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In Vitro Preclinical Evaluation Studies with the Echinocandin Antifungal MK-0991 (L-743,872)

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The echinocandin MK-0991, formerly L-743,872, is a water-soluble lipopeptide that has been demonstrated in preclinical studies to have potent activity against *Candida* spp., *Aspergillus fumigatus*, and *Pneumocystis carinii*. An extensive in vitro biological evaluation of MK-0991 was performed to better define the potential activities of this novel compound. Susceptibility testing with MK-0991 against approximately 200 clinical isolates of *Candida*, *Cryptococcus neoformans*, and *Aspergillus* isolates was conducted to determine MICs and minimum fungicidal concentrations MF(s). The MFC at which 90% of isolates are inhibited for 40 *C. albicans* clinical isolates was 0.5 μg/ml. Susceptibility testing with panels of antifungal agent-resistant species of *Candida* and *C. neoformans* isolates indicated that the MK-0991 MFCs for these isolates are comparable to those obtained for susceptible isolates. Growth kinetic studies of MK-0991 against *Candida albicans* and *Candida tropicalis* isolates showed that the compound exhibited fungicidal activity (i.e., a 99% reduction in viability) within 3 to 7 h at concentrations ranging from 0.06 to 1 μg/ml (0.25 to 4 times the MIC). Drug combination studies with MK-0991 plus amphotericin B found that this combination was not antagonistic against *C. albicans*, *C. neoformans*, or *A. fumigatus* in vitro. Studies with 0 to 50% pooled human or mouse serum established that fungal susceptibility to MK-0991 was not significantly influenced by the presence of human or mouse serum. Results from resistance induction studies suggested that the susceptibility of *C. albicans* was not altered by repeated exposure (40 passages) to MK-0991. Erythrocyte hemolysis studies with MK-0991 with washed and unwashed human or mouse erythrocytes indicated minimal hemolytic potential with this compound. These favorable results of preclinical studies support further studies with MK-0991 with humans.

MK-0991, previously known as L-743,872, a water-soluble, semisynthetic amine derivative of the natural product pneumocandin B_0 (L-688,786), belongs to a new generation of echinocandins that have been shown to have enhanced potency and an expanded spectrum of activity. Similar to other members of this class of compounds, the fungicidal mode of action of MK-0991 is via the inhibition of synthesis of 1,3-β-D-glucan, an essential cell wall polysaccharide providing structural integrity and osmotic stability for fungi, including *Pneumocystis carinii* cysts. MK-0991 is approximately 100-fold more potent against 1,3-β-D-glucan synthesis than the narrow-spectrum inhibitor L-688,786 (1, 13). MK-0991 has been demonstrated to have anti-*Candida*, anti-*Aspergillus*, anti-*Histoplasma*, and anti-*P. carinii* activity in vivo (3, 30, 34). Additionally, MK-0991 has been demonstrated to have excellent pharmacokinetics in rodents and nonhuman primates (19). It is currently in phase II clinical trials with humans.

In order to more clearly define the potential activities of MK-0991, a comprehensive in vitro biological evaluation was undertaken. More than 200 clinically relevant fungi consisting of *Candida*, *Cryptococcus*, and *Aspergillus* isolates were used for susceptibility testing. Susceptibility to MK-0991 also was determined for amphotericin B (AmB)-, flucytosine (5FC)-, fluconazole (FCZ)-, and ketoconazole (KTZ)-resistant fungal isolates. The potential for induction of resistance with a pathogenic *Candida albicans* strain was also conducted. Studies of the activity of the combination of MK-0991 and AmB against *C. albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* were performed. The effect of human or mouse serum on susceptibility was assessed, growth kinetic studies were performed, and the hemolytic potential of MK-0991 was evaluated in an a erythrocyte (RBC) hemolysis assay.

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MATERIALS AND METHODS

Compounds. MK-0991 (Fig. 1) was produced by the Department of Synthetic Chemical Research at Merck Research Laboratories, Rahway, N.J., and was shown by high-performance liquid chromatography to be >95% pure. The compound was dissolved and diluted in sterile distilled water. FCZ was obtained from Pfizer Central Research, Groton, Conn., and was formulated in sterile distilled water. AmB was purchased as Fungizone (Bristol-Myers Squibb & Sons, Inc. Princeton, N.J.) and was prepared according to the manufacturer's instructions. 5FC was obtained from Aldrich Chemical Company, Milwaukee, Wis., and was formulated in sterile distilled water.

Organisms. The activities of the antifungal agents were evaluated against a large battery of clinical isolates from the Merck Clinical Culture Collection. They included *Candida* spp. and *C. neoformans* and *A. fumigatus* isolates. *C. albicans* MY 1055 is a human isolate (obtained from the Williamsburg Community Hospital, Williamsburg, Va.), which was also used in the in vivo mouse studies. AmB-, 5FC-, FCZ-, and/or KTZ-resistant fungal isolates or isolates from patients with clinical failure after treatment with AmB, FCZ, and 5FC were graciously provided by Michael Rinaldi, Mycology Reference Laboratory, San Antonio, Tex.

Antifungal susceptibility assays. Antifungal susceptibility testing was performed by the broth microdilution assay with *Candida* spp. and *C. neoformans* strains to determine the MICs and minimum fungicidal concentrations (MFCs) of MK-0991 and AmB. The method was adapted from the reference method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (31) to a modified broth microdilution method, as reported previously (16). The inocula were standardized with a spectrophotometer (optical density, 530 nm) and were diluted to a final concentration of 0.5×10^3 to 2.5×10^3 in RPMI 1640 medium with L-glutamine, without sodium bicarbonate (Whittaker

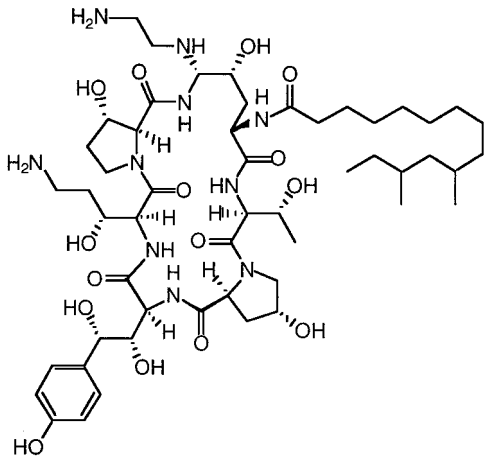

FIG. 1. Chemical structure of MK-0991.

Bioproducts, Boston, Mass.), and buffered with 0.165 M (34.54 g/liter) MOPS (morpholinepropanesulfonic acid) buffer (pH 7.0 at 35°C; Sigma, St. Louis, Mo.).

The test compounds were prepared as concentrated stock solutions, diluted in RPMI 1640 medium, and tested at concentrations ranging from 128 to 0.06 μg/ml. For serum studies the compounds were diluted in 0 to 50% fresh pooled human or mouse serum, and then RPMI 1640 medium was added prior to inoculation. The MIC was defined as the lowest concentration of compound that completely inhibited visible growth (absence of detectable turbidity). The MICs were recorded after 24 h of incubation at 35 to 37°C. After recording the MICs, microtiter plates were shaken and an MIC-2000 inoculator (Dynatech) was used to transfer a 1.5-μl sample from each well of the microtiter plate to a single reservoir plate containing 10 ml of Sabouraud dextrose agar (BBL, Cockeysville, Md.). The plates were incubated for 24 h (or 48 h for *Cryptococcus*) at 35 to 37°C. The MFC was defined as the lowest concentration of compound at which growth of fewer than four colonies occurred. The MICs and MFCs were determined for all *Candida* species and *C. neoformans* strains, while only MICs were determined for *Aspergillus* species.

The susceptibility of *A. fumigatus* strains to MK-0991 and AmB was also determined by a disk diffusion method on potato dextrose agar (Difco, Detroit, Mich.) adapted from the methods described in *Microbiological Assay, an Introduction of Quantitative Principles and Evaluation* (21). Briefly, 10 ml of potato dextrose agar was seeded with 10^6 CFU of *A. fumigatus* spores. The inoculum was prepared from saline-washed, 72-h slant cultures of *A. fumigatus* conidia and was standardized by hemocytometer counts and spectrophotometer reading (γ 660 nm with a 0.3 absorbance reading at a wavelength of 660 nm). Plate counts were used to confirm the numbers of viable cells in the final inoculum. Paper disks (1/4 in.; Schleicher & Schuell analytical paper disks; Difco) were impregnated with the test compounds at concentrations ranging from 128 to 0.06 μg/ml and were placed in duplicate on the agar surface. The plates were incubated for 24 h at 35°C, the zones of inhibition were measured, and the critical concentration (CC) of each compound was determined. The CC is the theoretically calculated concentration of compound at the edge of the zone of inhibition and is thus the concentration of compound at which a zone is no longer produced. The CC represents a measure of the susceptibility of a test organism and is similar but not identical to the MIC measured by dilution techniques that involve somewhat different test conditions. By plotting the natural logarithm of the concentration of the compound applied to the agar [ln (C_0)] against the square of the zone size produced (X^2), the intercept of the regression line on the logarithmic scale, where X^2 equals 0, is then equal to ln (CC), where CC is the concentration at which no zone is produced.

Geometric mean MICs and MFCs and the concentrations of the compound necessary to inhibit and kill 50% (MIC_{50} and MFC_{50}) and 90% (MIC_{90} and MFC_{90}) of the *Candida* and *Cryptococcus* isolates tested were determined, while only the MICs and CCs were determined for *Aspergillus* species.

Time-kill studies. Stock solutions of MK-0991 and AmB were prepared in sterile distilled water at a concentration of 1 mg/ml. To two tubes containing 49.9 ml of yeast nitrogen broth with dextrose (YNBD) was added 100 μl of these stock solutions. These were then serially diluted twofold (25 ml for each compound) to yield a concentration range of 2 to 0.06 μg/ml. A yeast extract malt broth for cultivation of yeasts and molds (YM) broth cultures of *C. albicans* MY 1055 and *Candida tropicalis* CLY 545 were grown overnight on a shaker (230 rpm) at 35°C. These cultures (≅10^8 CFU/ml) were diluted 1:1,000 and directly placed into each tube containing the antifungal agents (25 μl:25 ml), for a final concentration of 10^5 CFU/ml.

All tubes including a non-antifungal agent-containing growth control tube were placed in a shaker water bath with the temperature set at 35°C. At time points of 0, 1, 3, 4, 5, 7, 9, and 24 h, aliquots were removed from each tube, serially diluted 10-fold, and plated onto Sabouraud dextrose agar plates, and the plates were incubated for 24 h at 35°C. Colony counts were performed to determine the numbers of CFU remaining after each time point. Fungicidal activity was defined as 99% killing or a 2-log drop in the numbers of CFU compared to the numbers of CFU of the starting cell concentration of the growth control tube at the zero time point.

Resistance induction. The potential for resistance development was determined by recording MICs and MFCs after each of 40 serial transfers of *C. albicans* MY 1055 when the organism was incubated in the presence of subinhibitory concentrations of MK-0991. The test was conducted as described above for the microdilution method, except that the resistance induction experiments were performed in 10 ml of YNBD by the macrodilution methods recommended by NCCLS (31). Nonpassaged versus final-passage (40th passage) *C. albicans* cells were tested by the broth microdilution method described above.

Drug combination studies. Drug combination testing was performed by the broth microdilution checkerboard method to evaluate the activity of the combination of MK-0991 and AmB against *C. albicans*, *C. neoformans*, and *A. fumigatus* isolates (26). The in vitro interactions were calculated algebraically, and the results were interpreted as synergistic, indifferent, or antagonistic depending on whether the antifungal activity of the combination was greater than, equivalent to, or less than the activities of the individual agents, respectively. For each combination, the fractional fungicidal concentration (FFC) and the fractional inhibitory concentration (FIC) were calculated, as indicated below:

$$\text{FFC/FIC of agent A} = \frac{\text{MFC/MIC of agent A in combination}}{\text{MFC/MIC of agent A alone}}$$

$$\text{FFC/FIC of agent B} = \frac{\text{MFC/MIC of agent B in combination}}{\text{MFC/MIC of agent B alone}}$$

The summation of the FFC/FIC index (ΣFFC/FIC) for each combination was calculated as ΣFFC/FIC = FFC/FIC of agent A + FFC/FIC of agent B, and the results were interpreted as follows: synergism was ΣFFC/FIC of ≤0.5, indifference was ΣFFC/FIC of $0.5 < X < 1.0$, and antagonism was ΣFFC/FIC of > 1.0.

RBC hemolysis assay. A microtiter RBC hemolysis assay was used to determine the potential of MK-0991 to hemolyze human or mouse RBCs. A suspension of freshly drawn heparinized human or CD-1 mouse (Charles River, Wilmington, Mass.) whole blood (2 ml) was added to 50 ml of sterile 5% dextrose. A 4-mg/ml stock drug suspension was diluted by adding 0.2 ml of the stock drug suspension to 1.4 ml of sterile 5% dextrose. Test solutions were dispensed into microtiter wells and were serially diluted in 5% dextrose to yield final test concentrations of 400 to 0.20 μg/ml. Finally, 38 μl of the RBC suspension was added to each well. Hemolysis of RBCs was indicated by complete or partial clearing (hemolysis), and the concentration at which hemolysis occurred was defined as the minimum lytic concentration (MLC) of a test compound after 2 h at room temperature.

RESULTS

As indicated in Table 1, the MICs of MK-0991 and AmB did not differ more than twofold from the MFCs. When comparing MIC_{90}s, MK-0991 and AmB were comparable in activity against *C. albicans*, *C. tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, and *Candida* (*Torulopsis*) *glabrata* isolates. MK-0991 was more potent (fourfold) than AmB against *Candida lusitaniae* but was less potent than AmB against *Candida guilliermondii* and *C. neoformans* isolates. When tested in vitro against *A. fumigatus* by the NCCLS M27-T broth microdilution method (31), MK-0991 produced abnormal morphological effects but not complete inhibition. When tested by the disk diffusion method on solid *Aspergillus*-seeded agar, MK-0991 exhibited potent activity, with zones of inhibition (CC values) comparable to those for AmB at similar concentrations (Table 2).

Figures 2 and 3 demonstrate the fungicidal kinetics of MK-0991 against *C. albicans* and *C. tropicalis*. MK-0991 exhibited fungicidal activity (99% reduction in viability) within 6 to 8 h at concentrations ranging from 0.06 to 0.25 μg/ml (0.5 to 2 times the MIC). This supports the fact that glucan synthesis inhibition is a target against which action results in fungicidal activity (28). At the MFC, AmB was also fungicidal, but FCZ was fungistatic (Fig. 2). Although the killing rate for MK-0991 was slower than that for AmB, it was progressive and prolonged after a lag period of about 1 h and continued beyond 9 h. Rates

TABLE 1. MIC ranges, geometric mean MICs, MIC_{50}s, MIC_{90}s, and of MK-0991 and AmB for clinically relevant fungi

Organism (*n*)[a]	Antifungal agent	MIC (μg/ml)[b]				MFC (μg/ml)[c]			
		Range	50%	90%	Geometric mean	Range	50%	90%	Geometric mean
Candida albicans (40)	MK-0991	0.25–0.50	0.50	0.50	0.37	0.125–1.0	0.25	0.50	0.29
	AmB	0.125–0.50	0.25	0.25	0.25	0.06–0.50	0.125	0.25	0.18
Candida tropicalis (20)	MK-0991	0.25–1.0	0.50	1.0	0.54	0.25–1.0	0.50	1.0	0.57
	AmB	0.25–0.50	0.25	0.50	0.29	0.25–0.50	0.25	0.50	0.34
Candida parapsilosis (20)	MK-0991	0.25–1.0	0.50	0.50	0.52	0.25–0.50	0.50	0.50	0.44
	AmB	0.50–1.0	1.0	1.0	0.76	0.50–1.0	1.0	1.0	0.78
Candida lusitaniae (20)	MK-0991	0.125–0.50	0.25	0.50	0.30	0.06–0.50	0.25	0.50	0.28
	AmB	0.50–4.0	1.0	2.0	1.11	0.50–4.0	1.0	2.0	1.23
Candida guilliermondii (20)	MK-0991	0.25–2.0	1.0	2.0	1.19	0.25–4.0	2.0	2.0	1.41
	AmB	0.125–0.25	0.125	0.25	0.16	0.125–0.50	0.25	0.50	0.22
Candida krusei (20)	MK-0991	0.50–2.0	1.0	2.0	1.04	0.50–2.0	1.0	1.0	0.97
	AmB	0.25–0.50	0.25	0.50	0.30	0.25–0.50	0.25	0.50	0.28
Candida pseudotropicalis (20)	MK-0991	0.125–0.50	0.25	0.50	0.27	0.06–0.50	0.25	0.50	0.26
	AmB	0.25–0.50	0.25	0.50	0.28	0.125–0.50	0.25	0.50	0.29
Candida glabrata (20)	MK-0991	0.25–2.0	0.50	1.0	0.66	0.50–1.0	1.0	1.0	0.84
	AmB	0.125–0.50	0.25	0.50	0.25	0.125–0.50	0.25	0.50	0.26
Cryptococcus neoformans (19)	MK-0991	16.0–32.0	32.0	32.0	23.9	8.0–32.0	16.0	32.0	17.9
	AmB	0.125–0.50	0.25	0.50	0.27	0.125–0.50	0.25	0.50	0.26

[a] *n*, number of isolates tested.
[b] Broth microdilution method, RPMI 1640 medium, inocula of 0.5×10^3 to 2.5×10^3 CFU/ml, and incubation for 24 h at 35 to 37°C.
[c] Microtiter plates were shaken and 1.5-ml samples were transferred to 10-ml Sabouraud dextrose agar plates, and the plates were incubated for 24 to 48 h at 35 to 37°C.

of killing after the lag period were similar at 0.25 and 4 times the MFC of MK-0991 (data not shown), and MK-0991 showed a more rapid rate of killing between 3 and 4 h after the beginning of exposure. The viability of *C. tropicalis* was reduced more rapidly (99% reduction in 2 to 4 h), and as with *C. albicans*, the same prolonged killing was observed (Fig. 3).

The data in Table 3 demonstrate that MK-0991 was more effective than AmB against *C. albicans* and *C. tropicalis* isolates from patients who had failed treatment with AmB, 5FC, or FCZ, with MICs ranging between 0.125 and 1 μg/ml. MK-0991 was also more potent than AmB against the *C. lusitaniae* isolates (MICs, 1 to 2 μg/ml) but was as potent as AmB against the *C. glabrata* isolates. *C. neoformans* isolates from patients who had failed FCZ and 5FC treatment were 16- to 64-fold more susceptible to AmB than to MK-0991, as was observed with susceptible isolates. Human or mouse serum did not significantly affect the susceptibility of *C. albicans* to MK-0991 or AmB in YNBD or RPMI 1640 medium (Table 4).

TABLE 2. Susceptibility of *A. fumigatus* isolates to MK-0991 and AmB determined by agar disk diffusion

Culture no.	CC (μg/ml)[a]	
	MK-0991	AmB
MF5668	0.27	0.43
CLY 0315	0.38	0.04
CLY 0522	0.27	0.13
CLY 0523	0.47	0.12

[a] CC was calculated by regression analysis of disk diffusion inhibition data.

AmB combined with MK-0991 was not antagonistic against *C. albicans*, *C. neoformans*, or *A. fumigatus* strains. Interestingly, AmB combined with MK-0991 showed possible additive to synergistic effects against *C. neoformans* and *A. fumigatus*, with FICs and FFCs ranging between 0.39 and 0.66; against *C. albicans* the effects tended to be indifferent (FICs and FFCs, 0.74 to 0.90).

Repeated exposure (selection for resistance) of *C. albicans* MY 1055 to subinhibitory concentrations of MK-0991 did not significantly alter MICs or MFCs. The MIC of MK-0991 did not change significantly from the initial MIC (0.06 μg/ml) to the final MIC (0.125 μg/ml) after 40 passages in the presence of subinhibitory concentrations of MK-0991. The original *C. albicans* isolate and the isolate obtained after 40 passages were examined microscopically, and no apparent morphological changes were evident, nor did the MICs and MFCs change more than twofold when the MICs and MFCs were assayed by the broth microdilution method. Compared to AmB, MK-0991 was relatively nonhemolytic against human and mouse RBCs in an RBC hemolysis assay. The MLCs of MK-0991, AmB, and distilled water for human RBCs were >400, 25, and >400 μg/ml, respectively. The MLCs for mouse RBCs were 100 to 200, 3.0, and >400 μg/ml, respectively.

DISCUSSION

MK-0991 is a member of a new group of semisynthetic amine derivatives of pneumocandin B_0 (L-688,786). This compound was found to be significantly more potent than the narrow-spectrum echinocandins against clinically relevant, antifungal agent-susceptible and -resistant *Candida* isolates (6,

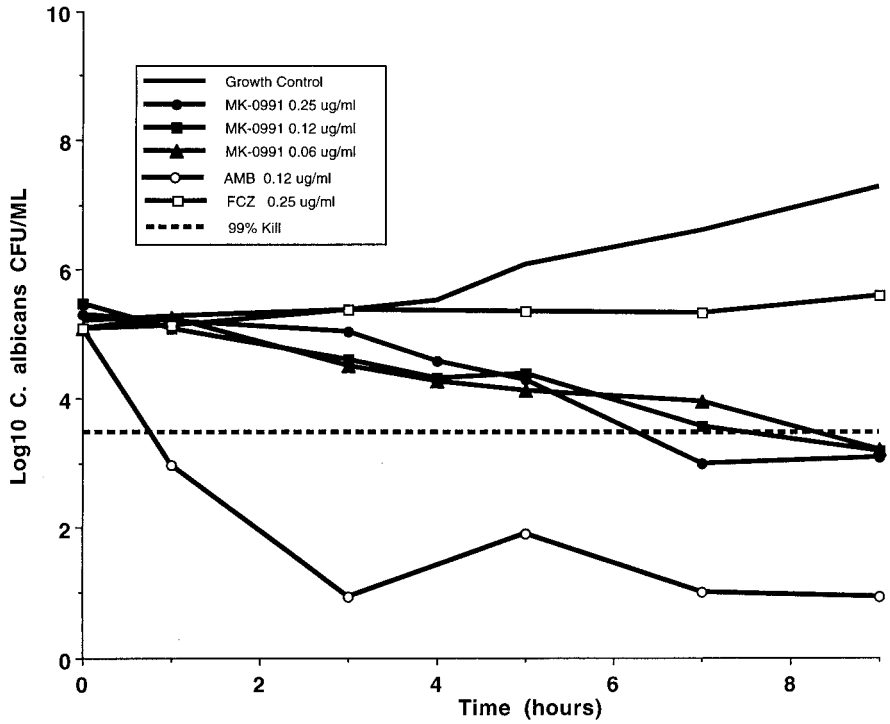

FIG. 2. *C. albicans* time-kill study.

32, 40). Because of the recent widespread emergence of resistance to antifungal agents, especially to FCZ (4, 5, 11, 13, 14, 18, 22, 25, 35), it is imperative that there be developed a chemotherapeutic agent that is effective against antifungal agent-resistant fungi. MK-0991 had equivalent activity against both susceptible and resistant isolates, suggesting its potential utility against these emerging pathogens (5, 38). In addition, MK-0991 did not induce resistance in vitro. Further-

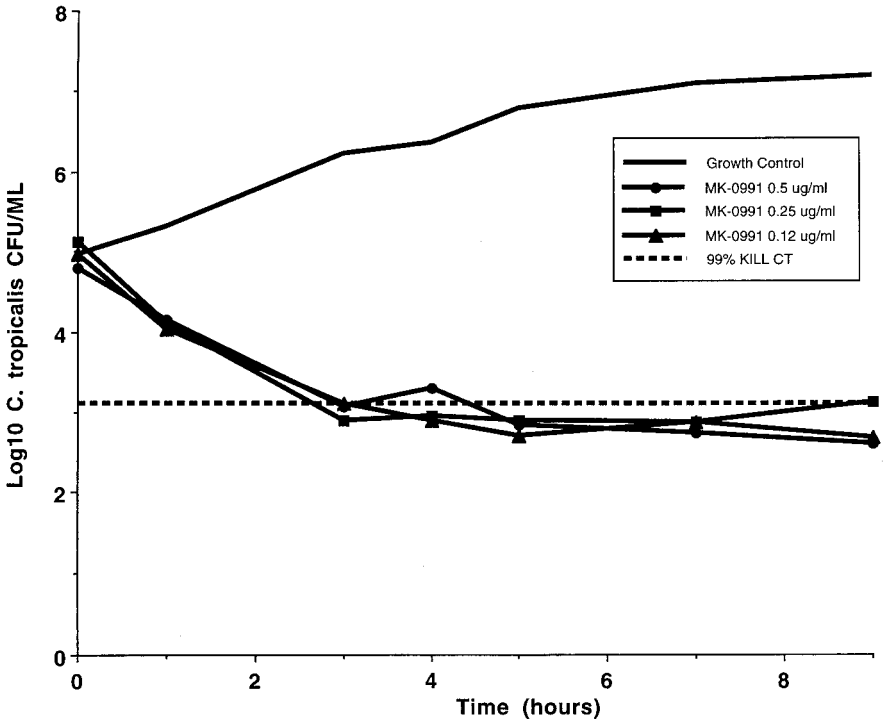

FIG. 3. *C. tropicalis* time-kill study.

TABLE 3. Susceptibilities of antifungal agent-resistant isolates or isolates from patients with clinical failure after treatment with AmB, FCZ, and 5FC

Organism	Resistance or failed outcome after treatment with the following drug:	Culture no.	MIC (μg/ml)			
			MK-0991[a]	AmB	FCZ	5FC
Candida albicans	Susceptible	489	0.25	0.50	0.06	0.06
	5FC	536	0.125	1.0	0.50	8.0
	5FC	544	0.125	0.50	0.50	>32.0
	AmB	537	1.0	4.0	1.0	1.0
	FCZ	538	0.125	0.50	2.0	0.06
	5FC and FCZ	543	0.25	0.50	2.0	>32.0
	FCZ	539	0.25	0.50	32.0	0.06
	FCZ	540	0.125	0.50	2.0	0.125
	FCZ	541	0.50	1.0	>32.0	0.06
	FCZ	542	0.125	0.50	32.0	1.0
Candida tropicalis	Susceptible	425	0.25	0.25	0.125	0.50
	FCZ	545	0.125	1.0	>32.0	0.06
Candida glabrata	FCZ	257	0.50	0.25	>32.0	0.03
	FCZ	494	0.50	0.50	>32.0	0.03
	5FC and FCZ	535	0.50	1.0	>32.0	>32.0
Candida lusitaniae	Susceptible	298	0.50	1.0	0.50	≤0.01
	AmB and 5FC	533	2.0	8.0	0.125	>32.0
	AmB	534	1.0	8.0	0.50	≤0.01
Cryptococcus neoformans	Susceptible	34	16.0	0.25	NT[b]	NT
	FCZ	525	32.0	0.50	8.0	1.0
	FCZ and 5FC	526	32.0	1.0	16.0	4.0
	FCZ	527	16.0	1.0	8.0	2.0
	FCZ	528	16.0	1.0	8.0	2.0
	FCZ	529	32.0	1.0	4.0	2.0
	FCZ	530	16.0	1.0	4.0	2.0
	FCZ	531	16.0	1.0	4.0	2.0
	FCZ	532	16.0	0.50	32.0	2.0

[a] Broth microdilution method, RPMI 1640 medium, inocula of 1×10^3 to 5×10^3 CFU/ml, incubation for 24 to 48 h at 35 to 37°C.
[b] NT, not tested.

more, naturally occurring echinocandin-resistant, virulent fungal isolates have not yet been described.

In an in vitro *C. albicans* 1,3-β-D-glucan synthase assay, semisynthetic analogs were 70- to 100-fold more potent than echinocandin B_0, with 50% inhibitory concentrations ranging between 1 and 10 μM (7, 8, 12). Importantly, MK-0991 showed demonstrable fungicidal activity. The echinocandins that were inhibitory to cell wall 1,3-β-D-glucan were fungicidal in growth inhibition assays, supporting the premise that glucan synthesis inhibition is a target against which action results in fungicidal activity (20). For MK-0991 there was an obvious lag period that lasted approximately 1 h, indicating that fungal growth and metabolism were required for killing to occur. Also, the rates and time to killing of *C. albicans* were not highly concentration dependent, as has previously been described by us and several investigators with echinocandins A_0 and B_0 and other echinocandins (7, 8, 17). The rate and time to killing for MK-0991 were significantly longer than those for AmB, which does not require cell growth for activity, but this observation did not appear to affect their comparative activities in vivo. AmB and MK-0991 were equally efficacious against disseminated *C. albicans* and *A. fumigatus* infections in animal models (3, 10, 37).

In most cases the susceptibility of *C. albicans* MY 1055 to MK-0991 or AmB was not significantly affected by the addition of human or mouse serum to either YNBD or RPMI 1640 medium. The only modestly elevated value was that of MK-0991 in RPMI 1640 medium with 50% mouse serum, which is not relevant in vivo.

Semisynthetic echinocandins like MK-0991 have been demonstrated to have a favorable spectrum of antifungal activity, especially against the clinically more prevalent fungal diseases (i.e., candidiasis and aspergillosis) (1–3, 29). Although there is no standard in vitro susceptibility assay for assessing the activities of compounds against *P. carinii*, the echinocandins proved to be highly potent and effective therapeutically against *P. carinii* pneumonia in immunosuppressed rats (36). The potency of MK-0991 against *P. carinii* was determined to be approximately 10-fold greater than that of echinocandin B_0.

The in vitro activity of MK-0991 against *A. fumigatus* isolates was not apparent by the broth dilution method, even though potent in vivo efficacy against *A. fumigatus* in rodents was demonstrated (3, 10, 37). It has generally been established that *Aspergillus* species are insensitive to the echinocandin class of antifungal agents in standard broth dilution susceptibility assays (9, 15, 23). Kurtz et al. (27) previously demonstrated that echinocandins can produce profound morphological changes in *Aspergillus* hyphae. Highly sensitive bioassay systems for echinocandins in which inhibition of *Aspergillus* growth on agar is used have been reported previously (17). In this study we also used the disk diffusion method as a means of determining the susceptibility of *A. fumigatus* isolates to MK-0991 and AmB. We found that MK-0991 possesses activity comparable

TABLE 4. Effects of human and mouse serum on susceptibility of *C. albicans* MY 1055 to MK-0991 and AmB[a]

Medium	MFC (μg/ml)	
	AmB	MK-0991
YNBD plus human serum		
Plain	0.25	≤0.06
10%	0.25	≤0.06
20%	0.25	≤0.06
30%	0.25	≤0.06
40%	0.25	≤0.06
50%	0.25	≤0.06
RPMI plus human serum		
Plain	0.25	≤0.06
10%	0.25	≤0.06
20%	0.25	0.125
30%	0.25	0.25
40%	0.25	0.125
50%	0.25	0.25
YNBD plus mouse serum		
Plain	0.25	≤0.06
10%	0.25	≤0.06
20%	0.5	≤0.06
30%	0.25	≤0.06
40%	0.25	≤0.06
50%	0.25	0.125
RPMI plus mouse serum		
Plain	0.25	≤0.06
10%	0.25	≤0.06
20%	0.25	≤0.06
30%	0.25	0.125
40%	0.25	0.25
50%	0.5	0.5

[a] The broth microdilution method was used. The compounds were diluted in fresh pooled human or mouse serum, and then RPMI 1640 medium was added before inoculation YNBD and RPMI 1640 medium (RPMI) contained the ingredients described in the text.

to that of AmB against the *A. fumigatus* isolates tested when the drugs are used at similar concentrations.

Only slight activity of MK-0991 was apparent against *C. neoformans* isolates. It has been postulated that *C. neoformans* may possess 1,6-β-glucan or other non 1,3-β-D-glucans (i.e., 1,3-α- or 1,6-α-glucans) in its cell wall, thus explaining its relative lack of sensitivity to echinocandins (20, 24, 33, 39). On the other hand, variable responses against *C. neoformans* may also involve penetration or access of the compound to the target, the metabolic state of the yeast in broth culture, or undefined resistance mechanisms.

It was also encouraging that in studies with combinations of AmB and MK-0991, no antagonistic effects against *Candida*, *Cryptococcus*, or *Aspergillus* isolates were observed. In fact, additive to synergistic effects against certain isolates were found. This may have substantial clinical significance, first, in situations in which patients may already be receiving an antifungal drug before the initiation of echinocandin therapy, second, in expanding the spectrum of activity to possibly include anti-*Cryptococcus* activity, and lastly, in potentially increasing the therapeutic index through synergistic action and reducting the doses of individual components, e.g., reduction of the AmB concentration.

MK-0991 was relatively nonhemolytic against human and mouse RBCs, which suggests that RBC hemolysis should not be a mechanistic factor for dose-limiting toxicity in vivo. AmB is highly hemolytic and does exhibit severe toxic manifestations clinically, but its dose-limiting toxicity may not be due solely to its hemolytic effects. Finally, since β-glucan is a selective target present only in fungal cell walls and not in mammalian cells, the mode of action of MK-0991 rules out the possibility of mechanism-based toxicity in the mammalian host (39).

The studies described here, combined with the preclinical efficacy studies with animal models performed thus far, support the need for the further evaluation of MK-0991 as a developmental antifungal candidate that possesses several potential advantages over the already marketed azoles and polyene agents.

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