BRAZILIAN JOURNAL OF MICROBIOLOGY

http://www.bjmicrobiol.com.br/

Medical Microbiology

Terpinen-4-ol, tyrosol, and β-lapachone as potential antifungals against dimorphic fungi

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ARTICLE INFO

Article history:
Received 8 July 2015
Accepted 11 February 2016
Available online 26 July 2016
Associate Editor: Luis Henrique Souza Guimarães

Keywords:
Dimorphic fungi
Terpinen-4-ol
Tyrosol
β-Lapachone
Inhibitory effect

ABSTRACT

This study aimed to evaluate the *in vitro* antifungal activity of terpinen-4-ol, tyrosol, and β-lapachone against strains of *Coccidioides posadasii* in filamentous phase ($n = 22$) and *Histoplasma capsulatum* in both filamentous ($n = 40$) and yeast phases ($n = 13$), using the broth dilution methods as described by the Clinical and Laboratory Standards Institute, to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of these compounds. The mechanisms of action of these compounds were also investigated by analyzing their effect on cell membrane permeability and ergosterol synthesis. The MIC and MFCf these compounds against *C. posadasii*, mycelial *H. capsulatum*, and yeast-like *H. capsulatum*, were in the following ranges: 350–5720 μg/mL, 20–2860 μg/mL, and 40–1420 μg/mL, respectively for terpinen-4-ol; 250–4000 μg/mL, 30–2000 μg/mL, and 10–1000 μg/mL, respectively, for tyrosol; and 0.48–7.8 μg/mL, 0.25–16 μg/mL, and 0.125–4 μg/mL, respectively for β-lapachone. These compounds showed a decrease in MIC when the samples were subjected to osmotic stress, suggesting that the compounds acted on the fungal membrane. All the compounds were able to reduce the ergosterol content of the fungal strains. Finally, tyrosol was able to cause a leakage of intracellular molecules.

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http://dx.doi.org/10.1016/j.bjm.2016.07.015

Introduction

Coccidioidomycosis and histoplasmosis are systemic mycoses reported in the Americas, especially in the United States, Mexico, and Brazil. These diseases are caused by the dimorphic fungi *Coccidioides* spp. and *Histoplasma capsulatum* var. *capsulatum*, respectively. In Brazil, coccidioidomycosis caused by the species *C. posadasii* has only been reported in the Northeast, while histoplasmosis has been reported throughout the country with a 40% lethality rate when associated with AIDS.[1,2] The treatment of these diseases involves azoles in cases with mild to moderate symptoms and amphotericin B in severe cases.[3,4] Although the standard antifungal therapies are effective for treating these mycoses, some disadvantages have been observed for these therapies, such as nephrotoxicity caused by amphotericin B,[5] the high cost of the lipid formulations of amphotericin B,[6] and the fact that only 50–60% of patients with coccidioidomycosis respond to the treatment with fluconazole and itraconazole[7] and 56–70% of patients with histoplasmosis respond to the treatment with ketoconazole.[8] Hence, a search for new therapeutic strategies against these pathogens is of great importance.

Terpinen-4-ol is a terpene alcohol that exhibits *in vitro* antifungal properties against *Candida* spp., *Cryptococcus neoformans*, *Malassezia* spp., *Rhodotorula* spp., *Trichosporon* spp., *Aspergillus* spp., *Penicillium* spp., and dermatophytes.[9–12] Tyrosol is a phenolic compound with antioxidant properties and an autoregulatory molecule of *C. albicans*[13,14] which has *in vitro* antifungal properties against *Candida* spp.[15] β-Lapachone is a quinone derived from lapachol with antifungal properties against *Candida* spp., *Cryptococcus neoformans*, and dermatophytes.[16–19] In the light of the earlier studies, this investigation aimed at evaluating the *in vitro* inhibitory effects of these compounds and investigating the mechanism of action of these compounds against the fungal strains, *C. posadasii* and *H. capsulatum*.

Materials and methods

Microorganisms

Twenty-two strains of *C. posadasii* in the filamentous phase (18 clinical, 3 environmental, and 1 animal) and 40 strains of *H. capsulatum* in the filamentous stage (38 clinical and 2 animal) were used for this study. Among the *H. capsulatum* strains, 13 were also evaluated in the yeast phase. All the fungal strains were obtained from the fungal collection of the Specialized Medical Mycology Center (CEMM, Federal University of Ceará, Brazil). The procedures for identification of the fungi included the classic mycological analysis, as described by Brilhante et al.[20] All the procedures were performed in a class II biological safety cabinet in a biosafety level 3 laboratory.

Antimicrobial agents

For the assays, terpinen-4-ol, tyrosol, and β-lapachone (all from Sigma Chemical Corporation, USA) were used. The traditional antifungal drugs, amphotericin B (AMB) (Sigma Chemical Corporation, USA) and itraconazole (ITC) (Janssen Pharmaceutica, Belgium) were used as control drugs. The stock solutions of terpinen-4-ol, ITC, and AMB were prepared in 100% dimethyl sulfoxide (DMSO); β-lapachone was dissolved in 80% DMSO; and tyrosol was dissolved in sterile distilled water.[15,21,22] All the stock solutions were stored at −20 °C until use. Serial dilutions of each compound were prepared in RPMI 1640 medium (Sigma Chemical Corporation, USA), supplemented with L-glutamine, buffered to pH 7.0 using 0.156 M MOPS (Sigma Chemical Corporation, USA). DMSO was included in the assays as control to confirm that the DMSO used to dilute the compounds did not interfere with fungal growth.[20]

Preparation of inoculum for antifungal susceptibility assays

The strains of *C. posadasii* were grown on potato agar and incubated for 7 days at room temperature (25–28 °C). To prepare the inoculum, 2 mL of sterile saline were added to each culture, and the surface of the mycelium was scraped with a microbiological loop. The suspensions were transferred to sterile tubes and allowed to stand for 5 min. The supernatant was read in a spectrophotometer at a wavelength of 530 nm, and its transmittance was set to 95%. The suspensions containing arthroconidia and hyphal fragments were diluted to 1:10 with RPMI 1640 medium to obtain inocula containing approximately 1×10^3–5×10^3 CFU/mL.[20]

H. capsulatum strains in filamentous form were grown on brain heart infusion (BHI) agar (Himedia, India) at 28 °C for 7 days. The inoculum was prepared as described earlier. *H. capsulatum* strains in the yeast phase were grown in Sabouraud agar or BHI agar supplemented with 10% sheep blood and incubated for 7 days at 35 °C. Then, an aliquot of the fungal colony was transferred to 2 mL of sterile saline. The absorbance of supernatant was measured in a spectrophotometer at a wavelength of 530 nm, and its transmittance was set to 95%. The suspensions containing arthroconidia and hyphal fragments were diluted to 1:10 with RPMI 1640 medium to obtain inocula containing approximately 1×10^3–5×10^3 CFU/mL.[20]

Antifungal susceptibility test

The susceptibility of *C. posadasii* strains to the compounds being tested was determined through the broth macrodilution method, according to the M38-A2 protocol standardized by the CLSI.[23] The susceptibility of *H. capsulatum* to the compounds was determined by the broth microdilution method, according to the M27-A3 protocol standardized by the CLSI.[24] The concentrations of the tested compounds for *C. posadasii* strains were as follows: Terpinen-4-ol (350–5720 μg/mL), tyrosol (250–4000 μg/mL), β-lapachone (0.48–7.8 μg/mL), AMB (0.0625–1 μg/mL), and ITC (0.0625–1 μg/mL). The concentrations of the compounds being tested against *H. capsulatum* strains (in both phases) were as follows: Terpinen-4-ol (10–5720 μg/mL), tyrosol (3.9–2000 μg/mL), β-lapachone (0.0312–16 μg/mL), AMB (0.0039–2 μg/mL), and ITC (0.00195–1 μg/mL).