Nystatin nanosizing enhances in vitro and in vivo antifungal activity against Candida albicans

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Objectives: In this study, we developed a nanoparticulate nystatin formulation and performed a comparative evaluation against a commercial nystatin preparation of its in vitro and in vivo antifungal activities.

Methods: A nystatin nanosuspension was prepared from a commercially available suspension by wet-media milling. The nanosuspension was characterized for particle size by laser diffraction and assayed for content by HPLC. Its in vitro activity was evaluated against Candida albicans strains SC5314 and LAM-1 (12.5–5000 µg/mL) using an agar plate assay and its in vivo efficacy was evaluated using a murine model of oral candidiasis. Briefly, DBA/2 mice were immunosuppressed with cortisone acetate, orally infected with C. albicans strain LAM-1, and treated for 14 days with conventional nystatin suspension, nystatin nanosuspension or saline control. Efficacy endpoints were oral fungal burden, mouse survival and organ histopathology. A single-dose pharmacokinetic study was also performed.

Results: The median particle size of the nystatin suspension was reduced from 6577 to 137 nm. The HPLC assay demonstrated a nystatin content of 98.7%±0.8% of the label claim. In vitro activity was superior to that of the conventional nystatin suspension at 100–5000 µg/mL concentrations. Beginning on day 3 of treatment, lower oral burdens of C. albicans were found in the nanosuspension group compared with the suspension and control groups. Mouse survival was also superior in the nanosuspension group. No systemic absorption was observed.

Conclusions: Taken together, these data reveal that nanonization of nystatin provides a novel approach to enhancing its efficacy in the treatment of oral candidiasis.

Keywords: oral candidiasis, nanosuspensions, drug delivery

Introduction

Oropharyngeal candidiasis (OPC) is a common human fungal infection characterized by an overgrowth of Candida species in the superficial epithelium of the oral mucosa.1,2 The vast majority of these infections are caused by Candida albicans. Immunocompromised patients, including those with HIV infection or cancer, are at enhanced risk of OPC.2–4 In addition, OPC can be triggered in healthy patients by transient risk factors such as antibiotic or corticosteroid treatment.5 The infection can be complicated by oesophageal candidiasis and, in the worst cases, fungal septicaemia.6 Although it is infrequent, disseminated candidiasis has a mortality rate of 47%.6,7

Nystatin and fluconazole are the most widely employed antifungal agents in the treatment of OPC. Nystatin is a polyene antifungal that possesses excellent in vitro fungicidal activity against C. albicans spp.7 Kovacic and Cooksy8 suggested that the mechanism of action of polyene antifungals is 2-fold. Firstly, polyenes bind to ergosterol, a component of C. albicans membranes, resulting in the formation of transmembrane pores, leakage of ions and sequestration of ergosterol. Secondly, induction of oxidative stress and damage also contribute to polyene antifungal activity.8,9

Poorly soluble nystatin is formulated as a suspension that patients swoosh and swirl in their buccal cavity, and either spit out or swallow.10,11 The drug is not bioavailable after oral administration and is eliminated in the faeces.10,11 Nystatin suspension has few side effects and no drug interactions have been reported,10 hence it remains a treatment of choice.11 Topical administration of nystatin provides sufficient efficacy in treating
OPC in immunocompetent patients. However, the clinical cure rate of oral candidiasis in immunocompromised HIV patients is only 52%, compared with 87% for fluconazole.12 Fluconazole, a systemically acting triazole antifungal, can cause adverse effects – including hepatic enzyme elevation and drug interactions – by inhibiting human cytochrome P450.13,14 In addition, cases of resistance to polyene drugs such as nystatin are very rare, while continuous fluconazole therapy may lead to azole resistance in C. albicans.15,16

Nanosizing is an attractive and novel approach to improving the efficacy of nystatin. Several marketed products incorporate this technology to improve the oral bioavailability of poorly soluble drugs.17,18 A limited number of studies have been conducted to assess the in vivo antimicrobial activity of nanoformulations of amphotericin B, a polyene antifungal structurally similar to nystatin.17–20 Interestingly, these studies demonstrated enhanced efficacy of amphotericin B nanoformulations in murine models of visceral leishmaniasis, amoebic encephalitis, cryptococcal meningitis, and pulmonary and disseminated aspergillosis. Other studies, using an agar plate assay, have demonstrated increased in vitro bactericidal activity of silver nanoparticles.21–23 No study has been conducted to evaluate the efficacy of polyene nanosuspensions on infections with Candida spp.

Nystatin exerts its effect when it comes into direct contact with Candida after topical application. When administered as an oral suspension, it is rapidly cleared by saliva.24 Mucoadhesive formulations have therefore been developed in an attempt to prolong contact with the oral mucosa.25,26 However, the tolerability of such formulations, especially among the paediatric population, has yet to be demonstrated. Furthermore, nystatin produces a biologically significant post-antifungal effect (PAFE).27 Indeed, 30 min exposure of C. albicans to nystatin at 25%–100% of the MIC will cause a PAFE of 5–6 h.28

Based on previous reports on amphotericin B and silver nanoparticles, we hypothesized that nanosizing would improve the efficacy of nystatin suspension. Here, we show that nanosized nystatin produces superior in vitro growth inhibition of C. albicans compared with conventional nystatin suspension, and is more efficacious in the treatment of experimental OPC in immunocompromised mice.

**Materials and methods**

**Materials and Candida strains**

Nystatin oral suspension USP was obtained from Pharmascience Inc. (Montreal, Canada; PMS-Nystatin, 100 000 IU/mL, Lot 420122). Nystatin USP powder was purchased from Medisca Inc. (Montreal, Canada, Lot 29303/M). All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA). All solvents were purchased from Laboratoire Mat (Montreal, Canada). Chemicals and solvents were used without further purification.

Sterile calcium alginate-tipped applicators (Calgiswabs) were purchased from Puritan (Guilford, ME, USA).

Unless otherwise specified, water was first distilled and purified using a Milli-Q system (Millipore, Billerica, MA, USA).

C. albicans strain SC5314 was originally isolated from a patient with disseminated candidiasis, and served as reference for the C. albicans genome sequencing project.29,30 C. albicans strain LAM-1 (serotype A) was originally isolated from the blood of a patient with systemic candidiasis.31

**Preparation of nystatin nanosuspensions**

Commercial nystatin oral suspension USP (60, 30 or 24 mL; 100 000 IU/mL) was diluted as necessary with water (0, 30 or 36 mL) to final concentrations of 100 000, 50 000 or 40 000 IU/mL in a final volume of 60 mL. The resulting suspension was wet milled with yttria-stabilized zirconia beads (0.8 mm, 144 mL) in a DynoMill ML (Glen Mills, Clifton, NJ, USA) equipped with a 300 mL milling chamber (4 h, 2389 rpm, 5°C). Control suspensions were prepared by diluting the commercial nystatin oral suspension (30 mL) with water (30 mL) to a final concentration of 50 000 IU/mL.

**Characterization of nanosuspensions**

Suspensions were assayed for nystatin by HPLC (Prominance HPLC, Shimadzu, Tokyo, Japan) as previously described.32 Particle size distribution was analysed in water at 22°C by laser diffraction (LS 13 320, Beckman Coulter, Mississauga, Canada).

**In vitro antifungal activity**

C. albicans cells were routinely grown at 30°C in yeast peptone dextrose (YPD; 1% yeast extract, 2% Bacto peptone, 2% dextrose plus 2% agar for solid medium). C. albicans SC5314 cells were resuspended in liquid YPD medium to an OD_600 of 0.1, and 150 μL of the cell suspension was spread on YPD Petri dishes. Whatman 3 mm CHR paper discs were then placed on each dish, and 12.5 μL of nystatin suspension or nanosuspension was added on top of the discs. The plates were incubated at 30°C, and growth inhibition diameters were measured at 18, 24 and 48 h. Eight concentrations of nystatin (12.5–5000 μg/mL) were each replicated nine times. The same tests were also performed in triplicate using C. albicans strain LAM-1.

**Efficacy in murine oral candidiasis**

Animal experiments were approved by the Animal Care Committee of the Université de Montréal, and conducted as described.33 Sixty male DBA/2 mice, 10 weeks old, were housed in sterilized individual cages equipped with air filter hoods, supplied with sterile water and fed with sterile mouse chow. Animals were immunosuppressed with cortisone acetate (225 mg/kg subcutaneously) on days –1, +1 and +3 of C. albicans inoculation.33 Oral inoculation with C. albicans LAM-1, assessments for signs of morbidity, and longitudinal quantification of C. albicans in the oral cavities of individual mice were conducted as described previously.34 Animals reaching predetermined morbidity endpoints were designated premortem and euthanized with ketamine.

The animals were divided into three groups (n = 20 per group): the first was treated with commercial nystatin suspension, the second with nystatin nanosuspension and the third with PBS as a negative control. Nystatin (80 000 IU/kg in 0.1 mL PBS) or PBS (0.1 mL) was administered intra-orally twice daily for 14 days, beginning 1 day after oral inoculation of C. albicans. The suspension was topically administered into the oral cavity using a 1.0 mL syringe equipped with a gavage needle. The mice were immobilized during dosing in a supine position without anaesthesia for 1 min, allowing the animals to ‘gargle’ the suspension and then swallow it. After completion of the 14 day treatment, the mice were monitored once daily to evaluate survival. In addition, to evaluate the extent of mucosal candidiasis and to assess the possible systemic spread of C. albicans in treated and untreated mice, histopathological examination of the tongue, kidneys and heart was performed on tissues harvested from mice (n = 3 per group) reaching morbidity endpoints on day 10 after infection. Tissue sections were stained with haematoxylin, phloxine and safranin, or by the Gomori–Grocott methenamine silver procedure.
Drug absorption

The extent of systemic absorption of nystatin after oral administration was evaluated using a fixed oral dose of 300000 IU/kg of nystatin nanosuspension (three mice) or of commercial nystatin suspension (two mice). Whole blood was collected 4 h post-dose, and nystatin was assayed by HPLC.32

Statistical analysis

Statistical analysis was carried out using SPSS version 20.0 software (IBM, Chicago, IL, USA). For in vitro activity results, an unpaired two-tailed Student’s t-test was used to determine statistical differences. Oral burdens of C. albicans were compared using a Welch one-way analysis of variance followed by a Games–Howell test for multiple comparisons of unequal variances. Kaplan–Meier modelling and a log-rank test were used to compare survival functions. P values for multiple comparisons were adjusted using the Bonferroni correction. A P value of ≤0.05 was considered significant.

Results

Nanosuspension characterization

A nanosuspension is characterized by its particle size distribution. Ninety percent of the particles must be smaller than 400 nm (x90 < 400 nm) and 50% of the particles must be smaller than 200 nm (x50 < 200 nm).33 As shown in Table 1, adequate nanosuspensions were obtained when the commercial product was milled at concentrations of 50000 and 40000 IU/mL. The relative nystatin content of the nanosuspensions, compared with before milling, was 98.7% ± 0.8%. The 50000 IU/mL nanosuspension was used to evaluate in vivo efficacy because it had the highest concentration of drug while still maintaining adequate particle size.

In vitro antifungal activity

Evaluating the in vitro activity of a novel formulation of an existing antifungal is a challenging task because current antifungal susceptibility testing methods focus on molecular activity.36 Antifungal susceptibility testing can be performed in liquid or on solid media.36,37 A solid medium method has previously been reported for the evaluation of the bactericidal activity of silver nanoparticles.21–23 Nystatin’s solubility (about 50 μg/mL) is higher than its MIC (about 2 mg/L). Therefore, a liquid medium cannot be used because the required dilution would actually dissolve the particles. No discrimination between the suspensions can be achieved once their particles are dissolved into solutions. Consequently, in vitro activity was determined using an agar plate assay.

When tested against C. albicans SC5314, the nystatin nanosuspension produced significantly greater growth inhibition diameters at concentrations ranging from 100 to 5000 μg/mL (Figure 1a). At lower concentrations (12.5–50 μg/mL) no growth inhibition of C. albicans was observed and therefore a comparison of the activity of the preparations could not be made (Figure 1b). Increases in growth inhibition diameters were not proportional to increasing nystatin concentrations, consistent with the agar plate assays. Similar results were obtained using C. albicans strain LAM-1 (data not shown).

In vivo efficacy

At day 2 after oral inoculation of C. albicans, one day after beginning treatment, the nystatin nanosuspension significantly reduced the oral burden of C. albicans compared with the PBS control (Figure 2). This early response to treatment was not observed with the commercial nystatin suspension. Indeed, a significant reduction in oral C. albicans burden in response to commercial nystatin suspension was delayed to the fifth day of treatment (Figure 2). Afterwards, both treatments were statistically superior to PBS through to day 9 of treatment, after which statistical analysis of C. albicans oral burdens was not performed due to mouse attrition. Of particular interest, the nystatin nanosuspension was significantly more efficacious than the commercial suspension in reducing the oral burden of C. albicans on treatment days 3, 4, 5, 6, 8, and 9 (Figure 2).

Mice were assessed for survival, and survivors were euthanized 32 days after oral inoculation of C. albicans (Figure 3). Mice treated with the nystatin nanosuspension had improved survival compared with animals receiving commercial nystatin suspension (P=0.003) or PBS (P<0.001). Although mice treated with the commercial nystatin suspension showed lower survival than those treated with the nanosuspension, their survival was nevertheless significantly enhanced compared with untreated PBS controls (P=0.03) (Figure 3).

Histopathology

Histopathology

Uninjected control mice consistently displayed extensive candidal infection of the stratified squamous epithelium of the entire dorsum of the tongue, accompanied by an abundant inflammatory cell infiltrate (Figure 4). In contrast, in mice treated with either commercial nystatin or nystatin nanosuspension, the density of Candida hyphae in the epithelium was sharply diminished, and, in limited areas, hyphae were entirely absent (Figure 4). At necropsy, typical Candida microabscesses indicative of systemic dissemination were uniformly observed on the surface of the kidneys and heart of all mice, treated or not with either of the nystatin formulations. However, fewer microabscesses (0–5) were observed on tissue
sections of these organs harvested from mice treated with either the commercial nystatin suspension or the nanosuspension, compared with untreated controls (>10). It was not possible to discern commercial nystatin from the nanosuspension due to the qualitative nature of histopathology.

**Drug absorption**

Nystatin was not detected in whole blood (limit of detection: 1 IU/mL) of mice 4 h after administration of a massive single oral dose of 30000 IU/kg of commercial nystatin or nystatin nanosuspension.
Discussion

Nystatin nanosuspensions were obtained by high-energy wet-mill media milling. The process was simple owing to the few steps required. In addition, no additional ingredients were needed when milling the commercial product. However, it was necessary to dilute the commercial suspension with water to reduce viscosity and facilitate milling. At higher concentrations of nystatin (100,000 IU/mL), particle size was not sufficiently reduced even at a higher milling speed. Nystatin assays demonstrated no degradation of the active pharmaceutical ingredient during the milling process, which can potentially occur during this procedure.

In vitro tests demonstrated greater diameters of growth inhibition of *C. albicans* in the presence of the nystatin nanosuspension compared with the commercial suspension. This increased activity could be explained by one or both of the following mechanisms: (i) particle size reduction facilitated the diffusion on or within the solid medium, resulting in the increased inhibition diameter; (ii) particle size reduction increased the specific surface area of the particles, increasing the intrinsic particle activity of the drug.

Evaluating antifungal efficacy in vivo is also potentially challenging because of the limited number of clinically relevant animal models of oral candidiasis suitable for this purpose. No animal model has been used to compare two oral formulations of nystatin. DBA/2 mice immunosuppressed with cortisone acetate were used in this study. These mice are deficient in complement component 5 (C5) and are susceptible to infection with *C. albicans*. Indeed, it has been shown that DBA/2 mice develop greater oral colonization with *C. albicans* than BALB/c mice (not C5 deficient). Although DBA/2 mice are susceptible to *C. albicans* oral colonization they are nevertheless able to clear the infection after 1 week. The mice were therefore immunosuppressed with cortisone acetate to prolong oral carriage of *C. albicans*, thus providing a clinically relevant time course for assessment of antifungal efficacy.

In vivo assessment showed superior efficacy of the nystatin nanosuspension in reducing the oral burden of *C. albicans*. The nanosuspension significantly reduced the oral burden of *C. albicans* as early as 24 h after starting treatment, in contrast to the fifth day of treatment for the commercial suspension, demonstrating a more rapid onset of the in vivo response to...
treatment. In addition, the nystatin nanosuspension was consistently more efficacious than the commercial suspension in the reduction of fungal burden from day 3 to day 9 of treatment, with the exception of day 7. Increasing the statistical power of the study would most likely also produce a significant difference at this timepoint. Starting on day 10 after inoculation, mouse attrition precluded a meaningful statistical analysis of the oral burden of C. albicans. Therefore, mouse survival was used as an endpoint of in vivo efficacy from day 10 until the conclusion of the experiment.

Systemic candidiasis was not expected because it was not reported in a previously described model of OPC in mice immunosuppressed with cortisone acetate. It is possible that the C. albicans LAM-1 strain is more virulent than the SC5314 and SANK51486 strains used in this previous study, and that the greater susceptibility of DBA/2 mice to C. albicans infection compared with ddY mice facilitated systemic dissemination. Mucosal invasion leading to systemic dissemination most likely occurred in the oral cavity, oesophagus or cardial-atrium fold of the stomach. Mice treated with the nanosuspension had improved survival compared with those treated with the commercial suspension or PBS. This improved survival probably resulted from a greater reduction of Candida burden at the mucosal portal of entry in the oral cavity and gastrointestinal tract, because we found that the nystatin nanosuspension is not absorbed after oral administration. This observed reduction in mortality provides a foundation for investigating the potentially added value of antifungal prophylaxis with nystatin nanosuspension in susceptible hosts such as allogeneic haematopoietic stem cell transplant recipients or those undergoing intensive resection-induction or salvage-induction chemotherapy for acute leukaemia, who routinely receive systemic antifungal prophylaxis against Candida infection.

Assessment of absorption following oral administration of the nystatin formulations was performed. Commercial nystatin suspension is not bioavailable due to poor solubility and permeability, and no a priori evidence suggested that nanosizing nystatin would result in systemic absorption. Indeed, nanosizing is only used to improve bioavailability of poorly soluble but permeable drugs. Nevertheless, a pharmacokinetic study was performed because systemic absorption of nystatin in its nanosized formulation, if present, could lead to renal toxicity. No detectable absorption was found after oral administration of a single massive dose of 300,000 IU/kg. By comparison, a dose of 80,000 IU/kg was used during the in vivo efficacy study.

In conclusion, nanosizing provides a promising approach to increasing the efficacy of topically administered nystatin in the treatment of OPC. This novel application of nanomilling could lead to the development of improved formulations of other antimicrobial agents.

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Transparency declarations

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