ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1994, p. 378–380
0066-4804/94/$04.00+0

Vol. 38, No. 2

In Vitro Susceptibility of the Opportunistic Fungus *Cryptococcus neoformans* to Anthelmintic Benzimidazoles

M. C. CRUZ,[1]* M. S. BARTLETT,[2] AND T. D. EDLIND[1]

Medical College of Pennsylvania, Philadelphia, Pennsylvania 19129,[1] and Indiana University School of Medicine, Indianapolis, Indiana 46202[2]

Received 4 May 1993/Returned for modification 11 June 1993/Accepted 16 November 1993

Ten benzimidazole derivatives and amphotericin B were tested in vitro against three isolates of *Cryptococcus neoformans*. Drug concentrations inhibiting 50% of growth (IC_{50}s) were determined. Four derivatives, including mebendazole and albendazole, had moderately high activities (IC_{50} = 0.1 to 0.3 μg/ml). Fenbendazole, however, was 10-fold more active (IC_{50} = 0.01 to 0.02 μg/ml) and also 2-fold more active than amphotericin B. Ten additional clinical isolates of *C. neoformans* were tested against fenbendazole, mebendazole, and albendazole; similar susceptibilities were observed. Drug concentrations lethal to 90% of the cells (LC_{90}s) were determined for two isolates. The LC_{90}s of albendazole and mebendazole were 0.92 to 2.1 μg/ml, and those of fenbendazole were 0.06 to 0.07 μg/ml; the latter are eight to ninefold lower than the LC_{90}s of amphotericin B that were obtained. Spontaneously arising mutants displaying partial resistance to fenbendazole arose at a low frequency (5×10^{-9}).

Cryptococcus neoformans, an encapsulated yeast-like basidiomycete, is the most common cause of systemic mycosis in patients with AIDS (4, 8, 11). The incidence of cryptococcosis in AIDS patients has been reported to be 6 to 9% in the United States and as high as 33% in areas of Africa. The disease typically presents as meningitis and, less frequently, as pneumonia. The currently recommended treatment for cryptococcal meningitis is a combination of amphotericin B and flucytosine (13, 15). However, treatment failures and toxicity are common with these agents. In addition, amphotericin B must be administered intravenously. There is a need for new oral anticryptococcal agents with low toxicity. Fluconazole satisfies these two criteria, although it may be less effective than amphotericin B (13).

Benzimidazoles are a large group of drugs developed primarily as anthelmintic agents in human and veterinary medicine (9). Benzimidazoles act by blocking the polymerization of microtubules in susceptible organisms, hence disrupting mitosis. Three derivatives, mebendazole, albendazole, and thiabendazole, are currently approved for human use in many countries and are generally well tolerated. Two derivatives with antifungal activity, benomyl and carbendazim, have been developed for use in agriculture. Interestingly, thiabendazole has activity against certain fungi as well, primarily dermatophytes (MIC ≤ 2 μg/ml) (14). *C. neoformans* was reported to be relatively resistant to this derivative (MIC = 20 to 40 μg/ml). However, thiabendazole is structurally distinct from most other benzimidazole derivatives, with a thiazole instead of a carbamate at carbon 2. In 1971, Maxwell and Brody (10) reported that *C. neoformans* was sensitive (MIC < 1 μg/ml) to parbendazole, one of the first benzimidazole carbamate derivatives. More recently, the inhibitory activities of certain benzimidazole carbamates have been shown to extend to two species of protozoa (2, 5) and the fungus-like *Pneumocystis carinii* (1). These observations prompted us to examine more closely the in vitro activity of this drug group against *C. neoformans*.

Twelve clinical isolates of *C. neoformans* were obtained from Indiana University Hospital, Indianapolis, Ind. They were isolated from blood, spinal fluid, or bronchoaveolar lavage specimens obtained from patients with known disseminated disease. The environmental *C. neoformans* isolate 14116 was obtained from the American Type Culture Collection. Organisms were streaked onto 1% yeast extract–1% peptone–2% dextrose (YEPD) agar, incubated at 30°C for 48 h, and stored at 4°C. Drugs were obtained from Sigma (St. Louis, Mo.), with the following exceptions: carbendazim and benomyl (Dupont, Wilmington, Del.), parbendazole and oxibendazole (Smith-Kline Beecham, Philadelphia, Pa.), fenbendazole (Hoechst-Roussel, Somerville, N.J.), and oxfendazole (Syntex, Palo Alto, Calif.). Drugs were dissolved in dimethyl sulfoxide and diluted so that the final concentration of this solvent was <0.1%. Susceptibilities were determined by a broth dilution method. Cells were diluted from fresh overnight cultures grown in YEPD at 30°C to a density of 10^4 cells per ml of YEPD and aliquoted to culture tubes. Drugs were added by 2-fold serial dilution. Cultures were incubated at 30°C with shaking for 20 h; similar susceptibilities were obtained at 37°C for several benzimidazoles tested (not shown). Cell numbers were determined with a hemacytometer. Typically, control cultures reached a density of 5×10^7 cells per ml after incubation. Drug concentrations inhibiting growth to 50 or 90% of control levels (IC_{50}s and IC_{90}s, respectively) were estimated from plots of cell number (percent control) versus drug concentration.

Initially, two clinical isolates (IU4 and IU8698) and one environmental isolate (14116) were tested for susceptibility to 10 benzimidazole derivatives. For comparison, unsubstituted benzimidazole and amphotericin B were also tested. The 11 benzimidazoles tested defined four groups in terms of activity (Table 1). Benzimidazole and thiabendazole were inactive at the highest concentration tested (4 μg/ml). Oxibendazole, oxfendazole, and the two antifungal agents were weakly active (IC_{50} = 1.0 to 3.2 μg/ml). Albendazole, mebendazole, parbendazole, and nocodazole were moderately active (IC_{50} = 0.09 to 0.31 μg/ml). Fenbendazole, in comparison with the other drugs tested, was highly active, with an IC_{50} of ≤0.019

* Corresponding author. Department of Microbiology and Immunology, Medical College of Pennsylvania, 2900 Queen Lane, Philadelphia, PA 19129. Phone: 215-991-8374. Fax: 215-848-2271.

TABLE 1. Activities of benzimidazole derivatives and amphotericin B against in vitro growth of *C. neoformans* isolates

Drug	IC (μg/ml) for indicated isolate(s)							
	IU4		IU8698		ATCC 14116		10 additional isolates	
	50%	90%	50%	90%	50%	90%	50%	90%
Fenbendazole	0.019	0.028	0.011	0.014	<0.016	<0.016	0.012–0.024	0.015–0.033
Nocodazole	0.13	0.22	0.09	0.12	0.20	0.29		
Parbendazole	0.16	0.23	0.16	0.23	0.16	0.22		
Mebendazole	0.23	0.43	0.18	0.32	0.19	0.40	0.12–0.31	0.22–0.45
Albendazole	0.16	0.25	0.31	0.45	0.30	0.45	0.12–0.18	0.22–0.45
Oxibendazole	2.3	3.2	1.0	2.1	1.4	2.1		
Carbendazim	2.0	3.6	1.5	2.0	1.6	2.3		
Benomyl	2.3	3.7	2.8	3.8	2.0	3.5		
Oxfendazole	2.5	>4.0	3.2	>4.0	2.8	4.0		
Thiabendazole	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0		
Benzimidazole	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0		
Amphotericin B	0.035	0.064	0.024	0.065	0.011	0.045		

μg/ml. This was twofold more active than amphotericin B. For the benzimidazoles, the slopes of growth inhibition versus drug concentration were especially steep. Consequently, in terms of IC_{90}s, fenbendazole was at least threefold more active than amphotericin B (Table 1).

Two additional yeast species were tested for susceptibility to the 10 benzimidazole derivatives. *Saccharomyces cerevisiae* W303-1A was moderately susceptible only to benomyl, carbendazim, and the antitumor agent nocodazole (IC_{50} = 0.5 to 2 μg/ml). *Candida albicans* ATCC 24433 was resistant to all derivatives at the highest concentration tested (4 μg/ml), in agreement with previous studies (10, 14).

Next, 10 additional clinical isolates of *C. neoformans* were tested for susceptibility to the highly active fenbendazole and the moderately active mebendazole and albendazole. IC_{50}s and IC_{90}s were similar for all 10 isolates to those presented above and varied over a narrow range (Table 1).

In addition to inhibiting growth, the ability of antimicrobial agents to kill their target organism is important, especially in the treatment of immunocompromised patients. Following incubation for 20 h in the presence of drug as described above, lethal activity was determined by plating 250 μl of the cultures on drug-free YEPD agar (25 ml); control experiments indicated that there was no carryover effect on plating efficiency following this 100-fold dilution of drug. Plates were incubated at 30°C for 72 h, and colonies were counted. The LC_{90} was defined as the drug concentration that reduced colony counts by 90% compared with the initial inoculum. For the two *C. neoformans* isolates tested, the LC_{90}s of albendazole and mebendazole were 0.92 and 2.1 μg/ml (Table 2). The LC_{90}s of fenbendazole were 15- to 30-fold lower (0.06 and 0.07 μg/ml); these are also 8- to 9-fold lower than the values obtained for amphotericin B. Higher levels of killing (99 to 99.9%) were observed with all three benzimidazoles at concentrations about twofold higher than the LC_{90}s given above; however, the determination of killing at those levels was less reproducible.

TABLE 2. In vitro lethal activities of selected benzimidazole derivatives and amphotericin B against two *C. neoformans* isolates

Isolate	LC_{90} (μg/ml) of:			
	Febendazole	Albendazole	Mebendazole	Amphotericin B
1KR	0.06	0.92	1.3	0.51
14116	0.07	2.1	2.0	0.54

Since the clinical usefulness of a drug may be limited by the frequency at which spontaneous resistance occurs, this frequency was estimated in vitro. Drug-containing YEPD agar plates were inoculated with 10^7 (albendazole and mebendazole) or 10^8 (fenbendazole) cells from a fresh overnight culture. For two *C. neoformans* isolates, colony formation on plates containing 5 μg of albendazole or mebendazole per ml occurred at a frequency of $\leq 3 \times 10^{-6}$. Colony formation on plates containing 1 μg of fenbendazole per ml occurred at a frequency of only about 5×10^{-9} (one colony on two plates). IC_{50}s were increased only 1.9-fold for three random mutants obtained from albendazole plates and 15-fold (to 0.18 μg/ml) for the mutant obtained from a fenbendazole plate.

In conclusion, certain anthelmintic benzimidazole carbamates have high inhibitory and lethal activities against *C. neoformans* in vitro. In agreement with earlier studies (10, 14), *C. neoformans* is relatively resistant to thiabendazole, the first benzimidazole developed for clinical use. This may have discouraged further testing of this drug group against this organism, although in retrospect this appears unwarranted since thiabendazole is structurally distinct from the carbamate derivatives. The basis for the anticryptococcal activities of anthelmintic benzimidazoles is not yet known but presumably resides within the microtubule components of this organism. Fungal mutants resistant to benomyl and thiabendazole have been isolated and shown to have altered β-tubulin, one of the major components of microtubules (7). Characterization of *C. neoformans* mutants resistant to fenbendazole, albendazole, and mebendazole is under way. This should help resolve the basis for the selective toxicity of these clinically important derivatives.

Our results suggest that benzimidazole activity against other pathogenic fungi should be reexamined. In all likelihood, the innate resistance of *C. albicans* that was recognized early on (10, 14), and which we confirmed above, discouraged further studies of benzimidazoles as antifungal agents. The basis for this resistance may be drug efflux, since a *C. albicans* gene conferring benomyl (and methotrexate) resistance on *S. cer-*

evisiae encodes an apparent homolog of multiple drug resistance transport proteins (3, 12). Nevertheless, fungal pathogens reported to be susceptible (MIC $\leq$ 2 μg/ml) to the anthelmintic agents thiabendazole and parbendazole or the agricultural agent benomyl include *Aspergillus* spp., *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis*, *P. carinii*, and various dermatophytes (1, 10, 14, 16). The testing of additional derivatives and the search for specific antifungal derivatives would appear to be justified.

Finally, the potential use of benzimidazoles as therapeutic agents for cryptococcal meningitis warrants consideration. Benzimidazoles are normally administered orally, an advantage over amphotericin B; however, they vary in their intestinal absorption (17). Mebendazole is poorly absorbed. Levels of fenbendazole in serum ranging from 0.2 to 4 μg/ml have been reported, depending on the animal and the dose used. These values exceed significantly both the IC_{90} and the LC_{90} of this derivative determined in vitro. In humans with neurocysticercosis, an oral dose of 15 mg/kg resulted in albendazole levels in cerebrospinal fluid of about 0.4 μg/ml (6), exceeding the IC_{90}s for most of the *C. neoformans* strains tested here. Therefore, our in vitro results would appear to justify the testing of benzimidazole derivatives in animal models of cryptococcosis.

This study was supported in part by Public Health Service grant AI-32433 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Bartlett, M. S., T. D. Edlind, M. M. Durkin, M. M. Shaw, S. F. Queener, and J. W. Smith.** 1992. Antimicrotubule benzimidazoles inhibit in vitro growth of *Pneumocystis carinii*. Antimicrob. Agents Chemother. **36**:779–782.
2. **Edlind, T. D., T. L. Hong, and P. R. Chakraborty.** 1990. Activity of the anthelmintic benzimidazoles against *Giardia lamblia* in vitro. J. Infect. Dis. **162**:1408–1411.
3. **Fling, M. E., J. Kopf, A. Tamarkin, J. A. Gorman, H. A. Smith, and Y. Koltin.** 1991. Analysis of a *Candida albicans* gene that encodes a novel mechanism for resistance to benomyl and methotrexate. Mol. Gen. Genet. **227**:318–329.
4. **Hay, R. J.** 1991. Clinical manifestations and management of cryptococcosis in the compromised patient, p. 85–115. *In* D. W. Warnock and M. D. Richardson (ed.), Fungal infection in the compromised patient, 2nd ed. John Wiley & Sons, New York.
5. **Juliano, C., M. G. Martinotti, and P. Cappuccinelli.** 1985. In vitro effect of microtubule inhibitors on *Trichomonas vaginalis*. Microbiologica **8**:31–42.
6. **Jung, H., M. Hurtado, M. Sanchez, M. T. Medina, and J. Sotelo.** 1990. Plasma and CSF levels of albendazole and praziquantel in patients with neurocysticercosis. Clin. Neuropharmacol. **13**:559–564.
7. **Jung, M. K., I. B. Wilder, and B. R. Oakley.** 1992. Amino acid alterations in the benA (beta-tubulin) gene of *Aspergillus nidulans* that confer benomyl resistance. Cell Motil. Cytoskeleton **22**:170–174.
8. **Kovacs, J. A., A. A. Kovacs, M. Polis, W. C. Wright, V. J. Gill, C. U. Tuazon, E. P. Gelman, C. Lane, R. Longfield, G. Overturf, A. M. Macher, A. S. Fauci, J. E. Parillo, J. E. Bennett, and H. Masur.** 1985. Cryptococcosis in the acquired immunodeficiency syndrome. Ann. Intern. Med. **103**:533–538.
9. **Lacey, E.** 1988. The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of resistance to benzimidazoles. Int. J. Parasitol. **18**:885–936.
10. **Maxwell, W. A., and G. Brody.** 1971. Antifungal activity of selected benzimidazole compounds. Appl. Microbiol. **21**:944–945.
11. **Panther, L. A., and M. A. Sande.** 1990. Cryptococcal meningitis in the acquired immunodeficiency syndrome. Semin. Respir. Infect. **5**:138–145.
12. **Paulsen, I. T., and R. A. Skurray.** 1993. Topology, structure and evolution of two families of proteins involved in antibiotic resistance in eukaryotes and prokaryotes—an analysis. Gene **124**:1–11.
13. **Powderly, W. G.** 1992. Therapy for cryptococcal meningitis in patients with AIDS. Clin. Infect. Dis. **14**:S54–S59.
14. **Robinson, H. J., R. H. Silber, and O. E. Graessle.** 1969. Thiabendazole: toxicological, pharmacological and antifungal properties. Tex. Rep. Biol. Med. **27**(Suppl. 2):537–560.
15. **Sugar, A. M., J. J. Stern, and B. Dupont.** 1990. Overview: treatment of cryptococcal meningitis. Rev. Infect. Dis. **12**:S338–S348.
16. **Summerbell, R. C.** 1993. The benomyl test as a fundamental diagnostic method for medical mycology. J. Clin. Microbiol. **31**:572–577.
17. **Van Den Bossche, H., F. Rochette, and C. Hörig.** 1982. Mebendazole and related antihelmintics. Adv. Pharmacol. Chemother. **19**:67–128.