Comparative antifungal activity of cilofungin (LY121019) against *Candida* species, including evaluation of susceptibility testing method

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AH CHAGLA, JH HII, DJ HOBAN, et al. Comparative antifungal activity of cilofungin (LY121019) against *Candida* species, including evaluation of susceptibility testing method. Can J Infect Dis 1992;3(5): 231-234. The in vitro activity of cilofungin against 100 *Candida* species was compared with 5-flucytosine, amphotericin B and ketoconazole by two laboratories independently and in a blinded fashion using a macrotitre dilution broth method in SAAM-F medium. Cilofungin showed good in vitro activity against *Candida albicans*, *Candida tropicalis* and *Candida glabrata* (90% minimal inhibitory concentration [MIC] 3.2 μg/mL) but was inactive against other *Candida* species. When testing the susceptibility of cilofungin, 5-flucytosine and amphotericin B at the two centres, approximately 90% of the *Candida* strains had MICs differing by fourfold or less. However, when testing susceptibility of ketoconazole, only 51% of the *Candida* strains had MIC differences fourfold or less. MIC susceptibility testing with cilofungin, 5-flucytosine and amphotericin B in SAAM-F medium is reproducible.

Key Words: *Antifungal susceptibility testing*, Candida *species*, *Cilofungin*, *LY121019*

Activité antifongique comparative du cilofungin (LY121019) contre des espèces *Candida* et évaluation des méthodes de mesure de la sensibilité

RÉSUMÉ: L'activité in vitro du cilofungin contre cent espèces de *Candida* a été comparée à celle de la 5-flucytosine, de l'amphotéricine B et du kétoconazole par deux laboratoires indépendants et à l'insu, à l'aide d'une méthode de microdilution dans un bouillon de culture SAAM-F. Le cilofungin a démontré une bonne activité in vitro contre *Candida albicans*, *Candida tropicalis* et *Candida glabrata* (concentration minimale inhibitrice [CMI] 90% à 3,2g/mL), mais s'est révélé inactif contre d'autres espèces de *Candida*. Lorsque l'on a analysé la sensibilité du cilofungin, de la 5-flucytosine et de l'amphotéricine B dans les deux centres, environ 90% des souches de *Candida* avaient des CMI quatre fois inférieures ou davantage. Cependant, lors d'épreuves de sensibilité avec le kétoconazole, seulement 51% des souches de *Candida* présentaient des différences de CMI quatre fois inférieures ou davantage. Les épreuves de sensibilité en vue de mesurer la CMI du cilofungin, de la 5-flucytosine et de l'amphotéricine B dans un milieu SAAM-F sont reproductibles.

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CILOFUNGIN (N-P-OCTYLOXYBENZOYLECHINOCANDIN B; LY121019) is a semisynthetic lipopeptide analogue of the polypeptide antibiotic echinocardin. It is believed to inhibit the biosynthesis of the beta-1-3 glucan component of the *Candida albicans* cell wall in nonstationary, metabolizing cells, thereby disrupting cell wall integrity (1,2). It is known to be active against certain *Candida* species. In vitro susceptibility tests show that minimal inhibitory concentrations (MICs) for cilofungin against *C albicans* and *Candida tropicalis* are lower than those against *Candida glabrata* and other *Candida* species (3-5). Animal studies show it to be 20-fold less toxic than amphotericin B (6).

In vitro susceptibility testing for fungi and yeasts has been unsatisfactory because of a lack of standard test criteria. Variations in inoculum preparation, medium composition, pH, duration of incubation, temperature and endpoint determination have all contributed to variable test results among laboratories (7). Specifically, various laboratories have reported a nearly eightfold difference in MICs for cilofungin against *C albicans*, with results differing from 0.31 to 2.5 μg/mL (3-6,8,9). Different media, inoculum size and temperature of incubation were used. Hall et al (8) have suggested that cilofungin activity was affected by the growth medium and inoculum size. In contrast, Strippoli et al (9) have noted that compositional differences in the medium and/or the presence of animal serum did not adversely affect susceptibility testing with cilofungin. The few studies which have analyzed the antifungal activity of cilofungin have compared results based on differing methodologies, while none has evaluated a method of choice to determine reproducibility between different laboratories.

The authors report a study on the in vitro activity of cilofungin against 100 clinical isolates of *Candida* species using a macrotitre broth dilution method with synthetic amino acid medium for fungi (SAAM-F) (10,11), and compare test results measured by MIC and minimum fungicidal concentration (MFC) of these strains obtained between two laboratories in Canada.

A total of 100 clinical isolates of *Candida* species were examined. Fifty strains recovered from blood cultures were collected at Health Sciences Centre in Winnipeg, Manitoba and the remaining 50 isolates, obtained from specimens of various body sites (50 isolates), were collected at Mount Sinai Hospital in Toronto, Ontario. Identification was based on microscopic morphology, germ-tube formation, chlamydospore development and biochemical tests using the API 20C system (Analytab Products, New York). These isolates included 57 strains of *C albicans*, 16 strains of *C tropicalis*, 19 strains of *C glabrata*, five *Candida parapsilosis* strains, and one each of *Candida qvilliermondii*, *Candida lusitaniae* and *Candida krusei* (collectively referred to in the text as other *Candida* species). Stock cultures were preserved in skim milk at –70°C and regularly maintained on 5% sheep blood agar or Sabouraud's dextrose agar.

SAAM-F is comprised of 16 amino acids, glucose, fumaric and pyruvic acid, ammonium acetate, potassium hydrogen phosphate, N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid (HEPES) and Tris buffer (11). Vitamins and other salts were added separately, and the medium adjusted to pH 7.4. All reagents were purchased from the Sigma Chemical Co (Missouri) and constituted independently at each reference laboratory.

Cilofungin, provided by Eli Lilly Research Laboratories (Indiana) dissolved in 50% ethanol. Stock solutions of amphotericin B, provided by ER Squibb and Sons (New Jersey), and ketoconazole, provided by Janssen Pharmaceutica Inc were dissolved in dimethyl sulphoxide. The 5-flucytosine, provided by Roche Laboratories (New Jersey), was dissolved in sterile distilled water. The stock solutions were stored at –72°C to retain potency. Serial twofold dilutions of antifungal agents were made with sterile distilled water to achieve working solution ranging from 0.05 to 200 μg/mL. A 0.5 mL working solution was further diluted by half with addition of 0.5 mL 2X (doubly concentrated) SAAM-F medium to obtain final drug concentrations ranging from 0.025 to 100 μg/mL.

Fungal cultures grown on blood agar for 48 h at 30°C were recovered and suspended in 5 mL sterile saline and standardized to 0.5 MacFarland. The cell suspension was diluted 100-fold, and an actual cell count was made on diluted suspension using a hemacytometer. A 50 μL aliquot was then used as the inoculum for susceptibility testing to obtain a final concentration of $1x10^3$ yeast cells/mL. Controls were used for medium alone and for medium with 2% ethanol. A sample of inoculum was placed on blood agar to check for contamination, inoculum quantitation and viability. The tubes were incubated unagitated for 48 h at 30°C. The MICs were determined after gentle agitation of the tubes and comparison with controls. The MICs were defined as the lowest concentration of antifungal agent tested which yielded no macroscopic turbidity compared with uninoculated control. To determine the MFC, 10 μL of agitated suspension, from one tube before and at least six tubes after MIC endpoints, was subcultured on 5% sheep blood agar or Sabouraud's dextrose agar, and incubated for 48 h at 30°C. Colonies were counted and the MFC was defined as the lowest concentration at which 99% of the initial inoculum was killed. An Eagle effect was not produced using the present testing method. Controls were performed with each test, using two reference strains of *C albicans* ATCC 10231 and LY-A26 provided by Eli Lilly Research Laboratories. The study was blinded and carried out independently at the two reference laboratories. Cultured isolates were given numbers prior to the study and exchanged between the two laboratories without any accompanying information concerning the source or strain identification.

TABLE 1
In vitro susceptibility of 100 clinical isolates of *Candida* species to cilofungin, 5-flucytosine, amphotericin B and ketoconazole*

Species (number of strains)	Antifungal agent	MIC (μg/mL) Range	MIC (μg/mL) 50%†	MIC (μg/mL) 90%†	MFC (μg/mL) Range	MFC (μg/mL) 50%†	MFC (μg/mL) 90%†
Candida albicans (57)	Cilofungin‡	0.8 to 12.8	1.6	3.2	1.6 to 25	3.2	6.4
	5-flucytosine	0.2 to 100	1.6	2.5	0.4 to 100	3.2	2.5
	Amphotericin B	0.1 to 1.6	0.8	0.8	0.2 to 1.6	0.8	1.6
	Ketoconazole	0.025 to ≥100	12.8	50	0.2 to ≥100	50	≥100
Candida tropicalis (16)	Cilofungin‡	0.4 to 50	1.6	3.2	0.8 to 50	3.2	12.8
	5-flucytosine	0.1 to 25	1.6	3.2	0.4 to 100	3.2	6.4
	Amphotericin B	0.1 to 1.6	0.8	0.8	0.2 to 1.6	0.8	1.6
	Ketoconazole	≤0.025 to 3.2	≤0.025	3.2	≤0.025 to ≥100	0.1	6.4
Candida glabrata (19)	Cilofungin‡	0.8 to 25	3.2	3.2	1.6 to 50	6.4	6.4
	5-flucytosine	≤0.025 to 100	0.4	0.8	0.1 to 100	0.4	0.8
	Amphotericin B	0.1 to 1.6	0.4	0.8	0.2 to 1.6	0.4	1.6
	Ketoconazole	0.2 to ≥100	12.8	50	0.8 to ≥100	50	≥100
Other *Candida* species (8)	Cilofungin‡	6.4 to ≥100	–	–	12.8 to ≥100	–	–
	5-flucytosine	0.8 to 100	–	–	1.6 to 100	–	–
	Amphotericin B	0.2 to 1.6	–	–	0.2 to 6.4	–	–
	Ketoconazole	≤0.025 to 0.8	–	–	≤0.025 to 50	–	–

*MIC Minimal inhibitory concentration; MFC Minimum fungicidal concentration; *Results of susceptibility testing performed at Mount Sinai Hospital, Toronto, Ontario; †50 or 90% of isolates; ‡The respective MIC_{90} and MFC_{90} for cilofungin tested at Health Sciences Centre, Winnipeg, Manitoba, against C albicans was 1.6 and 1.6 μg/mL; C tropicalis 3.2 and 12.8 μg/mL; C glabrata 3.2 and 6.4 μg/mL*

Each reference laboratory performed susceptibility testing on the 100 clinical isolates using a common protocol, and without prior knowledge of other laboratory results. Based on NCCLS guidelines, a difference of fourfold or less in MICs and MFCs was considered acceptable (12). An overall 95% confidence limit was computed, taking into consideration the comparison of MICs and MFCs for cilofungin to the other three drugs.

The MIC_{50}, MIC_{90} and MFC results of in vitro susceptibility testing of the 100 isolates using an inoculum of 10^3 yeast cells/mL are summarized in Table 1. Cilofungin showed good in vitro activity against *C albicans*, *C tropicalis*, and *C glabrata* (MIC_{90} 3.2 μg/mL), but was inactive against the other *Candida* species. Amphotericin B showed significant in vitro activity against all the *Candida* strains tested. A significant trailing effect was seen when ketoconazole was tested, with considerable disparity between partial and complete end-point inhibition. This may be the reason for interlaboratory variation in determining antifungal activity of ketoconazole, and has been confirmed by other investigators (13). The determination of end-points by measurement of turbidity and calculation of IC_{50} (concentration of antifungal agent that causes 50% decrease in turbidity) has been proposed as being more reliable than conventional (visual) scoring for absence of growth (14). In the present study trailing effect was not observed with other antifungal agents which were tested, and the results were based on determination of clear cut end-points.

Table 2 shows the variations in in vitro susceptibility testing of the four antifungal agents between the two

TABLE 2
Interlaboratory variability in antifungal susceptibility test results between two testing laboratories

Antifungal agent		Interlaboratory variability 0 dilution difference (cumulative %)	1 (cumulative %)	2 (cumulative %) (95% CI*)
Cilofungin	MIC	16 (16)	53 (69)	21 (90) (0.83, 0.97)
	MFC	21 (21)	25 (46)	28 (74) (0.63, 0.85)
5-flucytosine	MIC	46 (46)	33 (79)	12 (91) (0.84, 0.98)
	MFC	31 (31)	49 (80)	12 (92) (0.85, 0.99)
Amphotericin	MIC	29 (29)	47 (76)	16 (92) (0.85, 0.99)
	MFC	25 (25)	53 (78)	18 (96) (0.91, 1.00)
Ketoconazole	MIC	23 (23)	21 (44)	7 (51) (0.39, 0.63)
	MFC	9 (9)	6 (15)	21 (36) (0.24, 0.48)

*MIC Minimal inhibitory concentration; MFC Minimum fungicidal concentration; *CI Confidence interval = $p + Z_\alpha (p(1-p) + N^{0.5})$, where p = (number of strains with MIC or MFC with a dilution difference of 2 or less) + total number of strains tested; N=100; Z_α=2.45*

reference laboratories. Approximately 90% of the strains tested against cilofungin, 5-flucytosine and amphotericin B showed a difference in MIC and MFC by fourfold or less. Ninety per cent of cilofungin MICs had less than or equal to two well dilution differences, but only 74% had two or fewer well dilution differences. This variability could not be explained by a trailing effect, since end-points were clearcut. A discordance in both MIC and MFC was seen with ketoconazole between the two centres.

Antifungal testing with cilofungin has been performed variably using microtitre and macrotitre broth dilution methods, various media including SAAM-F, and various inoculum sizes. In these reports (3-6,8,12) there was a fourfold or greater variation in MIC endpoints between studies. In the present study, the authors carried out interlaboratory comparison of antifungal susceptibility results using a standardized method which included an inoculum of 10^3 yeast cells/mL and a constitutionally defined medium, SAAM-F, recommended for antifungal susceptibility testing (10). SAAM-F has only been used in two studies using a similar inoculum (3,15), and was thought to account for the loss of susceptibility to cilofungin in some isolates of *C albicans* and *C tropicalis*; an Eagle effect was also described with testing several isolates of *C tropicalis* (3). This problem was not observed in the current study.

An optimal inoculum of 10^3 yeast cells/mL was used in the present study, comparable to the inoculum used by others (4,6). This was determined by testing 27 representative strains (data not shown). An inoculum greater than 10^5 yeast cells/mL resulted in decreased activity of cilofungin, flucytosine and ketoconazole, but not of amphotericin B, in agreement with the finding of others (8,16). The disparity in MIC end-points by using high inocula may reflect an absolute increase in inoculum to drug concentration or, which is less likely, resistance to cilofungin (16).

Cilofungin demonstrated less activity against *C albicans*, *C tropicalis* and *C glabrata* than reported elsewhere (3-6,8). This may have resulted from testing in SAAM-F (3). In contrast to the present findings, Hobbs et al (3) report a MIC_{90} threefold higher for cilofungin against *C glabrata*, using a microtitre assay system in SAAM-F. In agreement with other reports (3-6,8), cilofungin has demonstrated little or no in vitro activity against the other *Candida* species tested.

Cilofungin (LY121019) demonstrates good in vitro activity against *C albicans*, *C tropicalis* and *C glabrata* with MICs well within achievable animal serum concentrations. A common problem with antifungal susceptibility testing has been an unacceptable discordance of results between various laboratories due to differing test conditions (7). However, in the present study the authors were able to provide reproducible interlaboratory MIC results when testing cilofungin, 5-flucytosine and amphotericin using a standardized methodology.

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