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Original article

Antiretroviral drugs saquinavir and ritonavir reduce inhibitory concentration values of itraconazole against *Histoplasma capsulatum* strains *in vitro*

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ABSTRACT

Recent studies have shown that some drugs that are not routinely used to treat fungal infections have antifungal activity, such as protease inhibitor antiretroviral drugs. This study investigated the *in vitro* susceptibility of *Histoplasma capsulatum* var. *capsulatum* to saquinavir and ritonavir, and its combination with the antifungal itraconazole. The susceptibility assay was performed according to Clinical and Laboratory Standards Institute guidelines. All strains were inhibited by the protease inhibitor antiretroviral drugs. Saquinavir showed minimum inhibitory concentrations ranging from 0.125 to 1 $\mu g\,mL^{-1}$ for both phases, and ritonavir presented minimum inhibitory concentrations ranging from 0.0312 to 4 $\mu g\,mL^{-1}$and from 0.0625 to 1 $\mu g\,mL^{-1}$ for filamentous and yeast phase, respectively. Concerning the antifungal itraconazole, the minimum inhibitory concentration values ranged from 0.0019 to 0.125 $\mu g\,mL^{-1}$ and from 0.0039 to 0.0312 $\mu g\,mL^{-1}$ for the filamentous and yeast phase, respectively. The combination of saquinavir or ritonavir with itraconazole was synergistic against *H. capsulatum*, with a significant reduction in the minimum inhibitory concentrations of both drugs against the strains ($p < 0.05$). These data show an important *in vitro* synergy between protease inhibitors and itraconazole against the fungus *H. capsulatum*.

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Introduction

Histoplasmosis is a systemic infection caused by the dimorphic fungus *Histoplasma capsulatum*. It is mainly associated with immunosuppression, especially in HIV patients.[1,2] This disease is characterized by a broad spectrum of clinical manifestations ranging from asymptomatic to disseminated forms.[3] Histoplasmosisis is widely distributed in the Americas.[4] In Brazil, the number of cases has increased in several regions. Some outbreaks have been recorded in the country, involving the states of Rio de Janeiro, São Paulo, Minas Gerais, Espírito Santo, Mato Grosso, and Rio Grande do Sul.[4–6] In Ceará state, a recent study reported 254 cases of histoplasmosis in patients with HIV in the period from 2006 to 2010, showing its high prevalence in this region.[7]

Treatment of histoplasmosis depends on the severity of infection, clinical manifestations and individual risk factors. The therapy indicated for mild to moderate cases is the administration of azoles, such as itraconazole. The use of amphotericin B is limited to severe cases because of its high toxicity. Due to the increase of histoplasmosis cases in recent years, particularly among HIV patients, associated with the occurrence of refractory and recurrent infections, there is a need to find new therapeutic approaches to control this mycosis.[8,9] Some studies have shown that certain drugs not routinely used to treat fungal infections have significant antifungal activity.[10] Among these, the antiretroviral drugs have demonstrated the ability to interfere with the viability and virulence of fungal cells. The protease inhibitors indinavir and ritonavir have shown *in vitro* and *in vivo* inhibitory effects against *Candida albicans*.[11] Indinavir also has shown activity against the fungus *Cryptococcus neoformans*, reducing its virulence and making it more susceptible to the killing activity of natural effector cells of the immune system.[12]

Thus, this study aimed to evaluate the *in vitro* susceptibility of *H. capsulatum* var. *capsulatum* to the antiretroviral drugs saquinavir and ritonavir, as well as their combination with the azole antifungal itraconazole.

Materials and methods

Microorganisms

We used a total of 20 clinical strains of *H. capsulatum* in the filamentous phase and 10 in the yeast phase, isolated from the Northeast and Southeast regions of Brazil. The samples came from the culture collection of the Specialized Medical Mycology Center, Federal University of Ceará, and were handled in a biosafety level 3 cabin.

Antifungal agents

Stock solutions of the antiretroviral drugs saquinavir (Roche Holding AG, Basel, Switzerland) and ritonavir (Abbott Laboratories, Chicago, USA) and the antifungal itraconazole (Janssen Pharmaceutica, Beerse, Belgium) were prepared in dimethyl sulfoxide (DMSO). These solutions were stored at −20 °C until use. Serial dilutions of each antimicrobial agent were prepared in RPMI 1640 (Sigma Chemical Corporation, St. Louis, MO, USA), supplemented with L-glutamine, buffered at a pH of 7.0 with MOPS 165 mmol l^{-1} (Sigma Chemical Corporation, St. Louis, MO, USA).

Preparation of fungal inoculum

To prepare the inoculum, fungal suspensions were prepared in saline from stock cultures after seven days of incubation, maintained on BHI (brain heart infusion) agar and incubated at 28 °C for the filamentous phase. The cultures were maintained on BHI agar supplemented with sheep blood at 10% and incubated at 35 °C to obtain the yeast phase. The inoculum was adjusted to 90–95% by transmittance spectrophotometry at a wavelength of 530 nm. After reading, the suspensions were diluted 1:10 in RPMI 1640 medium to obtain inoculums of approximately 0.5×10^3 to 2.5×10^4 cfu mL^{-1}.[13]

Susceptibility test

The *in vitro* antifungal activity was determined by the broth microdilution method in accordance with the protocol described in document M27-A3 and standardized by the Clinical Laboratory Standards Institute.[14] Initially, the minimum inhibitory concentration (MIC) was determined for each drug. Subsequently, the MIC values were used as the highest concentration to prepare drugs in combination. The concentration ranges of the drugs alone were: 0.0039–2 μg mL^{-1} for saquinavir, 0.0156–8 μg mL^{-1} for ritonavir, and 0.0009–0.5 μg mL^{-1} for itraconazole. The concentration ranges of the drugs in combination were: 0.0002–1 μg mL^{-1} for saquinavir, 0.00006–4 μg mL^{-1} for ritonavir and 0.000003–0.0625 μg mL^{-1} for itraconazole. The results were determined by visual readings after seven and four days of incubation at 35 °C for strains in the filamentous and yeast phase, respectively. The MICs were defined as the lowest concentration of drug able to inhibit 80% of the fungal growth for antiretroviral drugs and itraconazole, as well as for the combination of both.[13] Drug interaction was evaluated by calculating the fractional inhibitory concentration index (FICI), which was classified as synergistic (FICI $\leq$ 0.5), indifferent (0.5 < FICI < 4), or antagonistic (FICI $\geq$ 4).[15] The FICI values obtained for each drug combination against *H. capsulatum* were compared through Wilcoxon test ($p < 0.05$). The analysis was carried out using IBM SPSS ver. 21.0 software (IBM Co., Armonk, NY, USA). Standard strains of *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were included in each test as quality controls.

Results

The protease inhibitors saquinavir and ritonavir were capable of inhibiting the strains of *H. capsulatum*, with MIC values ranging from 0.125 to 1 μg mL^{-1} for saquinavir in both filamentous and yeast phase; and from 0.0312 to 4 μg mL^{-1} and from 0.0625 to 1 μg mL^{-1} for ritonavir in filamentous and yeast phase, respectively (Table 1). Concerning itraconazole, the MIC ranged from 0.0019 to 0.0625 μg mL^{-1} and from 0.0039 to