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Inhibition of filamentous fungi by ketoconazole-functionalized electrospun nanofibers

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ABSTRACT

Nanotechnology strategies have been used for delivery and controlled release of antimicrobial drugs. Electrospun nanofibers can be versatile vehicles to incorporate antimicrobials. In this work, poly-ε-caprolactone nanofibers functionalized with ketoconazole were produced by electrospinning and tested against filamentous fungi. Ketoconazole-free nanofibers were produced as controls. Functionalized nanofibers showed antifungal activity against *Aspergillus flavus*, *A. carbonarius*, *A. niger*, *Aspergillus* sp. A29, *Fusarium oxysporum* and *Penicillium citrinum* by agar diffusion test. Inhibitory zones ranging from 6 to 44 mm were observed, this larger inhibition was against *A. flavus*. The nanofibers were incubated in different simulant solutions to evaluate the ketoconazole release, which was only detected in the solution containing 5% (v/v) Tween 20. Electron microscopy images showed the nanofibers with ketoconazole presented mean diameters of 526 nm, and the degradation of the nanofiber structures could be observed by electron microscopy after incubation in simulant solution. Infrared and thermal analyses indicated that ketoconazole was dispersed without chemical interactions with the polycaprolactone matrix. These results suggest that polycaprolactone nanofibers incorporating ketoconazole may be an interesting alternative to control pathogenic fungi.

1. Introduction

Fungal infections are a serious public health problem, since they contribute substantially to human morbidity and mortality (Brown et al., 2012). Although most fungi are not dangerous, some strains can be harmful to health and fungi that are common in the environment can cause several fungal diseases (Hawksworth, 2001). Common filamentous fungi can represent a particular hazard to patients who are immunocompromised due to serious illnesses or undergoing immunosuppressive therapies (Brown et al., 2012). In addition, fungi that are normally found in air, soil or aquatic environments are likely associated with infections of skin wounds (Bartesteanu et al., 2014).

Azole antifungal drugs such as miconazole, econazole, oxiconazole, clotrimazole and ketoconazole are exceptionally water-insoluble, and therefore it is very difficult to administer and deliver these compounds to the infected site (Gupta and Cooper, 2008). Ketoconazole (Fig. 1A) is an antifungal agent with topical and systemic action that can be incorporated into several pharmaceutical forms. This triazole antifungal agent is used in the treatment of superficial and systemic fungal infections and for the treatment of seborrheic dermatitis. Ketoconazole has a high permeability, but low solubility in aqueous media, which is insufficient for the whole dose to be dissolved in the gastro-intestinal fluids under normal conditions (Van den Mooter et al., 2001). Some recent studies indicate that nanoparticles can be useful for improvement of ketoconazole delivery and bioavailability (Modi et al., 2013; Kakkar et al., 2015).

Nanotechnology has been used as an interesting tool for drug delivery and controlled release. A promise of nanostructured drug delivery is to reduce the drug amount and promote a more effective action, thus reducing the impact on human and animal health and the environment (Brandelli, 2015). Antimicrobial substances can be incorporated into several nanostructures, including nanospheres, nanovesicles, nanofibers and nanotubes (Brandelli, 2012; Brandelli and Taylor, 2015). Nanofibers have been mostly evaluated for their potential as wound dressings or scaffold for tissue engineering. However, nanofibers functionalized with antimicrobial agents can be a relevant alternative to protect against microbial pathogens (Heunis and Dicks, 2010; Wei and Wei, 2012). Among the processes used to nanofiber production, the electrospinning technique has been mostly used by its versatility, allowing the incorporation of antimicrobials and other bioactive molecules (Damasceno et al., 2013; Oliveira et al., 2014).

The capability to produce nanofiber-based thin films functionalized with antimicrobials has a huge potential for applications in fungal

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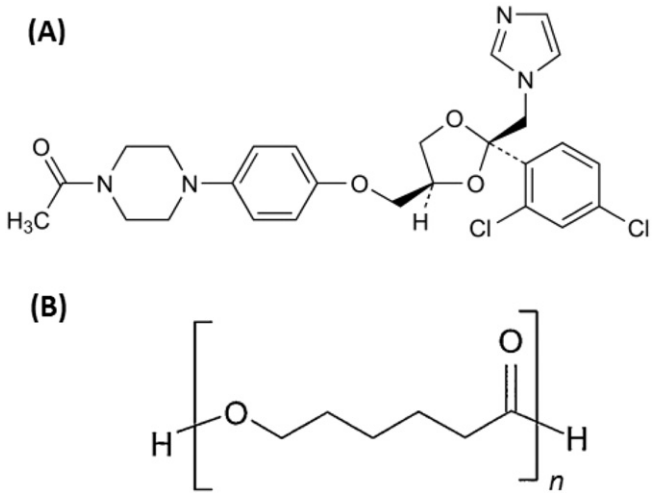

Fig. 1. Chemical structures of (A) ketoconazole and (B) poly-ε-caprolactone.

control. However, the incorporation of ketoconazole into nanofibers has not been reported. In this study, ketoconazole was incorporated in poly-ε-caprolactone (PCL, Fig. 1B) nanofibers and this nanostructure was characterized and evaluated for its capability of inhibiting pathogenic fungi.

2. Materials and methods

2.1. Chemicals

Poly-ε-caprolactone (PCL; average MW 80,000) was obtained from Sigma Aldrich (St. Louis, MO, USA). Pharmaceutical grade ketoconazole was purchased from Piramal Healthcare (Maharashtra, India). Tetrahydrofuran (THF), chloroform ($CHCl_3$) and HPLC grade acetonitrile were from Merck (Darmstadt, Germany). All solutions were prepared using ultrapure water obtained from a Milli Q device (Millipore, Billerica, MA, USA).

2.2. Microorganisms

Aspergillus carbonarius and *Penicillium citrinum* strains were kindly provided by Instituto de Tecnologia de Alimentos (ITAL, Campinas, Brazil). *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus* sp. A29 and *Fusarium oxysporum* strains were from the collection of the Laboratório de Toxicologia de Alimentos, Universidade Federal do Rio Grande do Sul (Porto Alegre, Brazil). The fungal cultures were maintained on potato dextrose agar (PDA; Merck, Darmstadt, Germany) slants at 4 °C.

2.3. Preparation of PCL/ketoconazole nanofibers by electrospinning

Electrospinning was carried out essentially as described previously (Castañeda et al., 2014). PCL (100 g/l) was dissolved in a solution of THF:$CHCl_3$ (3:1) containing 1 g/l ketoconazole. PCL solution without the antifungal was prepared as control. The electrospinning was carried out under the following conditions: voltage of 30 kV, feeding rate of 0.05 ml/min; needle of 0.5 mm inner diameter; distance to the collector 16 cm. The nanofibers were collected on an aluminum plate (15 × 15 cm). The process was developed at 25 °C.

2.4. Fungal inhibition assays

For antifungal activity tests, the fungal strains were cultured on PDA slopes for 7 days at 25 °C. A solution containing 0.05% (v/v) Tween 80 was poured on each of the colonies and the spores harvested with the aid of a Drigalski loop. The suspension was collected and transferred to a sterile tube. The concentration of spore suspension was determined with a Neubauer chamber and adjusted with sterile distilled water to 1×10^6 spores/ml. One milliliter of this suspension was added to 99 ml of sterile PDA to 45 °C homogenized and transferred to a plate. After solidification of the media, nanofibers were cut and placed on agar plates. A disk containing 20 μg ketoconazole was used as positive control. Zone diameters were measured after five days of incubation at 25 ± 2 °C.

2.5. Ketoconazole quantification

The quantification of ketoconazole was performed by high performance liquid chromatography (HPLC) essentially as described elsewhere (Jat et al., 2012), using a Shimadzu Prominence system equipped with a diode array detector (Shimadzu, Tokyo, Japan). Analyses were performed using a Shim-pack HR-ODS column (250 × 3.0 mm, 3 μm; Shimadzu). A 20 μl sample was injected onto the HPLC by an auto sampler at 25 ± 1 °C. The column was eluted with an isocratic mobile phase prepared with ultrapure water: acetonitrile: 10 mM phosphate buffer pH 6.8 (51:45:4 ratio), with a flow rate of 1 ml/min. The column temperature was adjusted to 25 ± 1 °C and the detection wavelength was 238 nm. Mobile phase was filtered through 0.45 μm nylon filters and degassed by sonication prior to use. A standard calibration curve was prepared in the mobile phase with 1, 2, 5, 10, 25, 50 μg/ml ketoconazole. Regression analysis for HPLC provided a linear relationship of concentration to absorbance to over this range with a R^2 value of 0.9987. The lower limit of detection under these conditions was determined to be approximately 0.1 μg/ml.

2.6. Drug load

Drug load was determined using three different samples of the nanofibers. Samples of 4 mg were added to a solution of 0.2 ml ultrapure water and 1.8 ml acetonitrile. After 30 min at 37 °C an aliquot was removed and the ketoconazole concentration was determined by HPLC. The drug load was calculated as DL = (released drug amount/sample weight).

2.7. Drug release assay

In order to describe the release of ketoconazole from the nanofibers, the migration assay was performed using different simulant solutions (Simoneau, 2009; Anvisa, 2010). These included distilled water (simulant A), 5% (v/v) Tween 20 (simulant B), 3% (v/v) acetic acid

Table 1
Inhibition of fungal growth by nanofibers containing ketoconazole.

Fungi	Diameter of inhibitory halo (mm)		
	Ketoconazole-functionalized nanofiber	Control nanofiber	Pure ketoconazole
Penicillium citrinum	20.0 ± 5.0 [a]	0.0	16.7 ± 0.6 [a]
Aspergillus carbonarius	6.3 ± 1.0 [b]	0.0	8.0 ± 1.0 [b]
Aspergillus flavus	44.3 ± 10.0 [c]	0.0	16.3 ± 0.6 [a]

[a,b,c] Results are the means ± s.e.m. of three independent experiments. Values followed by different superscript letters are significantly different ($P < 0.05$). Pure ketoconazole was used as positive control.