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Toxicity and Therapeutic Effects in Mice of Liposome-Encapsulated Nystatin for Systemic Fungal Infections

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The therapeutic activity of nystatin (NYS) incorporated in multilamellar liposomes (L-NYS) was studied in vivo. Hale-Stoner mice injected intravenously with various doses of L-NYS and free NYS showed a significant reduction in toxicity of NYS after the NYS was incorporated into liposomes (maximal tolerated doses, 16 and 4 mg/kg of body weight, respectively). The maximal tolerated dose of free NYS had no effect in the treatment of mice infected with *Candida albicans*, whereas L-NYS at an equivalent dose improved the survival of mice. A marked increase in survival was observed when L-NYS was administered in higher and multiple doses (total doses up to 80 mg/kg). Liposome encapsulation thus provided a means for intravenous administration of NYS, reducing its toxicity and making it an active systemic antifungal agent.

The treatment of fungal infections remains a major problem in spite of the availability of effective antifungal drugs such as polyenes (3). Most of the available polyene antibiotics have toxic side effects that limit their clinical application (5, 6). In addition, the hydrophobic nature of nystatin (NYS) has precluded its systemic administration. It has been used as suspensions prepared in various ways and administered to patients orally (1, 2, 7, 14, 17). However, most of these studies failed to document a beneficial effect of NYS administration against systemic fungal infections (2, 14, 17).

We have recently demonstrated that liposome encapsulation improves the therapeutic index of amphotericin B both against experimental murine candidiasis (10, 11) and in the treatment of fungal infections in patients with leukemia and lymphoma (9). NYS in liposomal form (L-NYS) was a good additional drug prototype to study antifungal activity because of the structural similarities NYS shares with amphotericin B. The present communication reports on the in vivo toxicity and antifungal efficacy of L-NYS as compared with those of the free drug.

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.

Liposome preparation. Multilamellar vesicles were prepared as described previously (11). NYS was solubilized in methanol (1 mg/ml) and stored at 4°C, protected from light. Phospholipids DMPC and DMPG (7:3) were mixed with a methanol solution of NYS, and the organic solvents were evaporated under vacuum by using a rotary evaporator. The dried drug-lipid film was suspended in 0.9% pyrogen-free saline and hand shaken, allowing liposomes to form. The suspension was then centrifuged at 100,000 × g for 1 h, and the pellet was resuspended in the saline. The amount of NYS incorporated in liposomes was determined by dissolving a known volume in methanol and measuring A_{306}. Doses were adjusted thereafter in appropriate volumes of saline for injections for various groups.

Animals and model of experimental candidiasis. Hale-Stoner mice, 6 to 8 weeks old (body weight, 20 to 25 g), were purchased from the University of Texas Science Park, Bastrop. The mice (eight per group) were injected via the tail vein with 0.2 ml of *Candida albicans* cell suspension containing 7×10^5 CFU. This concentration of cells consistently produced a disseminated infection in mice 48 h after injection, affecting liver, spleen, lungs, and kidneys primarily (11).

Toxicity in vivo. Groups of eight mice each were injected with various doses (ranging from 1 to 6 mg/kg of body weight), in 5% dimethyl sulfoxide (DMSO) diluted with saline, of L-NYS (range, 2 to 20 mg/kg), empty liposomes (400 mg/kg), or 5% DMSO as the control. The mice were observed for acute, subacute, and chronic toxicity, and the survival time of each animal in each group was noted (11). After 45 days, the surviving animals were sacrificed, and blood and tissue samples were obtained. Blood biochemistry examination included blood urea nitrogen, alkaline phosphatase, and lactic dehydrogenase. The organs (liver, spleen, lungs, and kidneys) were preserved in 10% Formalin. Tissue slices were processed for hematoxylin-eosin and Gomori methenamine silver stains.

Therapeutic experiments. (i) Single-dose trials. Groups of eight mice each were injected intravenously with various doses of free NYS (range, 1 to 4 mg/kg), L-NYS (range, 2 to 12 mg/kg), empty liposomes, or 5% DMSO 2 days after the injection of *C. albicans*. The survival of animals in each group was noted and compared with that in the untreated control group.

(ii) Multiple-dose trials. At 2 days after the intravenous injection of *C. albicans*, the mice were treated with daily doses of free NYS (4 mg/kg), L-NYS (range, 2.4 to 16 mg/kg), empty liposomes, or 5% DMSO controls for 5 consecutive days. The multiple-dose groups were also compared with appropriate cumulative single-dose groups (12 and 16 mg/kg). The animals were then observed for survival

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FIG. 1. Acute toxicity of free NYS versus L-NYS in vivo. The mice were injected intravenously with free NYS (○), L-NYS (●), 5% DMSO (△), or empty liposomes (*). The total lipid dose was 400 mg/kg.

FIG. 3. Effect of liposome encapsulation on the antifungal activity of NYS in mice; single-dose treatment. At 2 days after challenge with *C. albicans*, the animals were treated with free NYS (4 mg/kg) (⊙), or L NYS at doses of 2 (▽), 4 (▲), 8 (□), or 12 (■) mg/kg. ●, Untreated controls.

or for any toxicity pertaining to the treatment with 5 daily doses. These experiments were terminated after 60 days, and blood and tissue samples were collected from the surviving animals after sacrifice.

Survival curves were calculated by the method of Kaplan and Meier (8), and tests for differences in survival distributions were based on a generalized Wilcoxon test (4). Linear trend and the χ^2 test for differences in response rates among the groups and paired t tests were used to compare the means.

RESULTS

After we observed reduced toxicity and a maintained antifungal activity in experiments in vitro (13), we extended our studies to investigate the potential of L-NYS as an antifungal agent in vivo.

In vivo toxicology studies. The maximal tolerated dose (MTD) of free NYS was 4 mg/kg of body weight, and at a dose of 4.4 mg/kg, all the animals died immediately (Fig. 1). The MTD of L-NYS, on the other hand, was 16 mg/kg. Empty liposomes (400 mg/kg) and DMSO equivalent to the amounts present in the highest doses of L-NYS and free NYS, respectively, did not have any toxic effects, and the animals survived until the experiment was terminated (i.e., for 45 days). No subacute or chronic toxic reactions were observed in the surviving animals. No significant changes in the biochemistry pattern were observed. Histopathology studies failed to demonstrate the cause of death in these animals.

Single-dose treatment. In the first set of experiments, infected mice were treated with increasing doses of free NYS, ranging from 1 to 4 mg/kg of body weight (Fig. 2). None of the doses tested improved the survival of mice as compared with that in the control untreated group. DMSO (5%) did not affect the survival of mice. The next set of experiments included groups of mice treated with various doses of L-NYS (range, 2 to 12 mg/kg), free NYS (1 to 4 mg/kg), and empty liposomes (400 mg of lipid per kg) (Fig. 3). Empty liposomes did not show any effect on the survival of mice. No difference in the survival of mice was observed with an L-NYS dose of 2 mg/kg as compared with free NYS, whereas doses of 4 and 8 mg/kg showed improvement in survival ($P < 0.01$ and $P < 0.02$, respectively). Furthermore, a dose of 12 mg/kg showed a significant improvement in survival ($P < 0.003$) when compared with the MTD of free NYS. However, all mice died within 18 days regardless of treatment.

Multiple-dose treatment. Groups of mice in these experiments were injected with free NYS at a dose corresponding to the MTD or L-NYS for 5 consecutive days. The effects of these multiple doses were compared with that of the MTD of free or L-NYS given as a single dose (Fig. 4). The experiments also included groups of animals injected with the daily equivalent dose of empty liposomes or 5% DMSO, injected on 5 consecutive days; untreated controls were also in-

FIG. 2. Antifungal activity of free NYS in mice. At 2 days after being infected with *C. albicans*, mice were injected intravenously with free NYS at doses of 1 (■), 2 (△), or 4 (●) mg/kg. ○, Untreated controls.

FIG. 4. Effect of L-NYS on the survival of mice infected with *C. albicans*; multiple-dose treatment. The animals were treated with free NYS (five doses at each) (⊙) 4 mg/kg or L-NYS in a single dose of 12 (○) or 16 (◇) mg/kg or multiple doses (five doses of each amount [milligrams per kilogram]) as follows: 2.4 (▽), 6 (▲), 8 (⊡), 12 (■), 16 (△). ●, Untreated controls.

cluded. The animals in all the groups treated with single or multiple doses of free NYS died as rapidly as the untreated controls. L-NYS, on the other hand, produced significant improvement in the survival of mice. The 12-mg/kg dose given as a single or divided dose (five doses of 2.4 mg each) also produced a similar pattern of survival; a significant improvement over the groups treated with free drug was observed ($P < 0.003$). When given five times, the 12-mg/kg dose (total dose, 50 mg/kg) produced a dramatic increase in survival ($P < 0.007$), with 70% of the mice surviving at day 60, as compared with survival times of 18 and 10 days in groups treated with a single dose of L-NYS or single or multiple doses of free NYS, respectively. The medium-cumulative dose (five doses of 6 mg each) also produced a marked improvement in survival ($P < 0.001$). The increase in survival time was proportional to the total dose of L-NYS. The mice could also tolerate a dose of 16 mg/kg for 5 days (a total of 80 mg of NYS per kg), but only when it was given at a slow rate. This dose resulted in a 100% survival of mice for up to 60 days, when the experiment was terminated.

DISCUSSION

The results obtained demonstrate that after incorporation in liposomes, NYS became an active therapeutic agent in the treatment of systemic experimental candidiasis. Although free NYS had significant antifungal activity in vitro, it was toxic and noneffective when administered intravenously. Liposome encapsulation provided an injectable formulation of NYS which was observed to be significantly less toxic than the free drug. L-NYS was four times less toxic (MTD, 16 mg/kg) than the free NYS (MTD, 4 mg/kg) and was nontoxic also when multiple doses were injected (cumulative dose of up to 80 mg/kg). L-NYS at 4 mg/kg was effective in improving the survival of infected mice, whereas the equivalent dose of free NYS showed no therapeutic effect. Further increase in survival time was achieved when higher doses of L-NYS were administered in multiple-dose regimens.

Liposomes have been extensively used to modify the therapeutic index of known active drugs (11, 15, 16). The observation with most encapsulated drugs has been that the improvement of the therapeutic index was related to reduced toxicity of the drug after encapsulation in liposomes. NYS, on the other hand, has been shown to be active when administered orally (1, 7), but its hydrophobic nature precludes its intravenous administration, and therefore it cannot be used for treatment of systemic fungal diseases. The observed ineffectiveness of free NYS as a systemic antifungal may be due, in part, to inadequate delivery of the drug to affected sites. Liposome encapsulation allowed the systemic administration of NYS and its use as an effective antifungal agent in mice. We have previously demonstrated that liposomes enhance the delivery of amphotericin B to infected sites (12), thus promoting the drug-drug carrier interaction with the yeast. Other mechanisms, such as the extrusion of liposome-encapsulated drug through capillaries damaged by infection or secondary delivery by peripheral phagocytes to the site of inflammation, may also play a role and are being investigated in our laboratory.

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