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Cilofungin (LY121019), an Antifungal Agent with Specific Activity against *Candida albicans* and *Candida tropicalis*

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Cilofungin (LY121019) is an antifungal agent that interferes with β-glucan synthesis in the cells walls of fungi. The activity of this agent against 256 clinical isolates of yeasts was determined. It was found to be very active in vitro against *Candida albicans* (MIC for 90% of isolates [MIC_{90}], ≤0.31 μg/ml; minimal fungicidal concentration for 90% of isolates [MFC_{90}], ≤0.31 μg/ml) and *C. tropicalis* (MIC_{90}, ≤0.31 μg/ml; MFC_{90}, ≤0.31 μg/ml) and moderately active against *Torulopsis galbrata* (MIC_{90} and MFC_{90}, ≤20 μg/ml). All *C. parapsilosis*, *Cryptococcus*, and *Saccharomyces cerevisiae* strains were resistant. The activity of cilofungin was affected by medium and inoculum size. Antibiotic medium no. 3 was used as the standard medium. Isolates of *C. albicans* and *C. tropicalis* demonstrated a paradoxical effect in Sabouraud dextrose broth and yeast nitrogen base broth in that growth was partially inhibited at MICs equivalent to those in antibiotic medium no. 3, but growth continued, in many instances, throughout all concentrations tested. There was decreased activity of cilofungin with inocula > 10^5 CFU/ml. The temperature and duration of incubation did not affect its activity.

Treatment of local and systemic fungal diseases is limited to relatively few agents. Amphotericin B, a polyene antibiotic, has been the mainstay therapy, but its associated toxicity often makes it a less than desirable choice. Flucytosine, a fluorinated pyrimidine, cannot be used singly to treat fungal disease, since development of resistance is common with most fungal species. The imidazoles miconazole and ketoconazole act similarly to amphotericin B in affecting the permeability of fungal cell membranes. Less toxicity is associated with their use; however, they may be limited to use in certain fungal diseases. A wide variety of newer antifungal agents have been tested, mainly in vitro, with varying degrees of success. Some, such as the nikkomycins (10, 18) and polyoxins (1, 14), inhibit chitin synthesis; the allylamines inhibit ergosterol synthesis in the cell membrane, thus affecting permeability. Examples of the allylamines are naftitine (15) and tolnaftate (8). The papulacandins (11), aculeacins (13), and echinocandins (4, 16) inhibit β-glucan synthesis in the cell walls of certain fungi. In metabolizing yeast cells, this inhibition results in the formation of protoplasts and spheroplasts, which may then lyse, depending on the osmotic pressure of their external environment. No effects are seen in stationary phase cells.

Cilofungin (LY121019; *N-p*-octyloxybenzoyl-echinocandin-B) is an analog of echinocandin-B and, when compared with over 100 other analogs, has demonstrated increased efficacy and decreased toxicity. Gordee et al. (9) have reported a narrow spectrum of activity for cilofungin. The MICs for 50% and 90% of isolates (MIC_{50} and MIC_{90}, respectively) for 96 isolates of *Candida albicans* were 0.625 and 1.25 μg/ml, respectively, with a minimum fungicidal concentration for 90% of isolates (MFC_{90}) of ≤0.31 μg/ml for one quality control strain of *C. albicans*. *C. tropicalis* responded similarly. For other yeasts, such as *Cryptococcus* sp. and *Saccharomyces* sp., MIC_{90}s were >5 μg/ml. No activity was demonstrated against *Aspergillus* sp. (4). The toxicity of cilofungin was shown to be 20-fold less than that of amphotericin B when tested in dogs (9).

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The purpose of this study was to compare the in vitro activity of cilofungin, amphotericin B, flucytosine, and ketoconazole against a variety of yeasts; to determine both the fungistatic and fungicidal action of cilofungin; and to determine the effects of several variables, including media, temperature, and length of incubation, on the activity of cilofungin.

MATERIALS AND METHODS

Antifungal agents. Cilofungin powder was supplied by Eli Lilly & Co., Indianapolis, Ind., and kept at −70°C prior to use. The powder was dissolved in 95% ethanol at a concentration of 1,000 μg/ml and diluted for use at concentrations ranging from 0.04 to 40 μg/ml. Amphotericin B was obtained from E. R. Squibb & Sons, Princeton, N.J., and kept at −70°C. The powder was dissolved in dimethyl sulfoxide and diluted for use in concentrations ranging from 0.015 to 32 μg/ml. Flucytosine was obtained from Sigma Chemical Co., St. Louis, Mo., and kept at 4°C in a desiccator prior to use. It was dissolved in water at 1,280 μg/ml; dilutions thereof were prepared to give final concentrations ranging from 0.6 to 128 μg/ml. Ketoconazole was obtained from USP and stored at 4°C before use. The powder was dissolved in water and 0.2 N HCl to 1,280 μg/ml and further diluted for use as concentrations ranging from 0.25 to 32 μg/ml.

Organisms. The isolates were obtained from clinical specimens processed in the Mycology Laboratory of the Cleveland Clinic Foundation. All isolates had been stocked in 200-μl aliquots of sterile sheep blood and kept frozen at −70°C. For use, approximately 10 μl of the stock culture was plated out onto potato dextrose agar (PDA) and incubated for 48 h, and then a colony was transferred to another PDA plate and incubated for 24 h. The latter served as the source of the inoculum for most of the studies. All yeast isolates had been identified in the Mycology Laboratory by the Quantum Identification System (Abbott Laboratories, North Chicago, Ill.) and/or the API Yeast Identification System (Analytab Products, Plainview, N.Y.).

Media. Cilofungin was tested in three different media: antibiotic medium no. 3 (A-3; Difco Laboratories, Detroit,

Mich.) Sabouraud dextrose broth (SAB), and yeast nitrogen broth (YNB). A-3 was used as the reference medium. It consists of beef extract, yeast extract, peptone, glucose, NaCl, and both mono- and dipotassium phosphates (pH 6.8). SAB consists of 1% neopeptone (Difco Laboratories, Detroit, Mich.) and 2% dextrose (pH 5.5). YNB is a synthetic, defined medium composed of amino acids, vitamins, and salts. Asparagine and glucose were added to the basal medium (pH 6.9). Other antifungal agents tested were amphotericin B (in A-3), ketoconazole (in YNB), and flucytosine (in YNB).

Susceptibility testing procedure. The inoculum of each isolate was prepared from the 24-h PDA transfer plate. A suspension equal in density to a McFarland no. 4 standard was prepared in 8 ml of sterile saline and added to 16 ml of sterile water. This suspension was the inoculum. Microdilution trays containing the antimicrobial agents were stored at −70°C. On the day of the experiment, the trays were removed, thawed at room temperature for approximately 1 h, and inoculated by means of a Dynatech Inoculator (Dynatech Industries, Inc., McLean, Va.). The volume delivered was 0.001 ml of the above inoculum. Within 30 min of inoculation, a 0.001-ml calibrated loop was used to subculture the growth control well (after mixing) onto a PDA plate, which was incubated for 48 h. Colonies were counted to determine the initial inoculum size in each tray.

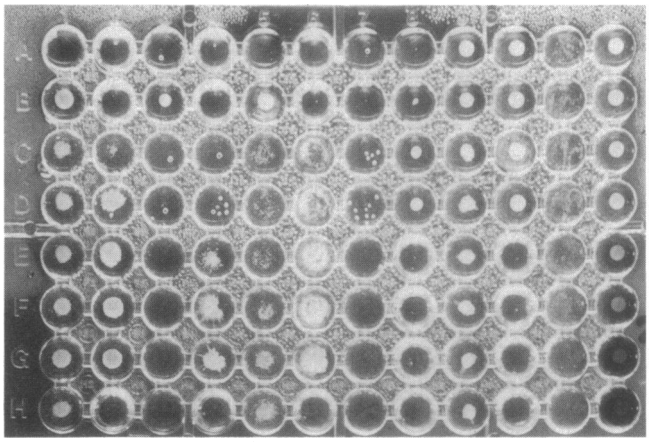
The microdilution trays were incubated for 48 h at 30°C. The MICs were determined by observing the wells for growth or evidence of turbidity. A comparison of the amount of growth was also made with the growth control well for each medium tested. Two endpoints were recorded as follows: a partial inhibition endpoint was recorded as the first well in which there was a decrease in the turbidity and/or growth pattern as compared with the growth control well; the complete inhibition endpoint was defined as the lowest concentration with no visible turbidity, particulate matter, or pellet.

The minimum fungicidal concentration (MFC) of cilofungin was determined by subculturing 0.1 ml from the first well demonstrating complete growth inhibition and from all wells that had no visible growth onto PDA plates that were incubated at 30°C for 48 h. Colonies were counted, and the MFC was defined as the lowest concentration at which 99% of the initial inoculum was killed.

Fifty isolates were tested in cilofungin under variable conditions: two microdilution trays were inoculated as described above; one was incubated at 35°C, and the other was incubated at 30°C. These trays were read at 24, 48, and 72 h. Additional trays were inoculated with various inocula of the same isolates: 10^4, 10^5, and 10^6 CFU/ml. The inocula of 10^4 CFU/ml were also prepared from two different sources: a 24-h PDA plate and a 24-h SAB culture. These plates were incubated at 30°C for 48 h. Quality control was performed each day by using a reference organism, *C. albicans* A-26, which was provided by Eli Lilly. The MIC of cilofungin was 0.156 to 0.312 μg/ml.

RESULTS

Of the 256 isolates tested, 164 (64%) were *C. albicans* or *C. tropicalis*. Another 28% (72 of 256) were other *Candida* or *Torulopsis* spp. The remaining 8% were *Cryptococcus* or *Saccharomyces* spp. Fifty-seven percent (147 of 256) of all isolates were from blood. Other sources included cerebrospinal fluid, peritoneal fluid, tissues such as kidney and lung, and urine.



	SAB 1	2	ANTI-3 3	4	YNB 5	6	7	8	9	10	11	12
A	S				S		0.25	0.125	1	0.5	0.25	6.25
B	G		G		G		0.5	0.06	2	0.25	0.5	12.5
C	1.25	0.625	1.25	0.625	1.25	0.025	1	0.03	4	0.125	1	25
D	2.5	0.312	2.5	0.312	2.5	0.312	2	0.015	8	0.06	2	50
E	5	0.156	5	0.156	5	0.156	4		16		4	150
F	10	0.078	10	0.078	10	0.078	8		32		8	200
G	20	0.039	20	0.039	20	0.039	16		64		16	400
H	40		40		40		32		132		32	800
	LY SAB	LY SAB	LY ANT-3	LY ANT-3	LY YNB	LY YNB	AMP ANT-3	AMP ANT-3	5-FLU YNB	5-FLU YNB	KETO YNB	RIF YNB

FIG. 1. Inhibition of *C. albicans* isolates by cilofungin. Abbreviations in key: LY, cilofungin; AMP, amphotericin B; 5-FLU, flucytosine; KETO, ketoconazole; RIF, rifampin; S, sterility control; G, growth control. Partial inhibition by cilofungin is shown in wells 2C, 4D, and 5C for SAB, A-3 (ANT-3), and YNB, respectively. Complete inhibition by cilofungin is seen only with A-3.

A-3 was used as the reference medium against which the others were compared. In this medium, there was <1 dilution difference between the wells of partial inhibition and complete inhibition for most isolates. With both SAB and YNB, partial inhibition by cilofungin was equivalent within 1 dilution of that found in A-3 for most isolates. Many isolates of *C. albicans* and *C. tropicalis* continued to grow in concentrations of cilofungin above that of partial inhibition in SAB and YNB. There was then a >1 dilution difference between the wells demonstrating partial and complete inhibition. This paradoxical effect seen with SAB and YNB is illustrated in Fig. 1. An inoculum of 0.5×10^4 to 4.9×10^4 CFU/ml was obtained 74% of the time. MICs for *C. albicans* and *C. tropicalis* did not appear to be uniformly affected by these differences until the inoculum was $>10^5$ CFU/ml.

The activities of cilofungin (in A-3), amphotericin B, flucytosine, and ketoconazole are shown in Table 1. Cilofungin was highly active against *C. albicans* and *C. tropicalis* and moderately active against *Torulopsis glabrata* and *C. krusei*. All strains of *C. parapsilosis* and *Cryptococcus neoformans* were resistant. Four strains each of *Cryptococcus* sp., non-neoformans cryptococci, and *Saccharomyces cerevisiae* were resistant (data not shown). In general, the partial and complete inhibition MICs of cilofungin (in A-3) agreed within ±1 dilution of each other. With the exception of one isolate of *C. albicans* (for which the MIC was 1 μg/ml) and one *Cryptococcus* isolate (for which the MIC was >32 μg/ml), all isolates were inhibited by ≤0.5

TABLE 1. Activities of antifungal agents against yeast isolates[a]

Organism (no.)	Antifungal agent	MIC (μg/ml)			MFC_{90} (μg/ml)[b]
		Range	50%	90%	
C. albicans (126)	Cilofungin	0.08–0.31 (0.16–2.5)	0.15 (0.31)	0.31 (0.31)	(0.31)
	Amphotericin B	0.03–1	0.06	0.12	
	Flucytosine	0.125–>128	0.25	8	
	Ketoconazole	0.25–32	0.25	8	
C. tropicalis (38)	Cilofungin	0.08–0.31 (0.16–0.31)	0.15 (0.15)	0.15 (0.31)	(0.31)
	Amphotericin B	0.15–0.125	0.06	0.12	
	Flucytosine	0.125–>128	0.25	1	
	Ketoconazole	0.26–16	0.25	8	
C. parapsilosis (26)	Cilofungin	10–>40 (20–>40)	40 (>40)	>40 (>40)	(>40)
	Amphotericin B	0.015–0.06	0.03	0.06	
	Flucytosine	0.06–1	0.25	1	
	Ketoconazole	0.25–0.5	0.25	0.5	
C. krusei (7)	Cilofungin	10 (10–20)	10 (10)		
	Amphotericin B	0.125–0.25	0.125		
	Flucytosine	16–64	64		
	Ketoconazole	4–16	4		
T. glabrata (32)	Cilofungin	1.2–5 (1.2–5)	2.5 (2.5)	5 (5)	(5)
	Amphotericin B	0.06–0.5	0.125	0.25	
	Flucytosine	0.06–>128	0.125	0.25	
	Ketoconazole	0.25–8	2	4	
C. neoformans (12)	Cilofungin	>40 (>40)	>40 (>40)	>40 (>40)	(>40)
	Amphotericin B	0.06–0.125	0.06	0.125	
	Flucytosine	2–32	8	16	
	Ketoconazole	0.25–4	1	2	

[a] Testing conditions: inoculum of 10^4 CFU/ml; 30°C; 48-h incubation. Cilofungin was used in A-3. Numbers in parentheses represent complete inhibition; those not in parentheses represent partial inhibition.
[b] MFC_{90}, MFC for 90% of isolates tested.

μg of amphotericin B per ml. Results with flucytosine were more variable, with 88% of the isolates being inhibited by ≤8 μg/ml. All *C. krusei* and *Cryptococcus* isolates required ≥16 μg of flucytosine per ml for inhibition. Ketoconazole inhibited 97% of all isolates at a concentration of ≤8 μg/ml. For only one isolate, a *C. albicans*, was the ketoconazole MIC >16 μg/ml.

When cilofungin was diluted in SAB or YNB, isolates of *C. albicans* and *C. tropicalis* were resistant, as demonstrated by the complete inhibition endpoint (Table 2). The activity of cilofungin against *C. krusei* and *T. glabrata* was not affected by the medium. *C. parapsilosis*, *Candida* sp., and *Cryptococcus neoformans* were resistant in all three media.

There was a ≤1 dilution difference between MIC_{90}s determined at 30 and 35°C and among those determined at 24, 48, and 72 h. With *C. albicans* and *C. tropicalis* in A-3, inocula of 10^5 CFU/ml increased the complete inhibition MIC in less than a third of the isolates tested. The partial inhibition MIC

TABLE 2. MIC_{50} and MIC_{90} of cilofungin against yeast isolates in different media[a]

Organism (no.)	MIC endpoint[b]	MIC_{50} (μg/ml) in:			MIC_{90} (μg/ml) in:		
		A-3	SAB	YNB	A-3	SAB	YNB
C. albicans (126)	p	0.15	0.31	1.25	0.31	0.62	1.25
	c	0.15	>40	>40	0.31	>40	>40
C. tropicalis (38)	p	0.15	0.31	0.62	0.15	0.62	1.25
	c	0.15	20	>40	0.31	>40	>40
C. parapsilosis (26)	p	40	20	>40	>40	40	>40
	c	>40	20	>40	>40	40	>40
C. krusei (7)	p	10	5	10			
	c	10	10	20			
Candida sp. (7)	p	>40	>40	>40			
	c	>40	>40	>40			
T. glabrata (32)	p	2.5	2.5	2.5	5	5	5
	c	2.5	5	5	5	5	5
C. neoformans (12)	p				>40	>40	>40
	c				>40	>40	>40

[a] Conditions of incubation: 30°C for 48 h with 10^4 CFU/ml.
[b] p, Partial inhibition endpoint; c, complete inhibition endpoint.

remained the same. However, at 10^6 CFU/ml, the MIC for complete inhibition of 2 of 4 *C. tropicalis* and 19 of 20 *C. albicans* isolates was >20 μg of cilofungin per ml. At inocula of $>4 \times 10^6$ CFU/ml, all isolates of *C. albicans* and *C. tropicalis* grew in all concentrations tested. Preparation of the inoculum of 10^4 CFU/ml from an overnight agar or broth culture did not affect the MIC.

DISCUSSION

Cilofungin is a semisynthetic antifungal agent shown to have both inhibitory and fungicidal activity against some members of the genus *Candida*. Its mode of action is directed against the cell wall, specifically inhibiting glucan incorporation into actively metabolizing cells. We have demonstrated that cilofungin possesses very good in vitro activity against clinical isolates of *C. albicans* and *C. tropicalis*, with MIC_{90}s ≤0.31 μg/ml when tested in A-3. For 164 strains of these two species, the MIC was higher (2.5 μg/ml) for only 1 isolate of *C. albicans*. These data are comparable to a report by Gordee et al. (9), who demonstrated an MIC_{90} of 1.25 μg/ml with 96 isolates of *C. albicans* tested in either SAB or YNB. The MIC_{90} for *T. glabrata*, on the other hand, was ≤5 μg/ml, with a very narrow range. The MIC range for *C. krusei* was also very narrow (MIC_{90}, ≤20 μg/ml for seven strains tested). With the exception of one isolate of *C. utilis*, all other *Candida*, *Cryptococcus*, and *Saccharomyces* isolates were resistant in vitro (MIC_{90}, ≥40 μg/ml).

1,3-β-D-Glucan is one of the major structural polymers in yeast cell walls; therefore, inhibition of its synthesis could result in lysis of growing cells (2). *C. albicans* has been shown to have a cell wall containing 39% β-glucan. In the presence of a mixed-membrane preparation of *C. albicans*, echinocandin 32528 reduced 1,3-β-D-glucan synthesis by 80% (16). Perhaps smaller amounts of β-glucan are present in other organisms found to be resistant. Chattaway et al. (5) have reported that the enzymatic digestion of cell wall fractions of *C. albicans* resulted in a greater breakdown of glucan in the blastospores than in mycelial fractions. They suggested that this might indicate a difference in cell wall structure between the two forms.

A-3 was originally described for susceptibility testing of yeasts with amphotericin B by Utz et al. (18). We found that use of this medium enabled us to make clear-cut endpoint determinations. With most isolates, there was a <1 dilution difference between the partial inhibition endpoint and a complete inhibition endpoint, definitions used when SAB or YNB was used for testing. We found that when partial inhibition readings were compared, MICs in SAB and YNB for *C. albicans* and *C. tropicalis* were equivalent to those found in A-3. However, a paradoxical effect was noted with cilofungin in SAB and YNB, in that the inhibitory effect observed at lower concentrations was followed by growth at higher concentrations often equivalent to that in the growth control well. If the wells between the partial inhibition endpoint and complete inhibition endpoints were subcultured, growth occurred. The MFC would thus be equivalent to the complete inhibition MIC.

To further examine the effect of cilofungin in SAB and YNB media at these higher concentrations, we examined the contents of the microdilution wells microscopically. At subinhibitory drug concentrations (≤0.312 μg/ml) the cells were normal, oval to round, budding, and producing pseudohyphae. In dilutions above the partial inhibition endpoint, we found that the cells tended to clump and that this tendency increased with increasing drug concentration. Also, no pseudohyphae could be demonstrated. Gordee et al. (9) noted that in the presence of cilofungin cells tended to clump by means of stringy attachments and no budding was seen. Some of the cells also seemed partially collapsed. Cassone et al., working with an echinocandin agent, also demonstrated cytologically that there was a thinning of the bud cell wall on exposure to the drug, resulting in unbalanced cell growth (4). In addition, convoluted membranous bodies consisting of concentric lamellar whorls of membranes were seen. These were found in budding areas but were also associated with nuclear, vacuolar, and mitochondrial membranes. For an echinocandin agent to kill a yeast, actively metabolizing cells are needed; there is no effect on stationary-phase cells (4). The reason for the difference between the activity of cilofungin in SAB or YNB versus A-3 is not yet known; however, metabolic activity in these media may differ. Mehta et al. (12) demonstrated a medium-related effect in testing a chitin synthesis inhibitor, polyoxin. The agent demonstrated no inhibition of growth of yeasts in SAB or TSB. When tested in a defined medium, yeast nitrogen base-glucose or yeast carbon base-lysine, the polyoxin activity was demonstrated. The differences were attributed to differences in transport of the agent across the membrane of the yeast cell, possibly involving a specific peptide carrier mechanism. Noninhibitory peptides such as tryptone may protect the cell from entry of polyoxins.

The inoculum used in this study was 10^4 CFU/ml, which is higher than the 10^3 CFU/ml used by others (9). We did find, however, that the inoculum achieved by matching the density to that of a no. 1 McFarland standard was variable and differed from that occurring with bacterial cells. We determined that for the majority of yeasts, a suspension matching the density of a no. 4 McFarland standard was needed to yield a colony count of 10^7 CFU/ml and hence a final inoculum of 0.5×10^4 to 4.9×10^4 CFU/ml in the microdilution trays. By using this approach, we were able to achieve the desired inoculum in 74% of the microdilution trays.

Because of well-known problems with antifungal susceptibility testing in general (J. N. Galgiani, Antimicrob. Newl. 3:17–22, 1986), we decided to examine the effects of other variables on cilofungin. Many authors have shown that the MIC of flucytosine can be changed by changing the type of buffer (3) used to obtain a specific pH, the types of media (7), and the size of the inoculum (6), to name a few. The type of medium also affects the activity of amphotericin B. These variables, as well as the temperature and duration of incubation, must be controlled and standardized for susceptibility testing of imidazoles, such as ketoconazole. Neither temperature nor duration of incubation affected the MICs of cilofungin in A-3 when tested against *C. albicans* and *C. tropicalis*, nor did they decrease the resistance seen with other isolates tested. Use of a direct technique versus overnight broth culture preparation of the inocula did not alter the results. In studies with an increased inoculum of 10^5 or 10^6 CFU/ml, decreased activity of cilofungin against isolates of *C. albicans* and *C. tropicalis* in A-3 medium was shown.

In comparing the activity of cilofungin in A-3 with those of other antifungal agents, certain patterns of resistance were noted. Ten percent (11 of 106) of the isolates of *C. albicans* were resistant to flucytosine. Stiller et al. (17) reported 11.5 to 15% resistance from four medical centers and 35% resistance from a fifth center, using >12.5 μg/ml to define resistance. Of all yeasts tested in the present report, 12% were resistant to flucytosine. Only one isolate, *Cryptococcus unigullatus*, was resistant to amphotericin B (MIC, >32 μg/

ml). This isolate was also resistant to flucytosine and cilofungin but was inhibited by ketoconazole at ≤8 μg/ml. Using >8 μg/ml to define resistance to ketoconazole, we found that six isolates of *C. albicans* were resistant and 8 (3%) of the total number of isolates tested, 256, were resistant. Had we used the complete inhibition endpoint for ketoconazole, a much higher level of resistance would have resulted.

Cilofungin appears to be an active drug, in vitro, specifically against *C. albicans* and *C. tropicalis*. Its ultimate utility will therefore depend not only on its pharmacokinetic properties, safety, and efficacy in vivo, but also on the prevalence of *Candida* species other than *C. albicans* and *C. tropicalis* in any particular setting.

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