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Original article

Improved yeast delivery of fluconazole with a nanostructured lipid carrier system

Hamid Reza Kelidari[a,d], Maryam Moazeni[b,c,**], Roghayeh Babaei[a], Majid Saeedi[d,f], Jafar Akbari[d,f], Parisa Islami Parkoohi[e], Mojtaba Nabili[a], Atefeh Abdollahi Gohar[a], Katayoun Morteza-Semnani[f], Ali Nokhodchi[g,h,*]

[a] Student Research Committee Center, Mazandaran University of Medical Sciences, Sari, Iran
[b] Invasive Fungi Research Center, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran
[c] Department of Medical Mycology and Parasitology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran
[d] Pharmaceutical Sciences Research Center, Mazandaran University of Medical Sciences, Sari, Iran
[e] Educational Development Center, Mazandaran University of Medical Sciences, Sari, Iran
[f] Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran
[g] Pharmaceutics Research Laboratory, School of Life Sciences, University of Sussex, Arundel Building, Brighton BN1 9QJ, UK
[h] Drug Applied Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran

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Despite the growing trends in the number of patients at risk for invasive fungal infections, management with current antifungal agents results in complications due to changes in the epidemiology and drug susceptibility of invasive fungal infections. In the present research fluconazole-loaded nanostructured lipid carriers were prepared using probe ultrasonication techniques and investigated the efficacy of the optimal formulation on a large number of *Candida* species. The morphology of the obtained nanostructured lipid carriers was characterized by transmission-electron microscopy. The minimum inhibitory concentrations (MIC) for the new formulations against strains of *Candida* were investigated using the Clinical and Laboratory Standards Institute document M27-A3 and M27-S4 as a guideline. The fluconazole-loaded nanostructured lipid carriers presented a spherical shape with a mean diameter, zeta potential and entrapment efficiency of 126.4 ± 15.2 nm, -35.1 ± 3.0 mV, and 93.6 ± 3.5%, respectively. The drug release from fluconazole-loaded nanostructured lipid carriers exhibited burst-release behavior at the initial stage followed by sustained release over 24 h. Using a new formulation of fluconazole led to a significant decrease in MICs for all *Candida* groups ($P < 0.05$). Furthermore, *C. albicans* isolates showed more susceptibility to fluconazole-loaded nanostructured lipid carriers than *C. glabrata* and *C. parapsilosis* ($P < 0.05$). The MIC_{50} drug concentration was obtained as 0.0625, 0.031 and 0.25 μg/ml for fluconazole-resistant strains of *C. albicans*, *C. glabrata,* and *C. parapsilosis*, respectively. In conclusion, a novel delivery system which can be used as part of a strategy to improve the antifungal activity of fluconazole against various Candida strains with different susceptibilities to conventional formulations of fluconazole was evaluated.

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1. Introduction

A variety of novel drug carrier systems have recently been proposed to improve the bioavailability and release of drugs [1]. These systems are aimed at maintaining local effects and enhancing drug accumulation in various strata of skin through liposomes or niosomes [2], gel formulation [3], lecithin-based organogel [4], hydrogel [5] or polymeric mucoadhesive films, [6] poly-lactic *co*-glycolic acid (PLGA) microspheres [7], solid lipid nanoparticles (SLNs) [8,9], and nanostructure lipid carriers (NLCs) [10]. In particular, NLCs have emerged as a promising drug delivery

Abbreviations: MIC, minimum inhibitory concentration; FLZ, fluconazole; FLZ-NLC, fluconazole loaded nanostructured lipid carrier; NLC, nanostructured lipid carrier.

* Corresponding author at: Pharmaceutics Research Laboratory, School of Life Sciences, University of Sussex, Arundel Building, Brighton BN1 9QJ, UK.
** Corresponding author at: Invasive Fungi Research Center, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran.
E-mail addresses: moazeni.maryam@gmail.com (M. Moazeni), a.nokhodchi@sussex.ac.uk (A. Nokhodchi).

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system for pharmaceutical and cosmetic molecules, especially for the delivery of lipophilic compounds [11–13]. Briefly, NLCs are colloidal nanocarriers in the submicron range (40–1000 nm) composed of a solid lipid matrix and a liquid lipid [13]. They have several desirable advantageous such as low toxicity of constituents, and the ability to protect the incorporated drug from degradation by immobilization in the solid/liquid particle matrix [14]. Moreover, NLCs have overcome problems associated with SLNs, including limited drug loading, risk of gelation, and drug leakage during storage caused by lipid polymorphism [14].

Fluconazole (FLZ), a first-generation triazole, is a broad-spectrum anti-fungal agent that inhibits cytochrome P450-dependent 14α-lanosterol demethylation, a vital step in cell membrane ergosterol synthesis [15]. Fluconazole is active against all *Candida* species except for *C. glabrata,* which has acquired resistance to fluconazole, and *C. krusie,* which is resistant to the drug intrinsically [16,17]. Despite its advantageous pharmacological activity, FLZ can cause several clinically significant side effects, including headache, hives, itching or skin rash, abdominal pain, and hematemesis [3]. While the prevalence and severity of side effects may be decreased by lowering the dose of FLZ, clinical efficacy may be reduced and resistance increased through this approach. This problem is important because FLZ is currently used for both prophylaxis and the treatment of broad spectrum infections of candidiasis, yet, the emergence of drug-resistant isolates continues to increase dramatically [18–20]. In response to this challenge, the use of new drug formulations and drug delivery systems to reduce resistance while maintaining or increasing clinical efficacy are urgently needed [21,22]. While there is limited evidence regarding the effectiveness of FLZ loaded NLCs (FLZ-NLC) [1] on *Candida albicans*, the activity of FLZ-NLCs against a broad range of *Candida* species, including *C. glabrata* and *C. krusie,* has not yet been studied. To this end, the purpose of the present study was to estimate the clinical efficacy of FLZ-NLCs on FLZ-resistant strains of certain Candida species.

2. Materials and methods

2.1. Materials

Fluconazole (FLZ, Pharmaceutica grade) was obtained from Arasto Pharmaceuticals Chemicals Inc. (Tehran-Iran). Compritol® 888 ATO (CO), Lipocire and Precirol® ATO 5 were supplied from Gattefossé (Saint-Priest, Cedex, France). Sabouraud dextrose agar (SDA), RPMI medium, stearic acid (SA), Oleic acid, Tween 80 (Tn80), Span 60 (Sn60) and Span 80 (Sn80) were purchased from Merck Co. (Germany). HPLC grade acetonitrile and methanol were supplied by the Merck (Germany). Morpholinepropanesulfonic acid (MOPS) was purchased from Sigma Chemical Co., St. Louis, MO (USA). Deionized water was purified using a Milli-Q system (Millipore, Direct-Q). All other reagents and solvents were either of analytical or high-performance liquid chromatography (HPLC) grades.

2.2. Screening of lipids

In order to determine the maximum amount of drug a lipid can hold, it is necessary to estimate the solubility of the given drug in the lipid. To accomplish this goal, we evaluated the solubility of FLZ 25%w/w in melted Compritol® 888 ATO, Lipocire, Precirol® ATO 5 and stearic acid (SA) (Merck, Germany). The solubility of FLZ was also assessed in melted solid lipid combined with oleic acid as a liquid lipid, in ratios of 90:10, 80:20 and 70:30. The lipid mixtures were stirred at 200 revolutions per minute for 10 min at 85 °C, using a hot plate magnetic stirrer (Unimax 1010, Heidolph, Germany). When the drug was dissolved in lipids with different concentrations, they were examined for the presence of the drug crystals in the lipid matrix to explore which lipids or lipid combinations were able to dissolve the drug completely.

2.3. Preparation of formulation

FLZ-NLCs were prepared and optimised via a process utilising an ultrasonic probe. The method has been adopted from the previously reported studies in the literature [10]. To prepare NLCs, a carrier lipid in its solid form (stearic acid/Compritol® 888 ATO) was melted at 85 °C in combination with liquid lipid (oleic acid) and a lipophilic surfactant (Span 80) based on the proportion in Table 2. The molten lipid phase was dispersed in a 1/3 of the aqueous solution of hydrophilic surfactant prepared by weighing out 0.84% w/w Tween 80 at the same temperature and sonicated by using a probe sonicator (Bandelin sonopuls, Berlin, Germany) for 5 min (Model HD 3200, Prob TT25, 50% power and 14.28 KJ, continuous) to form a pre-emulsion. At the end of the sonication, the mixture was dispersed into the remaining 2/3 of the hydrophilic surfactant solution maintained in an ice bath. The final mixture was sonicated again for 10 min (50% power and 43.21 KJ) whilst still immersed in the ice-bath (Table 2).

2.4. Characterization of the nanoparticles

2.4.1. Morphology

In order to determine the shape of FLZ-NLCs, transmission electron microscopy (accelerating voltage 100 kV; TEM, CM 30, Phillips, Netherlands) was used. First, the NLC samples were diluted two times with distilled water. One drop of the diluted sample was placed on a 200-mesh carbon-coated copper grid, stained with 2% phosphotungstic acid solution and dried at room temperature. Representative images of the sample were reported.

2.4.2. Particle size and zeta potential

Photon correlation spectroscopy (PCS) with a Malvern Zetasizer ZS (Nano ZA, Malvern Instruments, UK) was used to determine the particle size, and to profile the size distribution (polydispersity index, PDI) and zeta potential of the nanoparticles. In this method, the sample was measured at 25 °C with an angle detection of 90°. The concentration of the samples for analysis on the Zetasizer was 20–400 kilocounts per second (KCPS) and the intensity of diffraction was 100,000 counts per second.

2.4.3. High-performance liquid chromatography (HPLC) analysis of fluconazole

The HPLC assay was performed using an Agilent 1100 chromatograph, equipped with the Agilent Eclipse XDB-C18 column (5 μm, 4.6 mm × 250 mm). The mobile phase was composed of 10 mM sodium acetate buffer (adjusted to pH 5.0 with glacial acetic acid) and methanol (65:35) with a flow rate of 1 ml/min.

2.4.4. Determination of fluconazole entrapment efficiency

Entrapment efficiency (EE%) was determined to assess the extent of FLZ incorporation in the nanoparticles by measuring the concentration of the free unloaded FLZ in the aqueous phase of the nanoparticle suspension. To determine the entrapment efficiency (EE%) of FLZ in the NLCs, the FLZ-NLCs were subjected to centrifugation for 20 min at 25,000 rpm (HERMLE, Z36HK, Germany) and filtered (pore size: 0.22 μm). The amount of drug in the supernatant was determined by HPLC and the experiment was conducted in triplicate.

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