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Antiprotozoal Activities of Benzimidazoles and Correlations with β-Tubulin Sequence

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Benzimidazoles have been widely used since the 1960s as anthelmintic agents in veterinary and human medicine and as antifungal agents in agriculture. More recently, selected benzimidazole derivatives were shown to be active in vitro against two protozoan parasites, *Trichomonas vaginalis* and *Giardia lamblia*, and clinical studies with AIDS patients have suggested that microsporidia are susceptible as well. Here, we first present in vitro susceptibility data for *T. vaginalis* and *G. lamblia* using an expanded set of benzimidazole derivatives. Both parasites were highly susceptible to four derivatives, including mebendazole, flubendazole, and fenbendazole (50% inhibitory concentrations of 0.005 to 0.16 μg/ml). These derivatives also had lethal activity that was time dependent: 90% of *T. vaginalis* cells failed to recover following a 20-h exposure to mebendazole at 0.17 μg/ml. *G. lamblia*, but not *T. vaginalis*, was highly susceptible to five additional derivatives. Next, we examined in vitro activity of benzimidazoles against additional protozoan parasites: little or no activity was observed against *Entamoeba histolytica*, *Leishmania major*, and *Acanthamoeba polyphaga*. Since the microtubule protein β-tubulin has been identified as the benzimidazole target in helminths and fungi, potential correlations between benzimidazole activity and β-tubulin sequence were examined. This analysis included partial sequences (residues 108 to 259) from the organisms mentioned above, as well as the microsporidia *Encephalitozoon hellem* and *Encephalitozoon cuniculi* and the sporozoan *Cryptosporidium parvum*. β-tubulin residues Glu-198 and, in particular, Phe-200 are strong predictors of benzimidazole susceptibility; both are present in *Encephalitozoon* spp. but absent in *C. parvum*.

Microtubules are a characteristic feature of eukaryotic cells. They are major components of the mitotic spindle and, in some cells, the cytoskeleton and flagella or cilia. Microtubules form by polymerization of tubulin, a dimeric protein composed of α- and β-tubulin subunits, each approximately 440 amino acids long. Each subunit binds one molecule of GTP, and polymerization is followed by hydrolysis of the β-tubulin GTP. The mechanisms that control the rapid polymerization and depolymerization of spindle microtubules before and after mitosis are unclear. Most studies of microtubules have been limited to those isolated from mammalian brain, in which microtubules are abundant in the cytoskeleton. Microtubules involved in mitosis are much more difficult to isolate.

Besides GTP, the most well-studied effectors of microtubule polymerization are drugs, notably colchicine, vinca alkaloids, and benzimidazoles (for a review, see reference 22). Among these agents, the benzimidazoles are unique in being selectively toxic to certain lower eukaryotes. Biochemical and genetic analysis has identified the β-tubulin subunit as the primary benzimidazole target. Four different regions of the molecule have been implicated in benzimidazole activity by isolating resistant mutants of susceptible fungi (e.g., *Aspergillus nidulans*) and sequencing their β-tubulin genes (14, 17, 18, 25, 26). These regions include amino acid residues 6, 165 to 167, 198 to 200, and 241.

Initially, benzimidazole susceptibility appeared to be limited to helminths and fungi (for reviews, see references 7 and 27). In 1985, however, in vitro growth of the vaginal protozoan parasite *Trichomonas vaginalis* was reported to be inhibited by the anthelmintic derivatives mebendazole and flubendazole (16). Effects on morphology and mitotic index supported the notion of a microtubule target for these drugs. Subsequently, we and others described similar in vitro results for the intestinal protozoan *Giardia lamblia* (3, 5, 12, 23, 24). Furthermore, clinical trials with mebendazole (1) and albendazole (15) demonstrated efficacy in treatment of giardiasis. Two recent clinical studies suggest that albendazole is moderately active against the microsporidian *Enterocytozoon bieneusi*, which causes intestinal infections in AIDS patients (2, 10). In contrast, the apicomplexan *Plasmodium falciparum* was relatively resistant to several benzimidazoles tested (9).

In this study, we examined the susceptibilities of *T. vaginalis* and *G. lamblia* to 14 anthelmintic and antifungal benzimidazole derivatives. Furthermore, susceptibilities were examined for three unrelated protozoans not previously examined: the parasitic amoeba *Entamoeba histolytica*, the free-living amoeba *Acanthamoeba polyphaga*, and the kinetoplastid *Leishmania major*. To investigate the molecular basis for these results, partial β-tubulin sequences from these organisms (and from the AIDS-associated pathogens *Cryptosporidium parvum*, *Encephalitozoon hellem*, and *Encephalitozoon cuniculi*) were analyzed for the presence of five amino acid residues previously implicated in benzimidazole susceptibility. This analysis indicated that Glu-198 and, in particular, Phe-200 are correlated with benzimidazole susceptibility.

MATERIALS AND METHODS

For *G. lamblia* WB, *T. vaginalis* 30236 and Tv1:MCP, *E. histolytica* 200:NIH and HM1:IMSS, and *L. major* 1S, the source of organisms, culture conditions, and methods for determining drug susceptibility were as previously described (19). *G. lamblia* H/7 was kindly provided by T. Nash and was

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cultured and assayed identically to *G. lamblia* WB. *A. polyphaga* CDC:0187:1 was cultured aerobically at 23°C in proteose peptone-yeast extract-glucose medium (28). To assay susceptibility of this organism, benzimidazoles were diluted into 200 μl of medium containing 200 trophozoites in microtiter wells and cells were counted microscopically after 48 h. To estimate toxicity to mammalian cells, Vero (African green monkey kidney) cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 37°C in 5% CO_2. Benzimidazoles were added to microtiter wells containing 2×10^4 cells in 200 μl of medium. After 48 h of incubation, cells were counted microscopically. For all organisms, drug concentrations inhibiting growth to 50% of control levels (IC_{50}) were estimated by plotting percent control versus log concentration. The following benzimidazoles were kindly supplied by the indicated manufacturers: parbendazole, oxibendazole, and albendazole sulfoxide (SmithKline Beecham, Philadelphia, Pa., and Surrey, United Kingdom); flubendazole and cyclobendazole (Janssen, Beerse, Belgium); benomyl and carbendazim (Dupont, Wilmington, Del.); fenbendazole (Hoechst-Roussel, Somerville, N.J.); oxfendazole (Syntex, Palo Alto, Calif.); and cambendazole (Merck, Rahway, N.J.). All other drugs were obtained from Sigma (St. Louis, Mo.). Benzimidazoles were dissolved in dimethyl sulfoxide (DMSO) at 1 or 10 mg/ml and stored at −20°C. The final concentration of DMSO in cultures was ≤0.1%; control cultures were treated with DMSO alone.

Lethal activity was determined for *T. vaginalis* 30236 by exposing cells (4×10^6/ml) for various lengths of time to nocodazole, mebendazole, or fenbendazole at 0.04, 0.2, 1, or 5 μg/ml. Other conditions were as described previously (19). Final DMSO concentrations were ≤0.1%; controls received DMSO alone. Following drug exposure, cultures were mixed well before 2-μl aliquots were removed and diluted into 2 ml of drug-free medium (final benzimidazole concentration was ≤5 ng/ml). Diluted cultures were incubated for 48 h, cells were counted, and percent controls were calculated. Drug concentrations lethal to 50, 90, or 99% (LC_{50}, LC_{90}, or LC_{99}, respectively) of the cells were estimated by plotting percent control versus log concentration.

RESULTS AND DISCUSSION

Susceptibilities of *G. lamblia* and *T. vaginalis*. Juliano et al. (16) reported *T. vaginalis* susceptibility data for three benzimidazole derivatives: mebendazole, flubendazole, and thiabendazole. We (12) and others (3, 5, 23, 24) have previously reported *G. lamblia* susceptibility to a total of seven derivatives. The structures of the 14 derivatives tested here, plus unsubstituted benzimidazole, are shown in Table 1 and Fig. 1. With the exception of thiabendazole and cambendazole, all derivatives have a carbamate side chain at position 2. Similarly, with the exceptions of thiabendazole, carbendazim, and the prodrug benomyl, all derivatives have position 5 substitutions, which are variable. In Table 2, the IC_{50}s for single strains of *T. vaginalis* and *G. lamblia* are presented for each of the 15 compounds. Data are also presented for a second strain of each organism, with nine derivatives used.

Four derivatives had high activities (IC_{50} = 0.005 to 0.16 μg/ml) against both *G. lamblia* and *T. vaginalis*: nocodazole, flubendazole, mebendazole, and fenbendazole. Nocodazole is unusual in its broad-spectrum toxicity; it was originally developed as an antitumor agent (8). Flubendazole and mebendazole are closely related structurally. The fenbendazole susceptibilities of *T. vaginalis* varied between the two strains: the strain ATCC 30236 was less susceptible than the recent clinical isolate Tv1:MCP (IC_{50} = 0.16 versus 0.060 μg/ml). This variation was also seen with the structurally related oxfendazole (IC_{50} = 1.9 versus 0.38 μg/ml), which, in vivo, is a major fenbendazole metabolite.

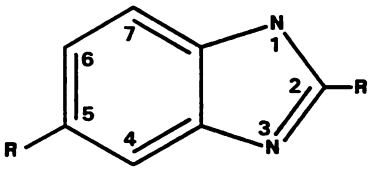

FIG. 1. Structure of benzimidazoles.

TABLE 1. Structures of benzimidazoles tested for antiprotozoal activity

Derivative	Side chain composition	
	R_2	R_5
Benzimidazole	-H	-H
Thiabendazole	-4-Thiazole	-H
Cambendazole	-4-Thiazole	$-NHCOOCH(CH_3)_2$
Carbendazim	$-NHCOOCH_3$	-H
Benomyl[a]	$-NHCOOCH_3$	-H
Albendazole	$-NHCOOCH_3$	$-SCH_2CH_2CH_3$
Albendazole sulfoxide	$-NHCOOCH_3$	$-SCH_2CH_2CH_3$ ‖ O
Cyclobendazole	$-NHCOOCH_3$	-C-cyclopropyl ‖ O
Fenbendazole	$-NHCOOCH_3$	-S-phenyl
Flubendazole	$-NHCOOCH_3$	-C-4-fluorophenyl ‖ O
Mebendazole	$-NHCOOCH_3$	-C-phenyl ‖ O
Nocodazole	$-NHCOOCH_3$	-C-2-thienyl ‖ O
Oxfendazole	$-NHCOOCH_3$	-S-phenyl ‖ O
Oxibendazole	$-NHCOOCH_3$	$-OCH_2CH_2CH_3$
Parbendazole	$-NHCOOCH_3$	$-CH_2CH_2CH_2CH_3$

[a] The prodrug benomyl includes a one-position modification [1-(butylamino) carbonyl] that is metabolically removed by cells, generating carbendazim.

G. lamblia was also highly susceptible (IC_{50} = 0.007 to 0.090 μg/ml) to albendazole, parbendazole, oxibendazole, cyclobendazole, and cambendazole. Moderate antigiardial activity (IC_{50} = 0.21 μg/ml) was observed for oxfendazole, while albendazole sulfoxide (the in vivo metabolite of albendazole) was active but in an atypical fashion: low levels of growth were still detectable at high drug concentrations. In contrast, these seven derivatives were all poorly active against *T. vaginalis* ($IC_{50} \geq 0.9$ μg/ml).

Carbendazim, benomyl, thiabendazole, and the parent compound, benzimidazole, all of which lack position 5 substitutions, have relatively little or no activity against both of these organisms ($IC_{50} \geq 0.7$ μg/ml).

The effects of benzimidazole exposure on *T. vaginalis* morphology have been described previously (16) and were noticeable here but were less pronounced than the previously described effects on *G. lamblia* morphology (4, 12).

Lethal activity against *T. vaginalis*. Previously, mebendazole

TABLE 2. In vitro activities of benzimidazole derivatives against two protozoan parasites

Derivative	IC_{50} (μg/ml)[a]			
	G. lamblia		*T. vaginalis*	
	WB	H/7	30236	Tv1:MCP
Nocodazole	0.005	0.007	0.009	NT[b]
Flubendazole	0.011	NT	0.033	NT
Fenbendazole	0.012	0.007	0.16	0.060
Albendazole	0.016	0.007	0.90	1.0
Mebendazole	0.018	0.009	0.029	0.080
Parbendazole	0.023	NT	2.2	>1.0
Oxibendazole	0.032	0.033	>3.0	>3.0
Cyclobendazole	0.047	NT	1.1	NT
Albendazole sulfoxide	0.088[c]	NT	>3.0	NT
Cambendazole	0.090	NT	>3.0	>3.0
Oxfendazole	0.21	0.20	1.9	0.38
Carbendazim	0.70	1.2	>3.0	>3.0
Benomyl	1.8	1.2	>3.0	>3.0
Thiabendazole	2.2	>3.0	>3.0	NT
Benzimidazole	>3.0	NT	NT	NT

[a] Datum points used to estimate IC_{50}s were averaged from two to five separate determinations.
[b] NT, not tested.
[c] Slope of inhibitory activity versus drug concentration was unusually shallow (i.e., IC_{90} = 0.70 μg/ml).

was shown to display lethal activity against *G. lamblia* after a 3-h exposure at a concentration (LC_{50} = 0.33 μg/ml) sevenfold above the inhibitory concentration (12). This was consistent with the steep slope of activity versus mebendazole concentration and suggests that this drug would be parasiticidal at adequate doses. Similarly steep slopes were generally observed in the studies reported here with *T. vaginalis*. To examine lethal activity, *T. vaginalis* was exposed to mebendazole, fenbendazole, or nocodazole for approximately 3, 8, and 20 h before dilution into drug-free medium and further incubation to assess its viability. The LC_{50} for mebendazole was 0.30 μg/ml after 3 h, a value very similar to that determined for *G. lamblia*. This is 10-fold above the inhibitory concentration. Lethality correlated with inhibitory activity: the ratio of lethal-to-inhibitory activity was only 6 for nocodazole (LC_{50} = 0.05 μg/ml), but it was 30 for fenbendazole (LC_{50} = 5 μg/ml) following a 3-h exposure.

However, lethal activity was observed to be strongly dependent on time as well as concentration. As illustrated with fenbendazole (Fig. 2), up to 55-fold increased killing was observed with longer exposure to the drug. After 20 h, LC_{90} and LC_{99} ratios of 0.80 and 4, 0.17 and 0.9, and <0.04 and 0.1 μg/ml were determined for fenbendazole, mebendazole, and nocodazole, respectively. The ratio of lethal-to-inhibitory activity approached 1.

Susceptibilities of *E. histolytica*, *A. polyphaga*, and *L. major*. Eleven of the fourteen benzimidazole derivatives (the exceptions were flubendazole, albendazole sulfoxide, and cyclobendazole) were tested for in vitro inhibitory activity against cultures of the parasitic amoeba *E. histolytica*. None were inhibitory at concentrations below 4 μg/ml. The lack of albendazole activity was reported previously (5). The same derivatives were tested against flagellated promastigotes of *L. major*, an agent of cutaneous leishmaniasis. None of the derivatives were inhibitory below 5 μg/ml, with two exceptions: benomyl and cambendazole were weakly active (IC_{50} = 1.2 and 1.6 μg/ml, respectively). Similarly, the free-living but occasionally pathogenic amoeba *A. polyphaga* was resistant to

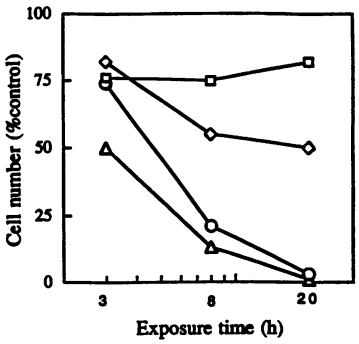

FIG. 2. Time and concentration dependence of fenbendazole lethal activity against *T. vaginalis*. Cells were exposed for approximately 3, 8, or 20 h to fenbendazole at 0.04 (□), 0.2 (◇), 1 (○), or 5 (△) μg/ml. Following dilution into drug-free medium, cells were incubated an additional 48 h before being counted.

albendazole, fenbendazole, mebendazole, and thiabendazole at concentrations of ≥6 μg/ml. Benomyl and carbendazim at 3 μg/ml inhibited growth to approximately 25% of control levels; lower concentrations were not tested. In summary, these three organisms lack the high-level benzimidazole susceptibility characteristic of *G. lamblia* and *T. vaginalis*.

Toxicity to mammalian cells. The selective toxicity of benzimidazoles is assumed to derive from a lack of inhibitory activity towards the mammalian (or plant) host cells. However, there is little published data to support this assumption. We therefore tested selected benzimidazoles for activity against the Vero line of African green monkey kidney cells. Thiabendazole and carbendazim had little or no activity (IC_{50} ≥ 10 μg/ml). Mebendazole, fenbendazole, and albendazole displayed inhibitory activity, but only at relatively high concentrations (IC_{50} = 3 to 6 μg/ml). Nocodazole, consistent with its initial development as a potential antitumor agent (8), was 10- to 20-fold more toxic (IC_{50} = 0.3 μg/ml). However, nocodazole activity against *G. lamblia* and *T. vaginalis* is 30- to 60-fold higher still (Table 2); thus, in terms of these two parasites, nocodazole can still be considered selectively toxic.

Correlations with β-tubulin sequence. Partial β-tubulin sequences (residues 108 to 259) have been determined following gene amplification for the five protozoan parasites studied as described above (18a, 20). Residues 151 to 250 of these sequences are aligned in Fig. 3. *G. lamblia* has three β-tubulin gene copies (21) which, over the region analyzed here, differ from each other in only two positions. *T. vaginalis* has six to seven gene copies which fall into three groups in terms of sequence and expression: (i) highly expressed *btub1*-like genes and (ii) moderately expressed *btub2*, which are identical in all but one position, and (iii) the poorly expressed, more divergent *btub3*. *A. polyphaga* has four gene copies: *btub1* and *btub2* are very similar, *btub4* is divergent, and *btub3* combines features of the other three. A single β-tubulin gene was detected in *E. histolytica*, while *L. major* most likely has a single gene which is tandemly repeated. For comparison, these partial β-tubulin sequences are also aligned (Fig. 3) with the corresponding regions of β-tubulin from two benzimidazole-susceptible (*A. nidulans* and *Caenorhabditis elegans*) and two benzimidazole-resistant (human and *P. falciparum*) organisms.

Six different β-tubulin residues have been implicated in benzimidazole activity through the characterization of fungi resistant to benomyl (14, 18, 25, 26) and, in one case, thiabendazole (17). Two of these, residues 6 and 241, are conserved in both benzimidazole-resistant and -susceptible organ-

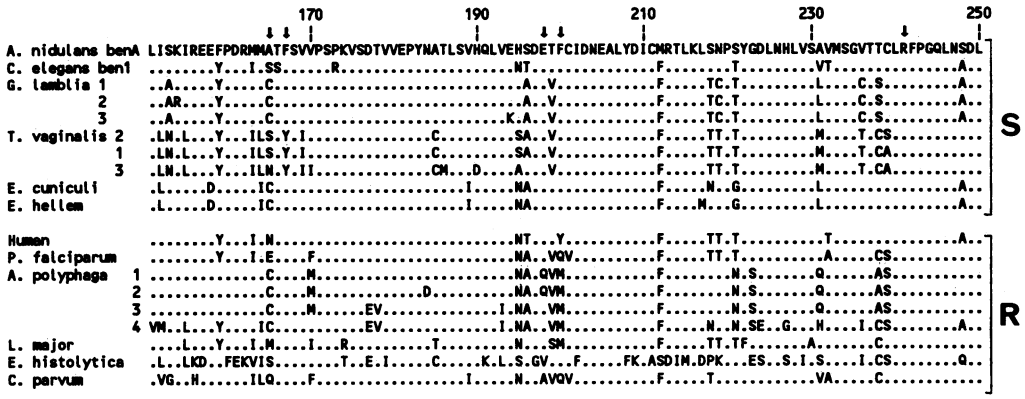

FIG. 3. Alignment of partial β-tubulin sequences (residues 151 to 250) from organisms demonstrated or predicted to be benzimidazole susceptible (S) or resistant (R). Single-letter amino acid abbreviations are used. Dots represent identity to the *A. nidulans* sequence. Arrows indicate five residues implicated in benzimidazole susceptibility by mutational analysis. Sequences were obtained from the GenBank database.

isms and hence are not predictive of benzimidazole activity. Residue 165, which is mutated from Ala to Gly in thiabendazole-resistant *A. nidulans*, is variable and not clearly correlated with benzimidazole susceptibility. Phe-167, as previously discussed (20), must not be a general requirement for benzimidazole activity since all *T. vaginalis* β-tubulins have Tyr-167. Phe-167 is also not predictive of benzimidazole activity. On the other hand, Glu-198 and, in particular, Phe-200 are predictive. β-Tubulins from all four organisms listed in Fig. 3 known to be benzimidazole susceptible (and many additional susceptible fungi and helminths) have Glu-198 and Phe-200. Of the five organisms listed which are benzimidazole resistant, two have alternative residues at position 198 and four have alternative residues at position 200; all five organisms (and many additional resistant organisms) lack one or both residues. The basis for this correlation between benzimidazole susceptibility and β-tubulin sequence is unknown. It can be hypothesized that residues Glu-198 and Phe-200 participate in benzimidazole binding; however, there is no experimental evidence as yet to support this.

Predictions of benzimidazole susceptibility from β-tubulin sequence. Included in Fig. 3 are partial β-tubulin sequences from the microsporidia *E. hellem* and *E. cuniculi* and the apicomplexan *C. parvum* (11). These intracellular protozoan parasites normally cause inapparent or self-limiting infections but have been implicated in severe intestinal or systemic infections in immunocompromised individuals, including AIDS patients. These infections do not respond to treatment with a wide variety of conventional antimicrobial agents (6, 13). The identification of new agents is hampered by the lack of routine in vitro culture systems for both the microsporidia and *C. parvum*. On the other hand, in clinical studies of AIDS patients, intestinal infections with the microsporidian *E. bieneusi* responded favorably to treatment with albendazole (2, 10). It was of interest therefore to analyze the β-tubulin sequences from *E. hellem*, *E. cuniculi*, and *C. parvum* to predict their benzimidazole susceptibility or resistance (*E. bieneusi* itself was not available for study). Both of the microsporidia and *C. parvum* possess a single β-tubulin gene copy (11, 18a). *C. parvum* β-tubulin lacks both Glu-198 and Phe-200 (Fig. 3) and is thus predicted to be benzimidazole resistant. *E. hellem* and *E. cuniculi* β-tubulins are very similar to each other over the region analyzed. In addition to Phe-167 and Arg-241, both β-tubulins include Glu-198 and Phe-200. Thus, these organisms are predicted to be benzimidazole susceptible.

ACKNOWLEDGMENTS

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