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Liposomal voriconazole (VOR) formulation for improved ocular delivery

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ABSTRACT

Treating infectious eye diseases topically requires a drug delivery system capable of overcoming the eye's defense mechanisms, which efficiently reduce the drug residence time right after its administration, therefore reducing absorption. In order to try to surpass such administration issues and improve life quality for patients with fungal keratitis, liposomal voriconazol (VOR) formulations were prepared. Formulations were composed of soy phosphatidylcholine (PC) containing or not 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and cholesterol. Liposomes were characterized by their drug entrapment efficiency (EE), drug recovery (DR), average diameter (size) and polydispersivity index (PdI). *In vitro* mucosal interaction and irritancy levels, *ex vivo* permeation, as well as the short-term stability were also assessed. Liposomal VOR formulation produced with 7.2:40 mM VOR:PC showed to be the most promising formulation: mean size of 116.6 ± 5.9 nm, narrow PdI (0.17 ± 0.06), negative zeta potential (~ -7 mV) and over 80% of EE and yield, remaining stable for at least 30 days in solution and 90 days after lyophilization. This formulation was classified as 'non-irritant' after HET-CAM's test and was able to deliver about 47.85 ± 5.72 μg/cm^2 of VOR into porcine cornea after 30 min of permeation test. Such drug levels are higher than the minimal inhibitory concentrations (MIC) of several fungi species isolated from clinical cases of corneal keratitis. Overall results suggest VOR can be effectively incorporated in liposomes for potential topical treatment of fungal keratitis.

1. Introduction

The treatment of ocular infections remains among the most challenging subjects of ophthalmology [1]. The eye is a small organ protected from the external environment and separated from the body by a complex structure of biological barriers and defense mechanisms (i.e. tight junctions of the corneal epithelium, blinking, tear secretion and even tear film composition), which efficiently reduce the drug residence time right after its administration, therefore reducing absorption [2,3].

The treatment of mycotic ocular infections is specially complicated due to epidemiological characteristics of this disease combined with sociodemographic indicators, i.e. more resistant fungi tend to prevail in hot and humid environments, affecting mainly rural workers who live in poor insanitary environment [4,5]. In addition, patients with mycotic keratitis generally delay seeking medical care from onset of complaints compared to patients with nonmycotic infections [6]. Hence, current therapies are mostly invasive or require the use of high doses of antimycotics, reducing the adhesion to the treatment and, so, increasing the chances of a bad prognosis [7,8]. In this scenario, an efficient biocompatible formulation capable of delivering a potent antifungal agent is essential for treatment effectiveness.

Abbreviations: % CV, coefficient of variation; % E, relative error; AD, drug added; ANOVA, analysis of variance; BCOP, bovine corneal opacity and permeability; CAM, chorioallantoic membrane; Chol, cholesterol; DLS, dynamic light scattering; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DR, drug recovery; EE, entrapment efficiency; EEi, initially recorded entrapment efficiency; EEs, subsequently recorded entrapment efficiency; FD, free drug; HET, hen's egg test; HPLC, high-performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantitation; LPC, liposome of phosphatidylcholine; LPCD, liposome of phosphatidylcholine and DOTAP; MIC, minimal inhibitory concentration; NaCl, sodium chloride; NaOH, sodium hydroxide; PC, phosphatidylcholine; PdI, polydispersivity index; Rem, % remaining entrapped drug; SD, standard deviation; SDS, sodium dodecyl sulfate; TD, total VOR in liposomal dispersion; TEM, transmission electron microscope; VOR, voriconazole.

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Voriconazole (VOR) is a second-generation antifungal agent from the 'azole' family, which possesses broad-spectrum activity even against resistant fungal species [9–11].

Although several adverse effects have been reported following VOR systemic exposure [12–15], animal and clinical studies have suggested excellent outcome on treating fungal keratitis by its topical, intrastromal and/or systemic applications [16–19]. In all these studies, researchers have improvised with off-label usage of commercially available product for injection in the form of lyophilized powder of cyclodextrin-voriconazole complex. So far, no topical ocular formulation of VOR has been available in the market, probably due to its poor aqueous solubility [20].

Nanoencapsulation techniques could be applied to overcome such drug physicochemical challenges and improve ophthalmic formulation performance, prolonging drug residence time, enhancing drug penetration into the cornea and improving sensorial felling, hence, patient compliance. Recently, a microemulsion of VOR was formulated to this end and indeed exhibited about 3-fold higher drug permeation trough excised cornea in comparison to the VOR suspension [21]. Such results encourage the development of lipid nanoparticles entrapping VOR for ocular delivery. Liposomes can be fairly ideal drug delivery systems for topical treatment of ocular infections, as they are considered to be non-toxic, biodegradable, can be easily produced and lyophilized and present good interaction with mucosal structures.

The dual nature of the cornea provided by its superficial layers, the lipophilic epithelium and the hydrophilic stroma, is a limiting factor which enables only a few molecules to suit as candidates for ocular drug delivery. In this way, liposomes represent an interesting approach, since they are able to carry different drug molecules across these main layers, enhancing drug bioavailability [22,23]. Accordingly, a recent study has shown topical liposomal fluconazole (2 mg/mL) as superior to fluconazole solution in eliminating experimental *Candida albicans* infection of the rabbit cornea [24].

In this way, this article proposes a liposomal VOR formulation for the treatment of mycotic ocular infections.

2. Materials and methods

2.1. Materials

Cholesterol (Chol), soybean phosphatidylcholine (PC) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Voriconazole (VOR) was purchased from Hangzhou Dayangchem Co., Limited (Hangzhou, China). Acetonitrile was purchased from J. T. Baker (Phillipsburg, USA). Water was purified using a Milli-Q system (Millipore, Billerica, USA) with a 0.22 μm pore end filter. All other chemicals and reagents were of analytical grade or superior.

2.2. Preparation of liposomal VOR formulation

The VOR liposomes were prepared by the thin-film hydration method. VOR and PC were dissolved in chloroform/methanol (4:1, v/v) in a round-bottom flask. The organic solvent was removed by evaporation promoting the formation of a thin lipid film on the glass wall. The dried lipid film was maintained overnight under reduced pressure to remove traces of solvent. The thin layer of lipid was then hydrated with 4 mL of HEPES buffer (pH 7.4), so that the total lipid concentration was 40 mM. The particle size of the crude liposomal dispersion was further decreased by 10 cycles of extrusion through 600-nm-pore polycarbonate filters and additional 6 cycles through 100-nm-pore filters using an extrusion device operated with compressed nitrogen (Lipex^{TM}, Northern Lipids Inc., Canada). Loading capacity of liposomes was evaluated by changing the drug: phospholipid molar ratio (3.6:40; 4.3:40; 5.0:40; 5.8:40; 7.2:40; 14.3:40 mM of VOR:PC). After choosing two VOR:PC ratios, cholesterol (10 mM) was added to the formulations to evaluate its ability to enhance the formulations' stability and entrapment efficiency.

Cationic liposomes with the basic composition of VOR:DOTAP:PC at a molar ratio of 7.2:20:40 and 7.2:20:20 were also obtained.

Formulations composed of VOR/PC containing cholesterol or not were represented by the code LPC. The code LPCD was used for formulations also containing DOTAP.

Liposomes further submitted to lyophilization process were produced by the same method presented with the addition of trehalose as cryoprotectant in the ratio of 1:5 (lipid:trehalose).

2.3. Characterization of liposomes

2.3.1. Particle size and zeta potential

Particle size and polydispersity index were determined by dynamic light scattering (DLS) (3 measurements/batch; the software automatically determined the number of runs in each measurement, 25 °C) after adequate dilution of an aliquot of the suspensions in purified water (Zetasizer Nanoseries, Malvern Instruments, Worcestershire, UK). Zeta potentials were determined using the same instrument at 25 °C after the dilution of the samples in 10 mM NaCl aqueous solution to assure conductivity values of approximately 2 mS/cm (3 measurements/batch; 10 runs/measurement, 25 °C).

2.3.2. Entrapment efficiency and drug recovery

VOR entrapment efficiency (EE) was determined by indirectly calculating the amount of entrapped drug inside the liposomes. Separation of free drug (FD) in liposomal dispersion from encapsulated VOR was performed by centrifuging 1 mL of the dispersion for 10 min at 3000 rpm (centrifuge model 3–18 K SIGMA, Osterode am Harz, Germany) using Amicon Ultra filtration tube (Millipore, USA, 300 kDa cutoff). The filtrate was collected and analyzed for free drug content by high-performance liquid chromatography (HPLC) (section 2.9). Filtration parameters were validated allowing for 100% drug recovery in the filtrate. Total VOR (TD) in liposomal dispersion (free + entrapped drug) was obtained by diluting 100 μL of liposomal dispersion in 900 μL of methanol, therefore rupturing the liposomes and releasing all VOR in solution. Entrapment efficiency was calculated according to Eq. (1).

$$EE\% = \frac{(TD - FD)}{TD} \times 100 \quad (1)$$

Drug recovery (DR) was obtained relating the total VOR in liposomal dispersion with the amount of drug added (AD) at the beginning of the process, according to Eq. (2).

$$DR\% = \frac{TD}{AD} \times 100 \quad (2)$$

2.3.3. Morphological analysis

The morphological analysis of the VOR liposomes was performed in a TEM (JEM 1011 Transmission Electron Microscope, JEOL, Tokyo, Japan – 100 kV) and the images were captured with a GATAN BioScan camera, model 820 (GATAN, PA, USA) using the Digital Micrograph 3.6.5 software (GATAN, PA, USA). Liposomal dispersion was diluted 100-fold with purified water. An aliquot of 20 μL was deposited on a Formvar-coated copper grid (300 mesh, Electron Microscopy Sciences, PA, USA) and air-dried for 10 min. The excess of formulation was absorbed with filter paper and the sample was then stained with a drop of a uranyl acetate solution 2% (w/v). The sample was air-dried for 10 min and the excess of reagent removed with filter paper.