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Comparison of In Vitro Activity of Liposomal Nystatin against *Aspergillus* Species with Those of Nystatin, Amphotericin B (AB) Deoxycholate, AB Colloidal Dispersion, Liposomal AB, AB Lipid Complex, and Itraconazole

KAREN L. OAKLEY,[1] CAROLINE B. MOORE,[2] AND DAVID W. DENNING[1,3,4]*

Departments of Medicine[1] and Microbiology,[2] Hope Hospital, Salford, and University of Manchester,[3] and Department of Infectious Diseases and Tropical Medicine, North Manchester General Hospital,[4] Manchester M8 6RB, United Kingdom

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We compared the in vitro activity of liposomal nystatin (Nyotran) with those of other antifungal agents against 60 *Aspergillus* isolates. Twelve isolates were itraconazole resistant. For all isolates, geometric mean (GM) MICs (micrograms per milliliter) were 2.30 for liposomal nystatin, 0.58 for itraconazole, 0.86 for amphotericin B (AB) deoxycholate, 9.51 for nystatin, 2.07 for liposomal AB, 2.57 for AB lipid complex, and 0.86 for AB colloidal dispersion. *Aspergillus terreus* (GM, 8.72 μg/ml; range, 8 to 16 μg/ml) was significantly less susceptible to all of the polyene drugs than all other species ($P = 0.0001$).

Aspergillus infections are the most prevalent non-*Candida* fungal infections, causing 70% of such infections in bone marrow transplantation patients in a recent study (10). Estimates of prevalence in different immunocompromised patient groups range from 25 to 40% in patients with chronic granulomatous disease and 20 to 25% in lung transplant and high-risk leukemia patients to as low as 1% in patients with systemic lupus erythematosus (2). The intravenous treatment of choice is amphotericin B (AB), although the use of this drug is problematic, because there are many toxicity problems, some dose related and others not (3).

The past decade has seen the emergence of liposomal formulations of AB which have reduced toxicity and comparable efficacy to that of conventional AB (8, 11). Nystatin is another polyene antifungal agent that has antifungal activity in humans, although its systemic use is limited by toxicity and it is not absorbed from the gastrointestinal tract. Consequently a liposomal formulation of nystatin has been developed to facilitate intravenous administration of this drug (9, 15).

In this study, we investigated the in vitro activity of liposomal nystatin (Nyotran).

A total of 60 *Aspergillus* clinical isolates, comprising 36 isolates of *A. fumigatus* and 8 each of *A. flavus*, *A. terreus*, and *A. niger*, were used in this study. All isolates were cultivated from frozen stock on Sabouraud dextrose agar (Oxoid, Basingstoke, United Kingdom) for 3 to 4 days at 30°C.

A stock concentration of 1,000 μg of liposomal nystatin per liter (Nyotran; Aronex Pharmaceuticals, Houston, Tex.) was prepared according to the manufacturer's instructions by adding 50 ml of 0.9% sterile NaCl to a vial containing 50 mg of drug powder. The vial was shaken vigorously for 60 s and placed in a 40°C water bath for 15 min. The vial was removed from the water bath, shaken vigorously for 60 s, and equilibrated to room temperature before use. AB lipid complex (Abelcet; The Liposome Company, Princeton, N.J.), liposomal AB (AmBisome; Nexstar Pharmaceuticals, San Dimas, Calif.), and AB colloidal dispersion (Amphocil, or Amphotec; Sequus Pharmaceuticals, Menlo Park, Calif.) were prepared as instructed by their respective manufacturers. All lipid-associated products were prepared and used within 24 h.

Conventional nystatin (Squibb, Middlesex, United Kingdom) was dissolved in dimethyl sulfoxide (Sigma, Poole, United Kingdom) to produce a stock concentration of 3,200 μg/ml. Itraconazole (Janssen Pharmaceuticals, Beerse, Belgium) was suspended in 1:1 acetone and 0.2 M HCl to produce a final concentration of 3,200 μg/liter. AB with deoxycholate (Squibb, Middlesex, United Kingdom) was dissolved in sterile distilled water to produce a stock solution of 3,200 μg/ml, after being adjusted for potency. Fluconazole (Pfizer, Sandwich, United Kingdom) was dissolved in sterile distilled water to produce a stock solution of 10,240 μg/ml. All drugs were then dispensed into samples and stored protected from the light at −20°C until required.

MICs were determined by a microtiter method with RPMI 1640 medium (Sigma, Poole, United Kingdom) supplemented with 2% glucose and buffered to pH 7.0 with morpholinepropanesulfonic acid (MOPS) (Sigma). Concentrations of most of the drugs used in this study ranged from 0.03 to 16 μg/ml, and those of fluconazole ranged from 0.125 to 1,280 μg/ml.

The inoculum was prepared by suspending *Aspergillus* conidia in phosphate-buffered saline containing 0.05% Tween 80. The conidia were counted with a hemocytometer and then diluted into the growth medium to a concentration of 10^6 conidia/ml. A positive control (drug free) was included for each isolate. Microtiter trays were incubated in moist chambers at 37°C for 48 h. The MICs were read visually and were defined by using a no-growth end point.

Minimum fungicidal concentrations (MFCs) of all drugs were also determined. One hundred microliters was removed from every well containing no growth and transferred to a blood agar plate. The sample was allowed to soak into the agar and when dry was streaked to separate any conidia if present and to remove them from the drug source. The plates were incubated at 37°C for 48 h. The MFC was defined as the lowest drug

* Corresponding author. Mailing address: Department of Infectious Diseases and Tropical Medicine, North Manchester General Hospital, Delaunays Road, Manchester M8 6RB, United Kingdom. Phone: 0161 720 2734. Fax: 0161 720 2732. E-mail: ddenning@fs1.ho.man.ac.uk.

TABLE 1. Summary of MICs for the *Aspergillus* species tested in this study

Antimicrobial agent	MIC (μg/ml)[a]														
	A. fumigatus			*A. terreus*			*A. flavus*			*A. niger*			Total		
	GM	50%	90%	GM	50%	90%	GM	50%	90%	GM	50%	90%	GM	50%	90%
Liposomal nystatin	1.9	2.0	2.0	8.7	8	8	2.4	2	4	1.4	1	2	2.3	2	8
Nystatin	7.7	8.0	16.0	32.0			12.3	16	16	5.7	4	8	9.5	8	>16
Liposomal AB	1.1	0.5	4	32.0	>16	>16	16.0	>16	>16	0.3	0.13	1	2.1	1	>16
AB lipid complex	1.6	1	>16	32.0	>16	>16	29.3	>16	>16	0.1	0.25	0.25	2.5	2	>16
AB colloidal dispersion	0.4	0.25	0.5	20.7	>16	>16	5.2	2	>16	0.2	0.25	0.25	0.9	0.25	>16
AB	0.6	0.5	1	5.7	16	16	2.6	2	4	0.3	0.5	0.5	0.9	0.5	4
Itraconazole	0.9	0.25	>16	0.1	0.13	0.13	0.5	0.25	1	0.5	0.5	0.5	0.6	0.25	>16
Fluconazole[b]	2,153	>1,280	>1,280	ND[c]	ND	ND	ND	ND	ND	ND	ND	ND	2,153	>1,280	>1,280

[a] 50% and 90%, MICs for 50 and 90% of isolates tested, respectively. In calculating the GM values, a MIC of ≤0.03, >16, or >1,280 is assumed to be 0.03, 32, or 2,560 μg/liter, respectively.
[b] Sixteen isolates were tested against fluconazole.
[c] ND, not done.

concentration that allowed the growth of five colonies or less (≥99.99% killing).

Twenty percent of isolates (12 of 60) were randomly selected and retested to assess the reproducibility of the susceptibility tests.

A summary of the MICs of all antifungal agents tested is shown in Table 1. The MICs of liposomal nystatin range from 0.5 to 16 μg/ml, with a geometric mean (GM) of 2.3 μg/ml. In comparison, the MICs of nystatin were fourfold higher, with a range of 2 to >16 μg/ml and a GM of 9.5 μg/ml. This was in contrast to the higher MICs seen with lipid-associated AB compared to conventional AB.

GM MICs of the AB formulations (both conventional and lipid associated) ranged from as low as 0.9 μg/ml for AB deoxycholate and AB colloidal dispersion to 2.6 μg/ml for AB lipid complex. Lipid incorporation of amphotericin yielded either the same GM MIC as conventional AB or a GM MIC two- to threefold higher. This was in contrast to the results with nystatin, in which lipid incorporation decreased the MICs. The MICs of itraconazole were the lowest MICs recorded, with a GM MIC of 0.6 μg/ml and a range of MICs from 0.06 to >16 μg/ml. Twelve isolates tested against itraconazole were resistant (i.e., MIC of 16 μg/ml), and the MICs of one or more of the liposomal AB formulations for 8 of these isolates were also elevated. *A. fumigatus* isolates, the only isolates for which there were MICs of 32 μg/ml of one or more lipid-based AB agents were also resistant to itraconazole, but not AB. This relationship was not apparent with the other species. However, this cross-resistance was not seen with liposomal nystatin, the MICs of which ranged from 1 to 4 μg/ml for these 12 isolates. The nystatin MICs for these 12 isolates were higher, ranging from 8 to >16 μg/ml.

In addition, a subset of 16 isolates were tested against fluconazole—the MICs ranged from 1,280 to >1,280 μg/ml for all isolates tested.

Differences in susceptibilities between species were observed for all drugs, with the exception of the azoles, for which there were no significant differences between species. There was very little difference between species in MICs of liposomal nystatin, with the exception of *A. terreus*, for which the MICs were significantly higher than those for all other species. For *A. terreus*, liposomal nystatin MICs ranged from 8 to 16 mg/liter, with an overall GM MIC of 8.7 μg/ml. The MICs for all other species ranged from 0.5 to 4 μg/ml. Elevated MICs for *A. terreus* were also seen with nystatin and all AB formulations. In addition to higher MICs for *A. terreus*, those for *A. flavus* were also significantly higher than those for *A. fumigatus* for all AB formulations ($P < 0.001$). In contrast, MICs of AB lipid complex for *A. niger* were lower than those for *A. fumigatus* ($P \leq 0.001$). For all AB preparations *A. fumigatus* was more susceptible than *A. terreus* and *A. flavus* ($P < 0.001$).

MFCs of all drugs were obtained, and a summary of the MFC results can be seen in Table 2. The MFCs of liposomal nystatin ranged from 8 to >16 μg/ml, with an overall GM MFC of 24.0 μg/ml. Higher MFCs were obtained with nystatin, with all MFCs being >16 μg/ml (GM MFC of 32 μg/ml) compared

TABLE 2. Summary of MFCs (99.99% killing) for *Aspergillus* species tested in this study

Antimicrobial agent	MFC (μg/ml)[a]														
	A. fumigatus			*A. terreus*			*A. flavus*			*A. niger*			All		
	GM	50%	90%	GM	50%	90%	GM	50%	90%	GM	50%	90%	GM	50%	90%
Liposomal nystatin	21.4	32	32	32	32	32	32	32	32	22.6	32	32	24	32	32
Nystatin	32	32	32	32	32	32	32	32	32	32	32	32	32	32	32
Liposomal AB	31	32	32	32	32	32	32	32	32	11.3	32	32	27.5	32	32
AB lipid complex	32	32	32	32	32	32	32	32	32	9.5	32	32	27.2	32	32
AB colloidal dispersion	27.4	32	32	32	32	32	32	32	32	4.4	10	32	22.4	32	32
AB	8.3	12	32	32	32	32	10.4	8.0	32	6.2	6	32	9.8	16	32
Itraconazole	25.4	32	32	14.7	32	32	5.7	16	32	17.4	32	32	18.4	32	32
Fluconazole[b]	2,560	2,560	2,560	ND[c]	ND	ND	ND	ND	ND	ND	ND	ND	2,560	2,560	2,560

[a] 50% and 90%, MFCs for 50 and 90% of isolates tested, respectively. In calculating the GM values, an MFC of >16 or >1,280 is assumed to be 32 or 2,560 μg/ml, respectively.
[b] Sixteen isolates were tested against fluconazole.
[c] ND, not done.

to liposomal nystatin. This pattern was not reflected for AB deoxycholate, for which the GM MFC was much lower than those of all lipid-associated AB formulations. The GM MFC of AB was 9.85 μg/ml, compared to 27.5, 27.2, and 22.3 μg/ml for liposomal AB, AB lipid complex, and AB colloidal dispersion, respectively. There were differences in MFC results between species. The MFCs for *A. terreus* and *A. flavus* were the highest of all drugs, except with itraconazole, for which they were the lowest.

The reproducibility of the susceptibility tests showed that for liposomal nystatin, itraconazole, AB deoxycholate, liposomal AB, and AB lipid complex, 11 of 12 isolates retested, and for nystatin and AB colloidal dispersion, 10 of 12 isolates retested were the same or within 1 dilution of the original MIC.

Antifungal agents are often prepared in liposomal formulations to enhance the activity and delivery of the drug and to either reduce or eliminate harmful side effects (4). AB is such an example and has been available in a variety of liposomal formulations for a considerable period of time (5, 13). The reduced toxicity, especially nephrotoxicity, of the new AB formulations is now widely recognized.

The polyene antifungal agent nystatin, a close relative of AB has known activity against a broad spectrum of fungi, including *Aspergillus* (12) and *Candida* (6). However, due to its toxicity, studies in the 1950s led to a preference for AB for treatment of systemic fungal infections, and nystatin is used primarily for superficial infections—either applied as a cream or given orally. The development of liposomal nystatin is an attempt to avoid some of the systemic toxicity problems.

In this study, the in vitro susceptibility tests have shown encouraging results for the liposomal formulation of nystatin. Liposomal nystatin was shown to have in vitro activity against the four species of *Aspergillus* tested. In comparison to the conventional formulation of nystatin, the MICs and MFCs were lower and were comparable to those of both liposomal and conventional AB. The MICs were higher than that of itraconazole, although liposomal nystatin was found to be active against all itraconazole-resistant *Aspergillus* isolates. No liposomal nystatin-resistant isolates were identified among *A. fumigatus*, *A. flavus*, and *A. niger* isolates. However the liposomal nystatin MICs for one species, *A. terreus*, were significantly higher than those for the other species tested. However, the MICs of all other polyene drugs tested for this species were high.

We have used a much more stringent criterion for fungicidality (99.99% killing) than that of Johnson et al. (95%) (7). In this context, 8 isolates were killed by liposomal nystatin at ≤8 mg/liter compared with none by nystatin, 3 (*A. niger*) by liposomal AB, 3 (*A. niger*) by AB lipid complex, 7 by AB colloidal dispersion (5 *A. niger*), 28 by AB (5 *A. niger*) and 8 by itraconazole (1 *A. niger*). Clearly *A. niger* is much more susceptible to killing by polyenes than other species, but none of the compounds is very active in this regard, with the possible exception of conventional AB, in which clinical results are mediocre and not entirely consistent with these data.

One possible reason for the relatively high MICs and MFCs of the lipid-based amphotericins is the variable and incomplete release in vitro of active AB into the medium. This is possible. However, the opposite effect was seen with liposomal nystatin, suggesting a more complex interaction. One possible explanation could be the release of AB or nystatin by phospholipases (1, 14). We have examined all 60 isolates for the production of extracellular phospholipase (after 60 h of incubation) (1), and although there was substantial interisolate variation, there was no relationship with the MIC (data not shown). More elaborate experiments are required to understand the dynamics of lipid-associated polyenes under in vitro test conditions.

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