Antimicrobial Agents and Chemotherapy, Jan. 2001. p. 327–330 Vol. 45, No. 1
0066-4804/01/$04.00+0 DOI: 10.1128/AAC.45.1.327–330.2001
Copyright © 2001, American Society for Microbiology. All Rights Reserved.

In Vitro Susceptibility Testing Methods for Caspofungin against *Aspergillus* and *Fusarium* Isolates

SEVTAP ARIKAN,* MARIO LOZANO-CHIU, VICTOR PAETZNICK, AND JOHN H. REX

Division of Infectious Diseases, Department of Internal Medicine, Center for the Study of Emerging and Reemerging Pathogens, University of Texas Medical School, Houston, Texas 77030

Received 5 June 2000/Returned for modification 11 July 2000/Accepted 18 October 2000

We investigated the relevance of prominent reduction in turbidity macroscopically (MIC) and formation of aberrant hyphal tips microscopically (minimum effective concentration; MEC) in measuring the in vitro activity of caspofungin against *Aspergillus* and *Fusarium*. Caspofungin generated low MICs and MECs against *Aspergillus*, but not for *Fusarium*. While MICs increased inconsistently when the incubation time was prolonged, MEC appeared as a stable and potentially relevant endpoint in testing in vitro caspofungin activity.

Downloaded from http://aac.asm.org/ on April 30, 2020 by guest

The echinocandins are a group of lipopeptide antifungal agents that contain a cyclic hexapeptide nucleus and act via inhibition of (1,3)-β-D-glucan synthase. Novel echinocandins, including LY303366, L-733,560, and caspofungin (formerly referred to as MK-0991 and as L-743,872) (1, 4, 5, 9–12, 15, 17, 19, 21–24) are currently under investigation.

A reproducible and clinically relevant method for susceptibility testing of echinocandins has not been fully established yet. One of the undetermined test parameters is the MIC endpoint to be used for measuring the in vitro activity. Echinocandins exhibit fungicidal or fungistatic activity against *Candida* spp. (8, 9). The MICs of echinocandins for *Candida* have been determined so far as either the least concentration of the drug that produces 100% inhibition of growth (21, 22) or that producing 80% reduction in turbidity (9, 13).

Testing the in vitro activity of echinocandins against *Aspergillus* spp. is more complicated. Echinocandins are active against *Aspergillus* both in vitro (5, 10, 20) and in vivo (1, 16). However, the assessment of in vitro activity requires distinct evaluation. Instead of a complete macroscopic growth inhibition, partial inhibition is seen in which the fungus microscopically produces short, stubby, and highly branched hyphae (6, 9, 16). Kurtz et al. (16) proposed that the drug concentration at which these morphological changes were first observed be called the minimum effective concentration (MEC). Nevertheless, neither the proper method to be used for the detection of the in vitro activity of caspofungin and other echinocandins against molds (5, 10, 20) nor the clinical significance of MEC has been fully defined.

This study was designed to comparatively evaluate the two endpoints, MIC and MEC, in the determination of the in vitro activity of caspofungin against clinical *Aspergillus* and *Fusarium* isolates. The effect of incubation period and test media on both MIC and MEC was also investigated.

(This work was presented in part at the 39th Interscience Conference on Antimicrobial Agents and Chemotherapy, 26 to 29 September 1999, in San Francisco, Calif., as abstr. no. J-160.)

The test organisms were comprised of 82 *Aspergillus* and 22 *Fusarium* strains. Two itraconazole-resistant *A. fumigatus* strains, kindly provided by D. W. Denning, were also included (7). Each isolate was tested in duplicate. One of the clinical isolates (strain no. 2-160; *A. fumigatus*) was included in each run for quality control.

Caspofungin was provided by Merck Research Laboratories (Rahway, N.J.) as a standard powder and tested in concentrations of 0.03125 to 16 μg/ml. Except as noted, susceptibility tests were performed according to the NCCLS M38-P microdilution methodology (18). In addition to the reference RPMI 1640 medium (RPMI; Sigma Chemical Co., St. Louis, Mo.), RPMI supplemented to 2% glucose (RPMI-2) and Antibiotic Medium 3 (BBL/Becton Dickinson lot JD4ZSG) buffered by adding 1 g of Na_2HPO_4 and 1 g of NaH_2PO_4 to each liter and supplemented to 2% glucose (AM3) were also used as test media.

Microdilution plates were prepared in bulk and stored at −70°C until use. In order to assure that caspofungin remains stable in microdilution plates stored at −70°C, stability control tests were performed initially. For this purpose, four quality control strains previously tested in our laboratory several times for caspofungin susceptibility were chosen (5W31 [*C. lusitaniae*], CL524 [*C. lusitaniae*], UTR-14 [*C. albicans*], and 707-13 [*C. albicans*], with caspofungin MICs at 24 h of 2, 2, 0.5, and 0.5 μg/ml, respectively). Caspofungin dilutions were prepared in microdilution plates using the test media, and the first susceptibility tests were performed on the same day. Keeping the plates at −70°C in the interim, this initial test was followed by its repetitions performed at 24 and 48 h and at 8, 9, 13, and 21 days following the day of plate preparation. MICs were read visually after both 24 and 48 h of incubation. The analysis of the data showed that, for each individual isolate, the MICs at 21 days either remained the same or changed by at most one twofold dilution compared to those obtained on the day of plate preparation. Based on these results, the plates were prepared in bulk and kept at −70°C until use but never longer than 21 days. Likewise, since the plates are incubated at 35°C for 48 h during susceptibility testing, the stability of caspofungin at 35°C was also investigated. For this purpose, the suscep-

* Corresponding author. Present address: Hacettepe University Medical School, Department of Microbiology and Clinical Microbiology, 06100 Ankara, Turkey. Phone: 90-312-3051562. Fax: 90-312-3115250. E-mail: sarikan@metu.edu.tr.

TABLE 1. MIC and MEC values obtained in RPMI after 24, 48, and 72 h of incubation

Species (*n*)	Incubation period (h)	MIC (μg/ml)		MEC (μg/ml)	
		GM	Range	GM	Range
A. flavus (27)	24	2.72	0.25–>16	0.27	0.25–0.5
	48	4	0.25–>16	0.31	0.25–0.5
	72	>16	>16	0.26	0.25–0.5
A. fumigatus (26)	24	0.73	0.25–>16	0.29	0.25–0.5
	48	27.7	0.25–>16	0.3	0.25–0.5
	72	17.45	0.25–>16	0.28	0.25–0.5
A. niger (17)	24	0.41	0.25–1	0.41	0.25–1
	48	0.44	0.25–2	0.42	0.25–1
	72	1.28	0.25–>16	0.42	0.25–0.5
A. terreus (9)	24	0.5	0.5	0.5	0.5
	48	0.5	0.5	0.5	0.5
	72	8.64	0.5–>16	0.5	0.5
A. nidulans (3)	24[a]				
	48	0.63	0.5–1	0.5	0.5
	72	0.63	0.5–1	0.5	0.5
F. solani (18)	24	22.6	16–>16	17.3	8–>16
	48	30.79	16–>16	27.43	16–>16
	72	>16	>16	30.8	16–>16
F. oxysporum (4)	24	16	16	19	16–>16
	48	>16	>16	>16	>16
	72	>16	>16	>16	>16

[a] No growth.

tibility tests performed at day 0 were repeated after the plates were stored at 35°C for 24 and 48 h. As with the long-term storage studies, the MICs did not vary more than one twofold dilution. These data show that caspofungin is stable both prior to and during the studies under the test conditions.

The plates were incubated at 35°C and the MIC results were read visually at 24, 48, and 72 h as the least concentration of drug that produced a prominent decrease in turbidity. MEC values were determined by microscopic examination of the microdilution plates. The least concentration of caspofungin causing abnormal hyphal growth with short abundant branchings was defined as the MEC (2, 16). The geometric mean (GM) and the ranges of MICs and MECs were analyzed to evaluate the in vitro activity of caspofungin. For computation of the GM values, high offscale MICs and MECs were converted to the twofold concentration just above the highest tested drug concentration.

GMs and ranges for the observed MIC and MEC values at 24, 48, and 72 h in RPMI, RPMI-2, and AM3 are shown in Tables 1, 2, and 3, respectively. In general, caspofungin MICs had a tendency to increase by one to five twofold dilutions at 48 and 72 h, compared to the 24-h MIC reading. MEC readings tended to be stable over this time period. Growth at 24 h was sufficient to permit reading an endpoint for all species but *A. nidulans*. Based on both MIC and MEC measurements, caspofungin did not have any meaningful activity against *Fusarium* isolates.

Among *Aspergillus* species, *A. flavus* yielded the highest MICs after 24 h in all test media, followed by *A. fumigatus*. However, the 48-h MICs obtained in RPMI and RPMI-2 for *A. fumigatus* were higher than those for *A. flavus*. On the other hand, all *Aspergillus* species generated similarly low MEC values. The itraconazole-resistant isolates had the same MIC and MEC values as other isolates of their species. Compared to RPMI, RPMI-2 gave slightly higher and AM3 generated slightly lower MICs and MECs for both *Aspergillus* and *Fusarium* spp. This effect was most pronounced with the MIC readings at 24 h.

MIC and MEC values were most similar at 24 h, but the correlation was limited overall. The MEC for *A. flavus* was 10-fold lower than the MIC at this time, and the MEC for *A. fumigatus* was approximately 3-fold lower than the MIC. As the incubation time was extended to 48 and 72 h, the discrepancies between the corresponding MIC and MEC values increased. The morphological changes seen at the MEC were relatively less affected by incubation time and did not vary more than one twofold dilution. On the other hand, MICs increased over time, but not in a consistent or predictable fashion.

Of note, a macroscopic (visible to the naked eye) correlate of the microscopic MEC was observed. At concentrations of caspofungin below the MEC, the colonies had a visibly filamentous growth pattern. At and above the MEC, the colonies became less filamentous and more granular. Thus, with practice it was possible to determine the MEC without the use of microscopic examination.

Data reported thus far on the activity of caspofungin against *Aspergillus* and *Fusarium* spp. are limited. Pfaller et al. (20) used the NCCLS microdilution methodology and reported MIC_{90} values after 72 h of incubation of 0.12 and 16 μg/ml, for *Aspergillus* and *Fusarium*, respectively, using an MIC endpoint of approximately 75% reduction in growth. Del Poeta et al. (5) used the macrodilution methodology proposed by NCCLS for yeasts (M27-A), an inoculum size of (0.5 to 2.5) $\times 10^3$ CFU/ml, and an MIC defined as the lowest drug concentration yielding visual turbidity less than or equal to that corresponding to 80% inhibition of growth. MICs were read when adequate growth was evident for each individual isolate. The GM MICs were 0.2, $\leq$0.09, and 75.78 μg/ml for *A. flavus*, *A. fumigatus*, and *F. oxysporum*, respectively. The values reported in both of

TABLE 2. MIC and MEC values obtained in RPMI-2 after 24, 48, and 72 h of incubation

Species (*n*)	Incubation period (h)	MIC (μg/ml)		MEC (μg/ml)	
		GM	Range	GM	Range
A. flavus (27)	24	3.17	0.25–>16	0.33	0.25–0.5
	48	4	0.25–>16	0.34	0.25–0.5
	72	>16	>16	0.34	0.25–0.5
A. fumigatus (26)	24	1.89	0.25–>16	0.31	0.25–0.5
	48	26.9	0.5–>16	0.31	0.25–0.5
	72	>16	0.5–>16	0.29	0.25–0.5
A. niger (17)	24	0.54	0.5–1	0.52	0.5–1
	48	0.64	0.5–2	0.54	0.5–1
	72	0.78	0.5–>16	0.54	0.5
A. terreus (9)	24	0.5	0.5	0.5	0.5
	48	1.26	0.5–>16	0.5	0.5
	72	8	0.5–>16	0.5	0.5
A. nidulans (3)	24[a]				
	48	0.5	0.5	0.5	0.5
	72	0.5	0.5	0.5	0.5
F. solani (18)	24	24.4	16–>16	22.63	8–>16
	48	>16	>16	28.51	16–>16
	72	>16	>16	>16	>16
F. oxysporum (4)	24	16	16	19.03	16–>16
	48	>16	>16	>16	>16
	72	>16	>16	>16	>16

[a] No growth.

Downloaded from http://aac.asm.org/ on April 30, 2020 by guest

TABLE 3. MIC and MEC values obtained in AM3 after 24, 48, and 72 h of incubation

Species (*n*)	Incubation period (h)	MIC (μg/ml)		MEC (μg/ml)	
		GM	Range	GM	Range
A. flavus (27)	24	2.27	0.125–>16	0.26	0.125–0.5
	48	3.7	0.125–>16	0.26	0.125–0.5
	72	>16	>16	0.26	0.125–0.5
A. fumigatus (26)	24	0.43	0.25–>16	0.31	0.25–0.5
	48	2.83	0.25–>16	0.31	0.25–0.5
	72	17.4	0.25–>16	0.31	0.25–0.5
A. niger (17)	24	0.23	0.125–1	0.2	0.125–0.25
	48	0.26	0.125–0.5	0.2	0.125–0.25
	72	0.32	0.25–1	0.27	0.25–0.5
A. terreus (9)	24	0.25	0.25	0.25	0.25
	48	0.25	0.25	0.25	0.25
	72	1.26	0.25–>16	0.25	0.25
A. nidulans (3)	24[a]				
	48	0.25	0.25	0.25	0.25
	72	0.25	0.25	0.25	0.25
F. solani (18)	24	19.4	16–>16	16.63	4–>16
	48	26.4	16–>16	26.4	16–>16
	72	>16	>16	>16	>16
F. oxysporum (4)	24	16	16	16	16
	48	26.91	16–>16	26.91	16–>16
	72	>16	>16	>16	>16

[a] No growth.

these studies are lower than what we observed for *Aspergillus* but similar for *Fusarium*. Espinel-Ingroff (10) defined the MIC as prominent inhibition of growth (≤50% of that of the growth control) and reported higher MIC values for *A. fumigatus* (GM of 2.15 μg/ml), lower values for *A. flavus* (0.5 μg/ml), and similar values for both *A. terreus* (0.5 μg/ml) and *Fusarium* spp. (≥16 μg/ml) in comparison to our results.

We also explored in detail the possible value of the MEC and its correlation with the MIC. Very few reports to date have used the MEC measurement. For *A. flavus* and *A. fumigatus* isolates, pneumocandin A_0 and pneumocandin B_0 MECs of 0.015 to 2 μg/ml (16) and LY-303366 MECs of 0.00125 to 0.005 μg/ml were reported (26). Whether the MEC has greater clinical relevance than the MIC has yet to be determined, but we think that the stability of the MEC measurement might make it a more reproducible endpoint to be used. Across multiple times of reading and medium conditions, the MEC values generally varied less than three twofold dilutions, while the MIC readings changed significantly and without obvious pattern as the incubation period was extended beyond 24 h.

The MEC is clearly a more labor-intensive measurement. We did, however, observe that the morphological changes observed microscopically are reflected in the macroscopic growth pattern. At and above the MEC, the abnormal hyphal structures yield colonies that are less filamentous and more granular. With experience, it did become possible to visually estimate the MEC. This, however, seemed to require considerable practice, and the interobserver reproducibility of this approach will require further study.

We also investigated the effect of time and medium on MIC and MEC. MICs generated in RPMI-2 were higher than those generated in RPMI, whereas AM3 tended to lower the values. Testing in AM3 has been shown to reduce the MIC of *Candida* isolates for both amphotericin B (25) and caspofungin (19). In other testing of *Aspergillus* and *Fusarium* isolates, we have shown that RPMI-2 MICs are higher than RPMI MICs for itraconazole and voriconazole and that AM3 MICs are lower for amphotericin B (3). Overall, these media effects are slight and have unclear meaning. In addition to the MICs, it has also been shown that the killing activity of LY303366 is influenced by the test medium used. The use of AM3 as the test medium instead of RPMI resulted in not only a significant decline in MICs but also in a more rapid fungicidal activity against *Candida* (13, 14).

The two itraconazole-resistant *A. fumigatus* isolates included in the study behaved similarly to others in terms of both caspofungin MICs and MECs. This finding is not unexpected, since the mechanisms of action for azoles and echinocandin antifungal agents are entirely distinct.

We conclude that MEC appears as a stable in vitro measurement for determining the activity of caspofungin against molds. Demonstration of its in vivo relevance awaits further studies.

This study was supported by a grant to Sevtap Arikan from Turkish Scientific and Technical Research Council (TUBITAK) and by a grant from Merck Research Laboratories.

REFERENCES

1. **Abruzzo, G. K., A. M. Flattery, C. J. Gill, L. Kong, J. G. Smith, V. B. Bikounis, J. M. Balkovec, A. F. Bouffard, J. F. Dropinski, H. Rosen, H. Kropp, and K. Bartizal.** 1997. Evaluation of the echinocandin antifungal MK-0991 (L-743,872): efficacies in mouse models of disseminated aspergillosis, candidiasis, and cryptococcosis. Antimicrob. Agents Chemother. **41:** 2333–2338.
2. **Abruzzo, G. K., A. M. Flattery, C. J. Gill, L. Kong, J. G. Smith, D. Krupa, V. B. Pkounis, H. Kropp, and K. Bartizal.** 1995. Evaluation of water-soluble pneumocandin analogs L-733560, L-705589, and L-731373 with mouse models of disseminated aspergillosis, candidiasis, and cryptococcosis. Antimicrob. Agents Chemother. **39:**1077–1081.
3. **Arikan, S., M. Lozano-Chiu, V. Paetznick, S. Nangia, and J. H. Rex.** 1999. Microdilution susceptibility testing of amphotericin B, itraconazole, and voriconazole against clinical isolates of *Aspergillus* and *Fusarium* species. J. Clin. Microbiol. **37:**3946–3951.
4. **Bartizal, K., C. J. Gill, G. K. Abruzzo, A. M. Flattery, L. Kong, P. M. Scott, J. G. Smith, C. E. Leighton, A. Bouffard, J. F. Dropinski, and J. Balkovec.** 1997. In vitro preclinical evaluation studies with the echinocandin antifungal MK-0991 (L-743,872). Antimicrob. Agents Chemother. **41:**2326–2332.
5. **Del Poeta, M., W. A. Schell, and J. R. Perfect.** 1997. In vitro antifungal activity of pneumocandin L-743,872 against a variety of clinically important molds. Antimicrob. Agents Chemother. **41:**1835–1836.
6. **Denning, D. W.** 1997. Echinocandins and pneumocandins—a new antifungal class with a novel mode of action. J. Antimicrob. Chemother. **40:**611–614.
7. **Denning, D. W., S. A. Radford, K. L. Oakley, L. Hall, E. M. Johnson, and D. W. Warnock.** 1997. Correlation between in-vitro susceptibility testing to itraconazole and in-vivo outcome of *Aspergillus fumigatus* infection. J. Antimicrob. Chemother. **40:**401–414.
8. **Ernst, E. J., M. E. Klepser, M. E. Ernst, S. A. Messer, and M. A. Pfaller.** 1999. In vitro pharmacodynamic properties of MK-0991 determined by time-kill methods. Diagn. Microbiol. Infect. Dis. **33:**75–80.
9. **Ernst, M. E., M. E. Klepser, E. J. Wolfe, and M. A. Pfaller.** 1996. Antifungal dynamics of LY 303366, an investigational echinocandin B analog, against *Candida* spp. Diagn. Microbiol. Infect. Dis. **26:**125–131.
10. **Espinel-Ingroff, A.** 1998. Comparison of in vitro activities of the new triazole SCH56592 and the echinocandins MK-0991 (L-743,872) and LY303366 against opportunistic filamentous and dimorphic fungi and yeasts. J. Clin. Microbiol. **36:**2950–2956.
11. **Franzot, S. P., and A. Casadevall.** 1997. Pneumocandin L-743,872 enhances the activities of amphotericin B and fluconazole against *Cryptococcus neoformans* in vitro. Antimicrob. Agents Chemother. **41:**331–336.
12. **Graybill, J. R., L. K. Najvar, M. F. Luther, and A. W. Fothergill.** 1997. Treatment of murine disseminated candidiasis with L-743, 872. Antimicrob. Agents Chemother. **41:**1775–1777.
13. **Klepser, M. E., E. J. Ernst, M. E. Ernst, S. A. Messer, and M. A. Pfaller.** 1998. Evaluation of endpoints for antifungal susceptibility determinations with LY303366. Antimicrob Agents Chemother. **42:**1387–1391.
14. **Klepser, M. E., E. J. Ernst, M. E. Ernst, and M. A. Pfaller.** 1997. Growth medium effect on the antifungal activity of LY 303366. Diagn. Microbiol. Infect. Dis. **29:**227–31.
15. **Krishnarao, T. V., and J. N. Galgiani.** 1997. Comparison of the in vitro

Downloaded from http://aac.asm.org/ on April 30, 2020 by guest

activities of the echinocandin LY303366, the pneumocandin MK-0991, and fluconazole against *Candida* species and *Cryptococcus neoformans.* Antimicrob. Agents Chemother. **41:**1957–1960.
16. **Kurtz, M. B., I. B. Heath, J. Marrinan, S. Dreikorn, J. Onishi, and C. Douglas.** 1994. Morphological effects of lipopeptides against *Aspergillus fumigatus* correlate with activities against (1,3)-β-D-glucan synthase. Antimicrob. Agents Chemother. **38:**1480–1489.
17. **Martinez-Suarez, J. V., and J. L. Rodriguez-Tudela.** 1996. In vitro activities of semisynthetic pneumocandin L-733,560 against fluconazole-resistant and -susceptible *Candida albicans* isolates. Antimicrob Agents Chemother. **40:** 1277–1279.
18. **National Committee for Clinical Laboratory Standards.** 1998. Reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi. Proposed standard. NCCLS document M38-P. National Committee for Clinical Laboratory Standards, Wayne, Pa.
19. **Nelson, P. W., M. Lozano-Chiu, and J. H. Rex.** 1997. In vitro growth-inhibitory activity of pneumocandins L-733,560 and L-743,872 against putatively amphotericin B- and fluconazole-resistant *Candida* isolates: influence of assay conditions. J. Med. Vet. Mycol. **35:**285–287.
20. **Pfaller, M. A., F. Marco, S. A. Messer, and R. N. Jones.** 1998. In vitro activity of two echinocandin derivatives, LY303366 and MK-0991 (L-743,792), against clinical isolates of *Aspergillus*, *Fusarium*, *Rhizopus*, and other filamentous fungi. Diagn. Microbiol. Infect. Dis. **30:**251–255.
21. **Pfaller, M. A., S. A. Messer, and S. Coffman.** 1997. In vitro susceptibilities of clinical yeast isolates to a new echinocandin derivative, LY303366, and other antifungal agents. Antimicrob. Agents Chemother. **41:**763–766.
22. **Pfaller, M. A., S. A. Messer, S. Gee, S. Joly, C. Pujol, D. J. Sullivan, D. C. Coleman, and D. R. Soll.** 1999. In vitro susceptibilities of *Candida dubliniensis* isolates tested against the new triazole and echinocandin antifungal agents. J. Clin. Microbiol. **37:**870–872.
23. **Uzun, O., S. Kocagoz, Y. Cetinkaya, S. Arikan, and S. Unal.** 1997. In vitro activity of a new echinocandin, LY303366, compared with those of amphotericin B and fluconazole against clinical yeast isolates. Antimicrob. Agents Chemother. **41:**1156–1157.
24. **Vazquez, J. A., M. Lynch, D. Boikov, and J. D. Sobel.** 1997. In vitro activity of a new pneumocandin antifungal, L-743,872, against azole-susceptible and -resistant *Candida* species. Antimicrob. Agents Chemother. **41:**1612–1614.
25. **Wanger, A., K. Mills, P. W. Nelson, and J. H. Rex.** 1995. Comparison of Etest and National Committee for Clinical Laboratory Standards broth macrodilution method for antifungal susceptibility testing: enhanced ability to detect amphotericin B-resistant *Candida* isolates. Antimicrob. Agents Chemother. **39:**2520–2522.
26. **Zhanel, G. G., J. A. Karlowsky, G. A. J. Harding, T. V. Balko, S. A. Zelenitsky, M. Friesen, A. Kabani, M. Turik, and D. J. Hoban.** 1997. In vitro activity of a new semisynthetic echinocandin, LY-303366, against systemic isolates of *Candida* species, *Cryptococcus neoformans*, *Blastomyces dermatitidis*, and *Aspergillus* species. Antimicrob. Agents Chemother. **41:**863–865.

Downloaded from http://aac.asm.org/ on April 30, 2020 by guest