Clotrimazole is highly effective in vitro against feline Sporothrix brasiliensis isolates

Thalita Gagini,1 Luana Pereira Borba-Santos,1,* Anderson Messias Rodrigues,2 Zoilo Pires de Camargo2 and Sonia Rozental1

Abstract

Purpose. Sporothrix brasiliensis, the most virulent species in the Sporothrix schenckii complex, is responsible for the ongoing epidemics of human and animal sporotrichosis in Brazil. Feline outbreaks are usually driven by S. brasiliensis and followed by extensive transmission to humans. Itraconazole is the first-line treatment for both feline and human sporotrichosis; however, reduced sensitivity is an emerging issue. Thus, we investigated the effect of the widely used antifungal clotrimazole – alone or in combination with itraconazole – against the pathogenic (yeast) form of feline and human S. brasiliensis isolates, in vitro.

Methodology. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values were determined for treatment with clotrimazole and itraconazole, as monotherapy or in combination. In addition, the effect of the drugs on neutral lipid levels and the yeast ultrastructure were evaluated by flow cytometry and transmission electron microscopy (TEM), respectively.

Results. The MIC and MFC values show that clotrimazole was more effective than itraconazole against feline S. brasiliensis isolates, while human isolates were more sensitive to itraconazole. Similarly to itraconazole, treatment with clotrimazole induced statistically significant neutral lipid accumulation in S. brasiliensis yeasts, and treated yeasts displayed irregularities in the cell membrane and a thicker cell wall when observed by TEM. Clotrimazole increased the antifungal activity of itraconazole in combination assays, with a synergistic effect for two feline isolates.

Conclusion. The strong activity of clotrimazole against feline S. brasiliensis isolates suggests that this drug is potentially a new alternative for the treatment of feline sporotrichosis, alone or in combination with itraconazole.

INTRODUCTION

Sporotrichosis is a chronic subcutaneous mycosis with worldwide distribution, although it is more common in tropical and subtropical areas. It is caused by thermo-dimorphic fungi from the Sporothrix schenckii complex [1]. In the last 15 years, epizooties of sporotrichosis in cats reached epidemic levels in the Brazilian state of Rio de Janeiro, where more than 4000 cats had been diagnosed by 2015 [2]. In this epidemic scenario, large-scale epizootic and zoonotic transmission occurred through bites and scratches from diseased animals, and the disease quickly spread to other urban areas with high feline population densities [3]. Measuring the impact of sporotrichosis in endemic areas is difficult due to the lack of reliable data on disease prevalence.

Sporothrix brasiliensis is the most virulent species from the Sporothrix schenckii complex [4, 5], and it is restricted to Brazil, showing high prevalence in the current epizooties among cats in the Southeast and South regions [2]. The predominance of S. brasiliensis in the Brazilian epizooties may have severe implications for the emergence of S. brasiliensis infections in humans, and may be linked to the more severe and atypical manifestations of human sporotrichosis reported recently [6].

The most common manifestations of feline sporotrichosis are ulcerated lesions on the skin (deep wounds) that do not heal and tend to evolve quickly [7]. The disease is typically more serious in cats than in humans, and frequently evolves to the systemic disseminated form, which is often fatal [8].
A major difference between human and feline sporotrichosis is that feline lesions have a higher yeast load, which facilitates both horizontal (cat-to-cat) and zoonotic (cat-to-human) transmission through bites or scratches. Veterinarians and cat owners are particularly at risk of acquiring sporotrichosis [9, 10].

Treatment of feline and human sporotrichosis is based on the use of itraconazole [8], a triazole antifungal that inhibits ergosterol biosynthesis by blocking the activity ofsterol 14 alpha-demethylase, a microsomal P-450-dependent enzyme [11], leading to the accumulation of toxic intermediates, and altering cell membrane permeability [12]. Sporotrichosis treatment of felines usually requires long-term (up to 9 months) therapy with itraconazole (5–10 mg kg⁻¹ day⁻¹), and cure rates vary considerably, with reports of therapeutic failures, adverse side-effects and relapses [8]. In addition, the recent emergence of isolates with reduced susceptibility to itraconazole has increased concerns about treatment failure [13–15]. Altogether, the side-effects of itraconazole, its therapeutic failures and the emergence of drug-insensitive isolates highlight the importance of searching for new alternatives to treat feline sporotrichosis.

Clotrimazole is an imidazole antifungal that is used extensively in the topical treatment of numerous fungal infections [16]. Clotrimazole is also effective in the treatment of some parasitic diseases, such as malaria, beriberi and Chagas disease [16–20]. Similarly to itraconazole, the mechanism of action of clotrimazole is the inhibition of sterol 14 alpha-demethylase activity, which blocks ergosterol biosynthesis [17]. Clotrimazole also inhibits the Ca²⁺-activated potassium channel and causes heme-induced membrane damage [16, 19, 21]. Its success and utility in topical treatments (while systemic treatment has important side-effects) granted clotrimazole a place on the list of essential medicines compiled by the World Health Organization (WHO) [16].

Clotrimazole shows broad-spectrum antifungal activity, inhibiting the growth of fungal species as diverse as Aspergillus spp., Candida spp., Trichophyton spp., Microsporum spp., Malassezia furfur, Microsporum canis and Epidermophyton floccosum, with MIC values ranging from 0.1 to 6.3 µg ml⁻¹ [22–25]. The filamentous form of S. schenckii is also susceptible to clotrimazole, with MIC values ranging from 0.5 to 8 µg ml⁻¹ [22, 26, 27]. These studies were conducted between the 1970 and 1980s (shortly after clotrimazole was first synthesized [28]), when no other Sporothrix species had been identified as pathogenic to humans [29]. Thus, the activity of clotrimazole against sporotrichosis agents has received little attention in recent years, as S. brasiliensis emerged as an important pathogenic species. Consequently, the antifungal activity of clotrimazole against S. brasiliensis yeasts has not been evaluated to date.

In this study, we determined the in vitro antifungal activity of clotrimazole against feline and human isolates of S. brasiliensis, in the pathogenic yeast form. We compared the effect of clotrimazole monotherapy with that of itraconazole, and also tested the effectiveness of combining both agents against S. brasiliensis. In addition, we determined whether treatment with clotrimazole increases neutral lipid levels in S. brasiliensis yeasts, as expected for ergosterol synthesis inhibition, and evaluated the ultrastructural alterations that exposure to clotrimazole effected in yeasts using transmission electron microscopy (TEM).

**METHODS**

**Fungal isolates and culture conditions**

Ten feline S. brasiliensis isolates from different Brazilian states (ATCC MYA 4823, Ss152, Ss153, Ss154, Ss247, Ss248, Ss249, Ss297, Ss299 and Ss300) [30, 31] and 10 human S. brasiliensis isolates from the Rio de Janeiro state (ATCC MYA 4824, B204, B428, B435, B702, B735, B758, B818, B848 and B972) [14] were used in this study. The isolates were stored in potato dextrose agar (PDA; Difco, USA) plates, in the mycelial form, at 4°C. The yeast form, used in all experiments, was obtained by cultivating conidia (mycelial form) in brain heart infusion broth (BHI; Difco) supplemented with 2% glucose (pH 7.8) at 36°C for 7 days, under orbital agitation (at 150 r.p.m.).

**Antifungals**

Clotrimazole and itraconazole (Sigma-Aldrich Co., USA) were diluted in DMSO to yield stock solutions of 1600 µg ml⁻¹, which were kept at −20°C. Further dilutions were prepared in RPMI 1640 medium, as recommended in the Clinical and Laboratory Standards Institute (CLSI) M27-A3 document. Itraconazole was used as a reference antifungal.

**Minimum inhibitory concentration (MIC) assay**

MIC values for each drug were determined using a broth microdilution method adapted for use with S. brasiliensis yeasts, and based on the M27-A3 document [32], with the following minor modifications: (i) RPMI 1640 medium was supplemented with 2% glucose (buffered with 0.165 M MOPS at pH 7.2); (ii) the final yeast concentration was exposed to concentrations of clotrimazole or itraconazole ranging from 0.03 to 16 µg ml⁻¹ for 48 h, at 35°C, in a humid atmosphere of 5% CO₂; (iii) the MIC was determined by spectrophotometric readings. Yeasts were exposed to concentrations of clotrimazole or itraconazole ranging from 0.03 to 16 µg ml⁻¹ for 48 h, at 35°C, in a humid atmosphere of 5% CO₂. MIC values – corresponding to concentrations that inhibited ≥90% of fungal growth relative to the untreated control – were determined by visual inspection in an inverted optical microscope, and confirmed by spectrophotometric readings at 490 nm or 492 nm, using a microtiter plate reader (Spectra Max Plus or EMax Plus, Molecular Devices). The percentage of inhibition was calculated according to the equation: 100–(A x 100/C), where A is the absorbance value of the wells containing the test compound and C is absorbance of the control (untreated) wells. The results are representative of two independent experiments made in duplicate. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc., USA) with Wilcoxon’s test (Student’s t-test), which was used to analyse differences in the susceptibility of isolates to...
antifungals in each group (feline and human isolates), and Mann–Whitney’s test (Student’s t-test), which was used to compare the antifungal efficacy of the two groups of isolates. Statistical significance was accepted when \( P<0.05 \).

**Minimum fungicidal concentration (MFC) assay**

To determine the MFC values, 15 \( \mu l \) aliquots were recovered from samples at the end of the MIC experiments, plated onto PDA drug-free medium, and then incubated for 5 days at 35 \(^\circ\)C before visual readings. The MFC was defined as the lowest drug concentration able to fully prevent fungal growth.

**Flow cytometry analysis**

The genome strain ATCC MYA 4823 (strain 5110 [33]) was used as a representative of the \( S. \) brassiliensis species in subsequent experiments. To quantify the lipid levels, yeasts were treated with sub-inhibitory concentrations (1/4 MIC) of clotrimazole or itraconazole for 24 h, and then \( 2\times10^7 \) yeasts \( ml^{-1} \) were stained with 20 \( \mu M \) of BODIPY 493/503 (Molecular Probes) for 30 min in the dark at room temperature. Then, cells were washed in PBS and fixed in 1 % formaldehyde for 40 min. The fluorescence intensity was measured in a BD Accuri C6 flow cytometer (Becton and Dickinson Biosciences, EUA) by acquiring 10,000 events/sample. The data were analysed using BD Accuri C6 software (Becton and Dickinson Biosciences). The results are representative of two independent experiments made in duplicate. Bonferroni’s test (one-way ANOVA, GraphPad Prism 5.0) was applied to analyse differences in fluorescence intensity. Statistical significance was accepted when \( P<0.05 \).

**Transmission electron microscopy**

\( S. \) brassiliensis ATCC MYA 4823 yeasts treated with sub-inhibitory concentrations (1/4 MIC) of clotrimazole or itraconazole for 24 h were washed in PBS and fixed with 2.5 % glutaraldehyde, 4 % formaldehyde and 5 mM \( \text{CaCl}_2 \) in 0.1 M cacodylate buffer for 24 h at 4 \(^\circ\)C. The post-fixed samples were washed in 0.1 M cacodylate buffer containing 1.25 % potassium ferrocyanide for 2 h at 4 \(^\circ\)C. The post-fixed samples were washed in 0.1 M cacodylate buffer, dehydrated in an ethanol series (30, 50, 70, 90 and 100 %) for 30 min in each step, and embedded in Spurr’s resin. Ultrathin sections were stained in uranyl acetate and lead citrate, and observed in a Zeiss EM 900 transmission electron microscope (Zeiss, Germany). The cell wall thickness was measured in 50 cells per sample, using Image J software (NIH, USA). The cell wall thickness values were compared using one-way ANOVA (Bonferroni’s test) in GraphPad Prism 5.0. Statistical significance was accepted when \( P<0.05 \).

**Combination assay between clotrimazole and itraconazole**

The combined activity of clotrimazole and itraconazole against 10 \( S. \) brassiliensis isolates was evaluated using the checkerboard microdilution method [34]. Yeasts were treated for 48 h with concentrations of clotrimazole ranging from 0.015 to 16 \( \mu g \) \( ml^{-1} \) and concentrations of itraconazole ranging from 0.002 to 16 \( \mu g \) \( ml^{-1} \), and MIC values were determined for each antifungal alone and for combinations. The most effective antifungal combinations were identified as those having the lowest fractional inhibitory concentration index (FICI), which was calculated according the equation: \( \text{FICI}=(\text{MIC}_{\text{clotrimazole \ alone}}+(\text{MIC}_{\text{itraconazole \ alone}})/(\text{MIC}_{\text{clotrimazole \ in \ combination/MIC}_{\text{itraconazole \ in \ combination}}) \). Interactions were considered to be synergistic if \( \text{FICI} \leq 0.5 \) [35].

**RESULTS**

**Clotrimazole was more active than itraconazole against feline \( S. \) brassiliensis isolates**

Clotrimazole is widely used, in its topical formulations, to treat a variety of fungal infections, but its activity against the important sporotrichosis agent \( S. \) brassiliensis had not yet been studied. To evaluate the inhibitory effect of clotrimazole against \( S. \) brasiliensis yeasts (the infective form) from feline and human sporotrichosis isolates, we determined the MIC and MFC values and compared them with those obtained for itraconazole, the current first-line treatment for sporotrichosis (Fig. 1, Tables 1 and S1, available in the online Supplementary Material). According to the MIC values, clotrimazole was more active than itraconazole against feline \( S. \) brassiliensis isolates (\( P=0.0220 \)) (Fig. 1), with MIC values ranging from 0.25 to 4 \( \mu g \) \( ml^{-1} \) (geometric mean=0.8 \( \mu g \) \( ml^{-1} \)), while the MIC values for itraconazole ranged from 0.5 to 16 \( \mu g \) \( ml^{-1} \) (geometric mean=1.8 \( \mu g \) \( ml^{-1} \)) (Table S1).

In contrast, human isolates were more susceptible to itraconazole than clotrimazole (\( P=0.0104 \)) (Fig. 1). Remarkably, two feline isolates (Ss153 and Ss248) with reduced in vitro susceptibility to itraconazole (MIC=16 \( \mu g \) \( ml^{-1} \)) were

![Fig. 1. Minimum inhibitory concentrations (MICs) of clotrimazole and itraconazole against human and feline isolates of Sporothrix brassilien-
sis in the pathogenic yeast form. Bars represent the geometric mean of MIC values. *\( P<0.05 \), by Wilcoxon’s test and Mann–Whitney’s test.](image-url)
Table 1. Fungicidal activity of clotrimazole and itraconazole against yeasts from Sporothrix brasiliensis isolates from cats and humans. Values represent the proportion of isolates to which a drug concentration was fungicidal (≤ MFC value).

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<th>Proportion of isolates with MFC value (µg ml⁻¹)</th>
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<td>Feline isolates</td>
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<td>Human isolates</td>
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<td>Itraconazole</td>
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Sensitive to clotrimazole (MIC ≤ 4 µg ml⁻¹) (Table S1). Overall, the feline S. brasiliensis isolates tested here were more sensitive to clotrimazole than the human isolates (P=0.0184). MFC analysis also showed that clotrimazole has strong fungicidal activity against S. brasiliensis yeasts, because it was able to kill yeasts at lower concentrations than itraconazole (Tables 1 and S1). Most of the feline and human isolates tested here (9/10 and 7/10 of isolates, respectively) were killed by treatment with up to 16 µg ml⁻¹ clotrimazole (Table 1), while itraconazole was not fungicidal against human S. brasiliensis isolates at this concentration.

Clotrimazole induced the accumulation of neutral lipids in S. brasiliensis yeasts, similarly to itraconazole

Clotrimazole and itraconazole alter lipid metabolism by inhibiting ergosterol biosynthesis, leading to the accumulation of neutral lipid intermediates in treated cells [12]. In light of the potent antifungal activity of clotrimazole against feline S. brasiliensis isolates, we evaluated the effects of this drug on neutral lipid accumulation in S. brasiliensis yeasts using a reference feline isolate (ATCC MYA 4823), and compared with the effects of itraconazole. Yeast cells treated with 0.125 µg ml⁻¹ clotrimazole or 0.25 µg ml⁻¹ itraconazole (corresponding to 1/4 MIC) were stained with the neutral lipid marker BODIPY 493/503 to estimate lipid accumulation. Clotrimazole increased the neutral lipid levels in S. brasiliensis yeasts considerably (by approximately two-fold) (P<0.0001), as demonstrated by the increase in BODIPY 493/503 fluorescence intensity by flow cytometry (Fig. 2). Clotrimazole induced similar lipid accumulation to itraconazole, at a much lower concentration (half that of itraconazole) (P>0.05).

Clotrimazole treatment altered cell membrane and cell wall structure

S. brasiliensis yeasts treated with sub-inhibitory concentrations of clotrimazole were also analysed by TEM to evaluate the alterations in intracellular morphology caused by this drug in comparison with itraconazole (used as a positive control). Untreated yeasts showed an electron-dense cytoplasm containing mitochondria and vacuoles, surrounded by a regular cell membrane and a compact cell wall, formed by a thin electron-lucent inner layer and a delicate fibrillary outer layer (Fig. 3a, b). The main alterations observed after clotrimazole or itraconazole exposure were changes in the cell membrane and cell wall morphology (Fig. 3c–f). Yeast treated with clotrimazole and itraconazole displayed frequent irregularities in the plasma membrane (arrows in Fig. 3d, f). The cell walls of the clotrimazole-treated yeasts were thicker than those of untreated yeasts (mean thickness of 263±53 and 119±21 nm for clotrimazole-treated and untreated, respectively) (P<0.0001). Itraconazole treatment also increased yeast cell wall thickness (to 240±54 nm) relative to that in the untreated control (P<0.0001). The cell walls of yeasts treated with clotrimazole (at half the concentration of itraconazole) were significantly thicker than those of yeasts treated with itraconazole (P<0.05).

Clotrimazole potentiated the inhibitory activity of itraconazole

To verify whether clotrimazole is able to improve the activity of itraconazole against feline and human S. brasiliensis isolates, we tested combinations of these antifungals against 10 isolates. When yeasts were treated with a combination of clotrimazole and itraconazole, lower concentrations of both antifungals were necessary to inhibit proliferation, as demonstrated by a reduction in MIC values for both drugs (Table 2). Although the combination of clotrimazole with
S. brasiliensis showed that clotrimazole is effective, expensive and require long-term treatment [36]. Here, we few therapeutic options to treat sporotrichosis, and they are reached epidemic levels [2]. However, there are currently the world, especially in Brazil, where recent epizooties Sporotrichosis is an important public health issue around DISCUSSION zole (Table 2).

lates when this drug was used in combination with clotrima-
values for itraconazole treatment were lower for all the iso-
/C20
itraconazole only had a synergistic antifungal effect (FICI
0.5) against two feline isolates, Ss 153 and Ss 154, the MIC 
values for itraconazole treatment were lower for all the iso-
ates when this drug was used in combination with clotrima-
zole (Table 2).

Fig. 3. Transmission electron microscopy analysis of Sporotrichosis brasi-
liensis ATCC MYA 4823 yeasts exposed to sub-inhibitory concentrations [1/4 minimum inhibitory concentration (MIC)] of clotrimazole or itracona-
zole. (a, c, e) Lower magnification images. (b, d, f) Higher magnifica-
tion images of the area marked by a white rectangle in the lower-
magnification image on the left. (a, b) Untreated yeasts displayed a 
cell wall [CW in (b)] with a thin electron-lucent inner layer, a delicate 
fibrillary outer layer and a regular plasma membrane that surrounded 
a homogeneous cytoplasm containing mitochondria (m) vacuoles (v) 
and a single nucleus (N). Yeast treated with 0.125 µg ml⁻¹ clotrima-
zole (c, d) or 0.25 µg ml⁻¹ itraconazole (e, f) displayed irregularities in 
the plasma membrane (arrows) and a thicker cell wall (d and f). Scale 
bars: 1 µm (a, c, e) and 0.25 µm (b, d, f).

S. brasiliensis isolates than itraconazole, the current first-
line treatment for sporotrichosis.

For our study we selected a set of S. brasiliensis isolates 
recovered from felines and humans with sporotrichosis in 
the main endemic areas of Brazil, i.e. Rio de Janeiro, Rio 
Grande do Sul and São Paulo [2] (Table S1). Remarkably, 
lower concentrations of clotrimazole were necessary to 
inhibit the proliferation of and kill S. brasiliensis yeasts from 
feline isolates (compared with human isolates), with a geo-
metric mean MIC value of 0.8 µg ml⁻¹, and an MFC <8 µg 
ml⁻¹ for most isolates. In contrast, treatment of feline 
S. brasiliensis isolates with itraconazole had a geometric 
mean MIC value of 1.8 µg ml⁻¹, and an MFC >16 µg ml⁻¹ 
for most isolates. The antiproliferative activity of clotrima-
zole against human S. brasiliensis isolates was lower than 
that of itraconazole (geometric mean MIC values of 2 and 
0.9 µg ml⁻¹ for clotrimazole and itraconazole, respectively); 
however, clotrimazole – but not itraconazole – was able to 
kill most human isolates at the highest drug concentration 
tested here (16 µg ml⁻¹).

Almeida-Paes et al. [37] reported that feline and human 
strains of S. brasiliensis had similar susceptibility to itracona-
zole, with a median MIC value of 1 µg ml⁻¹ [37]. These 
results support the suggestion that there is clonal transmis-
sion of sporotrichosis between cats and humans in Rio de 
Janeiro. Similarly, we observed no statistically significant 
differences in itraconazole MIC values between feline and 
human strains of S. brasiliensis (P=0.1787), although the 
geometric mean of the MIC values was different for the two 
groups (1.8 and 0.9 µg ml⁻¹ for clotrimazole and itraconazole, respectively). The feline isolates used in our study originated 
from three endemic areas in Brazil, and not just from Rio de 
Janeiro, and were tested in the yeast (pathogenic) form and 
not in the filamentous (saprophytic) form, which was used 
in the study by Almeida-Paes et al. [37].

For S. brasiliensis, an epidemiological ‘cutoff’ MIC value 
(ECV) of 2 µg ml⁻¹ for itraconazole was recently proposed 
by a multicentre study involving 17 laboratories worldwide 
[38]. Although the ECVs do not predict the clinical 
response to therapy, MIC values that are considerably 
higher than the ECV suggest that the isolate is less likely to 
respond to the antifungal [38]. Thus, isolates with MIC >2 
µg ml⁻¹ (non-wild-type isolates) exhibited reduced suscep-
tibility to itraconazole [38]. In our study, we observed two 
feline isolates with low susceptibility to itraconazole (MIC 
>16 µg ml⁻¹), which suggests that these isolates may be 
non-wild-type.

In general, our data showed that feline isolates had higher 
susceptibility to clotrimazole, in vitro, than human isolates. 
However, as ECVs have not yet been established for clotri-
maze, these data could not be correlated with the clinical 
response to therapy. We decided to focus our investigation 
on clotrimazole activity against feline S. brasiliensis isolates, 
which were more sensitive to this drug. Neutral lipid analy-
sis and electron microscopy data showed that clotrimazole


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induced cellular changes akin to those induced by itraconazole. Both azoles were able to increase the cellular neutral lipid levels significantly, and to a similar extent (−twofold vs the untreated control), in agreement with the expected mechanism of action based on the inhibition of sterol 14 alpha-demethylase [11, 39]. The concentration of clotrimazole tested was half that of the itraconazole concentration (0.125 and 0.25 µg ml⁻¹, respectively), corresponding to 1/4 MIC. These results suggest that both azoles can inhibit ergosterol synthesis, although clotrimazole was more efficient. After exposure to clotrimazole or itraconazole, Malassezia globosa displayed similar levels of neutral lipid accumulation to those observed here [40].

In a previous study, our group showed that treatment with itraconazole increases cell wall thickness in S. brasiliensis yeasts [14]. Here, using TEM, we showed that treatment with clotrimazole has a similar effect on S. brasiliensis yeasts, revealing that both clotrimazole and itraconazole induced changes in the cell membrane and increased cell wall thickness. The alterations in cell membrane morphology – the appearance of irregularities in the cell membrane – were in agreement with the inhibition of ergosterol synthesis, as indicated by neutral lipid accumulation. Cell membrane integrity is essential to maintain the cell wall structure [41]. Thus, the increase in cell wall thickness induced by clotrimazole, and also by itraconazole, is likely due to the loss of cell membrane integrity. Clotrimazole induced higher increases in cell wall thickness than itraconazole and, thus, may have a stronger negative effect on cell membrane integrity.

Clotrimazole has previously been tested in combination with botanicals, metabolites isolated from bacteria and even steroids showing synergetic effect [42–45]. However, few studies to date have evaluated combinations of clotrimazole with other antifungals [46, 47]. Given the stronger activity of clotrimazole compared with itraconazole against S. brasiliensis isolates, we tested whether the combination with clotrimazole could reduce the high MIC values for itraconazole observed for these isolates. For all of the strains tested here, the antifungal activities of both clotrimazole and itraconazole increased (i.e. MIC values decreased) when these drugs were used in combination, compared with monotherapy. It is important to point out that although the combination of clotrimazole with itraconazole only had a synergistic antifungal effect (FICI ≤0.5) against two feline isolates (Ss 153 and Ss 154), the MIC values for itraconazole were lower for all isolates when this drug was combined with clotrimazole.

Clotrimazole has well-known pharmacokinetics and pharmacodynamics, it has a well-known mechanism of action, it is available in various topical formulations (cream, solution and spray), and it is the drug of choice for the treatment of tinea pedis, tinea cruris and tinea corporis, as well as vulvovaginal and oropharyngeal candidiasis [48]. Currently, feline sporotrichosis treatment is limited by the drug options that are available, as well as by its high cost and side-effects. Our data suggest that the use of topical formulations of clotrimazole, alone or in combination with oral itraconazole, represents a potential therapy option for feline sporotrichosis, and a means of reducing cat-to-cat and cat-to-human transmission levels. Although clotrimazole seems to be safe and effective for the treatment of feline candiduria [49], as well as nasal aspergillosis in cats [50], further studies are required to evaluate the clinical responses of felines with sporotrichosis to clotrimazole.

In conclusion, our results show that clotrimazole has strong activity against feline S. brasiliensis isolates, suggesting that

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<th>MIC in combination</th>
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Table 2. Associations between clotrimazole and itraconazole against Sporothrix brasiliensis yeasts. The results are expressed in µg ml⁻¹ and FICI values ≤0.5 were considered synergistic.

MIC, minimum inhibitory concentrations values, corresponding to concentrations that inhibited ≥90 % of fungal growth relative to the untreated control, as determined by spectrophotometric readings. FICI (fractional inhibitory concentration index) = (MICClotrimazole in combination/MICClotrimazole alone) + (MICItraconazole in combination/MICItraconazole alone). The results correspond to the most frequent value obtained in two or more independent experiments.
the clotrimazole – alone or in combination with itraconazole – is potentially a new option for the treatment of feline sporotrichosis.

Funding information
This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ, Brazil) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil).

Acknowledgements
The authors thank Dr Leila Maria Lopes-Bezerra from Universidade Estadual do Rio de Janeiro (Rio de Janeiro, RJ, Brazil) for kindly providing the ATCC MYA 4823 and ATCC MYA 4824 strains, and Dr Marcio Nucci from the Universidade Federal do Rio de Janeiro (Rio de Janeiro, RJ, Brazil) for kindly providing the human S. brasiliensis isolates used in this study. The authors also thank Professor Wanderley de Souza and colleagues from the Laboratory of Cellular Ultrastructure Hertha Meyer (UFRJ, Brazil) for providing support on the use of microtitre plate readers, and on flow cytometry analysis. The authors are grateful to Beatriz Bastos Fonseca for technical support on electron microscopy copy experiments, and the Centro Nacional de Biologia Estrutural e Bioimagem (CENABIO, UFRJ, Rio de Janeiro, Brazil) for facilities and support on the use of transmission electron microscopy equipment.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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