Original Article

Nystatin Encapsulated Nanoliposomes: Potential Anti-infective against Candida Spp. Isolated from Candidiasis Patients

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Abstract

Background: Due to the pathogenic role of opportunistic fungi in immunodeficiency patients, many efforts have been made for developing effective treatment strategies to augment current practice standards. Nystatin, as one of the treatment candidates, is characterized by antifungal effects. In this study, we tried to use liposomal formulation as a nystatin carrier to increase its antifungal efficacy.

Materials and Methods: A total of 87 positive culture samples of yeast agents were applied to the study. Yeast species were identified by culturing on CHROMagar medium (HiMEDIA), culturing on NigerSide agar medium, and Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). Characterization of nanoparticles was examined by the size, zeta potential (ZP), scanning electron microscope (SEM), drug loading, and drug release rate. The standard method of broth microdilution according to CLSI M27-A and the quality control standard of *Candida parapsilosis* ATCC 22019 were used to evaluate the minimum inhibitory concentration (MIC) of nystatin and nystatin nanoliposomes.

Results: The particle size for liposomes containing nystatin was 100.8 ± 17.3 nm. Moreover, the ZP for liposomal formulation of nystatin was 21.14 ± 0.92 –mV. The formulation of nystatin in nanoparticles markedly increased the susceptibility of *Candida* species to nystatin at lower doses, which was statistically significant compared to free nystatin ($P \leq 0.05$).

Conclusion: Our results showed that liposomal formulation improves the efficiency of nystatin against *albicans* species. This formulation can be used to develop new antifungal agents to improve the delivery and absorption of hydrophobic drugs.

Keywords: Antifungal agents, Candida albicans, candidiasis, liposomes, macrolides, nano-delivery system, nanoparticles, nystatin

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Introduction

Nowadays, opportunistic pathogenic fungi are emerged as one of the most dangerous infections in immunosuppressed patients.[1] Yeasts and, to a lesser extent, *Candida* species are widely isolated from human infections, and despite advances in health care and treatment, the incidence of invasive systemic candidiasis is increasing significantly.[2,3] Although *Candida albicans* is the most common cause of infection in various clinical forms of candidiasis, other species belonged to the candida genus, including *C. tropicalis*, *C. glabrata, C. krusei, C. parapsilosis,* and *C. guilliermondii*, have also been isolated from patients.[4] The importance of non-albicans species has increased in recent years due to the relative resistance of some of these species, such as *Candida tropicalis* and *Candida glabrata*, to some antifungal drugs.[5] Therefore, production of new antifungal agents, as one of the attention attractive issues for mycologists, makes it necessary to perform several experiments such as determining the sensitivity, and the pattern of sensitivity to reveal key details and to avoid unwanted effects of various antifungal drugs.[6]

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Polyene antibiotics are the members of macrolide lactone family, which has been reported to have antifungal effects on a wide variety of fungi.[7] Recently, the higher incidence of acquired immunodeficiency syndrome (AIDS) and the wide use of immunosuppressive drugs made increased the prescription of these groups of antibiotics.[8] Nystatin, as the first identified polyene drug, is active against a wide variety of fungal pathogens including *Candida, Aspergillus, Histoplasma*, and *Coccidioides spp*.[9] Although nystatin has demonstrated broad spectrum of activity, due to the low solubility in aqueous solutions and toxic effects, it is still inefficient and is under heavy research to become a practical strategy for the treatment of systemic fungal infections.[10] Indeed, its clinical use and topical use resulted in adverse side effects and toxicity such as thrombophlebitis, fever, and rigors.[11]

Solid lipid nanoparticles are one of the novel drug delivery systems composed of degradable lipids and have a diameter of 50 to 1000 nm.[12,13] Recently, these particles have been used as carriers of antifungal drugs such as clotrimazole,[14] ketoconazole,[15] miconazole[16], and fluconazole.[17] The small size of lipid nanoparticles (higher surface-to-volume ratio) increases their bioavailability and, consequently, enhances penetration of the loaded drugs into the tissue.[18] In addition, lipid nanoparticles can store and deliver the loaded drugs slowly and provide sustained delivery of the drug.[19] Because of lipophilic nature, parenteral administration of nystatin to the patients is a serious problem. Therefore, its formulation into liposomes is one of the effective ways to increase its solubility.[20] In this study, nystatin-containing nanoliposomes were prepared, and then, the effects of nystatin-containing nanoliposomes on *Candida albicans*, *Candida parapsilosis*, *Candida glabrata*, *Candida tropicalis*, *Candida guilliermondii*, and *Candida krusei*, which were isolated from the patients, were investigated.

Materials and Methods

Sampling

A total of 87 positive culture samples of yeast agents isolated from cutaneous, mucosal, and systemic fungal lesions were collected from patients referred to Razi Hospital in Tehran, Iran, and Sina Hospital in Tabriz, Iran. Clinical specimens were taken from scabs, nails, mucosal secretions, and bronchoalveolar lavage (BAL) fluid. All experiments were carried out according to the ethical regulations of Islamic Azad University, Tabriz Branch (IR.TUMS.REC.1394.2008).

Isolation and identification of yeasts

Clinical specimens were cultured on sabouraud dextrose agar medium containing chloramphenicol. Yeast colonies were removed using a sterile loop after they reached stationary growth rate, transferred to microtubes containing sterile DW, and stored in a freezer at -20°C until testing. To identify yeast species, the yeasts isolated from the patients were cultured on CHROMagar medium (HiMEDIA) linearly and incubated for 48 hours at 35°C, and then, the plates were examined macroscopically. In this condition, *Candida albicans/ Dublinensis* produces green colonies, *Candida tropicalis* forms blue colonies, *Candida krusei* produces purple colonies, and other species produce white to cream colonies.

For green colonies, chlamydoconidia production and filamentous forms, mycelium, were examined using corn meal agar medium containing Tween-80 for 48 hours at 30°C. To distinguish *albicans* species from *dublinensis*, chlamydoconidia production by *dublinensis* species in NigerSide agar medium and the inability of *Candida albicans* to produce Chlamydoconidium were assessed. The isolated *Candida spp*., which were blue or purple colonies in CHROMagar candida medium, were identified by the PCR-RFLP method. This method is based on the amplification of ITS1-5.8S-ITS2 fragment in ribosomal deoxyribonucleic acid (DNA) by the polymerase chain reaction (PCR) method using universal primers ITS4, ITS1, and then cutting PCR-amplified products by HpaII restriction enzyme. PCR products were finally separated using electrophoresis on 1% agarose gel, and a 50 bp marker was used to confirm the size of products.

Preparation of nystatin liposomal formulation

Liposomal formulation of nystatin was prepared by the thin-layer hydration method[21]. Briefly, lecithin (Sigma, USA), cholesterol (Sigma, USA), and nystatin (Sigma, USA) with the ratio of 1:1:10 were used to prepare liposomes. A thin layer was formed by dissolving above-mentioned substances in 10 ml of chloroform–methanol organic solvent (1:1), and then, the resulted solvent was evaporated in a rotary evaporator (Buchi Rotavapor R-124, Germany) at 45°C to remove organic solvents and depositing a thin layer of dried on the inner part of the plates. Subsequently, the lipid film was slowly hydrated at 65°C by 9% sucrose. The samples were homogenized by a homogenizer at 20,000 rpm for 10 min at 70°C. Finally, sonication of liposomal samples was performed by a sonicator (Model 150 V/T, USA) 10 times with 1-min intervals under ice bath to produce monolayer of nanometer-scale liposomes. The separation of unloaded drug was performed by centrifugation at 4,000 rpm for 4 min through the 10-KD Amicon filter tube (Millipore, UK). The prepared solution was transported to the laboratory and used for MIC analysis. Blank lipid formulation was prepared according to the same method in the absence of the nystatin.

Determination of the size of nystatin nanoliposomes

The particle size distribution, mean diameter, and ZP of nanoliposomes were measured by laser light scattering-based zetasizer (Zetasizer Nano ZS, Malvern, UK).

Determination of the particle structure of nystatin liposome

SEM (MIRA3 FEG-SEM, Czech) was used for determining the particle structure of nystatin liposome. For this purpose, 200 µl of a nystatin liposome containing sample was placed on a 1 × 1 cm glass surface. It was then placed in an incubator at 37°C until the sample was completely dried. These particles were plated with gold and imaged at 20,000 X and 40,000 X.

Determination of the loaded and released drug in nystatin liposome

The amount of loaded nystatin in liposomes was quantified using ultraviolet–visible (UV/Vis) spectrophotometer (SCINCO, Korea) at 305 nm. A part of prepared nystatin liposomes was diluted with normal saline and then centrifuged at 21,000 g for 30 min at 4°C. Following the removal of the pellet, the remained supernatant was assessed by the spectrophotometer. Linear regression analysis data for the calibration plot showed a satisfying linear relationship between response and concentration in the range of 1 to 100 µg/ml. The obtained regression coefficient was 0.9989, and the linear regression equation was y = 0.0495× +0.0234 (n = 3). The absolute calibration curve method was used for calculation. The following formula was used to calculate the percentage of loaded nystatin.

$$EE(\%) = \left(\frac{W_{initialdrug} - W_{freedrug}}{W_{initialdrug}} \right) \times 100$$

EE: Entrapment efficiency

Microdialysis against phosphate-buffered saline (PBS) pH 7.4 at 37°C was used to determine the amount of released drug *in vitro*. An amount of 2 ml of prepared nystatin-contained nanoliposomes was poured into 10-KD cutoff membrane and suspended in 500 ml of PBS. Then, at different time points (2, 4, 6, 8, 15, 24, and 36), aliquot samples were withdrawn and were analyzed spectrophotometrically as described above.

Antifungal susceptibility testing

The broth microdilution based on CLSI M27-A and *Candida parapsilosis* ATCC 22019 were used as a standard method and quality control to evaluate the MIC of nystatin and nystatin nanoliposomes, respectively.[22] Briefly, the concentration of antifungal agents was selected from 0.125 to 512 µg/ml. Cell suspensions of *Candida spp.* were prepared in Roswell Park Memorial Institute (RPMI) 1640 medium containing 2% of glucose and phosphate buffer pH: 7 adjusted to give a final inoculum concentration of 0.5 McFarland standard (1.5×10^6 CFU/ml). Final concentration of fungi in individual tubes was adjusted to about 5×10^3 CFU/ml. In the case of liposomes, this amount was calculated according to the percentage of loaded drug. Finally, the possible fungal turbidity and MIC were examined after 48-h incubation at 35–37°C.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) software version 19 (SPSS Inc., USA) was used for data analysis, and one-way analysis of variance (ANOVA) was performed to compare statistical differences between groups. *P* values less than 0.05 were considered significant.

Results

All 91 isolated yeast colonies [Table 1] in this study were able to grow on *Candida* agar chromium medium and were identified according to the resulting color difference. *albicans* species were discriminated from *dublinensis* by detecting the production of chlamydoconidia by *dublinensis* species and lack of the production of Chlamydoconidium by *Candida albicans* in the NigerSide agar medium. The identification of those non-albicans species, which formed colonies with nonspecific color, PCR-RFLP method, was used [Table 2 and Figure 1].

Characterization of nystatin nanoparticles and the release rate of the drug

In this study, our goal was to obtain an optimized formulation with acceptable particle size distribution and nystatin encapsulation rate to evaluate its anti-yeast effects compared to the naked drug. As can be seen, the particle size for liposomes containing nystatin was 100.8 ± 17.3 nm. Moreover, the ZP for liposomal formulation of nystatin was obtained 21.14 ± 0.92 – mV [Figure 2]. The structure and shape of the nanoliposomes examined by SEM are shown in Figure 3. The nystatin released from nanocarriers within 36 h is shown in Figure 4. As can be seen, nystatin releasing increased time-dependently.

Drug susceptibility test

As demonstrated in Figure 5, the formulation of nystatin in nanoparticles has caused the susceptibility of *Candida species* to this substance at lower doses, which was statistically significant compared to free nystatin ($P \leq 0.05$).

Table 1: Number and colony type isolated from patient samples

Sample	Candida species (number)	Total (%)
Fingernail	*Albicans* (16), *Parapsilosis* (5), *Tropicalis* (3), *Glabrata* (1), *krusei* (2), *Guilliermondii* (2)	29 (31.8)
Toenail	*Albicans* (6), *Parapsilosis* (3), *Tropicalis* (2)	11 (12)
Hand	*Albicans* (9), *Parapsilosis* (3), *Tropicalis* (2), *krusei* (1)	15 (16.5)
Leg	*Albicans* (4), *Parapsilosis* (2)	6 (6.6)
Vagina	*Albicans* (15), *Glabrata* (3), *krusei* (1)	19 (20.9)
Groin	*Albicans* (4), *Parapsilosis* (2)	6 (6.6)
BAL	*Albicans* (2), *Tropicalis* (1), *Glabrata* (2)	5 (5.5)

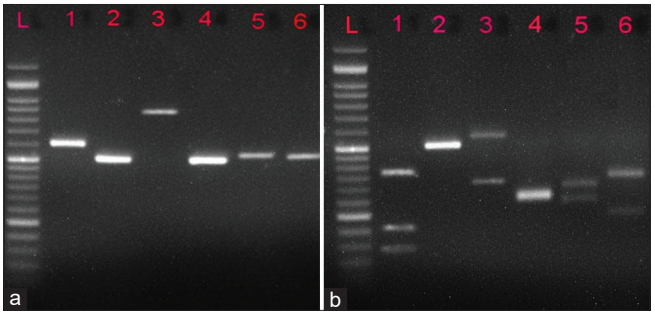

Figure 1: (a): PCR-RFLP products using electrophoresis after digestion with HpaII enzyme. L: marker (50 bp); 1: *Candida guilliermondii*; 2: *Candida parapsilosis*; 3: *Candida candida glabrata*; 4: *Candida krusei*; 5: *Candida albicans*; 6: *Candida tropicalis*. (b): Electrophoresis of PCR (ITS region amplification) product of some *Candida species*. L: marker (50 bp); 1: *Candida guilliermondii*; 2: *Candida parapsilosis*; 3: *Candida candida glabrata*; 4: *Candida krusei*; 5: *Candida albicans*; 6: *Candida tropicalis*

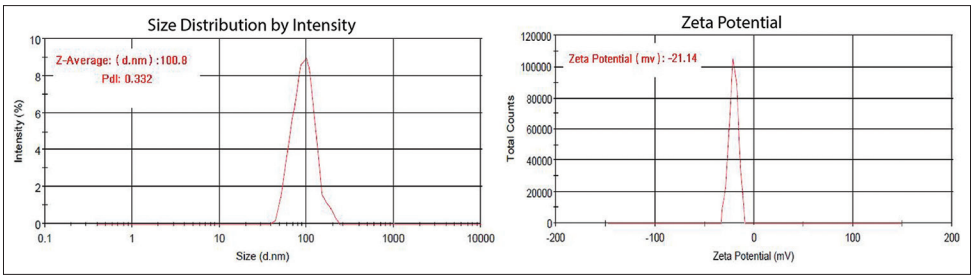

Figure 2: Size distribution and ZP of nystatin liposomal nanoparticles

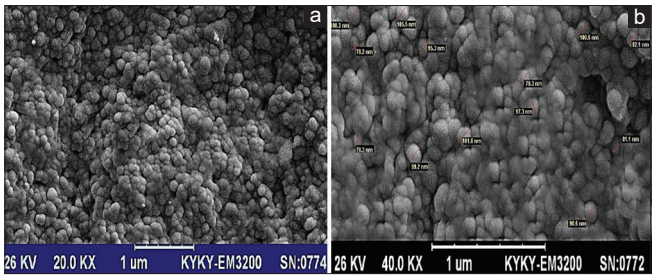

Figure 3: ESM images of structure and shape of the nanoliposomes. (a): Lower magnification to show the uniform particle size distribution of nanoliposomes. (b): Higher magnification to better display the uniform structure and spherical shape of nanoliposomes. Scale: 1 μm

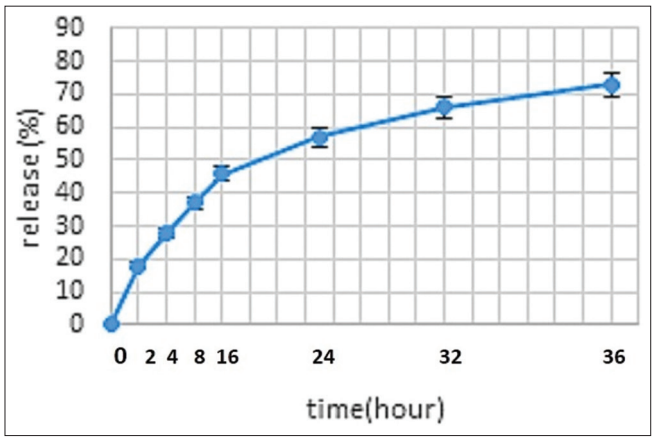

Figure 4: Nystatin release behavior from nanocarriers over 36 h

Discussion

In this study, a liposomal formulation was developed to load nystatin and investigate the antifungal effects of this formulation on *Candida* species isolated from patients in comparison with the free form of nystatin. In thin-layer hydration, several variables in size and load of liposomal formulation were decisive for the efficiency of the synthesis of liposomal formulation. In this study, we tried to set the appropriate variables and parameters as much as possible to obtain the optimal nystatin liposomes, which have the best size and drug-loading capacity.

ZP is considered one of the vital elements in determining the stability of the colloidal system and the surface electrical status of dispersions. In the liposomal formulation, which contains cholesterol together with lecithin, the ZP was negative. In the case of combined steric and electrostatic stability, a minimum ZP of 20% is desirable[23]. Ola H. El-Nesr *et al.* used multilayer liposomes containing fluconazole to the evaluation of the drug-loading capacity of several formulations with positive, neutral, and negative ZP.[21] This study showed that nanoliposome drug-loading capacity remarkably relays on the charge. Indeed, nanoliposomes with the positive, neutral, and negative charges had the highest, medium, and the minimum drug-loading capacity, respectively, which was probably due to the electrostatic force between the positive charge of stearylamine particles of liposomes and the partial negative charge of fluconazole. The drug-loading rate in liposomal formulation was 80%. In fact, a significant amount of nystatin was encapsulated inside the nanoliposomes. Due to the high molecular weight and hydrophobicity, the drug encapsulation on the outside of the nanoparticles was also observed.

Increasing the amount of cholesterol in lipid formulations causes more drug encapsulation in nanoparticles. The presence of cholesterol in nystatin-containing liposomes causes the retention of the drug in the liposomal particle nanocapsules. Increasing the level of loaded drug plays an important role in the releasing speed of the drug from nanocarriers, which means that the smaller the particle size, the faster the drug is released[24]. In our study, lipid nanoparticles released 73% of loaded nystatin within 36 h indicating the controlled and optimal release.

SEM figures obviously showed that particles formed spheroid assembly. According to the SEM results, the prepared particles had the same size with those obtained by the nano-zetasizer.

Table 2: Identification of non-albicans species

Species	Number (%)	Identification method
Candida albicans	56 (61.4)	Chrom Candida agar, chlamydoconidia production
Candida parapsilosis	15 (16.5)	PCR-RFLP
Candida tropicalis	8 (8.8)	Chrom Candida agar, PCR-RFLP
Candida glabrata	6 (6.6)	PCR-RFLP
Candida krusei	4 (4.4)	Chrom Candida agar, PCR-RFLP
Candida guilliermondii	2 (2.2)	PCR-RFLP
Total	91	

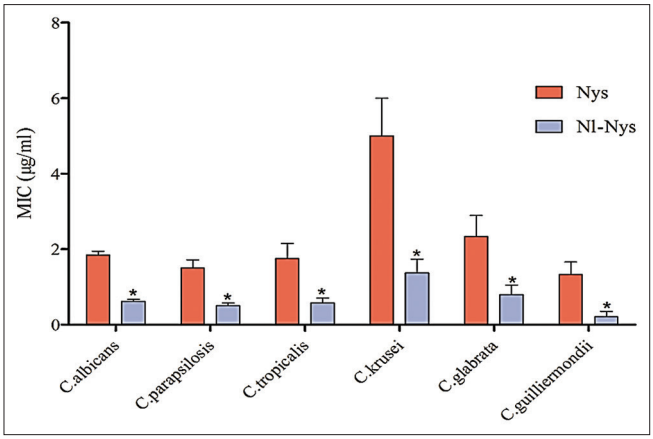

Figure 5: Susceptibility test results of nystatin nanoparticles and free nystatin

One of the main problems with the use of nystatin is the insolubility in distilled water and in many organic solvents. In this study, we tried to increase the solubility of the drug and also improve the antifungal effect of this drug. The encapsulation rate of nystatin in lipid nanoparticles was 80%, which is probably due to the hydrophobic bond between nystatin and the lipid chain and chemical structure. The use of nystatin liposomes significantly ($P < 0.05$) reduced the MIC of *Candida albicans, Candida parapsilosis, Candida tropicalis, Candida glabrata, Candida guilliermondii*, *and Candida krusei*. In a study conducted by Saadat *et al*.,[25] the results showed that the lipid formulation of nystatin was more effective than the free nystatin formulation. In another study by Quindós *et al*.,[26] the antifungal effects of nystatin lipid formulation were compared with free nystatin and the results showed that the MIC of nystatin lipid formulation was reduced compared to the nystatin. In a study by Oakley *et al*.,[27] on the antifungal effects of nystatin lipid formulation compared to free nystatin form on *Aspergillus* species, the same results were obtained. It seems that improvement of nystatin action against *Candida* species when it was used in the form of nanoliposome formulations compared to free nystatin is probably due to the better binding and penetration of the drug into the fungal cell. It has been shown that there are three main mechanisms for internalization of encapsulated nanoliposomes into the target cells. Through the first mechanism, nanoliposomes appear to merge with the membrane and then enter their content into the cells. During the second mechanism, nanoliposomes interact with and form pores in the cell membrane. The third mechanism suggests that nanoliposomes enter cells via endocytosis and release their content after endosome rupture[28]. In addition, similarity of the structure of the liposomes with the cell membrane helps in better penetration of nanoparticles into the target cells[26].

Conclusion

The use of nanotechnology for targeted drug delivery has been very effective, and researchers are trying to use this knowledge to overcome drug resistance and increase the effectiveness of drugs. The results of our study showed that liposomal formulation of nystatin increases efficiency and its effect on *albicans* species. This formulation can be a promising option to produce new antifungal agents.

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Author contribution

MA conceived and designed the experiments and revised the manuscript. ATM and KB contributed in sample collection, performed molecular analyses, and wrote the manuscript draft. All authors read and approved the final manuscript.

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Conflicts of interest

There are no conflicts of interest.

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