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Comparison of the In Vitro Activities of the Echinocandin LY303366, the Pneumocandin MK-0991, and Fluconazole against *Candida* Species and *Cryptococcus neoformans*

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Two new glucan synthesis inhibitors, the echinocandin LY303366 and the pneumocandin MK-0991 (formerly L-743,872), were studied for their antifungal activities in vitro in relation to each other and in relation to the activity of the triazole fluconazole. Systematic analysis of broth macrodilution testing by varying the starting inoculum size, medium composition, medium pH, temperature of incubation, length of incubation, or selection of endpoints failed to identify significant differences in antifungal activity for either LY303366 or MK-0991 in comparison to the activity under standard test conditions specified for other antifungal agents in National Committee for Clinical Laboratory Standards (NCCLS) document M27A. Under standardized conditions, both drugs exhibited prominent activity against *Candida* species including *Candida glabrata* and *Candida krusei* but showed little activity against *Cryptococcus neoformans*. This spectrum of activity differed from that of fluconazole, which exhibited marginal activity against *C. glabrata* and *C. krusei* but prominent activity against other *Candida* species and *C. neoformans*. For individual strains, broth microdilution MICs of LY303366 and MK-0991 were similar to but frequently higher than broth macrodilution results. In contrast, fluconazole broth microdilution MICs were often lower than broth microdilution results. We conclude that the test conditions specified in NCCLS document M27A are applicable to these two new glucan synthesis inhibitors and that systematic differences between broth microdilution procedures and the broth macrodilution reference standard will need to be addressed before the two test methods can be used interchangeably.

Inhibition of glucan synthesis is an attractive therapeutic strategy for systemic mycoses since the absence of homologous enzymes in humans may afford a high degree of selectivity for invading fungi (4, 13). Moreover, as a new class of therapeutic agents, inhibitors of glucan synthesis could possess activity against fungi that are resistant to drugs that act by other mechanisms (1, 5, 6, 12, 24). These considerations have led to the development of the echinocandin LY303366 by Eli Lilly & Company and the related pneumocandin MK-0991 (formerly designated L-743,872) by Merck Research Laboratories. Both of these compounds have been introduced into human experimental trials.

Prior to the approval of these new antifungal agents for general use, it would be useful to know if the standard reference method for testing yeasts, recently developed by consensus through the National Committee for Clinical Laboratory Standards (NCCLS) (16), is applicable to this agent. In the study described in this report, we analyzed the in vitro activities of LY303366 and MK-0991 by changing each of several test conditions that influence test results with other antifungal agents (2, 3, 10, 11, 14, 15, 23). On the basis of these studies, the NCCLS reference standard appears suitable for the testing of both agents, and therefore, we determined the susceptibilities of representative clinical isolates under those conditions.

MATERIALS AND METHODS

Drugs. LY303366, MK-0991, and fluconazole were provided by Eli Lily & Company, Merck Research Laboratories, and Pfizer Incorporated as their powders, which were stored at −70°C until use. MK-0991 was water soluble, and a stock solution was prepared in water at a concentration of 1.0 mg/ml and stored in 1.0-ml aliquots at −70°C until it was needed. LY303366, which is poorly soluble in water, was prepared in undiluted dimethyl sulfoxide (DMSO) in a similar fashion.

Yeast. Ten isolates (two each of *Candida albicans*, *Candida tropicalis*, and *Cryptococcus neoformans* and one each of *Candida lusitaniae*, *Candida parapsilosis*, *Candida glabrata*, and *Candida krusei*) were used for the initial experiments. Further experiments with 102 strains of yeast were conducted under a single set of standardized conditions. Of the 102 isolates, 15 were *C. albicans*, 12 were *C. lusitaniae*, 15 were *C. tropicalis*, 15 were *C. parapsilosis*, 15 were *C. glabrata*, 15 were *C. krusei*, and 15 were *C. neoformans*. Isolates were the generous gifts of M. A. Pfaller (69 genetically distinct strains from fungemic patients at the University of Iowa [19, 21]), Mahmoud A. Ghannoum (15 strains of *C. neoformans* [26]), J. H. Rex (11 strains recovered from fungemic patients during a clinical trial conducted by the National Institute of Allergy and Infectious Diseases Mycoses Study Group [22]), A. Espinel-Ingroff (5 strains of *C. krusei*), and J. C. Pottage (1 strain of *C. krusei* from St. Johns Hospital, Detroit, Mich. [25]). The remaining 10 isolates were from the clinical laboratory of the Veterans Affairs Medical Center in Tucson, Ariz. When not in active use, isolates were stored in yeast nitrogen base (YNB; Difco Laboratories, Detroit, Mich.) with 2% (wt/vol) glucose and 10% (vol/vol) glycerol at −70°C and were repeatedly subcultured on Sabouraud dextrose agar plates (Becton Dickinson Microbiology Systems, Cockeysville, Md.), with overnight incubation at 37°C before testing.

Media and buffers. RPMI 1640 (Sigma Chemical Co., St. Louis, Mo.) constituted with 0.2% (wt/vol) glucose and buffered with morpholinepropanesulfonic acid (MOPS; Sigma Chemical Co.) at a final concentration of 0.165 M to a pH of 7.0 was used in most studies. In selected studies, YNB or antibiotic medium 3 (Difco) was substituted for RPMI 1640, as indicated in the Results. In other studies, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; Sigma) or sodium phosphate was substituted for MOPS, and the pH was adjusted to between 3.0 and 7.0.

Susceptibility testing. NCCLS reference method M27A (16) was followed for the broth macrodilution susceptibility tests, with modification of the test conditions as described below for specific experiments. Briefly, twofold dilutions of the antifungal agents were prepared in medium to concentrations ranging from 0.015 to 8.0 μg/ml. For dilution procedures, the procedure described for reference method M27A was followed exactly. Yeast inocula were adjusted with a spectrophotometer (18) to 0.5×10^3 to 2.5×10^3 yeast cells per ml. Yeast inoculum (0.9 ml) and diluted drug (0.1 ml) were mixed (final volume, 1.0 ml) in polystyrene tubes, and the mixture was incubated at 35°C without agitation for 48 h (72 h for *C. neoformans*).

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TABLE 1. Effects of various test conditions on broth macrodilution susceptibility test results[a]

Variable	MK-0991					LY303366				
	MIC_0 (μg/ml)		MIC_2 (μg/ml)		$IC_{1/2}$ (μg/ml)	MIC_0 (μg/ml)		MIC_2 (μg/ml)		$IC_{1/2}$ (μg/ml)
	Day 1	Day 2	Day 1	Day 2		Day 1	Day 2	Day 1	Day 2	
Inoculum										
log 2	0.71	1.32	0.66	1.15	0.50	5.66	2.82	4.0	2.14	2.29
log 3	0.81	1.15	0.66	1.0	0.57	4.59	4.28	3.7	3.7	2.82
log 4	0.93	2.00	0.81	1.52	0.71	6.06	7.46	4.9	6.49	4.28
log 5	0.93	2.64	0.87	2.46	0.71	8.0	10.6	8.0	10.6	5.27
pH										
3	0.57	0.62	0.54	0.62	0.50	4.0	5.66	3.48	4.0	3.24
4	0.66	0.71	0.62	0.66	0.50	4.0	4.0	3.25	3.48	3.03
5	0.71	0.35	0.93	0.35	0.54	4.0	4.23	3.25	3.48	3.73
6	0.62	0.76	0.62	0.66	0.54	4.59	4.59	3.7	3.73	3.73
7	0.81	1.00	0.76	0.87	0.62	4.0	5.27	3.48	4.59	3.03
Temp										
30°C	1.0	1.15	1.0	1.07	0.57	4.0	4.0	3.2	3.24	3.24
35°C	1.0	1.15	1.0	1.07	0.57	4.0	4.0	3.2	3.73	3.48
37°C	0.93	1.15	0.81	1.0	0.62	3.73	4.0	3.73	3.24	3.48
Medium										
RPMI 1640	0.76	0.93	0.66	0.54	0.47	4.0	3.7	4.28	4.92	3.2
YNB	0.81	1.41	0.66	0.71	0.62	4.0	4.0	4.59	4.59	3.48
Antibiotic medium 3	0.76	1.23	0.76	0.81	0.54	3.73	4.0	4.92	4.59	3.48
Buffer										
MOPS	0.76	0.93	0.76	0.76	0.47	4.29	4.9	4.0	4.92	4.0
HEPES	0.81	1.41	0.71	0.81	0.62	3.48	4.0	3.25	3.48	2.83
Phosphate	0.76	1.23	0.71	0.76	0.54	4.0	4.59	4.0	4.28	3.24

[a] Results are geometric means for 10 isolates.

Endpoints for determining the MICs were defined as the lowest drug concentration which inhibited growth completely (MIC_0) or by 80% (MIC_2) after 48 h of incubation (72 h for *C. neoformans*). Under standardized conditions, MIC results for LY303366 and MK-0991 used the MIC_0 endpoint, and MIC results for fluconazole used the MIC_2 endpoint. In addition, a turbidimetric endpoint (50% inhibitory concentration [$IC_{1/2}$]) was determined during active growth as the lowest drug concentration that resulted in the following: $\%T > \%T$ control + [0.5(100 − $\%T$ control], where $\%T$ is the percent transmission and $\%T$ control is the turbidity in the drug-free control tube (9). MICs were determined by the broth microdilution method, in line with the recommendations in NCCLS document M27A, described previously (9). Briefly, 100 μl of fungal suspension was added to 100 μl of drug solution in each well of round-bottom 96-well microtitration plates, and the contents were mixed with a pipette before incubation at 35°C. MICs were read without agitation as the lowest drug concentration that prevented growth.

Statistical procedures. Kruskal-Wallis nonparametric analysis, as implemented by Systat (Systat, Inc., Evanston, Ill.), was used to test the effects of various test conditions. Differences between the results obtained by the broth macrodilution and the broth microdilution methods were analyzed by the Wilcoxon test. A P value of less than 0.05 was taken as significant.

RESULTS

Drug insolubility and effects of changing solvents. When DMSO, ethyl alcohol, polyethylene glycol 400 or water were used as solvents, MK-0991 dissolved in water but precipitated in other solvents and LY303366 precipitated in water but dissolved in other solvents. When the susceptibilities of two strains of *C. albicans* to LY303366 were tested with final concentrations of either DMSO, ethyl alcohol, or polyethylene glycol 400 of 1%, all results for each strain were within twofold (data not shown). On the basis of this survey, 1% DMSO was arbitrarily chosen as the solvent for LY303366 for all subsequent studies.

Effects of changing other test conditions. Systematic analysis of variations in the inoculum size, medium pH, buffer system, medium composition, incubation temperature, and time of reading are presented in Table 1. For both drugs, there was little or no effect of changes in test conditions on any of the endpoints measured. Statistical comparisons of results of tests for each variable showed no significant differences. These results indicate that the conditions specified by reference method M27A for other drugs (inoculum size, 10^2 cells/ml; RPMI 1640 medium buffered to 7.0 with MOPS and incubated at 35°C for reading on the second day) would be satisfactory for standardized testing of both LY303366 and MK-0991. Similarly, since our results did not indicate a difference between the two endpoints analyzed for this panel of strains, the more stringent endpoint definition of "complete absence of growth" was adopted for further studies.

Range and distribution of results under standard conditions. The MICs at which 50% of isolates are inhibited (MIC_{50}s), MIC_{90}s, and the range of susceptibility results for MK-0991, LY303366, and fluconazole are presented in Table 2. For both glucan synthase inhibitors, *C. albicans* was the most susceptible, *C. neoformans* was the least susceptible, and the other four species (*C. tropicalis*, *C. lusitaniae*, *C. parapsilosis*, and *C. glabrata*) were intermediate. Comparing MK-0991 to LY303366, the former was systematically more active. For individual isolates, MK-0991 had from 2- to 16-fold lower MICs

TABLE 2. Broth macrodilution susceptibility results determined under standard conditions

Species	No. of strains	MIC (μg/ml)								
		MK-0991			LY303366			Fluconazole		
		50%	90%	Range	50%	90%	Range	50%	90%	Range
C. albicans	15	0.25	0.25	0.062–0.50	0.25	0.5	0.25–1.0	1	2	0.5–2.0
C. krusei	15	0.25	1	0.125–1.0	0.5	1	0.125–1.0	16	32	8.0–32
C. tropicalis	15	0.25	0.5	0.125–1.0	1	2	0.50–2.0	1	2	0.5–2.0
C. glabrata	15	0.25	0.5	0.125–0.50	4	8	1.0–8.0	8	8	2.0–16.0
C. parapsilosis	15	0.5	2	0.25–2.0	>8.0	>8.0	>8.0–>8.0	1	2	0.25–4.0
C. lusitanae	12	1	2	0.125–2.0	8	>8.0	>8.0–8.0	1	2	0.5–2.0
C. neoformans	15	>8.0	>8.0	>8.0–>8.0	>8.0	>8.0	>8.0–>8.0	1	1	0.25–2.0

compared to those of LY303366. This spectrum of activity differed significantly from that of fluconazole, to which all species except *C. glabrata* and *C. krusei* exhibited susceptibility.

Broth microdilution test results. Broth microdilution test results were obtained for the same 102 strains that had been tested by the broth macrodilution method, and the relationship of the two procedures was analyzed for systematic differences (Table 3). For MK-0991 when broth microdilution test results were read at day 1, results for 73% of the strains agreed within a fourfold range with the results of the reference standard method. Day 2 readings agreed less well (64%) and were significantly different from broth macrodilution test results ($P = 0.22$) due to an increase in the broth microdilution MICs for some strains during the additional incubation. For LY303366, there was a similar but more pronounced upward systematic bias for both day 1 and day 2 broth microdilution test readings, so that fourfold agreements were only 38 and 35% for the 2 days, respectively. For fluconazole, a systematic bias in the reverse direction was observed. Broth microdilution test results tended to be lower than broth macrodilution test results so that fourfold agreements were 62 and 56% for days 1 and 2 of reading, respectively.

DISCUSSION

In this study, two new glucan synthase inhibitors, LY303366 and MK-0991, were studied under a variety of test conditions to determine which, if any, influenced the endpoint results obtained in a broth macrodilution assay. Virtually no significant differences were obtained by varying the starting inoculum size, medium pH, medium buffer, medium composition, incubation temperature, or time of reading of results. The results obtained with these new drugs are in contrast to those of a similar analysis of a previously studied inhibitor of glucan synthesis, cilofungin, for which changes in both pH and medium composition had prominent effects (14), and a similar analysis of other azole compounds, for which significant effects of changes in a variety of test conditions have been found (9, 15, 17). The design of our survey analyzed the effects of individual variables while holding others constant, as specified by NCCLS reference method M27A (16). Since we did not study all possible combinations of the several variables, it is possible that some combinations of variables might in fact elicit significant variations in test results not identified in this work. However, our studies do indicate that the test conditions specified by document M27A will not obscure the antifungal activity of either drug, and therefore, the reference method can be extended to both LY303366 and MK-0991 in future studies.

TABLE 3. Agreement of broth microdilution and broth macrodilution susceptibility test results

Ratio of microdilution broth MIC to macrodilution MIC, broth results[a]	No. of strains					
	MK-0991		LY303366		Flucanazole	
	Day 1[b]	Day 2[c]	Day 1[d]	Day 2[d]	Day 1[d]	Day 2[d]
32-fold more	0	0	3	2	0	0
16-fold more	0	1	9	9	1	0
8-fold more	6	3	26	19	0	0
4-fold more	10	20	25	33	2	4
2-fold more	31	30	27	22	14	9
No difference	18	21	9	11	15	16
2-fold less	24	17	3	3	34	32
4-fold less	10	7	1	2	32	27
8-fold less	3	5	0	0	2	7
16-fold less	0	0	0	0	2	7
32-fold less	0	0	0	0	0	1

[a] Results are the ratio of broth microdilution MIC at either day 1 or day 2 to the broth macrodilution MIC reading at day 2.
[b] $P = 0.034$.
[c] $P = 0.022$.
[d] $P < 0.0001$.

Under standard conditions, both LY303366 and MK-0991 displayed substantial in vitro activities against various species of *Candida*, most notably *C. albicans*. Particularly interesting were their activities against *C. glabrata* and *C. krusei*, two species that appear to be marginally susceptible or highly resistant to azoles such as fluconazole (Table 2). Overall, MK-0991 was slightly more active then LY303366 under the test conditions that we used. The relative efficacies of LY303366 and MK-0991 as therapeutic agents will ultimately require direct clinical trials. However, our results would support their development for the treatment of infections caused by *Candida* species. On the other hand, *C. neoformans* appears to be much less susceptible to both drugs in vitro. All isolates that we tested were not inhibited by 8.0 μg/ml, which is consistent with a recent report by Franzot and Casadevall (8), who found the MICs of MK-0991 for 18 strains of *C. neoformans* to range from 16 to 32 μg/ml. Therefore, in vivo studies which indicated a possible role for these agents in the treatment of cryptococcosis would be critical before these agents could be extended to clinical trials for this indication.

Although the broth macrodilution method of testing the susceptibilities of yeasts has played a critical role in developing consensus for a national reference standard (9), it has practical limitations when applied to routine applications in clinical laboratories. In this regard, broth microdilution methods are attractive and could potentially supplant broth macrodilution methods. The results reported here indicate, however, that broth microdilution MICs do not always exactly mimic those obtained by the broth macrodilution test. For both LY303366 and MK-0991, a systematic bias was found for broth microdi-

lution MICs, which were higher than broth macrodilution test results when the broth microdilution test results were read at day 2 and was also apparent with LY303366 when readings were done on either day. Fluconazole showed an opposite bias, with systematically lower broth microdilution MICs compared to the reference standard results. Lower broth microdilution MICs in relation to the broth macrodilution test results had also been reported for two other azoles, itraconazole and SCH-36592 (9). Further evidence for potential differences between broth microdilution and macrodilution test results was presented in recent reports by Pfaller and colleagues (7, 20), in which MICs obtained by the broth microdilution method were decreased when antibiotic medium 3 was substituted for RPMI 1640 in broth microdilution tests with LY303366. This is in contrast to the lack of effect from substituting the medium on the broth macrodilution test results reported here. These discrepancies do not preclude the eventual use of broth microdilution procedures. However, they do emphasize the need to either define accurately a broth microdilution method which precisely mimics the current reference standard or create a parallel standard specifically for broth microdilution testing. Determining which of these two approaches is most useful will be the focus of future work.

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