Effects of Amphotericin B and Three Azole Derivatives on the Lipids of Yeast Cells of *Paracoccidioides brasiliensis*

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Yeast cells of five different strains of *Paracoccidioides brasiliensis* were obtained for partial analysis of lipid composition, and sterol content was determined quantitatively and qualitatively. The determinations were conducted with cells cultured in the presence and absence of amphotericin B andazole derivatives at levels below the MIC.

Paracoccidioidomycosis is a chronic granulomatous disease with a diversity of clinical manifestations, and special emphasis has been placed on the pulmonary and mucocutaneous forms (29, 42). The disease is limited to Latin American countries, and it is considered the most prevalent systemic mycosis in Latin America (27–29, 38).

The dimorphic fungus *Paracoccidioides brasiliensis* is the single etiologic agent of paracoccidioidomycosis, which is acquired by inhalation of spores of the mycelial phase of the fungus (3, 38, 43, 45).

In the present study we assessed the effects of amphotericin B, ketoconazole, itraconazole, and fluconazole on the sterols of five different *P. brasiliensis* strains in order to better evaluate the in vitro efficacy of these drugs. The MIC of each drug and the effects of the compounds on total sterol content were determined.

**Fungal strains and culture conditions.** Five *P. brasiliensis* strains were studied. Of these, JT-1, a human isolate, corresponded to the reference strain ATCC 90659. Three other strains, called Pinguim, SN, and 18, were from the fungal collection of the Faculty of Medicine of the Universidade de São Paulo and were a kind gift from C. Fava-Netto (Universidade de São Paulo) and Patrícia Cisalpino (Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais). The environmental strain Pinguim was isolated from the feces of a penguin in the Antarctic and characterized as *P. brasiliensis* in mycologic and immunochemical studies (15). The human isolate RAJ-2 was a kind gift from Tomoko Tadano and originated from the Clinical Pathology Laboratory of the Julio Müller University Hospital, Cuiabá-MT, where it had been obtained from the sputum of a patient at the Julio Müller University Hospital.

All strains were maintained on solid Fava-Netto medium at 22 to 28°C with monthly subculture and converted to the yeast-like phase by incubation at 35°C. The full transition of the forms was confirmed by the observation of the macroscopic and microscopic aspects of the colonies obtained.

For the study of lipid composition and of the effects of amphotericin B and the three azoles, the microorganisms were adapted to solid McVeigh Morton (MVM) medium, modified as described previously (39).

**Antifungal agents.** All strains were tested in the presence of amphotericin B, ketoconazole, fluconazole, and itraconazole.

Amphotericin B was obtained from Sigma Chemical Company in pure form and was stored at 4°C in a dark glass bottle. The azole derivatives ketoconazole and itraconazole were kindly provided by Janssen in the form of pure salts and were stored at room temperature.

The studies involving fluconazole were carried out using a commercial preparation (Flucoral; Libbs) in 150-mg capsules. Single drug lots were used in all tests.

**Determination of MIC.** The MIC was determined by the technique of Shadomy et al. (44). The concentrations tested corresponded to the ranges reported in the literature as the standards for *P. brasiliensis* susceptibility, i.e., 0.0005 to 4 μg/ml for ketoconazole, fluconazole, and itraconazole and 0.062 to 4 μg/ml for amphotericin B (20, 21, 30, 32, 40). All concentrations were tested in duplicate. Drug-free and titrated solubilizing vehicle (dimethyl sulfoxide) controls were included.

Yeast cells in the exponential phase (31) were collected aseptically with a platinum loop and resuspended in Erlenmeyer flasks modified by the insertion of a lateral tube containing sterile 0.85% saline solution for readings with a photocolorimeter. After homogenization by vortexing, transmittance was measured at 520 nm and adjusted to 69 to 70%.

Mean cell counts were 10⁸ cells/ml and were obtained by counting the number of viable yeast cells in a Neubauer chamber starting from the 10-μl dilution of the fungal suspension in 1 ml of an isotonic solution (0.9% NaCl, 2% formaldehyde, 2% Tween 20) (25). A 0.1-ml aliquot of the suspension of each *P. brasiliensis* strain was added to 0.9 ml of MVM containing the serial drug solutions, providing a final concentration of 10⁴ cells/ml (7, 37). The tubes thus prepared were incubated at 35°C with constant shaking for 7 days.

In the present study, the MIC of amphotericin B was defined as the lowest drug concentration that did not permit any growth of the *P. brasiliensis* strains. For the three azoles, MICs were defined as the lowest concentration that resulted in 90% inhibition or more of visual turbidity compared with that produced by the growth control (0.1 ml of growth control plus 0.9 ml of uninoculated MVM).

Sterol analysis was performed after saponification according to the method of Breivick and Owades (2). A 25-μg sample of lyophilized cells was treated with 40% alcoholic KOH utilizing a condenser for 3 h at 80°C. The nonsaponifiable fractions were then extracted with n-heptane, and the solvent was removed by evaporation under a stream of nitrogen gas. Total
sterol content was determined as described by Moore and Baumann (36), and the results were expressed as milligrams of total sterols per 100 mg of lyophilized cells.

The sterols were detected and characterized by observation of the UV absorption profile (26) and by thin-layer chromatography (TLC). For UV spectrophotometry, the nonsaponifiable extracts were dissolved in twice-distilled ethanol, and the absorption spectra were recorded in a Beckman DB spectrophotometer from 220 to 310 nm along with the spectra of the control samples of ergosterol (Sigma). For TLC, nonsaponifiable extracts of the samples and commercially obtained sterols were dissolved in chloroform, applied to TLC aluminum sheets (Merck), and then chromatographed using an acetone-benzene (80:20, vol/vol) mixture as the solvent system. The separated components were detected under UV light and/or iodine vapor.

Effect of amphotericin B and azoles on the sterols of P. brasiliensis. Flasks containing 100 ml of MVM with amphotericin B at sub-MIC concentrations (0.5 times the MIC) were inoculated to an initial cell density of approximately $10^7$ cells/ml and incubated at $35^\circ$C for 7 days. The same procedure was used for theazole derivatives.

The cells were then harvested, washed, and lyophilized. Total sterol content was determined and the material was partially characterized by TLC and UV spectrophotometry.

The results are reported as the mean and standard deviation of three experiments for each P. brasiliensis strain. Statistical significance was evaluated using a two-tailed Student's t-test (23, 24) that uses no hypothesis about the form of the distributions except that the distributions have the same form. A P value of less than 0.05 was considered significant.

The MICs of amphotericin B for the five strains of P. brasiliensis ranged from 1.0 to 0.25 μg/ml. The MICs obtained were 0.5 μg/ml for JT-1 and Pinguim, 1.0 μg/ml for RAJ-1 and 18, and 0.25 μg/ml for SN.

Hamdan and Resende (20), in a study of the susceptibility of strain SN to amphotericin B, obtained a MIC of 0.2 μg/ml. The same strain was assessed in the present study, and the MIC was 0.25 μg/ml after 10 years of maintenance at room temperature in our laboratory by monthly subculture, demonstrating the stability of this strain in terms of this parameter.

On the basis of published data which define resistance to amphotericin B as the presence of growth at concentrations of 2 μg/ml, as a function of the mean drug doses that can be therapeutically applied to human hosts (34), the MICs obtained in the present study permit us to infer that none of the five strains tested were resistant to amphotericin B. Lacaz et al. (30) reported MICs of 0.06 to 0.243 μg/ml for three P. brasiliensis strains in the yeast phase. McGinnis et al. (32), in a study of 14 strains of the same fungus, determined MICs of 0.125 to 4.14 μg/ml but did not specify how many of those isolates may have been resistant.

The MICs of ketoconazole, itraconazole, and fluconazole for the five strains fell within the narrow ranges of 0.0009 to 0.015, 0.0009 to 0.5, and 0.125 to 0.5 μg/ml, respectively. Although the MICs determined in the present study agreed in general with those previously reported in the literature, direct comparisons of MIC data from one study to another are complicated with those previously reported in the literature, direct comparisons of MIC data from one study to another are complicated by variability in test methods and a lack of standardized approaches. In particular, there may be variations with respect to test format (i.e., agar dilution, disk diffusion, or broth dilution), growth medium used (including pH), inoculum size, incubation conditions (i.e., temperature, atmosphere, extent of aeration, and incubation length), and definition of the endpoint (7, 8, 12, 37). Furthermore, most studies on the development of standardized antifungal susceptibility assays have been conducted on yeasts (Candida spp. and Cryptococcus neoformans, in particular) and recent reports on dimorphic fungi have pointed out specific difficulties especially because of the slow and scarce growth of these microorganisms (13, 16, 41).

The MICs determined here demonstrated, on a weight basis, greater activity of ketoconazole, followed by itraconazole, while fluconazole had the lowest in vitro activity (i.e., highest MICs). In terms of data observed in vivo, the superiority of ketoconazole over itraconazole is not supported by reports on the treatment of paracoccidioidomycosis which refer to the latter compound as being more effective. Fluconazole MIC data agree with reports in the literature indicating that this drug has the lowest in vitro activity compared to other azoles (5, 35).

The data concerning total sterol contents (mg/100 ml) of five P. brasiliensis strains cultured in the absence (control) or presence of amphotericin B, or in the presence of ketoconazole, itraconazole, or fluconazole at one-half the MIC are presented in Fig. 1.

Effect of amphotericin B on the sterols of P. brasiliensis. Exposure of P. brasiliensis strains to sub-MIC concentrations of amphotericin B resulted in a substantial decrease in sterol content to 25 to 78% relative to control cells (Fig. 1). These findings are similar to those described by Hamdan and Resende (20) who observed a decrease in lipid (53%) and sterol (50%) content in the yeast phase of one strain of P. brasiliensis after exposure to amphotericin B. Likewise, Franzot and Hamdan (10) demonstrated that exposure to the same polynucleotide antibiotic resulted in substantial decreases in the lipid (33%) and sterol (52 to 94%) content of C. neoformans. Hamdan and Casali (19) observed a 49 to 97% decrease in sterol content compared to control values in Sporothrix schenckii strains exposed to one-half the MIC of amphotericin B.

Chromatographic analysis of the sterols of amphotericin B-treated cells revealed that ergosterol disappeared in strains SN and 18. In strains JT-1, RAJ-1, and Pinguim, lanosterol appeared in addition to ergosterol and squalene. UV spectrophotometry revealed the presence of ergosterol in all treated strains, although the absorption peaks typical of this compound were not as conspicuous as in the controls.

Our data are in agreement with those published by Hamdan and Resende (20) in a study in which spectrophotometry appeared to be more sensitive than TLC. On the other hand, using both procedures, Franzot and Hamdan (10) verified the total absence of ergosterol in amphotericin B-treated cells of C. neoformans.

Taken together, our results indicate a reduction of sterol content, especially considering the total sterol quantifications. These data could be interpreted as a possible effect of amphotericin B on sterol metabolism of the fungus. We suggest that the drug, used at sublethal concentrations, altered the lipid metabolism, preventing the synthesis of compounds (sterols in this case) upon which amphotericin B acts.

Effect of three azole derivatives on the sterols of P. brasiliensis. The amount of total sterol content ranged from 0.10 to 0.50 mg/100 ml. Statistical analysis of these data revealed that strain JT-1 contained a smaller amount of sterols than all other strains. Qualitative analysis of the sterols by chromatography revealed the presence of ergosterol, squalene, and lanosterol. The absorption spectra of the sterols extracted from the strains showed peaks at 274, 282, and 298 nm, which are typical of ergosterol and confirmed the presence of this sterol in the cells.

Exposure of P. brasiliensis strains to sub-MIC concentrations of ketoconazole, itraconazole, and fluconazole resulted in substantial changes in the sterol content relative to control cells.
These results are summarized in Fig. 1. The threeazole derivatives caused a decrease in total sterols in all strains. Comparative analysis of the effects of the three azoles on the sterol content of each strain showed that each strain presented a different profile in terms of sterol reduction after exposure to the three azoles, and it was not possible to clearly determine which of the three drugs was most effective in this respect (i.e., sterol depression).

Qualitative analysis by TLC of sterol extracted from azole-treated cells revealed the presence of ergosterol, lanosterol, and squalene. Lanosterol was not detected in untreated cells. The presence of ergosterol was confirmed by UV spectrophotometry.

In this study, we demonstrated that azole-treated cells contained significantly less sterol than controls, and we also showed the presence of lanosterol in these cells. Taken together, these findings seem to support the idea that azole antifungal agents act through the inhibition of ergosterol biosynthesis in fungi.

Costa and Campos-Takaki (6) reported that exposure of *P. brasiliensis* to ketoconazole resulted in reductions not only in total lipids and sterols but also fatty acid, triacylglycerol, and glycolipid levels.

When an attempt was made to compare the effects of the three azoles on the sterol content of the five strains, it was not possible to clearly distinguish which of the drugs was the most effective in terms of producing a decrease in total sterols. However, the MICs indicated that ketoconazole is considerably more active in vitro than both itraconazole and fluconazole. Thus, the differences in the effects of the three azoles on sterol content are difficult to reconcile with the differences in MICs. These findings are somewhat similar to those described by Ballard et al. (1) and Ghannoum et al. (17), who found no correlation between the sterol composition of fungi and susceptibility toazole derivatives.

The present results permit us to conclude that, regardless of origin (i.e., human or environmental), the different *P. brasiliensis* isolates presented the same general response when exposed to the azole derivatives, i.e., quantitative and qualitative changes in the lipid profile.

We have also shown that ketoconazole, itraconazole, and fluconazole had different activities on *P. brasiliensis* cells not only in terms of growth inhibition but also in terms of their effects on fungal lipids. Additional studies will be required to determine the role of lipid fractions in the mode of action of azole derivatives, as well as to analyze the individual sterol fractions of the fungus, in order to elucidate the precise changes in their patterns following exposure to these drugs.

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