intestinalis*. To determine if observed benzimidazole activity was directed against the parasite or was an indirect effect of host cell toxicity, IC₅₀[s] were determined for uninfected E6 cells (Table 1). For all derivatives, the E6 cells were clearly less susceptible than *E. intestinalis*. However, the ratio of the IC₅₀ for E6 to that for *E. intestinalis* varied from lows of 15 to 30 (parbendazole and nocodazole) to highs of >750 (oxfendazole and albendazole sulfoxide).

The use of benzimidazoles in the field as both fungicides and anthelmintics is seriously compromised by the emergence of resistant strains. The potential for development of benzimidazole resistance in *E. intestinalis* was examined by exposing E6 cultures infected with approximately 10⁷ spores to gradually increasing concentrations of albendazole (3, 6, and 9 ng/ml) over a period of 3 months. After extended exposure to 6 ng of albendazole per ml, cultures had approximately twofold fewer spores than controls. The IC₅₀ for these spores was determined to be 5 ng/ml, i.e., the same as that for unexposed spores (Table 1). After extended exposure to 9 ng of albendazole per ml, cultures appeared to be completely free of spores, and no viable *E. intestinalis* was recovered following a return to drug-free medium.

### DISCUSSION

Several laboratories have examined the in vitro activity of albendazole against rabbit-derived *E. cuniculi* and determined its IC₅₀ to be approximately 4 ng/ml (6, 12, 13). This is very close to the value presented here for *E. intestinalis* (5 ng/ml). Although the assays employed were different in several respects, it is likely that this agreement reflects the close taxonomic relationship between *E. cuniculi* and *E. intestinalis* revealed by rRNA analysis (3, 15) and, of particular relevance here, β-tubulin analysis (22). Franssen et al. (13) also reported results for two additional benzimidazole derivatives that were qualitatively similar to those obtained here; specifically, ox-

### TABLE 1. In vitro activities of benzimidazole derivatives against *E. intestinalis* and uninfected E6 host cells

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Side chain composition at position⁵:</th>
<th>IC₅₀ (ng/ml)⁶</th>
<th>E. intestinalis</th>
<th>E6 host</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nocodazole</td>
<td>NHCOCOCH₃</td>
<td></td>
<td>1</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Parbendazole</td>
<td>NHCOCOCH₃</td>
<td></td>
<td>2</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>Mebendazole</td>
<td>NHCOCOCH₃</td>
<td></td>
<td>3</td>
<td>&gt;3,000</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>Albendazole sulfoxide</td>
<td>NHCOCOCH₃</td>
<td></td>
<td>4</td>
<td>&gt;3,000</td>
<td>&gt;750</td>
</tr>
<tr>
<td>Oxibendazole</td>
<td>NHCOCOCH₃</td>
<td></td>
<td>5</td>
<td>&gt;3,000</td>
<td>&gt;430</td>
</tr>
<tr>
<td>Albendazole</td>
<td>NHCOCOCH₃</td>
<td></td>
<td>7</td>
<td>&gt;3,000</td>
<td></td>
</tr>
<tr>
<td>Carbendazim</td>
<td>NHCOCOCH₃</td>
<td></td>
<td>10</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>Fenbendazole</td>
<td>NHCOCOCH₃</td>
<td></td>
<td>10</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>Flubendazole</td>
<td>NHCOCOCH₃</td>
<td></td>
<td>10</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>Oxibendazole</td>
<td>NHCOCOCH₃</td>
<td></td>
<td>10</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>Thiabendazole</td>
<td>4-Thiazole</td>
<td></td>
<td>25</td>
<td></td>
<td>&gt;120</td>
</tr>
<tr>
<td>Cyclobendazole</td>
<td>NHCOCOCH₃</td>
<td></td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cambendazole</td>
<td>NHCOCOCH₃</td>
<td></td>
<td>75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benomyl⁶</td>
<td>NHCOCOCH₃</td>
<td></td>
<td>&gt;100</td>
<td></td>
<td>&gt;3,000</td>
</tr>
</tbody>
</table>

⁵ See Fig. 1.
⁶ Data are averages of at least two independent experiments.
⁷ NT, not tested.
⁸ Benomyl includes a labile 1-position side chain [1-(butylamino)carbonyl].
in vitro, there is little information on its pharmacologic prop-
use of this veterinary anthelmintic in humans. Similarly, while
comparison, were also highly toxic.
more common
And more rapidly metabolized, in this case to oxfendazole. Again,
zyme 1. This is not the case with several other opportunistic
activity could well exist.
REFERENCES
1. Aarons, E. J., D. Woodrow, W. S. Hollister, E. C. Canning, N. Francis, and
Pleskow, G. Desai, and C. A. Wanke. 1994. Clinical features of microspori-
Shadduck. 1995. Small subunit ribosomal DNA phylogeny of various micro-
Treatment of intestinal microsporiasis with albendazole in patients with
AIDS. AIDS 6:311–313.
the opportunistic fungus Cryptococcus neoformans to antihelmintic benzim-
Polymerase chain reaction and molecular confirmation of disseminated Enceph-
aloitoxum cuniculi in a patient with AIDS: successful therapy with albend-
10. Didier, E. S., L. B. Rogers, J. M. Orenstein, M. D. Baker, C. R. Vossbrinck,
T. van Good, R. Hartskeerl, R. Soave, and L. M. Beaudet. 1996. Charac-
terization of Entamoeba histolytica isolates cultured from nasal mucosa and
1994. Treatment with albendazole for intestinal disease due to Enterocyto-
Entamoeba histolytica to several drugs in vitro. Antimicrob. Agents Che-
mother. 39:1265–1269.
Discrepancy between in vitro and in vivo antifungal activity of albendazole,
abstr. B-69, p. 39. In Program and abstracts of the 35th Interscience Con-
cference on Antimicrobial Agents and Chemotherapy. American Society for
Microbiology, Washington, D.C.
Terpstra. 1994. Genetic and immunological characterization of the micro-
sporidian Septata intestinalis Cali, Kotler and Orenstein, 1993: reclassifica-
verification of Entamoeba histolytica (microsporidiosis) eradication fol-
18. Kamaiishi, T., T. Hashimoto, Y. Nakamura, F. Nakamura, S. Murata, N.
Okada, K. Okamoto, M. Shimizu, and M. Hasegawa. 1996. Protein phylog-
eny of the cytoskeletal protein, tubulin, in the mode of action and drug resistance to benzimidazoles.
Int. J. Parasitol. 16:885–936.
Saccharomyces cerevisiae β-tubulin: interaction between residue 167 and
Tubulin genes from AIDS-associated microsporidia and implications for
phylogeny and benzimidazole sensitivity. Mol. Biochem. Parasitol. 78:249–
295.
Derouin, and J. Modai. 1995. Disseminated microsporiasis due to Septata intestinalis in patients with AIDS clinical features and response to albend-
azole as a treatment for disseminated microsporiasis due to Septata intesti-
\nals in AIDS patients: a report of four patients. AIDS 7:840.
24. van Good, T., E. C. Canning, H. Gilis, M. A. van den Bergh Weerman, J. K.
Karakiewicz Schattenkerk, and J. Danzek. 1994. Septata intestinalis frequently
isolated from stool of AIDS patients with a new cultivation method. Para-
D. Ferguson, H. de Mora, S. Wallace, B. S. Slemenda, I. Tyrrell, D. F.
Moore, and J. Meador. 1995. In vitro culture and serologic and molecular

Acknowledgments
We are indebted to Gowinda S. Visvesvara and his colleagues for
providing parasites, host cells, and advice on their culture.
This work was supported by Public Health Service grant AI-32433
from the National Institute of Allergy and Infectious Diseases.

VOL. 41, 1997 BENZIMIDAZOLE SUSCEPTIBILITY OF
E. INTESTINALIS 2731

Downloaded from http://aac.asm.org/ on August 14, 2017 by guest


