Miltefosine is active against *Sporothrix brasiliensis* isolates with *in vitro* low susceptibility to amphotericin B or itraconazole

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Sporotrichosis is a common mycosis caused by dimorphic fungi from the *Sporothrix schenckii* complex. In recent years, sporotrichosis incidence rates have increased in the Brazilian state of Rio de Janeiro, where *Sporothrix brasiliensis* is the species more frequently isolated from patients. The standard antifungals itraconazole and amphotericin B are recommended as first-line therapy for cutaneous/lymphocutaneous and disseminated sporotrichosis, respectively, although decreased sensitivity to these drugs *in vitro* was reported for clinical isolates of *S. brasiliensis*. Here, we evaluated the activity of the phospholipid analogue miltefosine – already in clinical use against leishmaniasis – towards the pathogenic yeast form of *S. brasiliensis* isolates with low sensitivity to itraconazole or amphotericin B *in vitro*. Miltefosine had fungicidal activity, with minimum inhibitory concentration (MIC) values of 1–2 μg ml⁻¹. Miltefosine exposure led to loss of plasma membrane integrity, and transmission electron microscopy (TEM) analysis revealed a decrease in cytoplasmic electron density, alterations in the thickness of cell wall layers and accumulation of an electron-dense material in the cell wall. Flow cytometry analysis using an anti-melanin antibody revealed an increase in cell wall melanin in yeasts treated with miltefosine, when compared with control cells. The cytotoxicity of miltefosine was comparable to those of amphotericin B, but miltefosine showed a higher selectivity index towards the fungus. Our results suggest that miltefosine could be an effective alternative for the treatment of *S. brasiliensis* sporotrichosis, when standard treatment fails. Nevertheless, *in vivo* studies are required to confirm the antifungal potential of miltefosine for the treatment of sporotrichosis.

INTRODUCTION

Sporotrichosis is the most frequent subcutaneous mycosis in Latin America, and has high incidence rates in Brazil, especially in the Rio de Janeiro state, where zoonotic transmission prevails (Barros *et al.*, 2010). This disease is caused by dimorphic fungal species from the *Sporothrix schenckii* complex (Marimon *et al.*, 2007; 2008), including *Sporothrix brasiliensis*, the most virulent species (Arrillaga-Moncrieff *et al.*, 2009; Fernandes *et al.*, 2013) and the most prevalent in the clinical cases from the Rio de Janeiro state (Oliveira *et al.*, 2011; Rodrigues *et al.*, 2013a; Borba-Santos *et al.*, 2014). In zoonotic transmission, sporotrichosis is mainly acquired by scratches, bites or direct contact with lesion secretion from contaminated animals, where yeasts are the infective forms (Rodrigues *et al.*, 2013b).

The ‘gold standard’ treatment for cutaneous and lymphocutaneous forms of sporotrichosis is itraconazole, while amphotericin B is the first-line drug of choice for disseminated forms of the disease (Kauffman *et al.*, 2007). However, recent reports demonstrated that these drugs are less potent against *S. brasiliensis* isolates, which have high minimum inhibitory concentration (MIC) values *in vitro* for these antifungals (Ottonelli Stopiglia *et al.*, 2014; Rodrigues *et al.*, 2014; Borba-Santos *et al.*, 2014).

Miltefosine is a phospholipid analogue originally developed as an antitumour drug, but later shown to have potent...
antiparasitic activity, which led to its licensing for the treatment of cutaneous and visceral leishmaniasis in India and Colombia (Dorlo et al., 2012). Miltefosine induces changes in the lipid composition of membranes (among other cellular effects), leading to a disruption of membrane structure that affects intracellular signalling processes essential for survival and cell growth (Jimenez-Lopez et al., 2010). Recently, Brilhante et al. (2014) showed that miltefosine is active against the filamentous (i.e. sphaerophytic) form of S. schenckii complex species, including S. brasiliensis. However, these authors did not determine whether miltefosine is active against the pathogenic (yeast) form of S. schenckii complex species and did not examine the intracellular effects of miltefosine in these fungi.

Given the clinical and epidemiological importance of S. brasiliensis, and the limited repertoire of therapeutic options available to treat sporotrichosis by this highly virulent species, the aim of our study was to evaluate the in vitro activity of miltefosine against the pathogenic yeast form of clinical isolates of S. brasiliensis that have low susceptibility to amphotericin B or itraconazole (Borba-Santos et al., 2014). In addition, we analysed the cytotoxicity of miltefosine, and evaluated the alterations induced by exposure of yeast cells to this drug (by electron microscopy and flow cytometry), to clarify the mechanism of action of miltefosine in S. brasiliensis.

METHODS

Micro-organisms. In this study, we used 13 clinical isolates of S. brasiliensis (Ss 34, Ss 37, Ss 56, Ss 68, B91, B182, B628, B735, B484, B972, B1008, B1086, HE06) that displayed low susceptibility to amphotericin B or itraconazole (Borba-Santos et al., 2014). The genome strain ATCC MYA 4823 was used as a reference (Teixeira et al., 2014). The isolates were maintained in the filamentous (mycelial) form in potato dextrose agar (PDA; Difco) at 4 ºC until their use in experiments. The yeast phase was obtained from filamentous-form cultures by two successive 7 day passages in brain heart infusion (BHI, Difco) broth supplemented with 2 % glucose, at 36 ºC and with orbital agitation (Borba-Santos et al., 2014).

Drugs. Miltefosine (Cayman Chemical Company) was diluted in distilled water to obtain stock solutions of 2000 µg ml⁻¹ and maintained at −20 ºC. Amphotericin B and itraconazole (Sigma) were used as reference antifungals and kept as 1600 µg ml⁻¹ in DMSO stock solutions and stored at −20 ºC.

Minimum inhibitory concentration (MIC). MIC values for the treatment of S. brasiliensis yeasts with miltefosine were determined using broth microdilution methods adapted from the M27–A3 document from the Clinical and Laboratory Standards Institute (CLSI, 2008), as described in Borba-Santos et al. (2014). Briefly, yeast cells (0.5–2.5 × 10⁵ colony forming units or c.f.u. ml⁻¹) were treated with 0.01–16 µg ml⁻¹ miltefosine, in RPMI 1640 medium (Sigma) supplemented with 2 % glucose and buffered with 0.165 M 3-(N-morpholine) propane sulfonic acid (MOPS), pH 7.2. Samples were incubated at 35 ºC for 5 days and MIC was defined as the lowest concentration that prevents visible fungal growth in an inverted optical microscope (Axiovert 100, Zeiss).

Minimal fungicidal concentration (MFC). To determine MFC values, 10 µl aliquots were collected from MIC samples at the end of the 5 day incubation period, and were plated in drug-free PDA. Plates were incubated at 35 ºC for 7 days, and the lowest concentration of miltefosine that failed to yield fungal growth was defined as the MFC.

Drug interactions. Interactions between miltefosine and itraconazole were assayed for the reference strain (ATCC MYA 4823) and also for two clinical isolates (Ss 37 and B737) with high MIC values (≥ 4 µg ml⁻¹) for itraconazole, using the checkerboard microdilution method (Eliopoulos & Moellering, 1991). Concentrations of 0.03–16 µg ml⁻¹ for itraconazole and 0.25–16 µg ml⁻¹ for miltefosine were tested. MICs were determined for drugs alone and for their combinations, and the fractional inhibitory concentration index (FICI) was used to classify drug interactions. FICI values were determined using the equation: FICI = (MICin combination/MICa tested alone) + (MICb in combination/MICb tested alone), for drugs itraconazole (a) and miltefosine (b) tested against the same fungal strain (Eliopoulos & Moellering, 1991). Interactions were considered synergistic if FICI ≤ 0.5, absent if FICI >0.5 and ≤4 and antagonistic if FICI >4 (Odds, 2003).

Time kill assays. For time kill assays, S. brasiliensis ATCC MYA 4823 yeasts (10⁶ c.f.u. ml⁻¹ starting inoculum) were exposed to different concentrations of miltefosine (0, MIC/2, MIC, MIC × 3, MIC × 4, MIC × 8) in RPMI 1640 medium supplemented with 2 % glucose and buffered with 0.165 M MOPS (pH 7.2), for 24, 48, 72, 96 and 168 h, at 35 ºC. Treated cultures were diluted and 50 µl of diluted cultures was plated on PDA medium and incubated at 35 ºC for 7 days before c.f.u. counting. Fungalidal activity was defined as a reduction of ≥ 99.9 % in the number of c.f.u. relative to that found in the starting inoculum; otherwise, the activity was considered fungistatic (Klepser et al., 1998).

Membrane integrity assay. S. brasiliensis yeast cells (ATCC MYA 4823) were treated with subinhibitory concentrations (1/2 MIC) of miltefosine, amphotericin B or itraconazole for 24 h at 35 ºC. Untreated and treated yeasts were washed in PBS, and cells were incubated with 10 µM of SYTOX Blue dead cell stain (Molecular Probes) for 20 min at room temperature and in a dark chamber. After incubation, cells were washed in PBS and the fluorescence intensity was measured at 480 nm (excitation at 440 nm) using a Spectra-MAX 340 tuneable microplate reader (Molecular Devices).

Transmission electron microscopy. S. brasiliensis ATCC MYA 4823 yeast cells were treated with a subinhibitory concentration (1/2 MIC) of miltefosine for 24 h at 35 ºC. Untreated and treated cells were washed in PBS, fixed in 2.5 % glutaraldehyde and 4 % formaldehyde in 0.1 M cacodylate buffer (for 24 h at 4 ºC), and post-fixed in 1 % osmium tetroxide in 0.1 M cacodylate buffer containing 1.25 % potassium ferrocyanide and 5 mM CaCl₂ for 2 h at 4 ºC. Then, cells were dehydrated in ethanol and embedded in Spurr resin. Ultrathin sections were stained in uranyl acetate and lead citrate, and observed in a JEOL 1200 EX electron microscope. Cell wall thickness values – CW (thickness of the innermost cell wall layer) and ML (thickness of the outer microfibrilar layer of cell wall) – were measured in 20 cells for each sample, using Image J software (NIH).

Flow cytometry analysis. Untreated and miltefosine-treated S. brasiliensis ATCC MYA 4823 yeast cells (treated with subinhibitory concentration (1/2 MIC) for 24 h at 35 ºC) were washed in PBS, fixed in 4 % of formaldehyde in PBS, incubated with 1 % BSA for 30 min and then labelled with anti-melanin antibodies (0.1 mg ml⁻¹, for 30 min at room temperature) purified from sera obtained from chromoblastomycosis patients (Alviano et al., 2004). Then, samples were incubated with FITC-labelled anti-human IgG (Molecular Probes) for 30 min at room temperature and analysed in a BD Accuri C6 flow cytometer (10 000 events/sample). Data were analysed
using the BD Accuri C6 Software. Negative control samples were not incubated with melanin antibodies.

**Cytotoxicity test.** For cytotoxicity testing, aliquots of 100 μl of LLC-MK2 (Rhesus monkey kidney epithelial cells) cell suspensions at 5×10⁴ cells ml⁻¹ were dispensed onto a 96-well microtitre and incubated for 24 h. Then, monolayers were treated with different concentrations of miltefosine or amphotericin B (1–100 μg ml⁻¹) for 48 h, at 37 °C and 5% CO₂. Cell viability was analysed by the tetrazolium (XTT) reduction assay and selectivity indexes (SIs) were calculated using the formula: SI=CC₅₀/MIC. CC₅₀ values correspond to the concentration that killed 50% of LLC-MK2 cells.

**Statistical analysis.** Statistical analyses were performed using the GraphPad Prism 5.0 software. Wilcoxon’s tests were used to analyse differences in cell wall thickness values. Linear regression was used to analyse correlations between MIC values. One-way ANOVA was used (with Dunnett’s post hoc test) to compare membrane integrity evaluation results. A 5% significance level was adopted (P<0.05).

**RESULTS**

The yeast form of *S. brasiliensis* is sensitive to low concentrations (≤2 μg ml⁻¹) of miltefosine

The sensitivity of the infective yeast form of *S. brasiliensis* to miltefosine in vitro was established by determining MIC and MFC values, using reference protocols, and these values were compared to those reported previously for the standard antifungals amphotericin B and itraconazole (Table 1). *S. brasiliensis* clinical isolates displaying high MIC values (MIC ≥4 μg ml⁻¹) for amphotericin B or itraconazole (Borba-Santos et al., 2014) were sensitive to miltefosine (MIC ≤2 μg ml⁻¹), with MIC values between 1 and 2 μg ml⁻¹ for all isolates tested (Table 1). Miltefosine was more potent than amphotericin B (P=0.005) and itraconazole (P=0.03) against all clinical *S. brasiliensis* isolates tested, and the reference strain ATCC MYA 4823 (from feline sporotrichosis) was also susceptible to miltefosine treatment. No significant correlation was found between MIC values for amphotericin B, itraconazole and miltefosine (r<0.4).

According to MFC values, miltefosine was also more potent against *S. brasiliensis* than both amphotericin B and itraconazole (Table 1). Additionally, time kill assays performed using the reference strain (ATCC MYA 4823) showed that miltefosine has fungicidal activity towards *S. brasiliensis*, with 2 μg ml⁻¹ (MIC×2) of this drug resulting in complete culture growth elimination after as little as 24 h of treatment (Fig. 1). After prolonged treatment (168 h or 7 days) with miltefosine, 1 μg ml⁻¹ (MIC) and 0.5 μg ml⁻¹ (MIC/2) of this drug inhibited 100% and ~80% (1log₁₀) of starting inoculum growth, respectively (Fig. 1).

Combinations of itraconazole with miltefosine showed no synergistic effect against yeast cells from the isolates ATCC MYA 4823, Ss 37 and B735 (0.5<FICI ≤4).

**Exposure to miltefosine disrupts yeast membrane integrity, affects membrane-to-cell wall interactions and alters cell wall structure, increasing melanin content in the cell wall**

To identify early changes in fungal morphology after treatment with miltefosine, *S. brasiliensis* yeast cells were treated with subinhibitory concentration of this drug (0.5 μg ml⁻¹) for 24 h, and plasma membrane integrity and ultrastructural characteristics were analysed.

Miltefosine promoted loss of plasma membrane integrity in *S. brasiliensis* yeast cells, as demonstrated by the increase in SYTOX blue fluorescence intensity of cell populations (P<0.001) (Fig. 2). In contrast, no statistically significant loss of membrane integrity was observed after treatment with subinhibitory concentrations of amphotericin B and itraconazole (Fig. 2).

In transmission electron microscopy (TEM) images, untreated *S. brasiliensis* yeast cells displayed a cell wall with an outer microfibrillar layer (ML) and a compact inner layer (CW*), a plasma membrane with normal shape, and a cytoplasm containing a nucleus (N), mitochondria (m) and vacuoles (v) (Fig. 3a, b). *S. brasiliensis* yeast cells also exhibited an accumulation of electron-dense material in the cell wall (arrow in Fig. 3b). Teixeira et al. (2010) have described similar electron-dense material inside the cell wall as melanin deposits. Treatment with 0.5 μg ml⁻¹ of miltefosine induced changes in the ultrastructure of *S. brasiliensis* yeast cells, such as: decrease in cytoplasmic electron density (Fig. 3c), decrease in CW* thickness and an increase in ML thickness (Fig. 3e). The exposure to miltefosine also appeared to induce accumulation and aggregation of electron-dense material in the cell wall.

To confirm that miltefosine promoted melanin accumulation, yeasts treated with this drug were labelled with anti-melanin antibodies. Flow cytometry analysis of labelled cells revealed that yeasts treated with miltefosine had increased melanin content compared with untreated yeasts: fluorescence intensity mean equal to 232.7 ± 2.6 and 160.4 ± 1.3 to treated and untreated, respectively.

**Miltefosine is more selective than amphotericin B towards *S. brasiliensis* yeast cells**

The cytotoxicity of miltefosine towards host cells in vitro was determined by treating mammalian tissue culture cells (LLC-MK2) with different concentrations of the drug (Table 2). The miltefosine CC₅₀ (the concentration that reduced LLC-MK2 cell viability by 50%) was 5 μg ml⁻¹, a value similar to that obtained for amphotericin B (6.25 μg ml⁻¹). Despite the similarities between miltefosine and amphotericin B CC₅₀ values, the lower MIC values for the treatment of *S. brasiliensis* yeasts with miltefosine meant that the selectivity indexes (SI=CC₅₀/MIC) for this drug against *S. brasiliensis* were higher than those determined for amphotericin B (Table 2).
DISCUSSION

Sporotrichosis is an endemic subcutaneous mycosis acquired by traumatic inoculation and has gained medical and epidemiological importance in recent years (Barros et al., 2010). The development of new therapeutic alternatives against sporotrichosis, and particularly against the relatively drug-resistant and highly virulent species S. brasiliensis, is important to prevent further spread of the disease. In the present work, we showed that S. brasiliensis isolates that have low susceptibility to amphotericin B or itraconazole (Borba-Santos et al., 2014) were sensitive to the potent antiparasitic drug miltefosine, which induced loss of plasma membrane integrity and increase in the melanin content of fungal cells. In addition to its antifungal activity, miltefosine exhibited a higher selectivity towards the fungus when compared to the standard antifungal amphotericin B.

Table 1. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) for the treatment of Sporothrix brasiliensis yeasts with miltefosine, compared to reported values for amphotericin B and itraconazole (Borba-Santos et al., 2014). All values are in μg ml⁻¹.

<table>
<thead>
<tr>
<th>S. brasiliensis isolates</th>
<th>Miltefosine</th>
<th>Amphotericin B†</th>
<th>Itraconazole†</th>
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<tr>
<td></td>
<td>MIC</td>
<td>MFC</td>
<td>MIC</td>
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<tr>
<td>MYA 4823*</td>
<td>1</td>
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</tr>
<tr>
<td>B91</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>B182</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>B628</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>B735</td>
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<td>2</td>
<td>2</td>
</tr>
<tr>
<td>B848</td>
<td>2</td>
<td>&gt;16</td>
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<tr>
<td>B972</td>
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<td>2</td>
<td>4</td>
</tr>
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<td>B1008</td>
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<td>2</td>
<td>4</td>
</tr>
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<td>B1036</td>
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</tr>
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<td>4</td>
</tr>
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<td>4</td>
</tr>
<tr>
<td>Ss 56</td>
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<td>4</td>
</tr>
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*Reference (genome) strain, from feline sporotrichosis. All other strains are from human sporotrichosis.
†MIC and MFC values for amphotericin B and itraconazole reported in Borba-Santos et al. (2014).

Fig. 1. Time-kill plot showing the activity of miltefosine against the yeast form of Sporothrix brasiliensis ATCC MYA 4823. Yeast cell suspensions (10³ c.f.u. ml⁻¹) were treated with different concentrations of miltefosine at 35 °C, diluted and plated on PDA. Plates were incubated for 7 days at 35 °C, and c.f.u. numbers were determined by manual counting. Experiments were carried out in duplicate.

Fig. 2. Membrane integrity evaluation after treatment of Sporothrix brasiliensis (ATCC MYA 4823) yeast cells with miltefosine, amphotericin B and itraconazole. Cells were left untreated (control) or were treated with 0.5 μg ml⁻¹ miltefosine, or with 0.25 μg ml⁻¹ of amphotericin B or itraconazole, for 24 h, before staining with the exclusion dye SYTOX blue (Molecular Probes). Treatment with miltefosine only induced a statistically significant loss in plasma membrane integrity compared to untreated controls (**P<0.001).
Brilhante et al. (2014) have showed recently that miltefosine is active against the filamentous forms of S. brasiliensis. In a previous study, we demonstrated that the filamentous and yeast forms of S. brasiliensis isolates did not have the same susceptibility profile to antifungals (Borba-Santos et al., 2014). In the present work, we focus our studies on the yeast (pathogenic) form of S. brasiliensis that presented high MIC values for either of the standard antifungal agents itraconazole or amphotericin B (Borba-Santos et al., 2014). Here, we observed that miltefosine has a good antifungal activity towards the pathogenic yeast form of S. brasiliensis, and that MIC values for yeast forms of isolates that presented high treatment MIC values for either of the standard antifungal agents itraconazole or amphotericin B to miltefosine ranged from 1 to 2 μg ml\(^{-1}\) (Table 1).

Table 2. Analyses of miltefosine and amphotericin B cytotoxicity towards the epithelial cell line LLC-MK2 (CC\(_{50}\)) and selectivity index (SI) for the treatment of Sporothrix brasiliensis yeast cells

<table>
<thead>
<tr>
<th>Antifungal agents</th>
<th>LLC-MK2</th>
<th>Mean MIC* (μg ml(^{-1}))</th>
<th>CC(_{50}) (μg ml(^{-1}))</th>
<th>SI†</th>
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<tr>
<td>Miltefosine</td>
<td>2</td>
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</tr>
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<td>Amphotericin B</td>
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<td>6.25</td>
<td>1.56</td>
<td></td>
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</tbody>
</table>

*Mean MIC values for treatment of the yeast form of S. brasiliensis.  †SI=CC\(_{50}\)/MIC.

Fig. 3. TEM analysis of Sporothrix brasiliensis ATCC MYA 4823 yeast cells treated with miltefosine. Untreated yeasts (a and b) had a normal morphology, with a cytoplasm containing a nucleus (N), mitochondria (m) and vacuoles (v), as well as a compact cell wall, composed of an inner layer of cell wall proper (CW*') surrounded by an outer microfibrillar layer (ML) (b). Yeast cells treated with 0.5 μg ml\(^{-1}\) miltefosine for 24 h (c and d) exhibited a decrease in cytoplasmic electron density, aggregation of electron-dense material in the cell wall (arrow in d), decreased CW* thickness and increased ML thickness (d, and quantification in e). Scale bars, 1 μm (a, c) and 0.2 μm (b, d); *P<0.05.
larger spectrum of MIC values (0.5–4 μg ml⁻¹) was reported by Brilhante et al. (2014) for the treatment of the mycelial form of S. brasiliensis. The MIC values for miltefosine reported here are in line with the 2 μg ml⁻¹ value reported for the treatment of Candida albicans, Candida glabrata, Candida kruzei, Cryptococcus gatti, Cryptococcus neoformans, Aspergillus fumigatus and Trichophyton mentagrophytes with this drug (Widmer et al., 2006; Tong et al., 2007; Ravu et al., 2013).

We did not observe linear correlations between MIC values for miltefosine, amphotericin B and itraconazole (r<0.4), suggesting the absence of cross-resistance to these drugs. Moreover, low MFC values (in the 1–>16 μg ml⁻¹ range) confirm that miltefosine is potent against the tested isolates, and time kill experiments indicated that miltefosine has fungicidal activity against the yeast form of S. brasiliensis, in agreement with that previously described for other fungal species, including Candida sp., Cryptococcus sp. and Aspergillus sp. (Widmer et al., 2006; Ravu et al., 2013), and also for the mycelial form of S. schenckii complex species (Brilhante et al., 2014).

Synergistic effects of miltefosine-azoles combinations have been described for some fungal species (Biswa et al., 2013); however, we did not detect any interactions between itraconazole and miltefosine when treating S. brasiliensis yeast cells from the reference strain ATCC MYA 4823, or from two clinical isolates with high MIC values for itraconazole (Ss 37 and B735).

SYTOX blue staining of yeasts treated with miltefosine indicates that this phospholipid analogue promotes cell death due to pronounced disturbances in the plasma membrane, among other effects described previously. Miltefosine may have several cellular targets, due to its ubiquitous effects on cell membranes (Barratt et al., 2009). In Trypanosoma cruzi and Leishmania species, the drug alters the phospholipid and sterol contents of cells (Barratt et al., 2009), promoting a reduction in the amounts of phospholipids, ergosterol and fatty acids (Dorlo et al., 2012). Despite the antifungal activity of miltefosine and its potential as a therapeutic alternative to treat fungal infections, the mode of action of this drug in fungi is poorly understood. Zuo et al. (2011) demonstrated that miltefosine is rapidly incorporated into Saccharomyces cerevisiae yeast cells, where it crosses the mitochondrial inner membrane, penetrating the mitochondria and leading to an apoptosis-like cell death. In S. brasiliensis, miltefosine may act on the synthesis of membrane components, which may promote instability and loss of membrane integrity, ultimately leading to yeast cell death.

We also showed by TEM that the exposure to miltefosine promoted considerable changes in S. brasiliensis yeast cells, including: loss of electron density (Fig. 3c), accumulation of electron-dense material in the cell wall (arrow in Fig. 3d) and alterations in cell wall thickness (Fig. 3e). Alterations in cell wall components after miltefosine exposure were also described in C. albicans yeasts (Vila et al., 2013).

However, the aggregation of electron-dense material in the cell wall following miltefosine treatment had not been observed previously for other fungal species. This electron-dense material visualized by TEM is morphologically similar to the melain granules described by Teixeira et al. (2010). In addition, flow cytometry analysis using anti-melanin antibodies showed that miltefosine-treated cells had increased melanin content compared to untreated cells. Thus, our results suggest that miltefosine treatment could modulate melanin turnover (breakdown and/or production) in S. brasiliensis yeast cells, leading to the accumulation of aggregates of this pigment in the cell wall. Similarly, Teixeira et al. (2010) also showed that the amount of melanin in the cell wall of S. schenckii could be modulated by varying culture medium composition (Teixeira et al., 2010).

Although miltefosine displayed relatively high cytotoxicity towards kidney cells, this is comparable to that observed for amphotericin B, the only available treatment option for severe forms of sporotrichosis. Moreover, in vivo tests with BALB/c mice infected with L. amazonensis showed that miltefosine was toxic only at doses higher than 50 mg kg⁻¹ day⁻¹ (Godinho et al., 2012). Given the lower MIC values for miltefosine against S. brasiliensis in vitro, this drug shows higher selective indexes than amphotericin B for the isolates tested (Table 2). Thus, miltefosine is likely to have a wider ‘therapeutic window’ than amphotericin B against S. brasiliensis sporotrichosis, which should allow the use of lower effective doses of this drug, to limit toxicity. Another advantage of miltefosine treatment is that this drug can be administrated orally, whereas amphotericin B treatment is intravenous.

Miltefosine is already used to treat some of the clinical manifestations of leishmaniasis (Dorlo et al., 2012), which should facilitate its approval for use in the treatment of other infectious diseases. Interestingly, miltefosine was approved for the topical treatment of metastasized mammary carcinoma in Germany (Papazafiri et al., 2005), in 1992; thus, topical administration of miltefosine – which might be particularly useful for sporotrichosis treatment – may also be effective in patients. Although some resistance to miltefosine has been described in patients infected with Leishmania donovani, the main resistance mechanisms seem to be associated with a defect in translocation of the drug to the cytoplasm (Pérez-Victoria et al., 2003).

The dose of miltefosine used for the treatment of leishmaniasis in humans is 2.5 mg kg⁻¹ day⁻¹, for 28 days (Dorlo et al., 2012). The reported IC₅₀ value for the treatment of intracellular amastigotes of Leishmania amazonensis with miltefosine in vitro was 9 μM (3.6 μg ml⁻¹) (Santa-Rita et al., 2004), which is higher than the MIC values of 2.5–5 μM (1–2 μg ml⁻¹) reported here for the treatment of S. brasiliensis isolates. Therefore, our results indicate that lower miltefosine doses than those used against human leishmaniasis could be effective in the treatment of sporotrichosis.
In conclusion, our results show that miltefosine might represent an interesting alternative for the treatment of sporotrichosis caused by \textit{S. brasiliensis}, especially when standard treatment fails. Nevertheless, further \textit{in vivo} studies in experimentally infected animals are required to confirm the efficacy of miltefosine as an antifungal agent for the treatment of sporotrichosis.

**ACKNOWLEDGEMENTS**

The authors thank Dr Zoilo Pires de Camargo and Dr Anderson Messias Rodrigues from the Universidade Estadual de São Paulo (São Paulo, SP, Brazil), Dr Leila Maria Lopes-Bezerra from Universidade Estadual do Rio de Janeiro (Rio de Janeiro, RJ, Brazil) and Dr Marcio Nucci from the Universidade Federal do Rio de Janeiro (Rio de Janeiro, RJ, Brazil) for kindly providing the \textit{S. brasiliensis} isolates used in this study. This study was supported by the Brazilian funding agencies Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior and Conselho Nacional de Desenvolvimento Científico e Tecnológico.

The authors report no conflict of interests.

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