

Enhanced efficacy of pH-sensitive nystatin liposomes against *Cryptococcus neoformans* in murine model

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Objectives: To evaluate the efficacy of pH-sensitive liposomes of nystatin against *Cryptococcus neoformans* infection in a murine model.

Methods: In the present study, we investigated the antifungal activity of nystatin entrapped in pH-sensitive liposomes in a murine model. Mice infected with *C. neoformans* were treated with nystatin in neutral egg phosphatidylcholine (egg-PC) liposomes, as well as pH-sensitive nystatin liposomes. The anticryptococcal efficacy of liposomal formulations of nystatin was assessed by continued survival and colony-forming units (cfu) in liver and brain of the treated mice.

Results: pH-sensitive liposomes of nystatin showed better efficacy compared with its free or egg-PC liposome form against *C. neoformans* infection in BALB/c mice. Mice treated with pH-sensitive nystatin liposomes showed 80% survival with less fungal burden in liver and brain of treated mice. However, there was only 40% survival in the group of animals treated with egg-PC liposome-intercalated nystatin, whereas its free form had poor efficacy with 20% survival.

Conclusions: The enhanced anticryptococcal efficacy of the pH-sensitive nystatin liposomes can be attributed to the pH-dependent release of the drug in the low pH environment of lysosomes. The destabilization of the pH-sensitive liposomes in the acidic environment of macrophages results in the site-specific targeting of nystatin that improves its intracellular antifungal activity.

Keywords: lysosomes, drug delivery pH-sensitive liposomes

Introduction

In a manner similar to other opportunistic fungal pathogens like *Candida albicans*, *Aspergillus fumigatus*, etc., *Cryptococcus neoformans* afflicts patients with defective cell-mediated immunity.¹ The AIDS epidemic, cancer chemotherapy, pervasive use of antibiotics and immunosuppressive drugs used for organ transplantation have all contributed to the emergence of these pathogens.^{1,2} However, *C. neoformans* infection can occur in both immunocompetent, as well as immunocompromised individuals.³ Upon entering the host *C. neoformans* starts using host cells to disseminate more efficiently, evade the host immune response and under certain circumstances can survive in a dormant state until reactivation occurs upon sustained immunodeficiency.⁴

Polyene antibiotics, particularly amphotericin B and nystatin, have been found to be the most effective and extensively used drugs in treatment of both pre-systemic and systemic fungal infections. However, drug-induced nephrotoxicity has limited the clinical use of these most potent antifungals. The use of liposomal formulations

of these antibiotics have minimized the toxic manifestations of the drug and thus are extensively being used for treatment of deep-seated fungal infections.^{5,6}

For treatment of pathogens that adopt intracellular parasitism as a strategy to avoid host defence, the design of liposomal drug delivery systems that undergo controlled fusion with cellular or endosomal membranes seems to be an interesting proposition. Liposomes that can be triggered to release their contents or fuse in response to pH stimuli are of particular interest, as they can potentially respond to acidic environments *in vivo*.⁷ Such environments include those encountered in tumour tissue and primary endocytic vesicles. pH-sensitive liposomes are typically made of 1,2-dioleoylphosphatidylethanolamine (DOPE), a lipid that adopts the non-bilayer inverted hexagonal (H_{II}) phase in isolation, and an ionizable acidic lipid such as cholesteryl hemisuccinate. At pH values above the pK of the acidic lipid, the negatively charged form of the acidic lipid can stabilize the DOPE in the bilayer organization, thus allowing the formation of bilayer vesicles. These vesicles then fuse as the pH is reduced toward the pK of

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the acidic lipid. If the stabilizing lipid has a negatively charged head group with an appropriate pK_a , endosomal acidification can neutralize the lipid charge, reducing the bilayer-stabilizing effect. Bilayer destabilization in liposomes containing DOPE can result in both liposome fusion with adjacent membranes and content release.⁸

In the present work, we used pH-sensitive liposomes for delivery of the antifungal drug nystatin against *C. neoformans* infection in mice. It is anticipated that stability of these liposomes would be pH-dependent and at endosomal pH these liposomes would be destabilized resulting in the enhanced release of entrapped drug inside the macrophages.

Materials and methods

Chemicals

Egg phosphatidylcholine (egg-PC) was isolated and purified according to the published procedure.⁹ Cholesterol was bought from Centron Research Laboratory, Mumbai and used after crystallization with methanol. Nystatin, DOPE and cholesterylhemisuccinate (CHEMS) were purchased from Sigma Chemical Co., USA.

Preparation of nystatin liposomes

Egg-PC liposomes were prepared from egg-PC (49 μ mol) and cholesterol (21 μ mol) by sonication method.¹⁰ Similarly, pH-sensitive liposomes were prepared by mixing CHEMS and DOPE in the ratio of 2 : 3. All the ingredients, along with nystatin (drug/lipid, 1 : 20), were dissolved in a round-bottomed flask in a minimum volume of chloroform/methanol (1 : 1, v/v). The solvents were carefully evaporated under reduced pressure to form a lipid film on the wall of the flask. The final traces of the solvents were removed by subjecting the flask to vacuum overnight at 4°C. Subsequently, the dried lipid film (consisting of egg-PC/cholesterol, nystatin) was hydrated with 2 mL of 150 mM sterile saline with intermittent vigorous stirring followed by sonication (1 h, 4°C) in a bath type sonicator under N₂ atmosphere. The sonicated preparation was centrifuged at 10 000 g for 1 h at 4°C to remove traces of undispersed lipid and finally dialysed against normal saline for 24 h at 4°C in the dark. The liposomal preparation of nystatin was used in treatment of murine cryptococcosis.

Estimation of liposome-intercalated nystatin

The intercalation efficiency of nystatin in the liposomes was determined spectrophotometrically. A standard curve of nystatin was plotted at an absorbance of 320 nm. The amount of drug associated with liposomes was determined by dissolving the formulation in methanol and monitoring the absorbance at 320 nm using the corresponding amount of lipid in methanol as blank.¹¹ The amount of nystatin entrapped in liposomes was calculated from the standard curve. The intercalation efficiency of nystatin in plain egg-PC and pH-sensitive liposomes was of the order of 92 \pm 4% and 90 \pm 4%, respectively.

Animals

Male BALB/c mice weighing 20 \pm 2 g were used in the study. The animals were given a standard pellet diet (Hindustan Lever Ltd) and water *ad libitum*. Mice were checked daily for their mortality and morbidity. The techniques used for bleeding, injection, as well as sacrifice of animals were approved by the Animal Ethics Committee [Committee for the purpose of control and supervision of Experiments on Animals (CPCSEA), Government of India].

Test strain

The strain of *C. neoformans* (JMCR 102) was obtained from a leukaemia patient of the Jawaharlal Nehru Medical College (JNMC), Aligarh Muslim University, Aligarh. Sabouraud dextrose (SD) agar/broth was used for growing patient isolates of *C. neoformans*. The identity of the isolate was confirmed in the mycology section of the Department of Microbiology, JNMC, Aligarh, India.

Antifungal susceptibility testing

The MIC of nystatin was determined by broth macrodilution method described by NCCLS recommendations.¹² Nystatin was tested over the final concentration range of 0.05–5 mg/L. Testing was performed in 96-well round-bottom microtitre plates. Cell suspension of *C. neoformans* was prepared in RPMI-1640 medium and adjusted to give a final inoculum concentration of 2 \times 10³ cells/mL. The wells containing fungal inoculum with different concentrations of drug and proper controls were incubated for 48–72 h.

Preparation of *C. neoformans* cells for infection

Yeast cells were harvested from agar plates onto YPD (1% yeast extract, 2% peptone, 5% dextrose) medium at 37°C for 36 h. The cells were washed with normal saline at low speed centrifugation (2000 rpm) and diluted to the appropriate concentration in saline prior to use *in vitro* as well as *in vivo* studies. Each mouse was infected with 7 \times 10⁵ spores of *C. neoformans* by intravenous (iv) route.

Drug therapy

The infected animals (10 in each group) were grouped in the following manner:

- (i) Saline
- (ii) Empty PC liposomes
- (iii) Empty pH-sensitive liposomes
- (iv) Free nystatin (3 mg/kg)
- (v) Free nystatin (5 mg/kg)
- (vi) PC-lip-nystatin (3 mg/kg)
- (vii) PC-lip-nystatin (5 mg/kg)
- (viii) pH-lip-nystatin (3 mg/kg)
- (ix) pH-lip-nystatin (5 mg/kg)

As the average body weight of mouse used was 20 \pm 2 g; on average 60 \pm 9 and 100 \pm 12 μ g drug (3 and 5 mg/kg body weight) in 0.3 mL of liposomal suspension was injected intraperitoneally on days 1, 2 and 3 post-infection. The control groups of animals were treated with sham liposomes containing same amount of lipid without drug identically.

Assessment of anticryptococcal activity

The role of liposomal nystatin in protection against *C. neoformans* infection was assessed by survival data and fungal burden in the liver and brain of mice. The animals were observed until Day 40 post-infection. For colony-forming units (cfu) determination, three mice from each group were sacrificed and their brain and liver were analysed. Briefly, weighed portions of the given organs were homogenized in 5 mL of sterile normal saline and an aliquot of the suspension was plated on SD agar plates containing chloramphenicol and gentamicin after appropriate dilution. The plates were incubated for 48–72 h at 37°C. The numbers of fungal colonies (cfu) were counted and the fungal load in various organs was calculated by multiplying with the dilution factor.

Efficacy of pH-sensitive nystatin liposomes against *C. neoformans*

Statistics

Analysis of survival of animals was conducted using Kaplan–Meier curve, and various groups were compared by log-rank test. Fungal burden (cfu) in organs was analysed by two-way ANOVA followed by Bonferroni post-test using Graph Pad Prism software.

Results

In vitro antifungal susceptibility testing

The final fungal inoculum concentration of 2×10^3 cells/mL in each well containing different concentrations of drug and proper controls after incubation for 48 h were evaluated for the determination of MIC, which was defined, as the lowest concentration of drug at which there was complete inhibition of growth. By this method the MIC of *C. neoformans* was found to be 1.5 mg/L for nystatin.

Mice treated with nystatin entrapped in pH-sensitive liposomes showed enhanced survival

The efficacy of various nystatin formulations was evaluated in animals challenged systemically with 7×10^5 spores of *C. neoformans*. Treatment of *C. neoformans* infected mice with pH-sensitive nystatin liposomes, at the dose of 3 mg/kg body weight, was able to successfully treat 50% of the animals, whereas treatment with egg-PC-nystatin liposome resulted in 20% survival only ($P = 0.0016$). PC-nystatin liposomes were not found to be significantly effective over free nystatin in imparting cure to infected mice ($P = 0.1417$). The group of animals treated with nystatin entrapped in pH-sensitive liposomes showed increased survival among animals of all groups in the study followed by nystatin entrapped in egg-PC liposomes. The efficacy of nystatin was found to increase further upon treatment of animals with relatively higher dose (5 mg/kg). Mice treated with drug formulation in pH-sensitive liposomes showed ~80% survival, whereas those treated with PC-lip-nystatin showed ~50% survival rates as observed at day 40 post-infection ($P = 0.024$). Mice treated with same dose of free nystatin showed enhanced mean survival compared with those treated with saline or sham liposomes but died within 40 days of infection (Figure 1).

C. neoformans burden in vital organs

The efficacy of pH-sensitive nystatin liposomes in the elimination of *C. neoformans* infection from tissues was assessed by quantification of fungal burden (Day 9 post-infection) in vital organs, i.e. brain and liver. A remarkable reduction in fungal load was observed in the organs of mice treated with nystatin entrapped in pH-sensitive liposomes (Figure 2). Nystatin in pH-sensitive liposomes showed significantly higher activity in eliminating the pathogen from brain and liver tissues over neutral PC-nystatin liposomes or free nystatin ($P < 0.05$ and $P < 0.001$, respectively).

Residual fungal burden in liver of mice survived on day 40 post-infection

The elimination of fungal infection from tissues was assessed in treated mice on day 40 post *C. neoformans* infection. The cfu count was found to be insignificant in liver of mice treated with pH-sensitive nystatin liposomes, which shows infection had subsided. The cfu was found to be in the range of 50–350 in

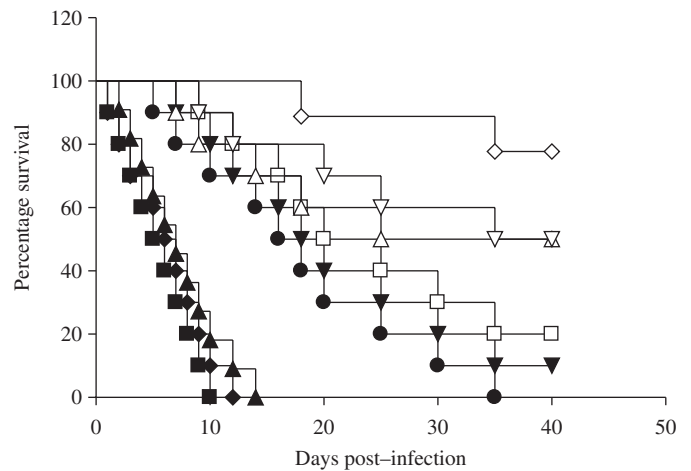


Figure 1. Survival rates of *C. neoformans* infected BALB/c mice followed by treatment with nystatin liposomes. Saline (closed squares), sham liposomes (closed diamonds), free nystatin (3 mg/kg) (closed circles), free nystatin (5 mg/kg) (closed inverted triangles), PC-nystatin liposomes (3 mg/kg) (open squares), PC-nystatin liposomes (5 mg/kg) (open triangles), pH-sensitive nystatin liposomes (3 mg/kg) (open inverted triangles), pH-sensitive nystatin liposomes (5 mg/kg) (open diamonds) and empty pH-sensitive liposomes (closed triangles). Mice were treated with nystatin formulations for three consecutive days 24 h post *C. neoformans* infection. Ten mice were taken in each group and experiment was repeated three times. Free nystatin versus lip-nystatin ($P = 0.1417$), free nystatin versus pH-lip-nystatin ($P = 0.0016$) and PC-lip-nystatin versus pH-lip-nystatin ($P = 0.024$).

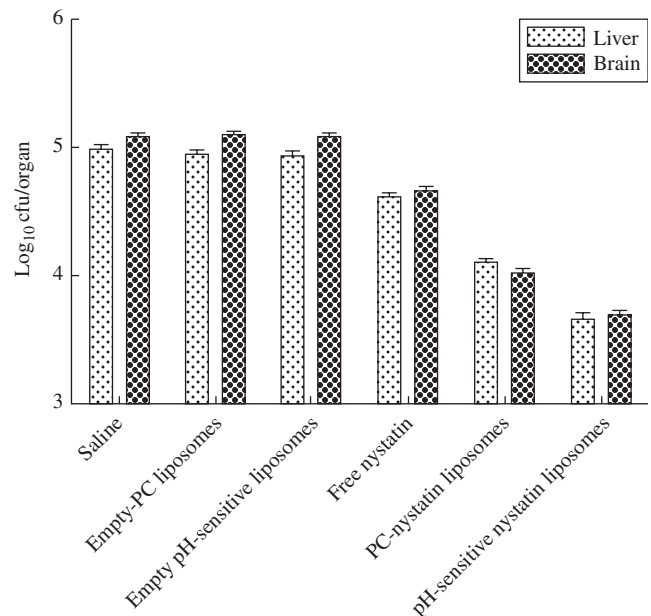


Figure 2. Nystatin in pH-sensitive liposomes shows enhanced anticryptococcal activity in murine model. Mice infected with *C. neoformans* (7×10^5 cfu/mouse) were treated with various formulations of nystatin for three consecutive days. On Day 9 post-infection three mice from each group were sacrificed and their liver and brain were taken out aseptically and processed as described in the Materials and methods section. The fungal load among various groups was compared by the two-way ANOVA followed by Bonferroni *t*-test. Free nystatin versus PC-lip-nystatin ($P < 0.001$), free nystatin versus pH-lip-nystatin ($P < 0.001$) and PC-lip-nystatin versus pH-lip-nystatin ($P < 0.05$).

mice treated with egg-PC-nystatin liposomes and 25–160 in those treated with pH-sensitive nystatin liposomes.

Discussion

Various liposomal formulations of polyene antifungal drugs viz. amphotericin B and nystatin have been found to impart cure against most common fungal pathogens.⁶ The liposomal formulations increase the therapeutic index of the drug by delivering higher concentrations to the infected tissues and therefore reducing the toxicity of the drug to the normal cells.⁶ The LD₅₀ of the iv deoxycholate micellar dispersion of amphotericin B is 1.2 mg/kg and increases (12 mg/kg) on liposomization,¹³ whereas the maximal tolerated iv dose for liposomized nystatin is 16 mg/kg.¹¹ These drugs become less toxic when administered intraperitoneally (LD₅₀: 200 mg/kg for free nystatin and 80 mg/kg for amphotericin B).¹³

The purpose of the present study was to evaluate the efficacy of a pH-sensitive liposomal antifungal delivery system in treatment of systemic murine cryptococcosis. The use of pH-sensitive liposomes significantly enhanced intracellular delivery of nystatin and thus showed enhanced antifungal activity in terms of increased survival rate, as well as reduced fungal burden in brain and liver (Figure 2). Mice treated with pH-sensitive nystatin liposomes showed enhanced survival (~80%) compared with those treated with neutral egg-PC-nystatin liposomes (~50%) or free nystatin (~10%) at equivalent doses (5 mg/kg). Mice treated with nystatin in pH-sensitive liposomes (3 mg/kg) showed survival (~50%) equal to those treated with nystatin in neutral liposomes (5 mg/kg). This clearly supports that pH-sensitive liposomes deliver substantial amount of drug to sites of infection compared with egg-PC liposomes.

Although neutral liposomes are also taken up by macrophages, their penetration into the endosomes is not as high as that of pH-sensitive liposomes. It has been demonstrated that pH-sensitive liposomes increase the efficacy of gentamicin against *Salmonella typhimurium* infection in mice.¹⁴ The dissociation of the drug from pH-sensitive liposomes and their rapid accumulation in the target organs (the liver and spleen) makes these pH-sensitive carriers ideal for *in vivo* evaluation in an antifungal as well as antibacterial efficacy model. This is also supported by cfu data of the present study that showed significantly lower fungal load in liver and brain of mice treated with pH-sensitive nystatin liposomes in comparison with mice of other groups ($P < 0.05$). Hence, owing to the high concentration of the nystatin inside the cells the pathogens find it difficult to escape from effective range of the drug.

C. neoformans after entering the host cells proliferates with ease in the low pH (~5) environment of the lysosomes. The delivery of nystatin using pH-sensitive liposomes immediately after infection results in the destruction of the pathogen because the environment, which was favourable for the survival of the pathogen, is loaded with the antifungal nystatin. As a result most of the infection is subsided owing to the use of pH-sensitive nystatin liposomes that release substantial drug at the pH favourable for *C. neoformans*. Use of pH-sensitive liposomes seems a good strategy to fight against intracellular pathogens, which are otherwise very difficult

to eliminate from their shelter sites. It can be concluded that nystatin, toxic in free form, can be made more effective and safe by entrapping it within pH-sensitive liposomes for treatment of fungal diseases.

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