

In Vitro Activities of Semisynthetic Pneumocandin L-733,560 against Fluconazole-Resistant and -Susceptible *Candida albicans* Isolates

JOAQUIN V. MARTINEZ-SUAREZ* AND JUAN L. RODRIGUEZ-TUDELA

Unidad de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, 28220 Majadahonda, Madrid, Spain

Received 21 December 1995/Returned for modification 19 January 1996/Accepted 6 March 1996

Lipopeptide L-733,560 is a water-soluble derivative of pneumocandin B₀ that exhibits enhanced anti-*Candida* activity. We investigated the in vitro activity of L-733,560 compared with those of amphotericin B, flucytosine, and itraconazole, against fluconazole-resistant ($n = 44$) and fluconazole-susceptible ($n = 46$) *Candida albicans* isolates. Tests were performed with a photometer-read broth microdilution method with RPMI-2% glucose and National Committee for Clinical Laboratory Standards reference strains. Except for those of itraconazole, MICs were not significantly different between the two groups of isolates, as expected for agents with different mechanisms of action. L-733,560 was the most active agent against *C. albicans*, with MICs for 50 and 90% of the strains tested of 0.01 and 0.06 $\mu\text{g/ml}$, respectively.

Development of resistance to azole antifungal drugs is an emerging trend that may threaten their clinical effectiveness (7, 14, 17). New classes of antifungal agents are needed to counter this resistance problem.

Echinocandins and pneumocandins are acyl-substituted cyclic hexapeptides (lipopeptides) that inhibit the synthesis of 1,3- β -D-glucan, a critical fungal cell wall component (6). A new generation of semisynthetic amine derivatives of the natural product pneumocandin B₀ is being developed. These compounds show enhanced potencies and expanded spectra of antifungal activity (6). Lipopeptide L-733,560 is a water-soluble pneumocandin analog that exhibits potent in vitro (5) and in vivo (1) anti-*Candida* activity, as well as in vivo anti-*Aspergillus fumigatus* (1, 9) and anti-*Pneumocystis carinii* (20) activities. The fungicidal mode of action of this compound, via inhibition of cell wall 1,3- β -D-glucan synthesis, is especially attractive (10).

In this study, we investigated the in vitro activity of L-733,560 against fluconazole-resistant and -susceptible *Candida albicans* isolates, comparing its activity with those of amphotericin B, flucytosine, and itraconazole.

A panel of 90 well-characterized clinical isolates of *C. albicans* was used in the study (11). Part of these strains were from documented fluconazole-susceptible or -resistant episodes of oral candidiasis in human immunodeficiency virus-infected patients (19). Four *C. albicans* reference strains were also tested (Table 1).

L-733,560 (Merck Research Laboratories, Rahway, N.J.) and flucytosine (Productos Roche, Madrid, Spain) were dissolved in sterile distilled water, and amphotericin B (Squibb Industria Farmacéutica, Madrid, Spain), fluconazole (Pfizer, Madrid, Spain), and itraconazole (Janssen Farmacéutica, Madrid, Spain) were dissolved in 100% dimethyl sulfoxide. All of these solutions were prepared as 100 \times stocks by adjusting the weight in accordance with the potency of the drug and were frozen at -70°C until they were used.

The assay medium was RPMI-2% glucose, an improved medium that facilitates the reading of growth inhibition by

azole drugs; preparation of RPMI-2% glucose has been previously described in detail (11).

MICs were determined by a microdilution test described previously (18), with minor modifications. Microtiter plates containing 96 flat-bottom wells each were used. Starting inocula of 10^6 CFU/ml were prepared by the spectrophotometric method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (13). Microtiter plates were inoculated with 10 μl per well to obtain final inocula of approximately 10^5 CFU/ml. Following incubation at 35°C for 24 h, the trays were shaken for 5 min (2) and turbidity was read spectrophotometrically at 405 nm (630 nm for amphotericin B) with a Mios Merck automatic plate reader (Merck Igoda, S.A., Madrid, Spain). For amphotericin B and flucytosine, the MIC endpoint was defined as the lowest drug concentration that allowed no visible growth (18) or MIC 100%. For azoles and pneumocandin L-733,560, the MIC was defined as the lowest drug concentration that reduced growth by 80% compared with the drug-free well (18), similar to the visual MIC 80% endpoint criterion recommended by the NCCLS (13). The MIC 80% endpoint was chosen for L-733,560 because some *C. albicans* isolates displayed partial inhibition of growth. This phenomenon hampered reading of the MIC 100%, although for most isolates the MIC 100% and MIC 80% were identical.

The MICs of the five antifungal agents for the four reference strains of *C. albicans*, as determined by the microbroth dilution method with RPMI-2% glucose, are shown in Table 1. Despite the numerous differences between this microbroth method and the NCCLS macrobroth reference method (13), including the larger inoculum and the 24-h incubation, there was close agreement between the results obtained with the two methods, at least for amphotericin B, flucytosine, and fluconazole (Table 1).

The in vitro activities of the five antifungal agents against 90 clinical isolates of *C. albicans* are given in Table 2. The itraconazole MICs for the group of isolates more resistant to fluconazole were significantly higher than those for the fluconazole-susceptible isolates (Table 2), as previously described by us (11) and others (4). For the other agents, including L-733,560, there was no difference between the two groups of

* Corresponding author. Phone: 34-1-6380011, ext. 248. Fax: 34-1-6391859. Electronic mail address: jmartinz@isciii.es.

TABLE 1. MIC results for four *C. albicans* reference strains

| Isolate | Comment (reference) | Antifungal agent | MIC ($\mu\text{g/ml}$) in: | |
|------------|---|------------------|------------------------------|------------------------|
| | | | Present study ^a | Reference ^b |
| ATCC 90028 | NCCLS M27-P reference strain (15) | Amphotericin B | 0.5 | 0.5–2.0 |
| | | Flucytosine | 1.0 | 0.5–2.0 |
| | | Fluconazole | 0.5 | 0.25–1.0 |
| | | Itraconazole | 0.12 | NA ^c |
| | | L-733,560 | ≤ 0.007 | NA |
| ATCC 24433 | NCCLS M27-P reference strain (15) | Amphotericin B | 0.25 | 0.25–1.0 |
| | | Flucytosine | 4.0 | 1.0–4.0 |
| | | Fluconazole | 0.5 | 0.25–1.0 |
| | | Itraconazole | 0.12 | NA |
| | | L-733,560 | ≤ 0.007 | NA |
| ATCC 64548 | Azole susceptibility proven in animal model (3) | Amphotericin B | 0.5 | 0.25 |
| | | Flucytosine | 0.12 | NA |
| | | Fluconazole | 0.5 | 0.5 |
| | | Itraconazole | 0.12 | NA |
| | | L-733,560 | ≤ 0.007 | NA |
| ATCC 64550 | Azole resistance proven in animal models (3) | Amphotericin B | 0.5 | 0.5 |
| | | Flucytosine | 1.0 | NA |
| | | Fluconazole | 32.0 | 32.0 |
| | | Itraconazole | 1.0 | NA |
| | | L-733,560 | 0.03 | NA |

^a Microdilution test described in the text.

^b NCCLS macrodilution method.

^c NA, not available.

isolates (Table 2). As expected, isolates of *C. albicans* with decreased susceptibility to azole drugs did not show cross-resistance to antifungal agents with a different mechanism of action, such as amphotericin B, flucytosine, or L-733,560. Pneumocandin L-733,560 was more active than amphotericin B, flucytosine, and systemic triazoles against *C. albicans* (Table 2).

The best method of performing antifungal susceptibility testing is a matter of debate (7, 16). In the present study, we used a previously described method (18) giving rise to results similar to those produced by the method proposed by the NCCLS (8). We consider the main differences between this method and the NCCLS reference method (microdilution format, RPMI–2% glucose medium, inoculum of 10^5 CFU/ml, 24 h of incubation, and spectrophotometric reading of MICs) improvements that

do not change the final results (Table 1). In addition, we found very good reproducibility when susceptibility tests were repeated with reference strains (data not shown).

The concordance of reference and RPMI–2% glucose microbroth results for amphotericin B, flucytosine, and fluconazole (Table 1) suggests that for L-733,560 results similar to those obtained with the microbroth method (Table 2) could be also achievable with the reference method. Previous results obtained for another cell wall inhibitor, cilofungin, with reference macrobroth or RPMI-microbroth tests were similar (12).

With L-733,560, as with the azoles, partial inhibition of the growth of some strains was seen. This phenomenon could be related to the increased osmotic protection of RPMI–2% glucose compared with other media. Nevertheless, for most isolates the MIC 80% and MIC 100% were identical, and for all

TABLE 2. In vitro susceptibility of *C. albicans* to pneumocandin L-733,560 and other antifungal agents

| Isolate | Agent | MIC ($\mu\text{g/ml}$) ^a | | |
|---|----------------|---------------------------------------|--------------------|--------------------|
| | | Range | For 50% of strains | For 90% of strains |
| Fluconazole-susceptible ^b <i>C. albicans</i> | Amphotericin B | 0.25–1.0 | 0.5 | 1.0 |
| | Flucytosine | 0.06–>16.0 | 0.12 | 1.0 |
| | Fluconazole | 0.12–0.5 | 0.25 | 0.25 |
| | Itraconazole | 0.03–0.25 | 0.12 | 0.12 |
| | L-733,560 | ≤ 0.007 –0.12 | 0.01 | 0.06 |
| Fluconazole-resistant ^c <i>C. albicans</i> | Amphotericin B | 0.12–1.0 | 0.5 | 1.0 |
| | Flucytosine | ≤ 0.03 –>16.0 | 0.12 | 2.0 |
| | Fluconazole | 8.0–>128.0 | 32.0 | 128.0 |
| | Itraconazole | 0.12–>8.0 | 1.0 | 2.0 |
| | L-733,560 | ≤ 0.007 –0.12 | 0.01 | 0.06 |

^a Microdilution test with RPMI–2% glucose described in the text.

^b MIC, ≤ 0.5 $\mu\text{g/ml}$ ($n = 46$).

^c MIC, ≥ 8.0 $\mu\text{g/ml}$ ($n = 44$).

of the strains tested the MICs of L-733,560 at 48 h did not differ more than twofold from reported MICs at 24 h (data not shown), as with amphotericin B.

These and previous results (5) predict a useful role for pneumocandin L-733,560 in the emerging resistance problem with the azole antifungal derivatives.

This work was supported in part by the Fondo de Investigaciones Sanitarias (grant 93/0002/01) and the Comisión Interministerial de Ciencia y Tecnología (grant BIO94-0181), both of Spain.

We thank Merck Research Laboratories, Productos Roche, Pfizer, Janssen Farmacéutica, and Squibb Industria Farmacéutica for supplying the antifungal powders.

REFERENCES

1. **Abruzzo, G. K., A. M. Flattery, C. J. Gill, L. Kong, J. G. Smith, D. Krupa, V. B. Pikounis, H. Kropp, and K. Bartizal.** 1995. Evaluation of water-soluble pneumocandin analogs L-733,560, L-705589, and L-731373 with mouse models of disseminated aspergillosis, candidiasis, and cryptococcosis. *Antimicrob. Agents Chemother.* **39**:1077–1081.
2. **Anaissie, E., V. Paetznick, and G. P. Bodey.** 1990. Fluconazole susceptibility testing of *Candida albicans*: microtiter method that is independent of inoculum size, temperature, and time of reading. *Antimicrob. Agents Chemother.* **35**:1641–1646.
3. **Anaissie, E. J., N. C. Karyotakis, R. Hachem, M. C. Dignani, J. H. Rex, and V. Paetznick.** 1994. Correlation between in vitro and in vivo activity of antifungal agents against *Candida* species. *J. Infect. Dis.* **170**:384–389.
4. **Barchiesi, F., A. L. Colombo, D. A. Mcgough, A. W. Fothergill, and M. G. Rinaldi.** 1994. In vitro activity of itraconazole against fluconazole-susceptible and -resistant *Candida albicans* isolates from oral cavities of patients infected with human immunodeficiency virus. *Antimicrob. Agents Chemother.* **38**:1530–1533.
5. **Bartizal, K., T. Scott, G. K. Abruzzo, C. J. Gill, C. Pacholok, L. Lynch, and H. Kropp.** 1995. In vitro evaluation of the pneumocandin antifungal agent L-733,560, a new water-soluble hybrid of L-705589 and L-731373. *Antimicrob. Agents Chemother.* **39**:1070–1076.
6. **Debono, M., and R. S. Gordee.** 1994. Antibiotics that inhibit fungal cell wall development. *Annu. Rev. Microbiol.* **48**:471–497.
7. **Espinel-Ingroff, A., and M. A. Pfaller.** 1995. Antifungal agents and susceptibility testing, p. 1405–1421. *In* P. K. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Tenover (ed.), *Manual of clinical microbiology*, 6th ed. ASM Press, Washington, D.C.
8. **Espinel-Ingroff, A., J. L. Rodríguez-Tudela, and J. V. Martínez-Suárez.** 1995. Comparison of two alternative microdilution procedures with the National Committee for Clinical Laboratory Standards reference macrodilution method M27-P for in vitro testing of fluconazole-resistant and -susceptible isolates of *Candida albicans*. *J. Clin. Microbiol.* **33**:3154–3158.
9. **Kurtz, M. B., E. M. Bernard, F. F. Edwards, J. A. Marrinan, J. Dropinski, C. M. Douglas, and D. Armstrong.** 1995. Aerosol and parenteral pneumocandins are effective in a rat model of pulmonary aspergillosis. *Antimicrob. Agents Chemother.* **39**:1784–1789.
10. **Kurtz, M. B., C. Douglas, J. Marrinan, K. Nollstadt, J. Onishi, S. Dreikorn, J. Milligan, S. Mandala, J. Thompson, J. M. Balkovec, F. A. Bouffard, J. F. Dropinski, M. L. Hammond, R. A. Zambias, G. Abruzzo, K. Bartizal, O. B. Mcmanus, and M. L. Garcia.** 1994. Increased antifungal activity of L-733,560, a water-soluble, semisynthetic pneumocandin, is due to enhanced inhibition of cell wall synthesis. *Antimicrob. Agents Chemother.* **38**:2750–2757.
11. **Martínez-Suárez, J. V., and J. L. Rodríguez-Tudela.** 1995. Patterns of in vitro activity of itraconazole and imidazole antifungal agents against *Candida albicans* with decreased susceptibility to fluconazole from Spain. *Antimicrob. Agents Chemother.* **39**:1512–1516.
12. **McIntyre, K. A., and J. N. Galgiani.** 1989. pH and other effects on the antifungal activity of clofungin (LY121019). *Antimicrob. Agents Chemother.* **33**:732–735.
13. **National Committee for Clinical Laboratory Standards.** 1992. Reference method for broth dilution antifungal susceptibility testing of yeasts. Proposed standard M27-P. National Committee for Clinical Laboratory Standards, Villanova, Pa.
14. **Odds, F. C.** 1993. Resistance of yeasts to azole-derivative antifungals. *J. Antimicrob. Chemother.* **31**:463–471.
15. **Pfaller, M. A., M. Bale, B. Buschelman, M. Lancaster, A. Espinel-Ingroff, J. H. Rex, M. G. Rinaldi, C. R. Cooper, and M. R. McGinnis.** 1995. Quality control guidelines for National Committee for Clinical Laboratory Standards recommended broth macrodilution testing of amphotericin B, fluconazole, and flucytosine. *J. Clin. Microbiol.* **33**:1104–1107.
16. **Rex, J. H., M. A. Pfaller, M. G. Rinaldi, A. Polak, and J. N. Galgiani.** 1993. Antifungal susceptibility testing. *Clin. Microbiol. Rev.* **6**:371–381.
17. **Rex, J. H., M. G. Rinaldi, and M. A. Pfaller.** 1995. Resistance of *Candida* species to fluconazole. *Antimicrob. Agents Chemother.* **39**:1–8.
18. **Rodríguez-Tudela, J. L., and J. V. Martínez-Suárez.** 1995. Defining conditions for microbroth antifungal susceptibility tests: influence of RPMI and RPMI-2% glucose on the selection of endpoint criteria. *J. Antimicrob. Chemother.* **35**:739–749.
19. **Rodríguez-Tudela, J. L., J. V. Martínez-Suárez, F. Dronda, F. Laguna, F. Chaves, and E. Valencia.** 1995. Correlation of in-vitro susceptibility test results with clinical response: a study of azole therapy in AIDS patients. *J. Antimicrob. Chemother.* **35**:793–804.
20. **Schmatz, D. M., M. A. Powles, D. McFadden, K. Nollstadt, F. A. Bouffard, J. F. Dropinski, P. Liberator, and J. Anderson.** 1995. New semisynthetic pneumocandins with improved efficacies against *Pneumocystis carinii* in the rat. *Antimicrob. Agents Chemother.* **39**:1320–1323.