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In Vitro Antifungal Activity of Clotrimazole (Bay b 5097)

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The in vitro antifungal activity of clotrimazole (Bay b 5097) was compared with those of amphotericin B, griseofulvin, nystatin, and pyrrolnitrin. The inhibitory activity of clotrimazole against most systemic pathogens was comparable to that of amphotericin B; minimal inhibitory concentrations of the two drugs for *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Sporothrix schenckii*, *Cryptococcus neoformans*, and *Coccidioides immitis* were in the range of 0.20 to 3.13 and 0.10 to 6.25 μg/ml, respectively. One isolate of *Allescheria boydii* was resistant to 100 μg of amphotericin B per ml but was inhibited by 6.25 μg of clotrimazole per ml. Clotrimazole was less active than amphotericin B against *Candida albicans* and *Aspergillus fumigatus*. The activity of clotrimazole against dermatophytes was comparable to that of pyrrolnitrin; 0.78 μg of either compound per ml was fungicidal for most isolates of *Trichophyton* sp., *Microsporum* sp. and *Epidermophyton floccosum*. Both griseofulvin and nystatin were less active than clotrimazole. The size of inoculum was shown to have a significant effect on the results of in vitro susceptibility testing with clotrimazole.

Clotrimazole, bis-phenyl-(2-chlorophenyl)-1-imidazolyl methane (Bay b 5097, Delbay Pharmaceuticals), is a chlorinated trityl imidazolyl with unique antifungal activity. Its in vitro spectrum includes yeasts, dermatophytes, dimorphic fungi, and dematiaceous species (4). Plempel et al. (4, 5) reported that it was inhibitory in vitro at concentrations of 4 μg or less per ml for most susceptible fungi and that many species, particularly *Trichophyton* and *Candida*, were inhibited by 1 μg/ml or less. However, it was said to be fungicidal only at concentrations greater than 20 μg/ml (4).

Published data regarding the clinical effectiveness of clotrimazole are limited. In one report, it was described as being effective in one patient with a pulmonary aspergilloma, in another with bronchial infection due to *Candida krusei*, and in a third with tinea barbae due to *C. albicans* (3). According to another report (2), it was effective in treatment of candidiasis in a limited number of pediatric patients. Unpublished data from clinical studies in 87 adults indicated that it was effective in approximately 80% of patients with acute or systemic candidiasis including septicemia, pneumonia, endocarditis, and renal infection. It also was described as being equally effective in dermatophytic infections but less effective or inactive in a small number of cases of pulmonary aspergillosis and chromoblastomycosis (A. Freis, IX Intersci. Conf. Antimicrob. Ag. Chemother., 1969).

The reported in vitro and in vivo activity of clotrimazole together with the initial clinical results stimulated much interest. A series of studies was initiated to define further the antifungal properties of this compound and to assess its potential in treatment of human mycotic disease. In the studies to be reported here, the in vitro activity of clotrimazole against systemic, dermatophytic, and opportunistic pathogenic fungi was compared with those of amphotericin B, griseofulvin, pyrrolnitrin, and nystatin.

MATERIALS AND METHODS

Cultures. Sixty isolates of pathogenic fungi were tested. These included *Allescheria boydii*, *Aspergillus fumigatus*, *Blastomyces dermatitidis*, *C. albicans*, *Cryptococcus neoformans*, *Coccidioides immitis*, *Epidermophyton floccosum*, *Histoplasma capsulatum*, *Madurella grisea*, *Microsporum gypseum*, *Microsporum ferrugineum*, *Microsporum fulvum*, *Microsporum canis*, *Sporothrix schenckii*, and various *Trichophyton* species. Tests were also performed with two standard bioassay organisms, *Saccharomyces cerevisiae* ATCC 9763 and *Paecilomyces variotii* MSCC 5605. All of the systemic and opportunistic pathogens, with the exception of *M. grisea*, *C. immitis*, and *A. fumigatus*, were isolated and identified in our laboratory. The

dermatophytes included both isolates recovered in our laboratory and cultures obtained from the Center for Disease Control, Atlanta, Ga.

Inocula used in susceptibility tests were prepared by harvesting mature or sporulating cultures with sterile saline. Suspensions of filamentous species were adjusted to a standard transmission of 85% measured at 530 nm, whereas those of yeastlike organisms were adjusted to 90%.

Media. Stock cultures were maintained on either modified Sabouraud agar (Difco) or on Sabhi agar (Difco).

In studies with filamentous species and mycelial phase cultures of dimorphic fungi, dilutions of drugs were prepared in sterile, modified, double-strength Brain Heart Infusion Broth (2X-BHIB/m) with the following composition (g/liter): BHIB (Difco), 18.5; neopeptone, 5; dextrose, 20. Serial dilutions of drugs were prepared at twice the final test concentrations in 2X-BHIB/m, and then diluted 1:1 with sterile, modified, Sabhi agar having the following composition (g/liter): Sabhi agar base, 29.5; BHIB, 18.5; neopeptone, 2.5; dextrose, 10. After mixing, the combined media were equivalent to single strength Sabhi base but with 0.38% agar.

Studies with *C. neoformans*, *C. albicans*, and *S. cerevisiae* were performed in Sabouraud broth (Difco).

Drug solutions. Five antifungal compounds were tested. These included amphotericin B (code 22-380, batch 38675-001, potency of 904 μg/ml, Squibb Institute for Medical Research); clotrimazole (Bay b 5097, Sch. 15335L, batch 1507/69, Delbay Pharmaceuticals); griseofulvin, "microsize" (B no. 1383LB, Ayerst Laboratories, Inc.); nystatin (code 1898A, batch 49921-002, potency of 4,300 units/mg, Squibb Institute for Medical Research); and pyrrolnitrin (preparation 182-414-008, Lilly Research Laboratories).

Stock solutions of amphotericin B, clotrimazole, and nystatin were prepared by dissolving 10.00 mg of drug, or its equivalent in active material, in 1 ml of 100% dimethylsulfoxide (DMSO). These solutions were then diluted in sterile broth to a concentration of 2,000 μg/ml. Griseofulvin was put into solution by dissolving 5.00 mg of drug in 5 ml of acetone; this solution was then diluted with an equal volume of water and then in broth to give a solution containing 100 μg/ml. Pyrrolnitrin was first dissolved in absolute alcohol and then diluted with sterile water to a concentration of 1,000 μg/ml.

Susceptibility testing. In studies with filamentous fungi, twofold serial dilutions of the test drugs were prepared in 2X-BHIB/m in sufficient amounts to provide 3 ml per dilution for each test organism. Solutions of amphotericin B, clotrimazole, and nystatin were first diluted 1:10 in broth containing 2% DMSO. The concentrations of the dilution serials for amphotericin B, clotrimazole, nystatin, and pyrrolnitrin ranged from 200 to 0.10 μg/ml; the range for griseofulvin was 100 to 0.05 μg/ml. The dilutions of drug in 2X-BHIB/m were then mixed in equal volumes with modified, half-strength Sabhi agar and dispensed, after mixing, in 2.0-ml volumes. The concentrations of the drugs in the finished semisolid media ranged from 100 to 0.05 μg/ml for all drugs except griseofulvin for which they ranged from 50 to 0.025 μg/ml.

Only amphotericin B and clotrimazole were tested in studies with yeastlike organisms. Stock solutions of the two drugs were first diluted in broth to a concentration of 100 μg/ml and then serially diluted in broth. Final concentrations ranged from 100 to 0.05 μg/ml. The drug dilutions were dispensed in 2.0-ml volumes with three tubes per dilution for each test organism.

Tubes of semisolid medium were inoculated by dropping 0.03- to 0.05-ml volumes of the prepared inocula onto the surface of the hardened medium. Tubes containing liquid medium were inoculated by introducing 0.05-ml volumes of inoculum directly into the broth. Three tubes were inoculated per each dilution of drug or control for each organism. Controls included uninoculated sterility controls, drug and solvent-free growth controls, and growth controls containing 1.0% of the appropriate solvent.

Inoculated tubes were incubated at 30 C until growth appeared in the growth control tubes. The minimum time of incubation was 48 hr. After determination of the minimal inhibitory concentrations, 0.05-ml volumes of agar or of broth were transferred from all tubes showing no growth and from the first tubes in which growth was detectable to plates of Sabouraud agar. These were incubated at 30 C for 72 hr or until growth was apparent on those areas inoculated from tubes containing visible growth.

The following criteria were employed in recording and evaluating the results. When tubes were examined after the initial incubation period, they were scored as negative, partially inhibited, and positive. The lowest concentration of drug which produced complete inhibition in at least two of three tubes was regarded as the minimal inhibitory concentration (MIC). When the plates were examined after the second incubation period, they were scored as negative, three colonies or less, and positive. The lowest concentration of drug for which subcultures from at least two of three tubes were negative or grew no more than three distinct colonies was regarded as the minimal fungicidal concentration (MFC).

RESULTS

The in vitro activity of clotrimazole against yeastlike pathogens and mycelial phase cultures of the dimorphic systemic pathogens was generally comparable to that of amphotericin B (Table 1). With the exception of the single isolate of *A. boydii* which was resistant to amphotericin B, all species were inhibited by both drugs.

Most isolates of *H. capsulatum*, *C. neoformans*, and *C. immitis* were inhibited by either drug at concentrations of 0.39 μg/ml or less. One isolate of *H. capsulatum* was not inhibited by less than 1.56 μg of amphotericin B per ml, whereas one of *C. neoformans* was resistant to less than 0.78 μg of clotrimazole per ml. Fungicidal activities of the two drugs against *H. capsulatum* and *C.*

TABLE 1. *In vitro susceptibility of systemic mycotic pathogens to clotrimazole and amphotericin B*

Test organisms	No. tested	Susceptibility to			
		Clotrimazole		Amphotericin B	
		MIC[a]	MFC[a]	MIC	MFC
Blastomyces dermatitidis[b]	5	0.78–3.13 (0.78)	0.78–>100 (1.56)	<0.05–0.39 (0.10)	0.39–1.56 (0.78)
Histoplasma capsulatum	4	0.10–0.39 (0.20)	0.10–0.39 (0.20)	0.20–1.56 (0.20)	0.20–1.56 (0.20)
Sporothrix schenckii	5	3.13–6.25 (3.13)	6.25–25 (6.25)	6.25–12.5 (6.25)	12.5–25 (25)
Cryptococcus neoformans[c]	3	0.20–0.78 (0.20)	6.25–25 (12.5)	0.20–0.39 (0.20)	0.39–0.78 (0.39)
Coccidioides immitis	3	0.10 (0.10)	0.10–1.56 (0.39)	0.20–0.39 (0.39)	0.39–0.78 (0.39)
Allescheria boydii	1	6.25	>100	>100	>100
Madurella grisea	1	0.39	0.39	0.20	1.56

[a] Minimal inhibitory (MIC) and fungicidal (MFC) concentrations, micrograms per milliliter, measured after 48 to 96 hr of incubation at 30 C with subcultures on Sabouraud agar incubated an additional 49 to 96 hr. Values in parentheses represent median.

[b] Dimorphic fungi tested in the vegetative phase.

[c] Tests with *C. neoformans* were performed in Sabouraud broth.

immitis were comparable, with 0.78 μg of either drug per ml being fungicidal for all but two isolates. These included the less susceptible isolate of *H. capsulatum* noted above and one isolate of *C. immitis* which was killed by amphotericin B at 1.56 μg/ml and by clotrimazole at 0.78 μg/ml. None of the three strains of *C. neoformans* was killed by clotrimazole at concentrations less than 6.25 μg/ml, whereas all three were killed by 0.78 μg of amphotericin B per ml.

Clotrimazole was both inhibitory and fungicidal for *B. dermatitidis* but at concentrations somewhat higher than those required for amphotericin B. Four of the five strains tested were inhibited by 0.78 μg of clotrimazole per ml, and the fifth was inhibited by 3.13 μg/ml. In contrast, four strains were inhibited by 0.20 μg or less of amphotericin B per ml and the fifth by 0.39 μg/ml. Three strains were killed by 1.56 μg or less of clotrimazole per ml, but the other two were not killed by less than 25 μg/ml. In contrast, four strains were killed by 0.78 μg of amphotericin B per ml and the fifth by 1.56 μg/ml.

S. schenckii was somewhat more susceptible to clotrimazole than to amphotericin B. However, neither drug was inhibitory at concentrations less than 3.13 μg/ml nor fungicidal at concentrations less than 6.25 μg/ml. Three strains were inhibited by clotrimazole at 3.13 μg/ml and the other two at 6.25 μg/ml. Fungicidal activity was obtained at 6.25 μg/ml with three strains, at 12.5 μg/ml with one strain, and at 25 μg/ml with the fifth. Amphotericin B was inhibitory for three strains at 6.25 and for two at 12.5 μg/ml; fungicidal activity was measured against two strains at 12.5 μg/ml and three at 25 μg/ml.

During the course of these studies, a biopsy specimen from a case of maduromycosis was received for cultural studies. This specimen yielded an isolate of *A. boydii* for which susceptibility data were obtained for clotrimazole, amphotericin B, hamycin, and 5-fluorocytosine. Clotrimazole was the only one of the four drugs active against this isolate with inhibition at 6.25 μg/ml but with no measurable fungicidal activity. Preliminary in vivo studies with this isolate have shown clotrimazole to be protective in infected mice treated orally with 100 mg/kg.

One isolate of *M. grisea* was received for susceptibility testing. It was found to be susceptible to both clotrimazole and amphotericin B at 0.39 μg/ml or less; it was also susceptible to 5-fluorocytosine, nystatin, and hamycin but to a lesser degree.

Both amphotericin B and clotrimazole were active against *C. albicans* and *A. fumigatus* (Table 2). Although the levels of activity of the two compounds against *A. fumigatus* were comparable, clotrimazole was much less active than amphotericin B against *C. albicans*. Amphotericin

TABLE 2. *In vitro susceptibility of opportunistic and bioassay organisms to clotrimazole and amphotericin B*

Test organisms	No. tested	Susceptibility to			
		Clotrimazole		Amphotericin B	
		MIC[a]	MFC[a]	MIC	MFC
Candida albicans	5	1.56–3.13 (1.56)	3.13–>100 (12.5)	0.39 (0.39)	1.56 (1.56)
Aspergillus fumigatus	3	0.39–1.56 (0.39)	3.13–>100 (6.25)	0.20–0.39 (0.39)	1.56–3.13 (3.13)
Paecilomyces variotti MSSC 5605		0.78	50	<0.05	12.5
Saccharomyces cerevisiae ATCC 9763		0.78–6.25	12.5–>100	0.10–0.39	0.78

[a] See footnote *a*, Table 1.

TABLE 3. *Effect of inoculum size on in vitro susceptibility testing with clotrimazole and Saccharomyces cerevisiae*

Approx. density of inoculum (cells/ml)	MIC[a] (μg/ml)	MFC[a] (μg/ml)
10^7	12.5	100
10^6	0.39	25
10^5	0.10	12.5
10^4	0.05	6.25
10^3	0.05	0.78
10^2	0.05	0.20

[a] Minimal inhibitory (MIC) and fungicidal (MFC) concentrations.

B was inhibitory for all eight isolates of *C. albicans* and *A. fumigatus* at a concentration of 0.39 μg/ml. In contrast, a similar concentration of clotrimazole was inhibitory for two of the three isolates of *A. fumigatus*, with the third isolate and all five isolates of *C. albicans* requiring concentrations of 1.56 μg/ml or more for inhibition. Amphotericin B was fungicidal for the five isolates of *C. albicans* and one of the three of *A. fumigatus* at 1.56 μg/ml and fungicidal for the remaining two isolates of *A. fumigatus* at 3.13 μg/ml. Fungicidal concentrations of clotrimazoles for these same organisms were higher and less constant, ranging from 3.13 to 100 μg/ml.

Neither *P. variotii* nor *S. cerevisiae* was sufficiently susceptible to clotrimazole to permit its use in bioassay procedures for this agent (Table 2).

S. cerevisiae was used as a control for testing with yeastlike fungi in these studies, and occasional fluctuations in inhibitory and fungicidal levels of clotrimazole for this organism were noted. These fluctuations were suspected to be due to variations in inoculum size. This was tested in one experiment in which inhibitory and fungicidal concentrations for *S. cerevisae* were determined by using a series of inocula of various densities. These were prepared by serial dilution of a dense suspension of *S. cerevisiae* with viable counts of each dilution being determined by a routine plate-counting procedure. The results clearly demonstrated a marked inoculum effect (Table 3). With densities of 10^4 cells per ml or less, clotrimazole was inhibitory at a concentration of 0.05 μg/ml and fungicidal at 0.20 to 6.25 μg/ml. In contrast, at a density of 10^6 cells per ml, the drug was not inhibitory at less than 0.39 μg/ml nor fungicidal at less than 25 μg/ml.

The antifungal activity of clotrimazole against 18 isolates of *Trichophyton* species was compared with those of griseofulvin, pyrrolnitrin, and nystatin (Table 4). Clotrimazole and pyrrolnitrin were the most active with 0.39 μg of either per ml being fungicidal for most isolates. Griseofulvin and nystatin were less active against most isolates of *Trichophyton*. Only six isolates were susceptible to griseofulvin at concentrations less than 0.78 μg/ml, and three were resistant to concentrations greater than 3.13 μg/ml.

The different species of *Trichophyton* varied in their susceptibility to the four compounds. *T. tonsurans* was less susceptible to clotrimazole than any of the other species, with two of these

TABLE 4. *In vitro susceptibility of Trichophyton species to four antifungal agents*

Test organisms	No. tested	Susceptibility to							
		Clotrimazole		Griseofulvin		Pyrrolnitrin		Nystatin	
		MIC[a]	MFC[a]	MIC	MFC	MIC	MFC	MIC	MFC
T. mentagrophytes	3	0.10–0.20 (0.20)	0.20–0.39 (0.20)	1.56–3.13 (3.13)	3.13 (3.13)	<0.05–0.10 (0.10)	0.05–0.20 (0.10)	3.13–25 (12.5)	3.13–25 (12.5)
T. rubrum	5	<0.05–0.39 (0.39)	0.05–0.39 (0.39)	0.20–0.78 (0.39)	0.39–0.78 (0.39)	0.10–0.39 (0.10)	0.05–0.78 (0.10)	0.39–1.56 (1.56)	0.39–1.56 (1.56)
T. tonsurans	3	0.05–1.56 (0.78)	0.05–1.56 (0.78)	0.10–1.56 (0.39)	0.20–0.78 (0.39)	<0.05–3.13 (0.10)	<0.05–0.78 (0.10)	0.20–3.13 (1.56)	0.20–3.13 (1.56)
T. schoenleini	3	0.10–0.20 (0.20)	0.10–0.20 (0.20)	0.78–25.0 (0.78)	0.39–25.0 (0.78)	<0.05–0.39 (0.20)	<0.05–0.39 (0.20)	0.39–1.56 (1.56	0.39–1.56 (1.56)
T. verrucosum	2	0.10–0.20	0.20	0.78	0.78	0.39	0.39	1.56–12.5	1.56–12.5
T. violaceum	1	0.10	0.20	0.10	0.20	0.10	0.20	0.78	0.78
T. concentricum	1	0.10	0.10	0.78	0.78	<0.05	0.05	0.78	0.78

[a] Minimal inhibitory (MIC) and fungicidal (MFC) concentrations, micrograms per milliliter, measured after 48 to 96 hr of incubation at 30 C with subcultures on Sabouraud agar incubated an additional 48 hr. Values in parentheses represent median.

TABLE 5. *In vitro susceptibity of Microsporum and Epidermophyton sp. to four antifungal agents*

Test organisms	No. tested	Susceptibility to							
		Clotrimazole		Griseofulvin		Pyrrolnitrin		Nystatin	
		MIC[a]	MFC[a]	MIC	MFC	MIC	MFC	MIC	MFC
M. canis	3	<0.05–0.10 (<0.05)	0.10–0.39 (0.39)	0.39–0.78 (0.78)	0.39–1.56 (0.78)	<0.05–0.20 (0.10)	0.10–0.20 (0.10)	0.78–1.56 (0.78)	0.78–1.56 (0.78)
M. fulvum	2	0.39–0.78	0.39–0.78	3.13	3.13	0.20–0.39	0.20–0.39	25.0	25.0
M. gypseum	3	0.10–0.39 (0.20)	0.10–0.78 (0.20)	0.78–3.13 (3.13)	0.78–3.13 (3.13)	<0.05–0.10 (0.10)	0.10–6.25 (0.39)	0.39–1.56 (0.78)	0.78–1.56 (0.78)
M. ferrugineum	1	0.05	0.05	0.20	0.20	1.56	1.56	0.39	0.39
E. floccosum	3	0.20 (0.20)	0.20–0.39 (0.20)	0.20–1.56 (0.78)	0.20–1.56 (0.78)	<0.05 (<0.05)	<0.05–0.10 (<0.05)	0.78–12.5 (1.56)	0.78–12.5 (1.56)

[a] See footnote *a*, Table 1.

isolates requiring 0.78 μg/ml or more for inhibition. In contrast, all seven faviform species were inhibited by 0.20 μg/ml. Three isolates of *T. mentagrophytes* were less susceptible to both griseofulvin and nystatin; two required 3.13 μg of the former drug per ml, and two required 12.50 μg or more of the latter per ml for inhibition. Similarly, several of the faviform isolates also were somewhat more resistant to these two drugs. One isolate of *T. schoenleini* was resistant to griseofulvin at concentrations less than 25 μg/ml, and one isolate of *T. verrucosum* was resistant to nystatin at concentrations less than 12.5 μg/ml.

Clotrimazole was the most active of the four drugs against species of *Microsporum*, and griseofulvin was the least active (Table 5). At a concentration of 0.39 μg/ml, clotrimazole was inhibitory for eight of nine isolates and fungicidal for seven. Griseofulvin was fungicidal for two isolates at this same concentration but required 3.13 μg/ml for either inhibition or killing of the other isolates. Pyrrolnitrin was the second most active drug, with seven isolates being inhibited and five being killed by concentrations less than 0.39 μg/ml. Only two isolates were susceptible to nystatin at this concentration.

Pyrrolnitrin was the most active compound against *E. floccosum* with 0.10 μg/ml being fungicidal for all three isolates tested (Table 5). Clotrimazole was slightly less active, with all three isolates being inhibited and two being killed at 0.20 μg/ml. Griseofulvin was fungicidal at 1.56 μg/ml. Nystatin was the least active with MIC and MFC values ranging from 0.78 to 12.5 μg/ml.

DISCUSSION

The results presented here confirm the earlier reports of Plempel et al. (4, 5) regarding the in vitro activity of clotrimazole against pathogenic fungi. This compound is, indeed, a "broad spectrum" antifungal agent; its inhibitory action against systemic and opportunistic pathogenic fungi compares favorably with that of amphotericin B. Minimal inhibitory concentrations for many of the fungi are within the range of reported blood levels in man. The only exceptions to this are *S. schenckii* and *A. boydii* which are inhibited only at concentrations of 3.13 μg or more per ml. In a recent study, single 40 mg/kg oral doses produced maximum serum levels of 2.5 μg/ml or more 4 to 6 hr postingestion (F. Falco, *personal communication*), whereas in an earlier study levels of 2.9 to 7 μg/ml were reported in individuals receiving 20 mg/kg three times daily (4).

The activity of clotrimazole against dermatophytes is comparable to that of pyrrolnitrin and superior to that of either nystatin or griseofulvin. More importantly, the ranges of inhibitory and fungicidal concentrations are narrow with little deviation either within or between species. In contrast, griseofulvin is inhibitory for *T. rubrum* at 0.20 μg/ml but not for *T. mentagrophytes* at concentrations less than 3.13 μg/ml. The data for griseofulvin are similar to those reported by Åberg and Thyresson (1), who demonstrated a positive correlation between speciation of *T. rubrum* and *T. mentagrophytes* on the basis of hair penetration vis a vis susceptibility to griseofulvin.

Clotrimazole has certain desirable properties as an antifungal agent. It is soluble in alcohols, stable to heat and moderate light, stable in alkaline solutions, quickly absorbed after oral administration and well distributed in tissues, devoid of toxicity in man at doses of 20 mg/kg three times daily, and active and well tolerated topically in animals. These properties combined with the broad spectrum of fungi affected suggest that it may be effective in treatment of human fungal disease. Such a suggestion, however, requires further clinical studies with this compound.

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