

Terbinafine inhibits *Cryptococcus neoformans* growth and modulates fungal morphology

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Cryptococcus neoformans is an encapsulated fungus that causes cryptococcosis. Central nervous system infection is the most common clinical presentation followed by pulmonary, skin and eye manifestations. Cryptococcosis is primarily treated with amphotericin B (AMB), fluconazole (FLC) and itraconazole (ITC). In the present work, we evaluated the in vitro effect of terbinafine (TRB), an antifungal not commonly used to treat cryptococcosis. We specifically examined the effects of TRB, either alone or in conjunction with AMB, FLC and ITC, on clinical *C. neoformans* isolates, including some isolates resistant to AMB and ITC. Broth microdilution assays showed that TRB was the most effective drug in vitro. Antifungal combinations demonstrated synergism of TRB with AMB, FLC and ITC. The drug concentrations used for the combination formulations were as much as 32 and 16-fold lower than the minimum inhibitory concentration (MIC) values of FLC and AMB alone, respectively. In addition, calcofluor white staining revealed the presence of true septa in hyphae structures that were generated after drug treatment. Ultrastructural analyses demonstrated several alterations in response to drug treatment, such as cell wall alterations, plasma membrane detachment, presence of several cytoplasmic vacuoles and mitochondrial swelling. Therefore, we believe that the use of TRB alone or in combination with AMB and azoles should be explored as an alternative treatment for cryptococcosis patients who do not respond to standard therapies.

Key words: *Cryptococcus neoformans* - terbinafine - fungal morphogenesis - cell differentiation

Cryptococcus neoformans is an encapsulated basidiomycetous fungus that causes disease in immunocompromised and, less commonly, in immunocompetent patients. Infection of the central nervous system (meningitis/meningoencephalitis) is the most common clinical manifestation of cryptococcosis and is followed, in decreasing frequency, by pulmonary, skin, eye and genitourinary disease. Cryptococcal meningitis/meningoencephalitis (CME) is a major cause of morbidity and mortality among immunocompromised individuals, especially patients with acquired immunodeficiency syndrome (AIDS) (Miceli et al. 2011). With the advent of highly active antiretroviral therapy, the incidence of cryptococcosis has decreased dramatically in developed countries. However, CME continues to be a major cause of mortality in developing countries where access to antiretrovirals is limited. Cryptococcosis is associated with AIDS and is the third most frequent neurological complication in AIDS patients (Del Valle & Piña-Oviedo 2006). The mortality rate of cryptococcosis in AIDS patients is estimated to be 55-70% in Latin America and Sub-Saharan Africa (Park et al. 2009). In Brazil, cryptococcosis is the second leading cause of death among systemic mycoses and 23.9 out of every 1,000 AIDS-related deaths are attributed to cryptococcal infection (Prado et al. 2009).

In addition, cryptococcosis is an opportunistic infection that afflicts a significant fraction of solid organ transplant (SOT) recipients (Singh et al. 2008). Approximately 33% of SOT recipients with cryptococcosis present with disease that is limited to the lungs. Pulmonary cryptococcosis may be incidentally detected in asymptomatic patients, or it may manifest as acute respiratory failure, which is associated with a poor prognosis. Cutaneous cryptococcosis can present as papular, nodular or ulcerative lesions, or as cellulitis. Cryptococcal skin disease usually indicates the presence of disseminated disease, but more recently it was proposed that primary cutaneous cryptococcosis is a distinct clinical entity. Infection of the skin appears to result from direct inoculation of *Cryptococcus* into the skin, which is the only affected tissue (Neuville et al. 2003).

Treatment for cryptococcosis is based primarily on amphotericin B (AMB), flucytosine and azole derivatives, such as fluconazole (FLC) and itraconazole (ITC) (Chayakulkeeree & Perfect 2006). AMB, a polyene agent whose mechanism of action is based on its affinity to sterols, has been used clinically for over 45 years. AMB induces pore formation by binding to membrane sterols, leading to altered permeability and eventual cell death (Ghannoum & Rice 1999). Although AMB is extensively used in the treatment of yeast and mould infections, it is both highly nephrotoxic and hepatotoxic and can cause severe anaemia (Tonomura et al. 2009).

Azole agents are the drugs of choice for prophylaxis and maintenance therapy for cryptococcal disease (Saag et al. 2000). FLC and ITC are triazole agents that exert microbial activity by inhibiting the cytochrome P-450-dependent 14 α -demethylase, which is a component of

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the ergosterol biosynthesis pathway. FLC exerts robust activity against yeasts, although some *Candida non-albicans* species are resistant to it (Chen & Sorrell 2007). ITC may be used as an alternative to FLC, but it is less effective because of its low bioavailability and poor penetration of the blood-brain barrier (Bicanic & Harrison 2004, Kon et al. 2008).

Despite the availability of antifungal agents possessing anticryptococcal activity, the mortality rates and incidence of treatment failure associated with cryptococcosis remain unacceptably high. Although *C. neoformans* is not known to be resistant to common antifungals, there is great concern that less-susceptible isolates will emerge as a result of the use of FLC in long-term or maintenance therapy (Brandt et al. 2001). This paucity of antifungal compounds available for the treatment of cryptococcosis, in combination with pharmacological restrictions, necessitates new research on treatment alternatives.

Terbinafine (TRB) is an antifungal drug belonging to the allylamine group that was first approved for the treatment of onychomycosis in UK and USA in the 1990s. TRB exerts its antifungal activity by inhibiting squalene epoxidase (SE), another component of the ergosterol biosynthesis pathway. The fungicidal activity of TRB derives from two mechanisms of action: (i) inhibition of SE, resulting in decreased synthesis of ergosterol, an essential component of the fungal cell membrane, and (ii) a toxic accumulation of intracellular squalene that interferes with cell membrane function and cell wall synthesis, ultimately contributing to cell death. TRB inhibits the enzymatic activity of fungal SE at a much lower concentration (non-competitive inhibition) than is required to inhibit its mammalian SE counterpart (competitive inhibition, requiring a 4,000-fold higher concentration). TRB has no effect on mammalian cholesterol synthesis *in vivo*, so it is safe for human use (Ryder 1992).

The aim of this study was to evaluate the *in vitro* activity of TRB, alone or in combination with FLC, ITC and AMB, against *C. neoformans* clinical isolates, including AMB and azole-resistant, as well as dose-dependent isolates. In addition, we analysed fungal morphology and differentiation before and after exposure to different antifungal agents.

MATERIALS AND METHODS

Fungal isolates - *C. neoformans* ATCC 28957 and *C. neoformans* ATCC 52817 (CAP 67, an acapsular mutant) were obtained from the American Type Culture Collection. *C. neoformans* T₁-444 was obtained from the Federal University of São Paulo, São Paulo (SP) (Brazil) and *C. neoformans* HEC3393 was provided by the Oswaldo Cruz Foundation, Rio de Janeiro (RJ) (Brazil); both are clinical isolates from patients with meningoencephalitis and AIDS. In addition, 16 *C. neoformans* isolates were obtained from human immunodeficiency virus (HIV)-positive patients at the Clementino Fraga Filho University Hospital, RJ. To ensure optimal growth, all yeast cells were subcultured at least twice in Sabouraud dextrose agar (Difco, USA) at 35°C for 48 h prior to any experiment.

Broth microdilution test - The broth microdilution test was performed as described in the M27-A3 docu-

ment, with some modifications (CLSI 2008). FLC (Pfizer, SP, Brazil) was diluted in water and AMB, ITC and TRB (Sigma Chemical Co, Missouri, USA) were diluted 100-fold in dimethyl sulfoxide to obtain stock solutions that were kept at -20°C. The antifungals were diluted in RPMI-1640 medium (Sigma Chemical Co, Missouri, USA) at pH 7.0 buffered with 0.16 M morpholinepropanesulfonic acid to obtain final concentrations ranging from 0.125-64 µg/mL for FLC and from 0.03-16 µg/mL for ITC, AMB and TRB. The diluted antifungal suspensions were then added to 96-well microtitre trays. Next, each fungal suspension was inoculated into the appropriate well at final concentrations ranging from 0.5 x 10³-2.5 x 10³ CFU/mL. The minimum inhibitory concentration (MIC) of each antifungal was determined by spectrophotometric reading at 492 nm following incubation at 35°C for 72 h. MIC₅₀ and MIC₉₀ values were defined as the lowest drug concentrations that resulted in a 50% and 90% decrease in absorbance relative to the growth control, respectively.

To interpret the broth microdilution results, we determined the MICs of each antifungal according to Clinical and Laboratory Standards Institute (CLSI) guidelines (2008). The MIC for AMB is defined as the lowest concentration that inhibits total growth (MIC₉₀), whereas the MIC for azole is the concentration that inhibits 50% of fungal growth (MIC₅₀).

Determination of the minimum fungicidal concentration (MFC) - Prior to spectrophotometric reading of the microtitre trays used in the broth microdilution test, the contents of each well were homogenised and an aliquot from each well was transferred onto Sabouraud dextrose agar drug-free plates. The plates were incubated at 35°C for 72 h and the MFC was defined as the lowest concentration that resulted in no observable fungal growth. A fungicidal effect was defined as the range from the MFC value equivalent to the MIC₅₀ (azoles) or MIC₉₀ (AMB) to four times that value. Above this upper limit, the antifungal effect is considered fungistatic (Pfaller et al. 2005).

Checkerboard assay - The checkerboard broth microdilution method was used to evaluate the effect of TRB combined with other antifungals on *C. neoformans* ATCC 28957 and HU2 isolates. The HU2 isolate was chosen for the checkerboard assays because it presented the highest FLC MIC₅₀ value and was characterised as AMB-resistant. Briefly, serial dilutions of TRB were dispensed into a 96-well microtitre tray together with serial dilutions of FLC, ITC or AMB. The plates were incubated at 35°C for 72 h and the absorbance was determined *via* spectrophotometer reading at 492 nm. The fractional inhibitory concentration (FIC) index was calculated for each antifungal combination as previously described (Jones 1990) and it was interpreted as follows: FIC < 0.5: synergism, 0.5 < FIC < 4: inconclusive and FIC > 4: antagonism. Two independent tests were performed for each isolate.

Optical microscopy - *C. neoformans* strain ATCC 28957 was treated with antifungal drugs at MIC₅₀ concentrations alone or in combination in RPMI-1640 me-

dium at 35°C. After 48 h, the antifungal-containing medium was replaced and the cells were incubated for an additional 72 h (for a total of 120 h in culture) at 35°C. Cells were collected at 48 h and 120 h and examined *via* light microscopy for any morphological alterations (Zeiss Axiostar Plus, Germany). The percentage of filamentous structures (morphological alterations) in relation to total cell number was calculated for at least 200 cells. Mean values and standard deviations (SD) were obtained from three independent experiments.

Calcofluor white and Nile red staining - *C. neoformans* (strain ATCC 28957) cells, either untreated or treated for 120 h with TRB alone or with combinations of TRB/FLC or TRB/AMB, were washed in phosphate buffered saline (PBS) pH 7.2 and fixed with 4% paraformaldehyde in PBS for 30 min. Subsequently, cells were adhered to poly-L-lysine-coated glass coverslips and incubated with one of two fluorescent stains: (i) lipophilic Nile red (Fluka, USA) at 5 µg/mL for 30 min or (ii) calcofluor white (Sigma Chemical Co, Missouri, USA), which binds to chitin in fungal cell walls, at 200 µg/mL for 20 min. The coverslips were then mounted in n-propyl gallate solution and observed with a Zeiss Axioplan epifluorescence microscope equipped with a rhodamine filter (Nile red fluorescence). Images were recorded with a C5810 Hamamatsu camera.

India ink staining - *C. neoformans* ATCC 28957 cells, either untreated or treated with antifungal drugs alone or in combination, were fixed in 4% paraformaldehyde and then negatively stained with India ink. Cells were visualised with a light microscope (Zeiss Axioplan), the images were recorded with a C5810 Hamamatsu camera and the capsule size was measured using ImageJ 1.4 software. Four different measurements were taken for each cell for a total of 30 cells per experimental condition. The mean and SD for each group were calculated and the capsule size of treated cells was compared with that of untreated cells.

Transmission electron microscopy (TEM) - *C. neoformans* strain ATCC 28957 cells were treated with TRB at the MIC₅₀ concentration at 35°C for 120 h, as previously described, and prepared for optical microscopy. The cells were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer for 1 h, after which they were post-fixed in 1% osmium tetroxide, 1.25% potassium ferrocyanide and 5 mM CaCl₂ in cacodylate buffer for 2 h. The cells were then dehydrated in crescent concentrations of acetone and embedded in Spurr epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and specimens were observed with a JEOL 1200 electron microscope equipped with a CCD camera (Mega View III model, Soft Image System, Germany). Images were processed using iTEM software (Soft Image System, Germany).

Statistical analyses - One-way ANOVA tests were performed with GraphPad Prism 4.0 software to compare the control group to the antifungal-treated groups. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Broth microdilution test - Independent of the origins of the clinical isolates, all 20 *C. neoformans* isolates tested were susceptible to TRB and FLC; however, 5% (1/20) were resistant to ITC and 35% (7/20) were susceptible in a dose-dependent manner (Table I). The resistance profile was considerably higher for AMB, as 60% of isolates (12/20) showed resistance, with growth inhibition occurring only at concentrations above 2 µg/mL (Table I). When we considered only the HU clinical isolates, this number increased to 75% (12/16). It is important to note that AMB was the first available treatment for HU patients, a situation that probably led to the development of some resistant isolates.

Interestingly, all ATCC strains and clinical isolates, including those resistant to AMB and ITC, were inhibited by TRB at MIC₅₀ values below 0.5 µg/mL (Table II). These values were considerably lower than those observed for AMB (4 µg/mL) and FLC (8 µg/mL), which are antifungals commonly used for cryptococcal therapy. In addition, all AMB-resistant HU isolates exhibited MIC₅₀ values ≤ 0.5 µg/mL for TRB. MFC values indicated a fungistatic effect for ITC, FLC and TRB and a fungicidal effect for AMB (Table I).

Checkerboard assays - All three antifungal combinations tested exhibited a synergistic effect *in vitro* (Table III). TRB/ITC and TRB/FLC combinations were more efficient *in vitro* than the TRB/AMB combination. Interestingly, TRB/FLC interactions were synergistic even for the HU2 isolate that presented the highest MIC value for FLC treatment alone (MIC₅₀ = 8 µg/mL). The most relevant finding was that the drug concentrations used for the TRB/FLC and TRB/AMB combination treatments were as much as 32 and 16-fold lower than the MIC values of FLC and AMB alone, respectively.

Optical microscopy - As revealed by optical microscopy, the most common morphological alteration in treated *C. neoformans* ATCC 28957 was the filamentation of approximately 10% of the yeast cells examined. These morphological alterations were quantified and are presented as a time-dependent profile (Fig. 1). After 48 h of treatment, no antifungal, alone or in combination, was able to significantly induce atypical filamentous morphologies compared to control cells. In contrast, 120 h of treatment with FLC, TRB/FLC or TRB/AMB induced atypical filamentation. Both FLC alone and TRB/FLC in combination induced filamentation more prominently, such that approximately 10% of cells showed this phenotype; however, there was no difference between these two treatments ($p > 0.05$). The effect of 120 h of TRB/FLC treatment was statistically higher than the effect of TRB treatment alone ($p < 0.01$). Interestingly, cells treated for 120 h with TRB/AMB showed substantial filamentation, but this effect was not significantly different from that of separate treatment with either AMB or TRB ($p > 0.05$) (Fig. 1). By staining the complete septum formation, calcofluor white staining allowed us to examine whether these treatments induced the development of true hyphal structures, as observed in TRB and TRB/FLC treatment (Fig. 2D, F). Interest-

ingly, treatment with TRB/AMB did not allow complete septum formation and resulted in pseudohyphae formation (Fig. 2H). Treatment with FLC or AMB alone also led to pseudohyphae formation, but no complete septum was observed (data not shown). Nile red staining of cells treated with TRB, TRB/FLC or TRB/AMB revealed cytoplasmic lipid inclusions (Fig. 3 D, F, H). The same

lipid inclusions were observed when cells were treated with FLC or AMB alone (data not shown).

Another important morphological alteration determined by optical microscopy and India ink staining was a change in capsule size (Fig. 4). Although neither AMB and TRB alone reduced capsule size after 48 h of treatment, the combination of these two drugs reduced cap-

TABLE I

In vitro antifungal activity of terbinafine, itraconazole, fluconazole and amphotericin B against *Cryptococcus neoformans* isolates

Isolates (n = 20)	Antifungal concentration ($\mu\text{g/mL}$)											
	Terbinafine			Itraconazole			Fluconazole			Amphotericin B		
	MIC ₅₀ ^a	MIC ₉₀ ^a	MFC	MIC ₅₀ ^a	MIC ₉₀ ^a	MFC	MIC ₅₀ ^a	MIC ₉₀ ^a	MFC	MIC ₅₀ ^a	MIC ₉₀ ^a	MFC
<i>C. neoformans</i> ATCC 28957	0.125	0.5	1	0.5 ^b	4	8	2	4	8	0.03	0.06	0.25
<i>C. neoformans</i> CAP 67	0.125	0.5	1	0.5 ^b	2	8	2	4	8	0.03	0.06	0.125
<i>C. neoformans</i> T ₁ 444	0.125	0.25	0.5	1 ^c	4	8	4	8	16	0.06	0.125	0.25
<i>C. neoformans</i> HEC 3393	0.25	0.5	1	0.5 ^b	2	8	2	4	8	0.06	0.125	1
<i>C. neoformans</i> HU1	0.5	1	4	0.06	0.25	1	4	4	16	1	2 ^c	4
<i>C. neoformans</i> HU2	0.5	1	16	0.06	0.25	8	8	16	32	1	2 ^c	4
<i>C. neoformans</i> HU3	0.25	1	8	0.03	0.125	1	4	8	16	0.5	2 ^c	8
<i>C. neoformans</i> HU4A	0.5	1	8	0.06	0.125	4	2	4	16	1	2 ^c	4
<i>C. neoformans</i> HU4B	0.5	1	4	0.03	0.125	4	2	4	8	1	2 ^c	4
<i>C. neoformans</i> HU4C	0.5	1	4	0.03	0.125	0.5	4	8	16	1	2 ^c	4
<i>C. neoformans</i> HU6	0.25	0.5	4	0.06	0.25	2	1	4	8	0.5	1	8
<i>C. neoformans</i> HU7	0.5	1	8	0.03	0.125	4	2	4	8	1	2 ^c	8
<i>C. neoformans</i> HU8	0.25	0.5	4	0.03	0.06	1	1	4	32	1	2 ^c	8
<i>C. neoformans</i> HU9	0.5	1	8	0.06	0.125	1	2	8	32	0.5	1	4
<i>C. neoformans</i> HU10	0.5	1	16	0.06	0.125	1	4	16	32	0.5	1	4
<i>C. neoformans</i> HU11	0.5	1	16	0.06	0.125	0.25	4	8	16	0.25	0.5	1
<i>C. neoformans</i> HU12	0.25	0.5	2	0.25 ^b	0.5	1	4	8	16	2	4 ^c	8
<i>C. neoformans</i> HU13	0.25	0.5	4	0.25 ^b	1	2	4	8	64	2	4 ^c	8
<i>C. neoformans</i> HU14	0.5	1	8	0.25 ^b	0.5	1	4	16	32	1	4 ^c	8
<i>C. neoformans</i> HU15	0.25	1	2	0.25 ^b	0.5	1	2	4	32	2	4 ^c	16

a: the antifungal minimum inhibitory concentration (MIC) was determined using MIC₉₀ for amphotericin B and MIC₅₀ for fluconazole, itraconazole and terbinafine as recommended by CLSI (2008); b: susceptible dose-dependent; c: resistant.

TABLE II

Cumulative percentage of susceptibility of the 20 *Cryptococcus neoformans* isolates to different antifungals

Antifungal ($\mu\text{g/mL}$) ^a	Cumulative (%) inhibited at MIC ($\mu\text{g/mL}$)											
	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64
Terbinafine	0	0	15	50	100	-	-	-	-	-	-	-
Itraconazole	25	60	60	80	95	100	-	-	-	-	-	-
Fluconazole	0	0	0	0	0	10	50	95	100	-	-	-
Amphotericin B	0	10	20	20	25	40	80	100	-	-	-	-

a: the antifungal minimum inhibitory concentration (MIC) was determined using MIC₉₀ for amphotericin B and MIC₅₀ for fluconazole, itraconazole and terbinafine.

sule size significantly in comparison to control cells ($p < 0.01$). In addition, after 48 h of treatment, TRB/FLC and TRB/AMB reduced capsule size relative to any of the drugs administered alone; this difference was statistically significant. Importantly, all antifungals tested decreased capsule size after 120 h of treatment compared to untreated cells ($p < 0.01$).

TEM - TEM of *C. neoformans* ATCC 28957 cells revealed that antifungal treatment caused several ultrastructural alterations. Untreated yeast cells exhibited a round profile with a well-preserved capsule, compact cell wall, normal juxtaposition of the plasma membrane to the fungal cell wall and a regular cytoplasmic profile (Fig. 5A). Treatment with TRB for 120 h (Fig. 5B) induced the detachment of the cell membrane from the cell wall, which probably resulted in membrane impairment (white arrow and inset in Fig. 5B) and an increase of cytoplasmic vacuoles that could account for the lipid accumulation and mitochondrial swelling observed previously. Combinatorial treatment with TRB/FLC (Fig. 5C) severely altered the structure of the cell wall, increasing its thickness (inset in Fig. 5C) and leading to the appearance of electron-dense granules in the fungal cytoplasm. Treatment with TRB/AMB (Fig. 5D) induced the most drastic changes; it led to substantial alterations in cell structure as a result of cell wall rupture and the loss of cytoplasmic contents (arrow in Fig. 5D). All treatments led to a release of capsule components that was not observed in the electron micrographs (Fig. 5B-D).

DISCUSSION

As there are currently no breakpoints defined for *C. neoformans* regarding the clinical use of antifungals, the MIC values for FLC and ITC observed in this study were interpreted based on breakpoints previously established for *Candida* spp (CLSI 2008). Regarding FLC activity, the samples observed in this study showed MIC₉₀ values as high as 4 µg/mL (MIC₉₀ is the MIC of 90% of the isolates tested). However, no isolate was resistant to FLC, including those obtained from patients who had been previously treated with FLC (isolates HU4, 10, 11

and 14). The MIC values observed in this study were similar to those reported in previous studies, which reported MIC₉₀ values for clinical isolates of *C. neoformans* between 4-8 µg/mL (Pfaller et al. 2005, Souza et al. 2005). In the present study, ITC showed robust activity in vitro, exhibiting low MIC values similar to those previously described (Alves et al. 2001, Espinel-Ingroff 2003). However, one isolate was characterised as resistant and seven were characterised as susceptible to dose-dependent treatment. As is common for azoles, FLC and ITC showed a fungistatic effect against *C. neoformans* isolates (Datta et al. 2003).

Previous studies of AMB resistance in *C. neoformans* have reported MIC₉₀ values greater than or equal to 2 µg/mL (Lozano-Chiu et al. 1998). In the present study, the resistance profile was much higher than previously reported for AMB, as 60% of isolates showed resistance. When we considered only the HU clinical isolates, this number increased to 75% (Table I). However, AMB exhibited a fungicidal effect for 90% of susceptible isolates (no visible colony growth until 4 x the MIC₉₀ value was applied) (Pfaller et al. 2005).

It is important to note that the *C. neoformans* isolates used in this study were predominantly isolated from HIV-positive patients who had undergone treatment with AMB and/or FLC; this could explain the reduced AMB-susceptibility of the isolates. Because of the possibility of long-term treatment effects, this also demonstrates the importance of surveilling resistance in clinical isolates.

No breakpoint has been defined for TRB, but some consider a MIC > 8 µg/mL to be indicative of resistance for *Candida* spp (Ryder et al. 1998). Our data show that TRB exerts potent activity against *C. neoformans* isolates relative to the other antifungals tested, as 100% of the isolates were susceptible to concentrations lower than 0.5 µg/mL. Moreover, TRB appeared to exert a fungistatic effect, as opposed to the fungicidal effect usually described for dermatophyte fungi. Similarly, previous studies have shown good activity of TRB against *C.*

TABLE III
In vitro combination of terbinafine (TRB) with itraconazole (ITC), fluconazole (FLC) or amphotericin B (AMB)

	Isolates	
	<i>Cryptococcus neoformans</i> ATCC 28957 (µg/mL) (FIC index ^a)	<i>Cryptococcus neoformans</i> HU2 (µg/mL) (FIC index ^a)
TRB/ITC	0.015/0.005 (0.17)	0.06/0.03 (0.3)
TRB/FLC	0.015/0.06 (0.25)	0.25/0.12 (0.26)
TRB/AMB	0.125/0.06 (0.37)	0.5/0.12 (0.5)

a: fractional inhibitory concentration (FIC) < 0.5: synergistic; 0.5 < FIC < 4: indifferent; FIC ≥ 4: antagonistic.

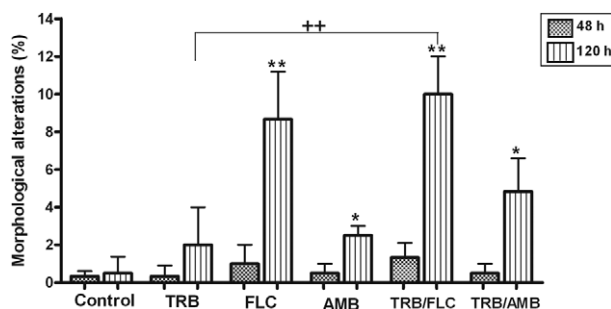


Fig. 1: the percentage of *Cryptococcus neoformans* ATCC 28957 cells showing pseudohyphae/true hyphae formation (morphological alterations) was evaluated after treatment with antifungals alone or in combination with terbinafine (TRB) for 48 h and 120 h and compared to control cells from each group. Results are plotted as means and the bars represent standard deviation. Statistical analysis: one-way ANOVA [*: $p < 0.05$; **: $p < 0.01$; +: comparing the drugs used in association and the drugs alone ($+ < 0.05$ and $++ < 0.01$)]. AMB: amphotericin B; FLC: fluconazole.

neoformans and *Candida* spp in vitro (Ryder et al. 1998, Jessup et al. 2000, Garg et al. 2006, Li et al. 2008).

TRB is an allylamine antifungal agent that has been available for more than a decade. TRB is currently used to treat dermatophytic infections and onychomycosis. Despite several studies demonstrating the efficacy of TRB against non-dermatophytic infections, including azole-resistant candidiasis, invasive aspergillosis, chronic chromoblastomycosis, disseminated fusariosis and scedosporiosis, the role of TRB in the management of these infections remains greatly underappreciated. Krishnan-Natesan (2009) published a brief review of TRB pharmacodynamics and pharmacokinetics that provides insight into the use of TRB as a potential adjunct in combination with azoles and polyenes for the management of severe drug-resistant or refractory mycoses. Despite its lack of intrinsic fungicidal activity against several non-dermatophytes, when used in combination with other antifungals and particularly with azoles, TRB has demonstrated good antifungal efficacy that could be exploited in clinical settings.

In recent years, combination therapy has generated considerable interest with regard to difficult-to-treat fungal infections. The potential advantages of combination therapy include a broad spectrum of activity, synergy between combined drugs and an improved safety profile with lower dosages. To date, most combination studies have examined agents with complementary mechanisms of action. Azoles and TRB are both inhibitors of ergosterol synthesis, but they act upon two different targets in the biosynthetic pathway. When used simultaneously, these agents could potentially complement each other by inhibiting multiple enzymes in the same biosynthetic pathway (Odds et al. 2003).

Likewise, our results demonstrate a synergistic effect for each of the combinations tested in vitro against two isolates of *C. neoformans* (i.e., TRB/FLC, TRB/ITC and TRB/AMB). These isolates were selected for checkerboard experiments because they are representative of two different profiles. The HU2 isolate exhibited in vitro resistance to AMB and displayed a high MIC₅₀ value for FLC; in contrast, ATCC 28957 was susceptible to AMB and to ITC in a dose-dependent fashion. In both cases, drug combinations enhanced antifungal potency and, more importantly, lowered the requirement for drug concentration in each case; these effects could minimise

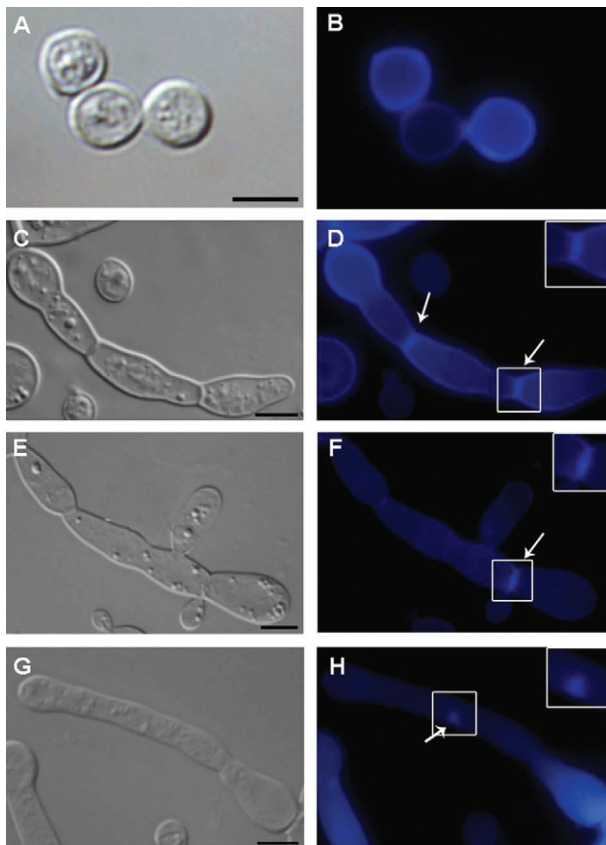


Fig. 2: calcofluor white staining (B, D, F, H) of *Cryptococcus neoformans* ATCC 28957 strain revealed the incidence of true hyphae (complete septum structure; arrows and inset in D and F) and pseudohyphae structures (incomplete septum structure; arrow and inset in H). Untreated cells (A, B), cells treated for 120 h with minimum inhibitory concentration 50 of terbinafine (TRB) (C, D), TRB/fluconazole (E, F) and TRB/amphotericin B (G, H). Differential interference contrast microscopy images (A, C, E, G). Bars = 5 μ m.

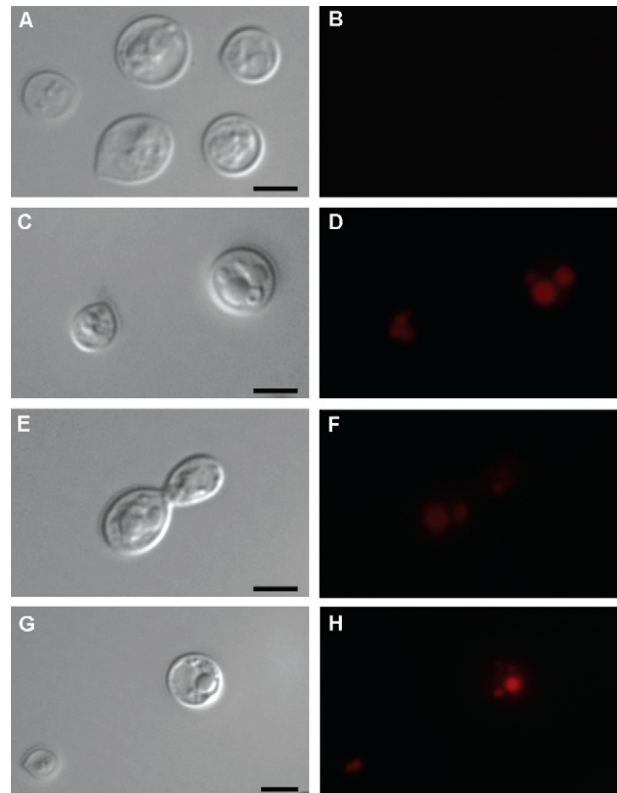


Fig. 3: *Cryptococcus neoformans* strain ATCC 28957. Differential interference contrast microscopy images (A, C, E, G) and Nile red staining (B, D, F, H). Untreated cells (A, B) and cells treated for 120 h with minimum inhibitory concentration 50 of terbinafine (TRB) (C, D), TRB/fluconazole (E, F) or TRB/amphotericin B (G, H). Nile red staining was observed only in treated cells (D, F, H). Bars = 4 μ m.

toxicity to the host and limit the emergence of antifungal resistance. The concentrations of FLC and AMB used in the TRB/FLC and TRB/AMB combinations were up to 32 and 16-fold lower than the respective MIC values of the drugs when used alone.

Although *C. neoformans* is not traditionally considered a dimorphic fungus, because its hyphal form is usually present only during mating (Wickes et al. 1996), atypical hyphal and pseudohyphal forms have been occasionally reported in hosts (Williamson et al. 1996, Bemis et al. 2000, Gazzoni et al. 2009). Several pathogenic fungi have the ability to undergo dimorphism between yeast forms and true hyphae or pseudohyphae during infection; examples of these include *C. albicans*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Blastomyces dermatitidis*, *Sporothrix schenckii*, *Coccidioides immitis* and *Penicillium marneffei* (Maresca & Kobayashi 1989, Klein & Tebbets 2007).

In the present paper, we report for the first time that antifungal treatment of *C. neoformans* yeasts with TRB, FLC, AMB, either alone or in combination, induced filamentation (formation of true hyphae or pseudohyphae). In addition, calcofluor white staining revealed that true hyphae with complete septum formation were detected only in cells treated with TRB alone or in combination with FLC and AMB. The induction of pseudohyphae in fungi that normally do not present dimorphism can usually be attributed to stress factors. For example, *Saccharomyces cerevisiae* cells have been shown to produce pseudohyphae when grown in low nutrient conditions (Wickes et al. 1996). Therefore, it seems likely that prolonged antifungal incubation can serve as a stress factor that is capable of inducing filamentation in *C. neoformans*. In addition, it is important to emphasise that pseudohyphal forms of *C. neoformans* are less virulent, as they are incapable of establishing infection in mice (Neilson et al. 1978, 1981, Zaragoza et al. 2003).

Moreover, antifungal treatment led to reductions in capsule size. Because *C. neoformans* capsule enlargement is believed to be involved in the inhibition of complement-mediated phagocytosis and to protect against several stress conditions, this may indicate reduced virulence in yeast cells (Zaragoza et al. 2003, 2008).

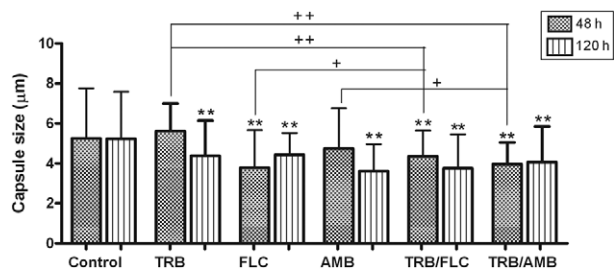


Fig. 4: capsule size of *Cryptococcus neoformans* strain ATCC 28957 after treatment with antifungals alone or in combination with terbinafine (TRB) for 48 h and 120 h. Results are plotted as means and the bars represent standard deviation. Statistical analysis: one-way ANOVA [**: $p < 0.01$; +: comparing the drugs used in association and the drugs alone (+ < 0.05 and ++ < 0.01)]. AMB: amphotericin B; FLC: fluconazole.

Because TRB is an inhibitor of the ergosterol biosynthesis pathway, *C. neoformans* cells treated with TRB were expected to show cellular membrane alterations; this was confirmed by TEM analyses. In addition, alterations in cell wall structure were observed; these were probably the result of altered plasma membrane ergosterols that also alter the composition and function of the cell wall. These ultrastructural alterations have also been reported by Ishida et al. (2009), who reported that *C. albicans* treated with compounds targeting the ergosterol biosynthesis pathway exhibited increased cell wall thickness as a secondary effect. Moreover, the increased number of cytoplasmic vacuoles could be related to alterations in lipid metabolism induced by antifungal treatment; this may induce cytoplasmic accumulation of biosynthesis pathway intermediates, such as squalene, as evidenced by Nile red staining of TRB, TRB/FLC and TRB/AMB-treated cells. These data suggest that these treatments can lead to the accumulation of lipids in the cytoplasm.

At present, the major niche for TRB in the antifungal armamentarium is as an adjunct therapeutic in the management of various refractory/resistant fungal infections. Although TRB demonstrates broad-spectrum antifungal activity, its efficacy is considered inferior to that of azole agents. Therefore, most in vitro studies and subsequent case reports have focused on the use of TRB in combination with other classes of antifungal agents, including polyenes and azoles (Cantón et al. 2005, Krishnan-Natesan 2009). The advantages of combination therapy

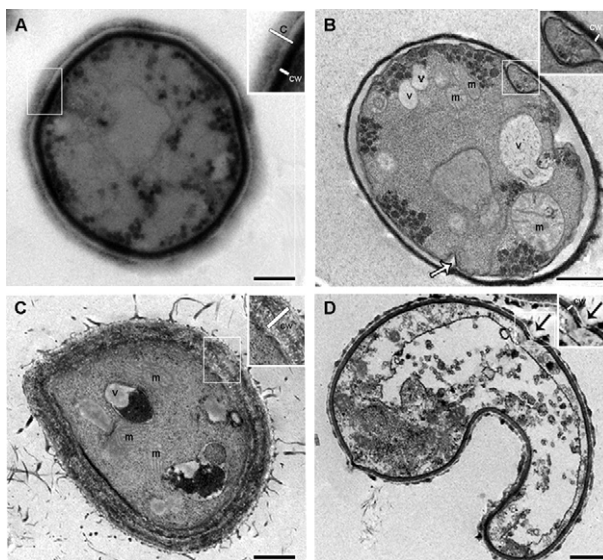


Fig. 5: transmission electron microscopy of *Cryptococcus neoformans* ATCC 28957 strain untreated cells (A) and cells treated with terbinafine (TRB) (B), TRB/fluconazole (C) and TRB/amphotericin B (D) for 120 h at 35°C. Untreated cells presented normal capsules (c) (inset in A), a compact cell wall (cw) and a well-preserved membrane and cytoplasm ultrastructure. Treatments caused an increase in the number of cytoplasmic vacuoles (v), mitochondrial swelling (m) (B-C) and membrane detachment from the cell wall (white arrow in B). Treatment with TRB/AMB also induced cell wall rupture (black arrow in C). Bars = 0.5 µm.

include a broad range of antifungal specificity, enhanced potency and decreased dosage of the individual agents; these effects may reduce toxicity and help to prevent the emergence of drug resistance. Despite these plausible benefits, there are few clinical trials supporting these conclusions. Combining TRB with polyenes (different targets) and azoles (inhibition of different enzymes in the ergosterol pathway) has been attempted with various fungi, resulting in successful clinical outcomes.

In the present study we showed that TRB, alone or in combination with FLC and AMB, possesses potent *in vitro* activity against *C. neoformans* clinical isolates, including those resistant to AMB. Furthermore, TRB induced important morphological alterations, such as the induction of pseudohyphae and true hyphae when used alone or in combination with AMB or FLC. In addition, these treatments led to a decrease in capsule size and caused several ultrastructural alterations. In light of the limited therapeutic options that are currently available and because rare and resistant fungal pathogens continue to emerge in immunocompromised patients, we believe that the use of TRB, alone or in combination with AMB and azoles, should be explored as a clinical alternative for cryptococcosis patients who do not respond to standard therapy. In addition, further *in vivo* studies with animal models should be performed to obtain vital information regarding the appropriate use of TRB in this context.

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