The Brazilian Journal of
INFECTIOUS DISEASES
www.elsevier.com/locate/bjid

Original article

Simvastatin inhibits planktonic cells and biofilms of *Candida* and *Cryptococcus* species

Raimunda Sâmia Nogueira Brilhante[a,b,*], *Erica Pacheco Caetano*[a], *Jonathas Sales de Oliveira*[a], *Débora de Souza Collares Maia Castelo-Branco*[a], *Elizabeth Ribeiro Yokobatake Souza*[a], *Lucas Pereira de Alencar*[a], *Rossana de Aguiar Cordeiro*[a,b], *Tereza de Jesus Pinheiro Gomes Bandeira*[a], *José Júlio Costa Sidrim*[a,b], *Marcos Fábio Gadelha Rocha*[a,c]

[a] *Centro Especializado em Micologia Médica, Programa de Pós-Graduação em Microbiologia Médica, Universidade Federal do Ceará (UFC), Fortaleza, CE, Brazil*
[b] *Programa de Pós-Graduação em Ciências Médicas, Universidade Federal do Ceará (UFC), Fortaleza, CE, Brazil*
[c] *Programa de Pós-Graduação em Ciências Veterinárias, Universidade Estadual do Ceará (UECE), Fortaleza, CE, Brazil*

ARTICLE INFO

Article history:
Received 2 April 2015
Accepted 1 June 2015
Available online 26 June 2015

Keywords:
Candida
Cryptococcus
Simvastatin
Antifungal activity
Biofilm

ABSTRACT

The antifungal activity of some statins against different fungal species has been reported. Thus, at the first moment, the *in vitro* antifungal activity of simvastatin, atorvastatin and pravastatin was tested against *Candida* spp. and *Cryptococcus* spp. Then, in a second approach, considering that the best results were obtained for simvastatin, this drug was evaluated in combination with antifungal drugs against planktonic growth and tested against biofilms of *Candida* spp. and *Cryptococcus* spp. Drug susceptibility testing was performed using the microdilution broth method, as described by the Clinical and Laboratory Standards Institute. The interaction between simvastatin and antifungals against planktonic cells was analyzed by calculating the fractional inhibitory concentration index. Regarding biofilm susceptibility, simvastatin was tested against growing biofilm and mature biofilm of one strain of each tested yeast species. Simvastatin showed inhibitory effect against *Candida* spp. and *Cryptococcus* spp. with minimum inhibitory concentration values ranging from 15.6 to 1000 mg L^{-1} and from 62.5 to 1000 mg L^{-1}, respectively. The combination of simvastatin with itraconazole and fluconazole showed synergism against *Candida* spp. and *Cryptococcus* spp., while the combination of simvastatin with amphotericin B was synergistic only against *Cryptococcus* spp. Concerning the biofilm assays, simvastatin was able to inhibit both growing biofilm and mature biofilm of *Candida* spp. and *Cryptococcus* spp. The present study showed that simvastatin inhibits planktonic cells and biofilms of *Candida* and *Cryptococcus* species.

* *Corresponding author at*: Rua Barão de Canindé, 210, Montese, CEP: 60425-540 Fortaleza, CE, Brazil.
E-mail address: brilhante@ufc.br (R.S.N. Brilhante).

http://dx.doi.org/10.1016/j.bjid.2015.06.001
1413-8670/

Introduction

The incidence of invasive fungal infections, especially those caused by opportunistic fungi of the genus *Candida* and *Cryptococcus*, has proportionally increased with the increase in the number of hosts with impaired immunity.[1–4] In addition, *in vitro* resistance to antifungal drugs among *Candida* spp. and *Cryptococcus* spp. strains recovered from humans and animals has been reported.[5–11]

This scenario motivates the search for new compounds with antifungal potential. Originally, the first statins were described as metabolites of microorganisms with the ability to lower blood cholesterol.[12] Later, it was demonstrated that these compounds reduce the growth of several fungal species,[13–15] including the yeasts *Candida* spp. and *Cryptococcus neoformans*[16] and the filamentous fungi *Mucor* spp. and *Rhizopus* spp.[17] In addition, it has also been reported that the administration of statins to hospitalized patients increases survival[18] and decreases *Candida* burden in diabetic patients.[19]

Although the antifungal potential of statins has already been addressed in previous reports, studies involving the effect of statins on fungal biofilms are needed to obtain a better knowledge on the antifungal potential of these compounds. Hence, this study evaluated the effect of the statins simvastatin, atorvastatin, and pravastatin on planktonic cells of *Candida* spp. and *Cryptococcus* spp. Considering that the best results were obtained for simvastatin, this drug was evaluated in combination with antifungal drugs against planktonic growth. In addition, simvastatin was tested against biofilms of *Candida* spp. and *Cryptococcus* spp.

Materials and methods

Microorganisms

For this study, 51 strains of *Candida* spp. (16 *Candida albicans*; 12 *Candida tropicalis*; 11 *Candida krusei*; 12 *Candida parapsilosis sensu lato*), and 25 strains of *Cryptococcus* spp. (13 *C. neoformans* – serotypes A, D and AD; and 12 *Cryptococcus gattii* – serotypes B and C) isolated from animals were used. The isolates belong to the culture collection of the Specialized Medical Mycology Center, Brazil. The purity and identity of the *Candida* spp. strains were confirmed by growth on chromogenic medium and microscopical and biochemical features.[20] For the *Cryptococcus* spp. strains, capsule formation, melanin production, and biochemical testing were evaluated and the serotype of each strain was assessed by PCR.[21]

Susceptibility testing of planktonic cells

Susceptibility assays were performed using the broth microdilution method, as described by the document M27-A3 of the Clinical and Laboratory Standards Institute.[22] The tests were performed in duplicate and visually read after 48 h of incubation at 35 °C. The strains *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were included as quality control for all tests.[22]

Inocula were prepared to obtain a final concentration of 0.5–2.5 × 10^3 cells mL^{-1}.[22] The statins simvastatin (Medley Indústria Farmacêutica Ltda, Campinas, SP, Brazil), atorvastatin (Laboratórios Pfizer Ltda, São Paulo, SP, Brazil), and pravastatin (Bristol-Myers-Squibb, Nova York, NY, USA) and the antifungal drugs amphotericin B (Sigma Chemical Corporation, St Louis, USA), itraconazole (Janssen Pharmaceutica, Beerse, Belgium), and fluconazole (Pfizer Pharmaceuticals, New York, USA) were tested against all strains.

To obtain the stock-solutions of each drug, atorvastatin, pravastatin, and fluconazole were diluted with sterile distilled water, and amphotericin B and itraconazole were diluted with dimethylsulfoxide (DMSO). Simvastatin was activated from its lactone prodrug form through hydrolysis in ethanolic NaOH (15% (v/v) ethanol, 0.25% (w/v) NaOH), at 60 °C, for 1 h.[15] The concentration range tested was 3.9–2000 μg mL^{-1} for simvastatin, 19.5–10,000 μg mL^{-1} for atorvastatin, 97.6–50,000 μg mL^{-1} for pravastatin, 0.0312–64 μg mL^{-1} for amphotericin B and itraconazole, and 0.25–256 μg mL^{-1} for fluconazole. The minimum inhibitory concentration (MIC) was defined as the lowest drug concentration able to inhibit 100% of fungal growth for amphotericin B and 50% inhibition of fungal growth when compared to the free-drug azoles[22] and statins control.

As simvastatin provided the best antifungal results, we evaluated the interaction between this drug and the antifungal drugs against the tested yeasts. For drug interaction studies, simvastatin was tested with each azole by broth microdilution method, using the MIC of each tested drug alone as the highest concentrations tested in combination. The concentrations of the drugs in combination ranged from 0.03 to 1000, 0.00024 to 2, 0.00006 to 64 and 0.00048 to 256 μg mL^{-1} for simvastatin, amphotericin B, itraconazole and fluconazole, respectively. The reading criteria were the same as for the antifungal drugs alone, namely 100% inhibition when combined with amphotericin B and 50% inhibition when combined with azoles. The interaction between the drugs was analyzed by calculating the fractional inhibitory concentration index (FICI), with values ≤0.5 indicating synergism.[23]

Susceptibility test of sessile cells

Simvastatin was tested against growing biofilms and mature biofilms of *Candida* spp. and *Cryptococcus* spp. Amphotericin B and itraconazole were used in all tests as control drugs for biofilm inhibition. The tests were performed in triplicate using one biofilm-producing strain of each tested fungal species (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. neoformans* and *C. gattii*), according to the methodology described by Chatzimoschou et al.,[24] with some modifications. Briefly, strains were grown on Sabouraud dextrose agar for 48 h at 30 °C and then subcultured into Sabouraud dextrose broth for 24 h, at 30 °C, under agitation at 150 rpm. After this period, the suspensions were centrifuged at 3000 rpm for 10 min, the supernatant was discarded, and the pellet was washed twice with sterile PBS. Then, the pellet was resuspended in RPMI 1640 medium (Gibco-BRL, USA), reaching a concentration of 1 × 10^6 cells mL^{-1}. Tests were performed in 96-well polystyrene plates.

To evaluate the effect of simvastatin, amphotericin B, and itraconazole on growing biofilm, 100 μL of the fungal suspension was exposed to 100 μL of simvastatin and incubated at