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Formulation, Toxicity, and Antifungal Activity In Vitro of Liposome-Encapsulated Nystatin as Therapeutic Agent for Systemic Candidiasis

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Multilamellar vesicles containing nystatin (NYS) were compared with vesicles containing the free drug for toxicity to erythrocytes and for antifungal activity in vitro. Liposomal nystatin was as active as free NYS was against a wide variety of yeasts and fungi. The antifungal activity against *Candida albicans* was maintained with different liposome compositions and without sterols. Liposome encapsulation also protected the erythrocytes from the toxicity of free NYS.

Polyene antibiotics are a group of macrolide lactones that have antifungal activity against a wide variety of fungi. The antifungal properties of polyenes are related to their degree of selectivity for ergosterol and cholesterol in biological membranes (9, 12), particularly to their high affinity for ergosterol in fungal membranes. Nystatin (NYS), a tetraene-diene polyene antibiotic, has a broad spectrum of activity, but problems in its solubilization in injectable solvents and its toxicity (1, 2) limit its use in the treatment of systemic fungal infections. On the basis of our earlier studies on amphotericin B (L-AmB) (4, 6, 7), we used liposomes as carriers of NYS in an attempt to reduce its toxicity while maintaining its antifungal activity. This report presents the formulation, toxicity, and antifungal activity of liposomal nystatin (L-NYS). Its toxicity and antifungal activity are compared with those of the free drug in vitro.

MATERIALS AND METHODS

Drug, lipids, and reagents. NYS (bulk powder) was obtained from Lederle Laboratories, Pearl River, N.Y. Chromatographically pure dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) were purchased from Avanti Polar Lipids, Birmingham, Ala. Methanol for high-performance liquid chromatography (HPLC), dimethyl sulfoxide (DMSO), and *N*,*N*′-dimethylformamide were purchased from Fisher Scientific Co., Fair Lawn, N.J. Human AB serum was from MA Bioproducts, Walkersville, Md. Human erythrocytes were obtained from healthy volunteers.

HPLC of NYS. The HPLC system consisted of M6000A and M45 solvent delivery systems with U6K Universal LC injector (Waters Associates, Inc., Milford, Mass.), a Waters automated gradient controller and programmer and model 441 fixed-wavelength absorbance detector, and an Omniscribe recorder (Houston Instruments, Houston, Tex.). A Waters μBondapak C-18 reverse-phase column (0.45 mm by 30 cm) was used for the analysis. The mobile phase consisted of methanol-water (70:30) pumped at a flow rate of 2 ml/min, and the A_{280} of the eluant was monitored (0.02 absorbance units, full scale). The purity was calculated as percent peak area corresponding to NYS divided by peak areas of total number of peaks in each chromatogram.

Liposome preparation and standardization. Multilamellar vesicles were prepared as described previously, with slight modifications (6). NYS was solubilized in methanol (1 mg/ml) and stored, protected from light, at 4°C. Phospholipids DMPC-DMPG (7:3), at a constant amount, were mixed with increasing amounts of the drug, and the organic solvents were evaporated under vacuum by using a rotary evaporator. The dried lipid-drug film was suspended in phosphate-buffered saline (PBS) and hand shaken, allowing the film to form liposomes. The suspensions were then centrifuged at 100,000 × *g* for 1 h. The pellets were resuspended in PBS, and the amount of NYS incorporated in liposomes was determined by dissolving a known volume in methanol and measuring A_{306}. Similarly, liposomes composed of phospholipids and sterols at a ratio of 9:1 were also prepared. The stability of NYS liposomes was assessed by incubating equal amounts of L-NYS with PBS and human AB serum at 37°C. At various times, samples were removed and centrifuged at 10,000 × *g* for 15 min, and the NYS concentration in the pellet was measured as described above.

Organisms, cultures, and assay of antifungal activity in vitro. All strains of yeast were grown overnight at 37°C on Sabouraud dextrose agar plates (6). All molds were grown at 30°C on Sabouraud dextrose agar for 3 to 10 days prior to the collection of spores. The yeasts or spores were then processed for susceptibility testing as described previously (3). A twofold serial dilution method (11) adapted to microtiter plates was used to determine the MICs of the drugs. MICs of L-NYS were compared with those of free NYS. Empty liposomes and 5% DMSO were used as controls.

Assay of toxicity to human erythrocytes in vitro. Lysis of human erythrocytes was quantitated by measuring the release of hemoglobin in the supernatants at 550 nm, as described previously (7). Various concentrations of L-NYS were incubated with freshly washed human erythrocytes at 37°C for 45 min. Free NYS dissolved in dimethylformamide was added to the assay at a final solvent concentration of

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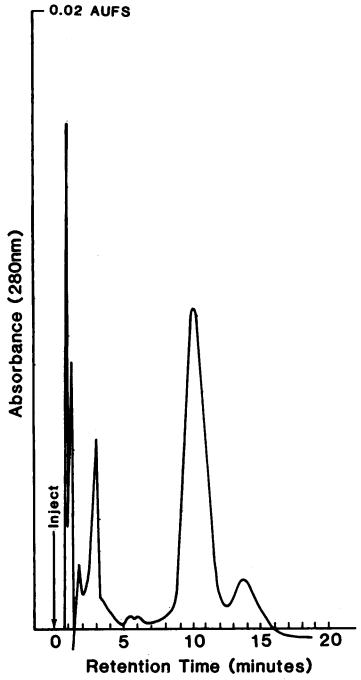

FIG. 1. Chromatogram (HPLC) of NYS. The peak at 10 min corresponds to 3 μg of NYS. AUFS, Absorbance units, full scale.

3%. Appropriate solvent controls, empty liposomes, and empty liposomes plus free drug were also included in each experiment. Release of hemoglobin by hypotonic lysis of the same number of human erythrocytes by water was taken as 100% positive control, while cells treated with PBS were used as negative controls.

RESULTS

NYS was soluble in methanol (1 mg/ml), dimethylformamide (15 mg/ml), and DMSO (40 mg/ml). A typical chromatogram of HPLC analysis of NYS is shown in Fig. 1. Optimal separation of the drug was achieved by using 70% (vol/vol) methanol as the mobile phase and other conditions

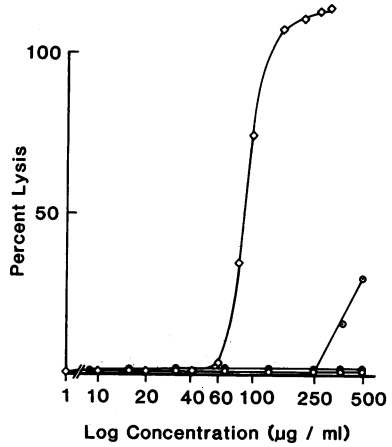

FIG. 2. In vitro toxicity of free NYS versus L-NYS to human erythrocytes. The human erythrocytes were incubated at 37°C for 45 min with free NYS (◇), L-NYS (●), empty liposomes (○), or empty liposomes plus free NYS (⊙).

TABLE 1. Encapsulation efficiency of NYS in liposomes[a]

Amt of drug (μg/10 mg of PL)	Drug/PL	Drug incorporated in MLV (μg/10 mg of PL)	Encapsulation efficiency[b]
10	1:1,000	10	100
20	1:500	20	100
40	1:250	40	100
60	1:167	60	100
80	1:125	80	100
100	1:100	95	95
200	1:50	180	90
400	1:25	400	100
600	1:16.7	600	100
800	1:12.5	640	80
1,000	1:10	628	62.8

[a] Liposomes were multilamellar vesicles (MLV) composed of DMPC and DMPG in a molar ratio of 7:3. PL, Total phospholipid consisting of both DMPC and DMPG. The NYS content was measured by the A_{306} of known volumes dissolved in methanol.

[b] Percentage of drug encapsulated in multilamellar vesicles (the pellet) per total amount of drug added.

as specified below. The retention time of NYS was 10 min, and the purity was calculated to be 72%.

The encapsulation efficiencies of different batches of liposomes prepared with a fixed amount of phospholipids and increasing doses of NYS are presented in Table 1. The maximum incorporation was obtained at 600 μg of NYS per 10 mg of phospholipids (drug/phospholipid ratio, 1:16.7), and it decreased thereafter.

Liposomes containing 500 μg of NYS per 10 mg of phospholipids were prepared, divided into aliquots, and incubated with PBS or human AB serum for various times. The amount of drug recovered in the pellet after incubation for each time interval is shown in Table 2. Liposomes were stable up to 72 h in PBS but were less stable in serum, with only 30% of the drug recovered in the pellet after a 72-h incubation.

In vitro antifungal activity. The MICs of free or L-NYS for a variety of yeasts and fungi are presented in Table 3. The L-NYS was as effective as free NYS was against all the species of yeasts and fungi tested. The effect of sterols on the activity of L-NYS against *Candida albicans* 336 is shown in Table 4. This was the strain used in further experiments to produce systemic candidiasis in mice; results are communicated in the accompanying report (8). The antifungal activity (MIC) of NYS did not change with the presence or absence of sterols in liposome preparations. Empty liposomes and DMSO at concentrations equivalent to those used with drug samples did not inhibit growth.

Toxicity to human erythrocytes in vitro. A linear increase in

TABLE 2. Stability profile of NYS liposomes at 37°C

Incubation time (h)	% NYS retained in pellet after treatment with[a]:	
	PBS	Human AB serum
1	100	100
2	100	95
4	100	75
24	100	65
48	100	
72	100	30
168	50–70	

[a] Each value is the percentage of NYS recovered in a sample as compared with an identical sample at 0 h taken as 100% controls.

lysis of human erythrocytes was observed with free-NYS concentrations ranging from 60 to 120 μg/ml, with a 100% lysis produced at 120 μg/ml (Fig. 2). In contrast, L-NYS did not cause any lysis with doses of up to 500 μg/ml. Empty liposomes alone did not affect the human erythrocytes; in addition, they protected against the effect of free NYS to some extent. Free NYS when present at a concentration of 375 μg/ml with empty liposomes produced only 15% lysis, while a 30% lysis was observed with 500 μg of free NYS per ml. Dimethylformamide at concentrations equivalent to those used with free NYS did not affect human erythrocytes.

TABLE 3. In vitro antifungal spectrum of free and L-NYS

Fungal strain	MIC (μg/ml)[a]	
	Free NYS[b]	L-NYS[c]
Candida albicans		
1	2.0	2.0
2	2.0	1.0
3	4.0	2.0
4	4.0	2.0
5	8.0	2.0
6	4.0	2.0
7	8.0	2.0
8	8.0	2.0
9	4.0	2.0
10	4.0	2.0
Candida tropicalis		
1	16.0	8.0
2	8.0	2.0
3	4.0	2.0
4	4.0	2.0
Candida parapsilosis		
1	2.0	2.0
2	2.0	2.0
3	4.0	2.0
4	4.0	2.0
Torulopsis glabrata		
1	4.0	2.0
2	2.0	2.0
3	4.0	2.0
4	4.0	2.0
Cryptococcus neoformans		
1	4.0	2.0
2	4.0	2.0
Aspergillus spp.		
1	4.0	1.0
2	16.0	8.0
Aspergillus fumigatus	8.0	8.0
Aspergillus niger	4.0	2.0
Aspergillus flavus	1.0	0.5
Fusarium spp.	62.5	62.5
Rhizopus spp.	4.0	4.0
Mucor spp.	2.0	1.0
Alternaria spp.	2.0	1.0
Allescheria spp.	16.0	16.0
Curvularia spp.	2.0	0.5
Chryosporium spp.	1.0	0.5
Cunninghamella elegans	8.0	1.0

[a] Values are representative of two different experiments performed in duplicate.
[b] Free NYS was dissolved in DMSO and diluted further with saline, which contained 5% DMSO at a concentration of 1 μg/ml.
[c] Liposomes were prepared by using DMPC and DMPG at a ratio of 7:3.

TABLE 4. Effect of sterols on antifungal activity of free versus L-NYS in vitro

Preparation	MIC (μg/ml)[a]
Free NYS[b]	1.0
Free-NYS with PL[c]	1.0
L-NYS with PL:C[d]	1.0
L-NYS with PL:E[e]	2.0
DMSO[f]	Nil
Empty liposomes[f]	Nil

[a] MIC was determined against *C. albicans* 336.
[b] Free NYS was dissolved in DMSO and diluted further with saline, which contained 5% DMSO at a concentration of 1 mg/ml.
[c] Phospholipids (PL) (DMPC-DMPG, 7:3) only used to prepare liposomes.
[d] Liposomes contained phospholipids and cholesterol at a ratio of 9:1.
[e] Liposomes were composed of phospholipids and ergosterol at a ratio of 9:1.
[f] Dilutions of DMSO or empty liposomes at an equivalent dose were the same as dilutions of free or L-NYS.

DISCUSSION

The results obtained show that NYS could be formulated into a stable liposome preparation which incorporated 100% of the drug up to a drug-to-phospholipid ratio of 1:16.7. The stability of L-NYS in serum up to 2 h is suitable for intravenous administration, since multilamellar liposomes have been reported to have a very rapid blood clearance (10).

L-NYS was found to be as active as free NYS was against a wide variety of yeasts and molds. Interestingly, L-NYS has a better spectrum of activity than that observed with free or L-AmB; L-AmB did not inhibit the growth of some molds tested (unpublished data). Moreover, the presence of sterols in liposomal membranes did not alter the antifungal activity (MIC) of L-NYS against *C. albicans* 336. We have previously shown that the presence of sterols in liposomes decreases the antifungal activity (minimal fungicidal concentration) of AmB (3). We then observed that sterol-containing L-AmB does not affect the antifungal activity in vivo (5). Therefore, the minimal fungicidal concentrations of L-NYS were not determined in this study. The toxicity of NYS to erythrocytes was abrogated after liposome encapsulation, which is similar to results of our earlier studies with L-AmB (7). Addition of empty liposomes to free NYS protected erythrocytes from lysis, which may be due to the binding of NYS to the liposome surface, thus preventing its full activity. However, at higher concentrations, some toxicity was observed, presumably because of extra free drug available at these concentrations after liposome surfaces are saturated. These results thus show that liposome encapsulation of NYS, a highly toxic and insoluble polyene, reduces the toxicity of the drug to mammalian cells, while allowing full expression of its antifungal activity in vitro. These findings suggest that some other polyenes may also be suitable for liposome encapsulation and drug delivery.

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