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Research Paper

Antifungal activity of topical microemulsion containing a thiophene derivative

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Abstract

Fungal infections have become a major problem of worldwide concern. Yeasts belonging to the *Candida* genus and the pathogenic fungus *Cryptococcus neoformans* are responsible for different clinical manifestations, especially in immunocompromised patients. Antifungal therapies are currently based on a few chemotherapeutic agents that have problems related to effectiveness and resistance profiles. Microemulsions are isotropic, thermodynamically stable transparent systems of oil, water and surfactant that can improve the solubilization of lipophilic drugs. Taking into account the need for more effective and less toxic drugs along with the potential of thiophene derivatives as inhibitors of pathogenic fungi growth, this study aimed to evaluate the antifungal activity of a thiophene derivative (5CN05) embedded in a microemulsion (ME). The minimum inhibitory concentration (MIC) was determined using the microdilution method using amphotericin B as a control. The formulations tested (ME- blank and ME-5CN05) showed physico-chemical properties that would allow their use by the topical route. 5CN05 as such exhibited moderate or weak antifungal activity against *Candida* species (MIC = 270-540 $\mu g.mL^{-1}$) and good activity against *C. neoformans* (MIC = 17 $\mu g.mL^{-1}$). *Candida* species were susceptible to ME-5CN05 (70-140 $\mu g.mL^{-1}$), but *C. neoformans* was much more, presenting a MIC value of 2.2 $\mu g.mL^{-1}$. The results of this work proved promising for the pharmaceutical industry, because they suggest an alternative therapy against *C. neoformans*.

Key words: antifungal susceptibility, *Cryptococcus neoformans*, microemulsion, thiophene derivatives.

Introduction

Fungal infections have become an issue of great concern around the world; it was estimated that over 40 million people do in fact suffer fungal infections both in developed and in developing countries (Güngör, Erdal and Aksu, 2013). The incidence and prevalence of invasive fungal infections have increased since the 1980s, especially in immunocompromised patients and those hospitalized with serious underlying diseases (Espinel-Ingroff , 2009).

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Candida yeasts are microorganisms belonging to the normal microbiota of healthy individuals, mainly in the oral mucosa, the gastrointestinal tract and the female genitourinary tract (Shao, Sheng and Zhang, 2007). However, these fungi are responsible for different clinical manifestations, especially in immunocompromised patients, ranging from skin and mucous infections to systemic infections (Sardi *et al.*, 2013). Their importance comes from the high frequency with which they colonize and infect the human host (De Bernardis *et al.*, 2004), being the fourth most common pathogens associated with cases of nosocomial infection (Wisplinghoff *et al.*, 2004).

Cryptococcus neoformans is an encapsulated fungus capable of causing infections in immunocompromised patients (Carroll *et al.*, 2012; Guerra *et al.*, 2012). Inhalation of its basidiospores or cells present in the environment can result in lung infection and its subsequent spread to the central nervous system, causing meningoencephalitis, recognized as one of the most important opportunistic infections in patients with HIV with a worldwide incidence of approximately 957,000 cases per year (Leongson *et al.*, 2013).

Conventional treatments of fungal diseases are currently based on a few chemotherapeutic agents such as azoles and polyenes, which have serious problems related to effectiveness, activity spectrum, toxicity, low power and inadequate pharmacokinetics (Scotti *et al.*, 2012). Moreover, the occurrence of microbial resistance to antimicrobial agents is shown to be a growing public health problem worldwide and the biggest obstacle to the success of a treatment, as it continues to reduce the number of drugs available in the market (Oliveira and Silva, 2008; Benghezal *et al.*, 2007). This scenario demonstrates the need for the development of new antifungal agents as therapeutic alternatives to control fungal infections.

Organic compounds containing aromatic heterocyclic rings such as thiophene are widely distributed in nature and are generally of great importance in many biochemical processes, having a broad spectrum of pharmacological properties (Mohammad *et al.*, 2012). The versatile applicability in syntheses and the biological activity of thes e heterocycles make them important structural fragments in synthetic medicinal chemistry, serving as a basis for planning and implementation of new therapeutic agents (Meotti *et al.*, 2003). For the present work, it was synthesized a derivative of 2-amino thiophene, namely the 2-[(3,4-dichloro-benzylidene)-amino]-5,6-dihydro-4H-cyclopenta[*b*]thiophene-3-carbonitrile, referred to as 5CN05 (Figure 1). This compound belongs to a series of synthetic tiophene derivatives with well-proven antifungal activity *in vitro* (Mendonça Junior *et al.*, 2011). Its biopharmaceutical characteristics are not yet well understood, especially with respect to permeability. However, the high value of its partition coefficient (logP = 5.98) suggests that it has poor water solubility and permeability.

A microemulsion (ME) is a thermodynamically stable and optically transparent system made up of a mixture of two immiscible liquids stabilized by an interfacial surfactant film. The specific properties of microemulsioned systems are primarily related to their low interfacial tension, large interfacial area, small size of droplets, low viscosity and high solubilization power for lipophilic, hydrophilic and amphiphilic drugs (Damasceno *et al.*, 2011; Fanun, 2012; Silva *et al.*, 2009b). MEs usually contain high concentrations of surfactants, an essential factor for successful incorporation of poorly water soluble drugs (Damasceno *et al.*, 2012; Djekic *et al.*, 2012). These systems have the ability to deliver water insoluble compounds by carrying the drugs in their oil core or on the water-oil interface. This biological property allows the use of MEs as biotechnological carriers for large water insoluble molecules such as those of the tiophene derivatives.

The treatment of fungal infections by topical dosage forms shows some advantageous features such as possibility of action at the application site and reduction of the risk of systemic side effects, increased efficacy of the therapy and its acceptance by the patients (Güngör, Erdal and Aksu, 2013). In topical formulations, MEs have proven effective in increasing the absorption of both hydrophilic and lipophilic drugs by the skin when compared to conventional systems such as emulsions and aqueous solutions (Grampurohit, Ravikumar and Mallya, 2011). The use of MEs may also increase the local or systemic action of drugs by different mechanisms, thereby representing an interesting alternative to antifungal drugs (Jadhav, Shetye and Kadam, 2010).

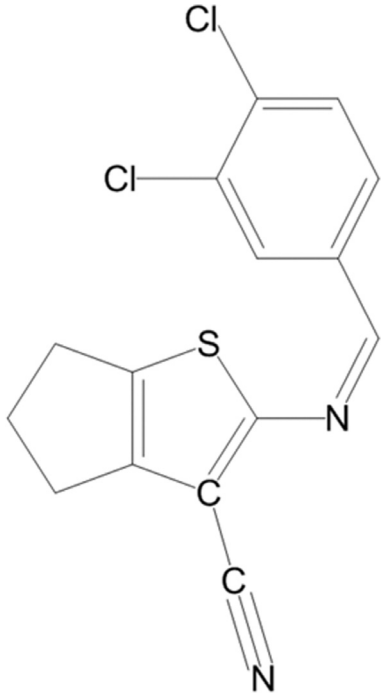

Figure 1 - Chemical structure of 5CN05 molecule.

In this context, the present study aims to evaluate the antifungal activity of 5CN05 embedded in a ME for topical use, by determination of its minimum inhibitory concentration against pathogenic yeast species.

Materials and Methods

Preparation of microemulsion formulation and incorporation of 5CN05

To obtain microemulsions (MEs), pseudoternary phase diagrams (PTPD) were constructed using a surfactant blend of LAS® (Brasquim, São Paulo - SP, Brazil) and Plurol Oleique® (Gattefossé, Saint-Priest, France), and isopropyl myristate as oil phase (Via Farma, São Paulo - SP, Brazil). Purified water was produced by a reverse osmosis purification system, model OS10LX (Gehaka, São Paulo - SP, Brazil).

For 5CN05 incorporation in ME, such a drug was initially dispersed in the oil phase, and the remaining components were added, including water. This multiphase mixture was then subjected to three 1 min-alternate homogenization cycles by an ultrasound probe, model DES500 (Unique, Indaiutaba - SP, Brazil), and the excess air bubbles were removed by 1 min-treatment in an ultrasound bath, model USC-3300 (Unique). The formulation obtained, called ME-5CN05, was characterized for its macroscopic appearance, pH, electrical conductivity and refractive index, and compared to that without drug (ME-blank).

Target microorganisms

The antifungal activity of ME-5CN05 and ME-blank systems was assessed using standard strains from the American Type Culture Collection (ATCC), namely *Candida albicans* ATCC 18804, *Candida parapsilosis* ATCC 22019 and *Candida tropicalis* ATCC 13803, provided by the Laboratory of Drug Development and Testing (LABDEM) belonging to the State University of Paraíba. Their activity was also assessed against *Cryptococcus neoformans* (LM10), provided by the Mycology Laboratory of the Federal University of Paraíba.

Determination of Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration (MIC) of MEs was determined by the microdilution method using 96-well microplates, consisting of serial dilution either of drug and or the ME-5CN05 and ME-blank formulations, prepared following the guidelines of the Clinical and Laboratory Standards Institute, rule CLSI M27-A3 for yeasts. 5CN05 was dissolved in LAS® at a concentration of 2175 $\mu g.mL^{-1}$ and then tested at concentrations ranging from 8 to 1088 $\mu g.mL^{-1}$. Amphotericin B (AmB) was used as reference at concentrations from 2 to 0.015 $\mu g.mL^{-1}$. The possible activity of LAS® was evaluated as well, using the broth without inoculum as a negative control.

To prepare inocula, microbial suspensions were standardized in tubes containing 5 mL of 0.9% sterile saline solution and adjusted spectrophotometrically to 90% transmittance at 530 nm, corresponding to a concentration of 106 $cfu.mL^{-1}$. Samples were serially diluted in the microdilution wells containing 100 µL of Sabouraud-Dextrose broth (Hi Media, Sasti - Maharashtra, India). After addition of inoculum (100 µL) in separate plates for each yeast, the plates were incubated at 35 ± 1 °C for 24 h and visually evaluated. The MIC corresponded to the lowest sample dilution capable of providing growth inhibition of yeasts. All tests were performed in duplicate.

Results

Figure 2 shows the phase diagram characterizing the phase behavior of surfactants blends, namely the oil and water phases. It is noteworthy the existence of a variety of states, highlighting the presence of microemulsion formation areas, which are regions where the energy used and the components proportions were suitable to reduce the interfacial tension until the formation of a homogeneous, clear and translucent system (Silva *et al.*, 2009a).

The composition of MEs was 35.3% w/w LAS®, 17.5% w/w Plurol Oleique®, 5.9% w/w isopropyl myristate and 41.2% w/w water. The ME-blank formulation was shown to be a translucent, slightly yellowish solution with slightly viscous appearance, while the addition of the drug at a concentration of 560 $\mu g.mL^{-1}$ to prepare the ME-5CN05 formulation did not modify its translucent appearance and consistency (as confirmed by rheological characterization studies - data not shown), differing mainly in the color. Table 1 summarizes the main results of physicochemical characterization tests performed on both formulations.

As expected, the tests of Minimum Inhibitory Concentration (MIC) either for the drug or MEs, whose results are listed in Table 2, showed a clear growth of all the yeasts only in the controls containing the inocula in addition to the culture medium, hence confirming the suitability of the selected conditions.

All the microorganisms studied proved sensitive to 5CN05, which showed moderate activity against *Candida* species (MIC = 270-540 $\mu g.mL^{-1}$) and an activity more than one order of magnitude greater against *Cryptococcus neoformans* (MIC = 17 $\mu g.mL^{-1}$), while the MIC value for the positive control (AmB) was only 0.5 $\mu g.mL^{-1}$.

As far as the MEs are concerned, microorganisms did not show any sensitivity to ME-blank, while ME-5CN05 proved excellent activity against *Candida* species, mainly *C. albicans* and *C. parapsilosis* (MIC = 70 $\mu g.mL^{-1}$), and excellent activity against *C. neoformans* (MIC = 2.2 $\mu g.mL^{-1}$).

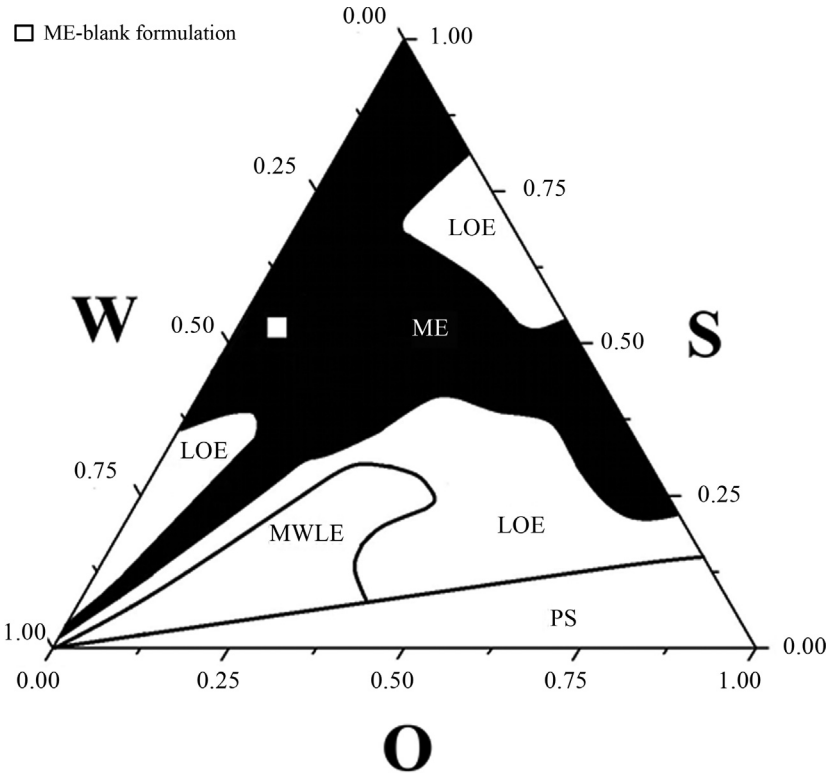

Figure 2 - Pseudo ternary phase diagram for the LAS®/Plurol Oleique® surfactant blend (S) at the proportion of 2:1, isopropyl myristate (O) and water (W) system. The marked areas represent: PS - phase separation; MWLE - milky white liquid emulsion; OLE - opaque liquid emulsion; ME - microemulsion; - ME-blank formulation.

Table 1 - Physico-chemical caracterization of ME-blank and ME-5CN05.

Parameters	Formulations	
	ME-blank	ME-5CN05
Macroscopic appearance	Limpid	Limpid
Color	Slightly yellowish	Strong yellowish
pH	6.60 ± 0.03	6.58 ± 0.03
Conductivity ($\mu S.cm^{-1}$)	105.00 ± 5.80	97.23 ± 1.38
Refractive index	1.410 ± 0.02	1.412 ± 0.01

Table 2 - Values of MIC determined either for the drug alone (5CN05) or for MEs with (ME-5CN05) and without (ME-blank) the drug.

Microorganism	MIC ($\mu g.mL^{-1}$)		
	5CN05	ME-blank	ME-5CN05
Candida albicans ATCC 18804	270	R	70
C. parapsilosis ATCC 22019	270	R	70
C. tropicalis ATCC 13803	540	R	140
Cryptococcus neoformans LM10	17	R	2.2

R = resistant.

Discussion

All MEs were handled and maintained at room temperature for 48 h before their characterization. Under these conditions, all formulations exhibited a homogeneous appearance, without any evidence of phase separation or precipitate formation. As is well known, the optimum pH of a topical formulation must be selected so as to maximize the stability of active components and the tolerance of the skin, being acceptable values between 5.5 and 8.0 (Silva *et al.*, 2009a). The pH values shown for ME-blank and ME-5CN05 were within this range, which would allow the topical application of the ME-5CN05 system. Moreover, this parameter was not influenced by the addition of 5CN05.

The use of topical MEs to improve the antifungal activity of drugs is a strategy adopted by some researchers. For instance, Jadhav, Shetye and Kadam (2010), developing MEs containing topical fluconazole, observed greater activity of their formulations than conventional gels available in the market, a fact that was attributed to the better diffusion of the drug provided by the presence of surfactants and oil phase. El-Hadidy *et al.* (2012), investigating the application of MEs as topical carriers for voriconazole, observed that their antifungal activity against *C. albicans* was considerably higher than that of a supersaturated solution of the same drug.

5CN05, either as such or in ME, showed greater activity against *C. neoformans* than against *Candida* spp. This activity profile is in agreement with the results of Mendonça Junior *et al.* (2011), who found that tiophene derivatives have moderate or weak antifungal activity against *Candida* strains and high activity against *C. neoformans*.

Nowadays, fungal infections are treated using a limited number of therapeutic agents, among which the main are echinocandins, polyenes (AmB), antimetabolites (flucytosine) and azoles (fluconazole), but only the last three have activity against *C. neoformans* (Hast *et al.*, 2011). Despite its wide use, AmB has high nephrotoxicity and hepatotoxicity and may cause severe anemia (Tonomura *et al.*, 2009). Azoles are the drugs of choice in the therapy of infections caused by *Cryptococcus* spp., but, although there is no report, as far as we are aware, on *C. neoformans* resistance to usual antifungals yet, it is certain that less susceptible strains will arise as a result of the extensive use of fluconazole in long-term or maintenance therapies (Guerra *et* al., 2012).

One of the most important factors of *C. neoformans* virulence is its polysaccharide capsule that has in its composition two different polysaccharides (90-95% of glucuronoxylomannan and 5-8% of galactoxylomannan) and mannoproteins (Vecchiarelli and Monari, 2012). This fungus has the ability to modulate the structure and size of its capsule during the infection so as to allow its adaptation to different environments and to make its dissemination in different tissues easier (Charlier *et* al., 2005). Such structural changes in the capsule have been reported as an important factor for the microorganism crossing of the blood-brain barrier (Guerrero *et al.*, 2006; Jain, Guerrero and Fries, 2006). Therefore, one can assume that the activity of 5CN05, after its incorporation into a ME, is probably related to the interaction between lipid components of the system and fungal capsule, thereby increasing the contact area and making the drug access to the microorganism easier. Finally, we believe that the ME-5CN05 formulation developed in this work may act as a facilitator of drug permeation through the capsular material of *C. neoformans.*

The results of this work could be of some concern for the pharmaceutical industry, because they suggest an alternative therapy against *C. neoformans*.

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