

## Liposomal nystatin against experimental pulmonary aspergillosis in persistently neutropenic rabbits: efficacy, safety and non-compartmental pharmacokinetics

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The activity of liposomal nystatin against invasive pulmonary aspergillosis was investigated in persistently neutropenic rabbits. Treatment groups included liposomal nystatin at dosages of 1, 2 and 4 mg/kg/day intravenously, or amphotericin B deoxycholate 1 mg/kg/day administered intravenously after normal saline loading. As compared with untreated controls, liposomal nystatin administered at 2 and 4 mg/kg/day prolonged survival and reduced fungus-mediated tissue injury and excess lung weight at post-mortem in a similar manner to amphotericin B. Although amphotericin B was superior in clearing infected lung tissue, treatment with all regimens of liposomal nystatin led to a significant reduction in pulmonary fungal tissue burden. During treatment, ultrafast CT-scan demonstrated ongoing resolution of pulmonary lesions at 2 and 4 mg/kg/day, but not at 1 mg/kg/day. With the exception of mild increases in blood urea nitrogen (BUN) and serum creatinine values during treatment at 2 and 4 mg/kg/day, which were similar to those found in amphotericin B-treated rabbits, liposomal nystatin was well tolerated. Preliminary pharmacokinetic studies in non-infected animals established linear drug disposition of liposomal nystatin in plasma over the investigated dosage range and peak plasma levels above the MIC for the test strain after multiple daily dosing for 7 days. Liposomal nystatin increased survival and provided reduced tissue injury, effective microbiological clearance and tolerable side effects in experimental pulmonary aspergillosis in persistently neutropenic rabbits, thus providing a rational basis for further investigations in clinical trials.

### Introduction

Invasive pulmonary aspergillosis has emerged as an important cause of morbidity and mortality in profoundly neutropenic patients.<sup>1–4</sup> Standard front-line therapy with high-dose intravenous amphotericin B deoxycholate is greatly limited by toxicity and overall poor response rates in the absence of bone marrow recovery.<sup>5</sup> The available lipid formulations of amphotericin B, while being better tolerated, are only indicated for second-line therapy<sup>6</sup> and the role of investigational third-generation triazoles

remains to be defined. Thus, there is a continuing demand for safe agents with improved therapeutic activity.

Nystatin, a tetraene diene macrolide, was discovered as the first polyene antibiotic in the early 1950s.<sup>7</sup> The primary mechanism of action is identical to that of amphotericin B and, like amphotericin B, nystatin possesses potent, broad-spectrum fungicidal activity *in vitro*.<sup>8</sup> Although the compound has been widely used topically for both treatment and prevention of superficial fungal infections, problems with solubilization and toxicity after parenteral administration have limited its use for systemic treatment.<sup>8</sup> More

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recent studies, however, have shown that incorporation of nystatin into a stable multilamellar liposome preparation of 0.1–3  $\mu\text{m}$  particle diameter consisting of dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) in a 7:3 molar ratio significantly reduces its toxicity to mammalian cells while preserving its antifungal activity *in vitro*.<sup>9</sup> This liposomal formulation demonstrated promising in-vivo activity in murine models of both disseminated candidiasis<sup>10</sup> and disseminated aspergillosis,<sup>11</sup> and it was well tolerated at dosages of up to 8 mg/kg/day in a phase I clinical trial in patients with haematological malignancies and refractory febrile neutropenia.<sup>12</sup> Little is known, however, about the antifungal efficacy of liposomal nystatin in this patient population.

In order to define better the potential clinical usefulness of this novel formulation in neutropenic patients, we investigated the safety, pharmacokinetics and efficacy of liposomal nystatin in a persistently neutropenic rabbit model of invasive pulmonary aspergillosis.

## Materials and methods

### Animals

Female New Zealand White rabbits (Hazleton, Denver, PA, USA) weighing 2–3 kg at the time of inoculation were used in all experiments. They were individually housed and maintained with water and standard rabbit feed *ad libitum* according to National Institutes of Health guidelines for laboratory animal care<sup>13</sup> and in fulfilment of American Association for Accreditation of Laboratory Animal Care criteria. For repeated non-traumatic administration of medications and sampling of blood, vascular access was established in each rabbit before experimentation by the surgical placement of a central venous silastic catheter. The catheter was inserted in the right external jugular vein, positioned into the right atrium and externalized via a subcutaneous tunnel at the interscapular region with a heparin lock device under general anaesthesia as previously described.<sup>14</sup>

### Immunosuppressive regimen and supportive care

Cytosine arabinoside (kindly provided by Upjohn, Kalamazoo, MI, USA) was administered intravenously at 525 mg/m<sup>2</sup> on days 1–5 and at 484 mg/m<sup>2</sup> on days 8 and 9 to produce profound and persistent neutropenia, such as is encountered in patients at highest risk for invasive fungal infections. Body weight  $W$  (in kg) was converted to body surface area  $A$  (in m<sup>2</sup>) using the following formula:  $A = 0.05W + 0.05$ .<sup>15</sup> Granulocyte counts were closely monitored and maintained below  $0.5 \times 10^9/\text{L}$ ; they were  $<0.1 \times 10^9/\text{L}$  from day 5 onwards. Concomitant platelet counts ranged from  $1 \times 10^{10}$  to  $2.5 \times 10^{10}/\text{L}$ . Starting on day 4, ceftazidime (Glaxo, Research Triangle Park, NC, USA)

75 mg/kg iv twice daily, gentamicin (Baxter Health Care Corp., Deerfield, IL, USA) 5 mg/kg iv every other day, and vancomycin (Eli Lilly, Indianapolis, IN, USA) 15 mg/kg iv daily were administered to prevent the occurrence of invasive bacterial infections during neutropenia.

### Organism and preparation of inoculum

On day 2 of the experiment, after the second dose of cytosine arabinoside, rabbits were inoculated intratracheally with a well characterized strain of *Aspergillus fumigatus* (NIH4215) obtained from a fatal case of pulmonary aspergillosis.

The inoculum was prepared from a frozen isolate that was subcultured onto potato dextrose slants, which were incubated for 24 h at 37°C and then kept at room temperature for 5 days. Conidia were harvested under a laminar air flow hood with a solution of 0.025% Tween 20 (Fisher Scientific, Fair Lawn, NJ, USA) in normal saline, transferred to a 50 mL conical tube and counted in a haemocytometer. The concentration was adjusted to give each rabbit a predetermined inoculum of  $1 \times 10^8$  conidia of *A. fumigatus* in a volume of 200–350  $\mu\text{L}$ . The concentrations of the inoculum were confirmed retrospectively by serial dilution and culture on Sabouraud dextrose agar check plates.

Inoculation was performed under general intravenous anaesthesia with 0.5–1.0 mL of a 2:1 (v/v) mixture of ketamine 100 mg/mL (Fort Dodge Labs, Fort Dodge, IA, USA) and xylazine 20 mg/mL (Moby Corp., Shawney, KA, USA) for analgesia, amnesia and muscle relaxation. Anaesthetic dosage was adjusted according to body weight to obtain similar depths of general anaesthesia. Once satisfactory anaesthesia had been reached, a Flagg 0 straight-blade laryngoscope (Welch-Allyn, Skaneateles Falls, NY, USA) was inserted and the inoculum was given intratracheally with a tuberculin syringe attached to a 13.3 cm Teflon catheter (Becton Dickinson, Sandy, UT, USA).

### In-vitro antifungal susceptibility

The susceptibility of the experimental isolate to liposomal nystatin, free nystatin (Lederle Laboratories, Pearl River, NY, USA) and amphotericin B deoxycholate was tested by broth macrodilution and microdilution with an inoculum of  $0.5\text{--}2.5 \times 10^3$  cfu/mL in RPMI 1640 medium as previously described.<sup>16</sup> With that method, the MICs of liposomal nystatin, free nystatin and amphotericin B deoxycholate were 2, 8 and 1 mg/L, respectively, and the minimum fungicidal concentrations (MFCs) were the same as the MICs.

### Antifungal therapy

Liposomal nystatin, administered daily in a single dose, was studied at three different dosage levels: six rabbits received

1 mg/kg, ten received 2 mg/kg and 16 received 4 mg/kg. Ten animals treated with amphotericin B deoxycholate at 1 mg/kg daily in a single dose and ten untreated, but infected animals served as controls.

Liposomal nystatin (Nyotran; 50 mg vials; 50 mg nystatin USP incorporated into a mixture of 350 mg DMPC and 150 mg DMPG; Aronex Pharmaceuticals, The Woodlands, TX, USA) was provided as a lyophilized powder and maintained at 4°C. The drug was freshly reconstituted before use with 50 mL of sterile normal saline to a 1 mg/mL solution. Following addition of normal saline, vials were vigorously shaken for 1 min, placed in a 40°C water bath for 15 min, removed from the water bath and vigorously shaken for a further 60 s. The reconstituted drug was administered at ambient temperature as slow intravenous infusion at a rate of 2 mg/min. Amphotericin B deoxycholate (50 mg vials of Fungizone; Bristol Myers-Squibb, Princeton, NJ, USA) was reconstituted with 10 mL distilled water, maintained at 4°C and diluted 1:4 (v/v) with sterile 5% dextrose in water immediately before use. Amphotericin B preparations were sheltered from light and administered intravenously at a rate of 0.2 mg/min after normal saline loading (10 mL/kg body weight) to decrease nephrotoxicity.

Antifungal treatment was begun 24 h after endotracheal inoculation and was administered daily throughout the experiment. Surviving rabbits were killed on day 12 after inoculation.

### Outcome variables

All experiments were evaluated according to the following outcome variables:

**Survival analysis.** Duration of survival, in days post-inoculation, was recorded for each rabbit. Surviving rabbits were killed by pentobarbital anaesthesia on day 12 after inoculation.

**Morphological and microbiological post-mortem studies.** The entire heart-lung block was carefully dissected and removed at autopsy. The heart was then dissected away from the lungs, leaving an intact tracheobronchial tree and lung preparation. The lungs were weighed (Mettler Instrument, Hightstown, NJ, USA) and inspected by two observers blinded to the treatment group who recorded the number and type of lesions, if any, in each separate lobe.

Bronchoalveolar lavage (BAL) was performed on each lung preparation by the instillation and subsequent withdrawal of 10 mL of sterile normal saline three times into the clamped trachea. An aliquot of 0.1 mL of this fluid was cultured on Sabouraud-glucose agar.

Thereafter, a representative region of each lobe was excised for cultures and histopathological examination. Each fragment reserved for culture was weighed individ-

ually, minced with sterile scissors and, depending on the weight, homogenized with 2 mL or 5 mL of sterile saline for 1 min per tissue sample in reinforced sterile polyethylene bags (Stomacher 80; Tekmar, Cincinnati, OH, USA).<sup>17</sup> Lung homogenates in 10<sup>-2</sup> and 10<sup>-4</sup> dilutions were prepared in sterile saline, and aliquots of 100 µL were plated on Sabouraud-glucose agar and incubated at 37°C for the first 24 h and then at room temperature for another 24 h. After this, the cfu of *A. fumigatus* were counted and recorded for each lobe and the cfu/g were calculated. One colony of *A. fumigatus* was considered positive. In addition, the percentage of culture-positive lobes was calculated for each rabbit.

Specimens reserved for histopathological examination were sectioned and preserved in 10% neutral-buffered formalin. The fixed specimens were then embedded in paraffin, sectioned and stained with haematoxylin-eosin (HE), periodic acid-Schiff (PAS) and Gomori methenamine silver stains (GMS) for microscopic examination. Haemorrhagic infarcts (dark-red, consolidated lesions) corresponded microscopically to coagulative necrosis and intra-alveolar haemorrhage.

**Radiographic studies.** Serial ultra-fast computerized tomography (UFCT) was performed in actively treated rabbits in order to monitor the effects of antifungal treatment on the course of the infection during life. The radiographic course of untreated, but infected rabbits monitored by UFCT has been previously described and consists of a continuous progression of pulmonary lesions until death.<sup>18</sup>

Computerized tomography (CT) was performed with a C-100XL ultrafast electron beam CT scanner (Imatron, Oyster Point, CA, USA).<sup>18</sup> Rabbits were transported between the laboratory animal facility and the UFCT scanner under general anaesthesia. An induction dose of 0.3 mL of a 2:1 mixture (v/v) of ketamine 100 mg/mL and xylazine 20 mg/mL was administered intravenously. Anaesthesia was maintained during the procedure by an additional dose of 0.2 mL of that mixture, if necessary. In virtually all cases, 30 slices were sufficient to scan the entire thorax of the rabbit, allowing a scanning time of <5 min per animal.

The radiographic course of experimental infection was followed on the day after inoculation and on days 3, 6 and 9 after inoculation, if feasible. At each session a mean pulmonary lesion score was established by evaluating each of the six lung lobes from each rabbit. Each lobe was evaluated independently using a score ranging from 0 to 3 with increments of 0.5, where 0 signified the absence of an infiltrate and 3 opacification of the entire lobe. Ultimately, the mean pulmonary lesion score for a given day and dosage level represents the mean of all lobes of all rabbits treated at that dosage level and scanned on that day. All CT scans were scored by the same observer, who was blinded to the identity of the study group of each rabbit.

### Toxicity studies

Blood samples were collected from each rabbit on days 5, 7 and 9 after inoculation. Plasma samples were stored in Sarsted tubes (Sarsted Inc., Newton, NC, USA) at  $-80^{\circ}\text{C}$  and were processed simultaneously. Levels of blood urea nitrogen (BUN), serum creatinine, potassium and hepatic transaminases were tested on the last sample drawn from each rabbit before death (Anilytics, Gaithersburg, MD, USA).

### Pharmacokinetic studies in plasma

Three groups of four uninfected rabbits each were studied. Animals received liposomal nystatin at 1, 2 and 4 mg/kg body weight once daily for a total of eight doses as a slow intravenous infusion (2 mg/min). On days 1 and 8, plasma samples were drawn immediately before administration of the compound and then at 10 min, 30 min and 1, 2, 4, 6, 8, 12 and 24 h after administration. An accurate, sensitive, reproducible and specific reversed phase high-performance liquid chromatographic (HPLC) method was used for quantification. The sample preparation involved addition of 2:1 (v/v) methanol to standard, quality controls, or unknown rabbit plasma and incubation at  $4^{\circ}\text{C}$  for 20 min. This was followed by two centrifugation steps (2000g for 10 min and 10,000g for 4 min, respectively), and a final centrifugation step before injection at 4000g for 4 min using  $0.2\ \mu\text{m}$  centrifugal filters (Ultra-Free MC, Millipore, Bedford, MA, USA). The mobile phase consisted of 10 mM sodium phosphate, 1 mM EDTA, 30% methanol and 30% acetonitrile (Fisher Scientific, Fair Lawn, NJ, USA) delivered at 2 mL/min. During analysis, samples were maintained at  $4^{\circ}\text{C}$ ; the injection volume was 200  $\mu\text{L}$ . Nystatin was detected by UV absorbance at 305 nm using a  $\text{C}_{18}$  analytical column maintained at  $30^{\circ}\text{C}$  ( $\mu\text{Bondapak C}_{18}$ , Waters Corp., Milford, MA, USA) in conjunction with an in-line precolumn filter (NewGuard RP-18, Perkin Elmer, Norwalk, CT, USA). Quantification was based on the peak area of the first main isomer, which eluted at 7.5–8.5 min, and the peak area–concentration response of the external calibration standard. Ten-point standard curves were linear from 0 to 25 mg/L with  $R^2$  values  $> 0.99$ . The lower limit of quantification was 0.1 mg/L. Using current guidelines for validation of analytical methods,<sup>19</sup> accuracies were within  $\pm 12\%$  and intra- and interday variability (precision) was  $\leq 7.5$ . The stability of extracted plasma samples maintained in the autosampler over 10 h at  $4^{\circ}\text{C}$  ranged from 90% to 94%, and accuracies of spiked plasma samples after storage for 3 months at  $-80^{\circ}\text{C}$  were within  $\pm 9\%$ .

Standard model-independent techniques were used to calculate the area under the plasma concentration–time curve from 0 to 24 h ( $\text{AUC}_{0-24}$ ), half-life ( $t_{1/2}$ ), plasma clearance ( $Cl$ ) and volume of distribution ( $V_d$ ).<sup>20</sup> Levels in plasma obtained at 10 min were used as maximum concentrations ( $C_{\text{max}}$ ).

### Statistical analysis

Survival curves were estimated by the Kaplan–Meier product limit method and differences between groups were analysed by the Mantel–Haenszel  $\chi^2$  test. Comparisons between proportions were done by the  $\chi^2$  or Fisher's exact test, and group-to-group comparisons of continuous variables were analysed by the Kruskal–Wallis ANOVA test with Dunn's correction for multiple comparisons or the Mann–Whitney  $U$ -test, as appropriate. Values are stated as means  $\pm$  S.E.M. Except for survival analysis, all  $P$  values were two-sided. A  $P$  value of  $\leq 0.05$  was considered statistically significant.

## Results

### Survival

Survival among the five study groups was analysed over 12 days using Kaplan–Meier analysis (Figure 1). Only one of ten untreated control rabbits survived beyond day 7 after inoculation. In contrast, four of ten rabbits (40%) treated with amphotericin B at 1 mg/kg/day survived for 12 days ( $P = 0.05$  vs controls). Similarly, survival in rabbits treated with either 2 or 4 mg/kg/day of liposomal nystatin was greater than that of untreated controls ( $P < 0.05$  for both dosage levels) with seven (70%) of ten and six (37.5%) of 16 rabbits surviving for 12 days after inoculation. In contrast, survival in animals treated with liposomal nystatin at 1 mg/kg/day (one of six at day 12) did not differ from that of untreated controls. There were no statistically significant differences in survival between rabbits treated with liposomal nystatin at 2 and 4 mg/kg/day or amphotericin B, or among the three dosage regimens of liposomal nystatin.

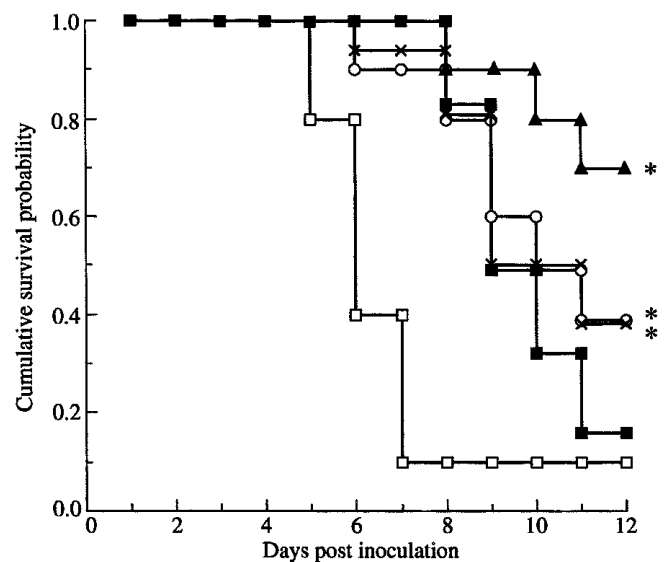
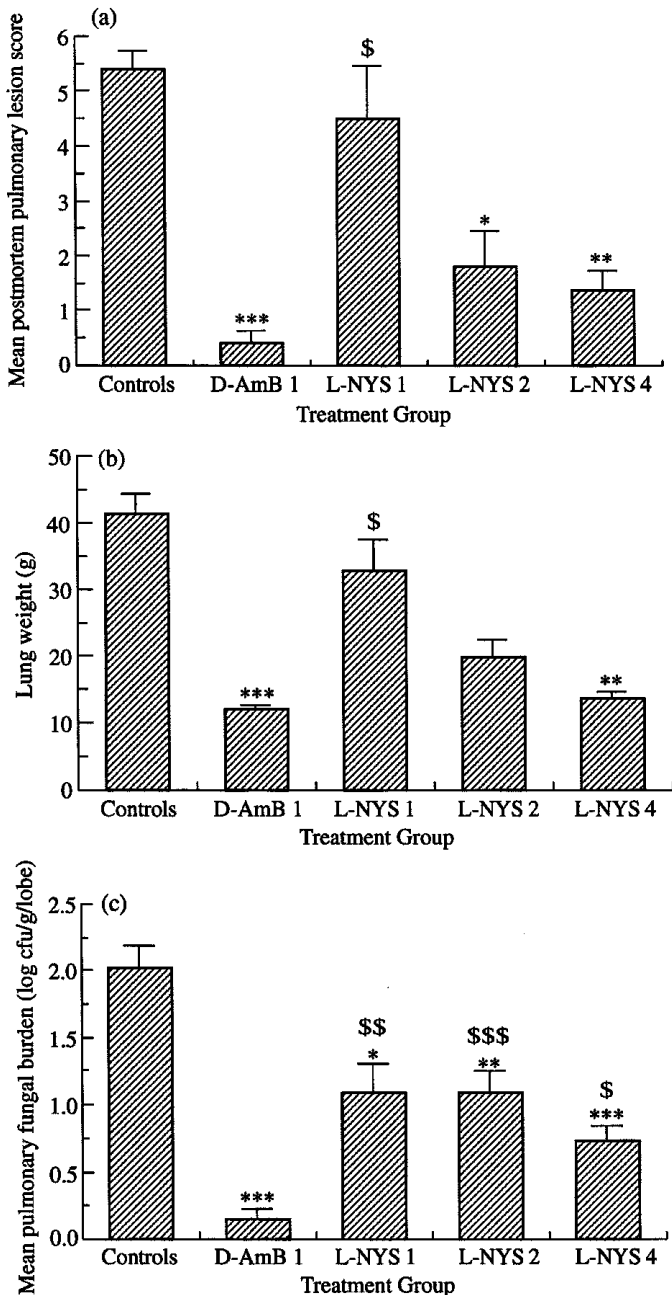


Figure 1. Cumulative survival probability in rabbits treated with liposomal nystatin 1 mg/kg (■), 2 mg/kg (▲) or 4 mg/kg (×), or amphotericin B 1 mg/kg (●) versus untreated control animals (□). \*,  $P \leq 0.05$  vs controls.

### Post-mortem morphological and microbiological studies

As depicted in Figure 2a, rabbits treated with either amphotericin B ( $P < 0.001$ ) or liposomal nystatin at 2 mg/kg ( $P < 0.05$ ) and 4 mg/kg ( $P < 0.01$ ) had significantly fewer haemorrhagic infarcts of lung tissue than untreated



**Figure 2.** Effects of antifungal treatment with liposomal nystatin (L-NYS) at the three selected dosage levels vs those of amphotericin B (D-AmB) and no treatment. (a) Mean post-mortem pulmonary lesion score; (b) mean lung weight at post-mortem; and (c) mean post-mortem lung tissue burden of *A. fumigatus*. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  vs untreated controls; \$ $P < 0.05$ , \$\$ $P < 0.01$ , \$\$\$ $P < 0.001$  vs amphotericin B-treated rabbits.

controls. No statistically significant differences in the mean number of haemorrhagic infarcts were observed between animals treated with liposomal nystatin at 2 or 4 mg/kg and amphotericin B-treated animals. Rabbits receiving liposomal nystatin at 1 mg/kg, however, did not have fewer haemorrhagic infarcts than untreated controls and had significantly more infarcts than amphotericin B-treated animals.

The mean lung weights in rabbits treated with amphotericin B ( $P < 0.001$ ) and liposomal nystatin at 4 mg/kg ( $P < 0.001$ ) were lower than those in untreated controls, reflecting a reduction in excess lung weight due to haemorrhage and oedema (Figure 2b). Treatment with liposomal nystatin at 1 or 2 mg/kg was not associated with a significantly reduced lung weight at post-mortem. Again, except for the 1 mg/kg dosage level of liposomal nystatin, there were no differences between liposomal nystatin-treated and amphotericin B-treated rabbits.

Compared with untreated controls, rabbits treated with either daily amphotericin B ( $P < 0.001$ ) or liposomal nystatin at 1 ( $P < 0.05$ ), 2 ( $P < 0.01$ ) and 4 mg/kg/day ( $P < 0.001$ ) had a significant reduction in mean log(cfu/g/lobe) (Figure 2c). Similarly, the percentage of culture-positive lobes was 76.6% in untreated controls, 6.6% in rabbits treated with amphotericin B ( $P < 0.001$ ) and 47.2% ( $P < 0.01$ ), 45.0% ( $P < 0.001$ ) and 31.2% ( $P < 0.001$ ) in rabbits treated at 1, 2 and 4 mg/kg/day of liposomal nystatin, respectively. Nevertheless, treatment with amphotericin B at the standard dose of 1 mg/kg/day was significantly more effective in reducing fungal burden than either of the investigated regimens of liposomal nystatin ( $P < 0.01$ ,  $P < 0.001$  and  $P < 0.05$  vs the 1, 2 and 4 mg/kg/day of liposomal nystatin, respectively), and was associated with a significantly lower percentage of infected lobes ( $P < 0.001$  for each of the investigated dosage levels of liposomal nystatin).

Cultures from BAL fluid obtained at post-mortem yielded *A. fumigatus* in 70% of untreated control animals vs 10% in those treated with amphotericin B ( $P < 0.05$ ). In comparison with untreated controls, a significant reduction in positive BAL cultures was noted only in rabbits treated with 2 mg/kg/day (to 20%) and 4 mg/kg/day (to 12.5%) of liposomal nystatin ( $P < 0.05$  and  $P < 0.01$ , respectively; Fisher's exact test). No differences were observed between nystatin-treated and amphotericin B-treated rabbits, or among the different dosage regimens of liposomal nystatin.

### Radiographic studies

Serial monitoring of pulmonary findings by UFCT in rabbits treated with amphotericin B or liposomal nystatin at 2 and 4 mg/kg, respectively, revealed marked improvement of pulmonary infiltrates in the course of the experiment (Figure 3). In contrast, there was no evidence for resolution of pulmonary lesions in rabbits treated with liposomal nystatin at 1 mg/kg; indeed, the mean pulmonary

lesion score on day 9 in that group was significantly greater than that in amphotericin B-treated rabbits ( $P < 0.05$ ). UFCT scanning was well tolerated without any deaths related to procedure or anaesthesia.

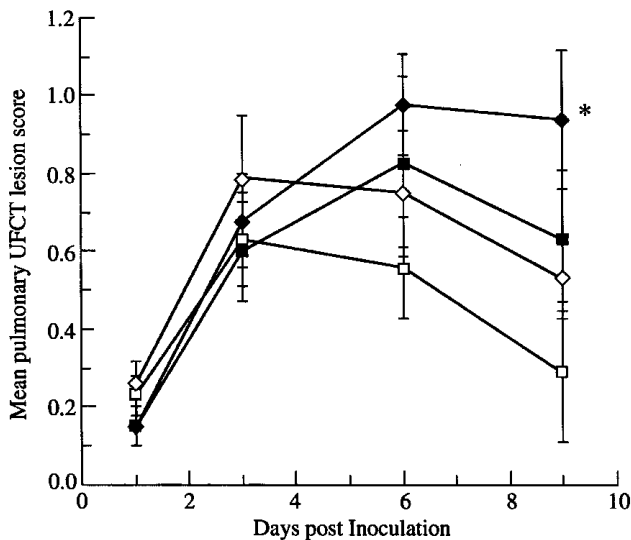
**Toxicity**

Rabbits treated with either amphotericin B (with prior saline-loading) or liposomal nystatin at 2 and 4 mg/kg (without saline-loading) had significantly greater mean serum creatinine and BUN levels when values determined from the last sample drawn in each individual rabbit were compared with untreated controls (Table). However, there were no statistically significant differences between

amphotericin B-treated animals and animals treated with liposomal nystatin at 2 and 4 mg/kg. No differences were observed between liposomal nystatin-treated rabbits, untreated controls and rabbits treated with amphotericin B with regard to serum potassium and hepatic transaminase levels.

**Pharmacokinetic investigations**

The plasma concentration–time curves of liposomal nystatin after oral administration to non-infected, normal rabbits over 8 days and the pharmacokinetic parameters derived from these curves are shown in Figure 4. Over the investigated dose-range, liposomal nystatin demonstrated linear disposition, with rapid initial distribution and a relatively short elimination half-life. At all dose levels, near-peak levels were at least two-fold greater than the MIC for the isolate used in the efficacy studies.



**Figure 3.** Mean ultrafast CT-scan (UFCT) lesion scores during the course of the experiments in rabbits treated with liposomal nystatin 1 mg/kg (◆), 2 mg/kg (■) or 4 mg/kg (◇), and amphotericin B 1 mg/kg (□).

**Discussion**

The results of this study indicate dose-dependent anti-fungal activity of liposomal nystatin in an experimental model of invasive pulmonary aspergillosis in persistently neutropenic rabbits. Whereas, in comparison with untreated controls, liposomal nystatin administered at 1 mg/kg was only effective in reducing fungal tissue burden, liposomal nystatin given at 2 or 4 mg/kg also prolonged survival and reduced fungus-mediated tissue injury and excess lung-weight at post-mortem. However, although treatment with all three regimens of liposomal nystatin led to a significant reduction in fungal tissue burden, amphotericin B was unequivocally superior in clearing infected lung tissue. Similar trends were noted in the microbiological analysis of BAL fluid obtained *post mortem*. UFCT

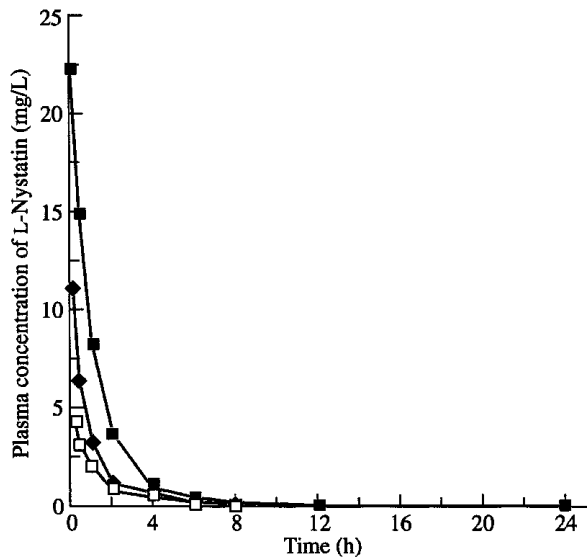
**Table.** Effects of treatment with liposomal nystatin on serum creatinine, blood urea nitrogen (BUN), potassium, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) compared with untreated but infected controls and amphotericin B-treated rabbits

	<i>n</i>	Creatinine (μmol/L)	BUN (mmol/L)	Potassium (mmol/L)	AST (U/L)	ALT (U/L)
Controls	9	93.70 ± 3.53	7.53 ± 0.82	2.93 ± 0.20	11.00 ± 1.59	36.44 ± 4.64
D-AmB 1 mg/kg	9	166.19 ± 18.56 <sup>a</sup>	21.79 ± 2.53 <sup>e</sup>	2.63 ± 0.11	5.33 ± 0.33	26.22 ± 3.33
L-Nys						
1 mg/kg	6	99.99 ± 3.53 <sup>b</sup>	8.50 ± 1.28 <sup>f</sup>	3.13 ± 0.41	21.67 ± 11.44	50.50 ± 11.90
2 mg/kg	10	190.06 ± 51.27 <sup>c</sup>	17.24 ± 4.96	3.10 ± 0.16	10.70 ± 3.03	29.30 ± 5.72
4 mg/kg	15	195.36 ± 25.63 <sup>d</sup>	19.13 ± 3.85 <sup>g</sup>	3.16 ± 0.12	4.47 ± 0.65	18.80 ± 1.78
Normal rabbits	12	69.83 ± 2.65	6.96 ± 0.35	4.24 ± 0.06	21.25 ± 3.14	41.73 ± 4.79

D-AmB, amphotericin B deoxycholate; L-Nys, liposomal nystatin.

All values are given as mean ± S.E.M.; values for normal rabbits are presented at the bottom of each column.

<sup>a</sup> $P < 0.01$  vs controls; <sup>b</sup> $P < 0.05$  vs AmB; <sup>c</sup> $P < 0.05$  vs controls; <sup>d</sup> $P < 0.001$  vs controls; <sup>e</sup> $P < 0.001$  vs controls; <sup>f</sup> $P < 0.05$  vs AmB; <sup>g</sup> $P < 0.05$  vs controls.



Drug dose (mg/kg)	$C_{max}$ (mg/L)	$AUC_{0-24}^*$ (mg·h/L)	$V_d$ (L)	$Cl$ (L/h)	$t_{1/2\beta}$ (h)
1	4.4 ± 0.5	6.02 ± 0.8	0.61 ± 0.1	0.56 ± 0.09	2.35 ± 0.2
2	11.3 ± 1.7	10.88 ± 0.7	0.69 ± 0.1	0.60 ± 0.04	2.58 ± 0.1
4	22.3 ± 1.2	26.24 ± 1.9	0.61 ± 0.1	0.57 ± 0.06	2.71 ± 0.4

**Figure 4.** Concentration–time profiles and noncompartmental pharmacokinetics of liposomal nystatin in normal rabbits after daily dosing at 1 (□), 2 (◆) and 4 mg/kg (■) for 8 days. Values are given as means ± S.E.M. from four (1 and 2 mg/kg) and three (4 mg/kg) rabbits each.  $C_{max}$ , near peak plasma level;  $AUC_{0-24}$ , area under the concentration–time curve from 0 to 24 h;  $V_d$ , volume of distribution;  $Cl$ , clearance;  $t_{1/2\beta}$ , terminal elimination half-life. \* $P = 0.7381$  for departure from linearity by plotting the ratio of  $AUC:dose$  vs dosage levels and performing linear regression.

demonstrated significant resolution of pulmonary lesions during treatment with liposomal nystatin at 2 and 4 mg/kg, but not at 1 mg/kg. In comparison with saline-loaded, amphotericin B-treated rabbits, rabbits treated with liposomal nystatin at 2 and 4 mg/kg developed similar elevations of BUN and serum creatinine. Of note, although not statistically significant, rabbits treated with liposomal nystatin appeared to have less potassium loss.

Preliminary, noncompartmental pharmacokinetic studies in uninfected animals established linear disposition of liposomal nystatin in plasma over the observed dose range after multiple dosing over 8 days. As previously described for multilamellar liposomal preparations,<sup>21</sup> the compound reached relatively high peak levels and was then rapidly distributed and eliminated from plasma with a relatively short half-life. These findings are consistent with pharmacokinetic data obtained from HIV-infected patients, who received the drug at dosages ranging from 0.25 to 7 mg/kg.<sup>22,23</sup> Taken together, both experimental and clinical data indicate a pharmacokinetic profile of lipo-

somal nystatin which is markedly different from that of amphotericin B deoxycholate and the three currently available lipid formulations of amphotericin B.<sup>6</sup> Notably, to the best of our knowledge, no data have ever been published on the plasma pharmacokinetics of free nystatin after intravenous administration.

Peak plasma levels were above both the MIC and MFC for the aspergillus strain used in the infection model, and above the MICs recently reported for various clinical fungal isolates.<sup>24,25</sup> Due to the linearity of pharmacokinetics and antifungal effects, no general conclusions can be drawn regarding whether maximum plasma concentrations or the amount of drug circulating in plasma over time are more important to the pharmacodynamics of liposomal nystatin. For a primarily localized infection, such as invasive pulmonary aspergillosis, sufficient tissue levels are certainly major determinants of efficacy.<sup>26,27</sup> Indeed, unpublished studies in normal rabbits performed in our laboratories revealed lung tissue levels 10- to 15-fold above the MIC for our test strain shortly after the last of multiple doses of 2 and 4 mg/kg/day, respectively. However, the ultimate distribution of free drug into infected tissue and into the fungal cell membrane is unclear.

The finding that the composite outcome at 1 mg/kg of liposomal nystatin was poorer than that in both higher dosage groups, despite similar residual fungal burden at post-mortem, might be explained by slower accumulation of free compound in lung tissue to critical levels. That amphotericin B-treated rabbits had relatively poor survival despite exhibiting the highest antifungal efficacy among all treatment groups may be explained by the compound's nephrotoxicity, and possibly, drug-associated anaemia. It may be somewhat surprising that rabbits treated with liposomal nystatin at 2 mg/kg had a trend towards improved survival over amphotericin B-treated animals despite being significantly less effective in clearing infected lung tissue from the organism. Nevertheless, there may be subtle differences in renal toxicity as indicated by lower mean BUN and higher mean potassium levels in the nystatin-treated animals; similarly, although this has only been demonstrated *in vitro*,<sup>9,28</sup> liposomal nystatin may also be less haemolytic than amphotericin B *in vivo*. These factors collectively may confer a survival benefit in the state of severe disease.

Liposomal nystatin was more active *in vitro* than its free counterpart, but less active than conventional amphotericin B against the *A. fumigatus* isolate used in our experiments. These observations are in accordance with the overall trend of two larger *in vitro* studies on the comparative activity of liposomal nystatin against various clinical isolates.<sup>24,25</sup> The improved *in vitro* activity of the multilamellar liposomal formulation of nystatin over free drug might indicate enhanced delivery to the fungal cell membrane.<sup>29</sup> Of note, the small unilamellar liposomal formulation of amphotericin B was overall considerably less active *in vitro* than conventional amphotericin B (and liposomal

nystatin) in one study,<sup>25</sup> although it was equally active in another.<sup>30</sup> These still very limited findings indicate the complexity of in-vitro susceptibility testing of lipid polyene formulations, for which carrier effects<sup>29</sup> and pharmacokinetic properties<sup>6</sup> are thought to be the major determinants of antifungal efficacy *in vivo*. They also underscore the need for evaluating these novel formulations in well designed and clinically relevant animal models.

Thus far, the antifungal efficacy of liposomal nystatin has been evaluated in two survival models. In non-neutropenic mice with disseminated candidiasis and in transiently neutropenic mice intravenously inoculated with *A. fumigatus*, respectively, treatment with liposomal nystatin at dosages ranging from 2 to 16 mg/kg/day conferred a significant increase in survival when compared with untreated control animals.<sup>10,11</sup> While the latter study demonstrated in-vivo efficacy of the compound against *Aspergillus* spp. by the endpoint of survival, our model was designed to mimic the clinical situation of patients at highest risk for invasive aspergillosis, i.e. persistent and profound neutropenia and respiratory route of infection. Beyond survival analysis, it provides important data on direct effects of treatment such as organism-mediated tissue injury, residual fungal burden and laboratory toxicity in the situation of severe illness. Furthermore, by incorporating UFCT and BAL, it allows for the correlation of tissue data with these clinically most relevant non-invasive diagnostic studies. Indeed, the model has been highly predictive for the clinical activity of other lipid polyene formulations, such as liposomal amphotericin B<sup>31</sup> and amphotericin B colloidal dispersion.<sup>32</sup>

Although treatment with liposomal nystatin was clearly associated with laboratory signs of renal toxicity in our study, this toxicity was mild and apparently not dose-proportional and there were no significant differences in comparison with rabbits receiving amphotericin B with prior normal saline loading. In unpublished toxicity studies performed by the manufacturer in dogs, hydration had a beneficial effect on kidney function following administration of liposomal nystatin (T. Wallace, personal communication). It is thus possible that administration of normal saline to liposomal nystatin-treated rabbits would have reduced renal toxicity and, if death was in part related to renal impairment, might have impacted on survival. Independent of these considerations, liposomal nystatin was tolerated without reaching the maximum tolerated dose (MTD) at dosages of up to 8 mg/kg and day in a phase I study in 32 patients with haematological malignancies and refractory febrile neutropenia who received the drug for a median of 8 days (range, 2–75 days); elevations of serum creatinine levels occurred in ten patients (31%) altogether, but never exceeded Grade II toxicity.<sup>12</sup>

In conclusion, this study demonstrates that liposomal nystatin at dosages of 2 and 4 mg/kg/day increases survival and provides reduced tissue injury, effective microbiological clearance and tolerable nephrotoxicity in a model of invasive pulmonary aspergillosis in persistently granulo-

cytopenic rabbits. Our findings add to the understanding of both pharmacokinetics and pharmacodynamics of liposomal nystatin as it is being developed for treatment of patients with proven or suspected invasive fungal infections.

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