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ARTICLE

Antifolates inhibit *Cryptococcus* biofilms and enhance susceptibility of planktonic cells to amphotericin B

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Abstract The *Cryptococcus neoformans* species complex contains the most important agents of fungal meningoencephalitis. Therapeutic choices are limited and issues related to toxicity and resistance to antifungals have been described. The present study evaluated the inhibitory effect of the antifolate combinations sulfamethoxazole–trimethoprim (SMX/TMP) and sulfadiazine–pyrimethamine (SDZ/PYR) against planktonic cells and biofilms of *C. neoformans* and *C. gattii*. The influence of the antifolate combinations on the amphotericin minimum inhibitory concentration (MIC) of planktonic cells was also investigated. In addition, the effect of these combinations on the cellular ergosterol content of planktonic cells was studied. Strains of *C. neoformans* (n=15) and *C. gattii* (n=15) obtained from environmental or clinical sources were evaluated by the broth microdilution method. SMX/TMP and SDZ/PYR showed antifungal activity against free living cells and sessile cells of *Cryptococcus* spp. Moreover, planktonic cells showed increased susceptibility to amphotericin B after pre-incubation with sub-inhibitory concentrations of SMX/TMP or SDZ/PYR. The drug combinations SMX/TMP and SDZ/PYR were able to prevent the biofilm formation and showed inhibitory effect against mature biofilms of both species. Additionally, the study showed that antifolate drugs reduced the ergosterol content in *C. neoformans* and *C. gattii* planktonic cells. Our results highlight the antifungal potential of antifolate drugs.

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Introduction

Cryptococcosis is a life-threatening disease caused by the *Cryptococcus neoformans* species complex, which is formed by *C. neoformans* (serotypes A, D, and A/D) and *C. gattii* (serotypes B and C), commonly acquired by inhalation of conidia from the air and bird droppings [1]. Primary infection usually results in pneumonia or dissemination to the central nervous system [2]. Cryptococcal meningitis is considered to be one of the most important human immunodeficiency virus (HIV)-related opportunistic infections, and it is estimated that around 1 million cases occur globally per year in acquired immunodeficiency syndrome (AIDS) patients [2, 3].

The treatment of cryptococcal meningoencephalitis relies mainly on amphotericin B, alone or associated with 5-fluorocytosine [2, 4]. However, nephrotoxicity and renal dysfunction have often been reported among patients under therapeutic regimens with this antifungal [4]. In order to overcome these issues, fluconazole is considered as an alternative for induction and consolidation, maintenance, or prophylactic therapy [2, 5]. Although antifungal resistance in *Cryptococcus* is not considered to be a main problem worldwide [6], some studies have shown that resistance can be a problem in developing countries [5, 7]. Moreover, the

heteroresistance to fluconazole and its linkage to virulence among clinical *Cryptococcus* strains have gained attention in recent years [5, 8]. To address these problems, much effort has been devoted to searching for new antifungals against *Cryptococcus* [9–11].

Additionally, many studies have shown that *Cryptococcus* spp. have the ability to colonize and form biofilms in medical devices [12–15]. Fungal biofilms are formed by microbial communities associated with an extracellular matrix and their development is controlled by complex molecular events [13]. These structures have been associated with infection with high levels of therapeutic refractoriness—due to their intrinsic antifungal resistance—as well as being associated with significant mortality [13]. However, despite the great importance of the topic, few studies have investigated strategies to control and eradicate *Cryptococcus* biofilms [14–16]. The insertion of medical devices, such as ventriculoatrial shunt catheters [17, 18], prosthetic valves [19], and prosthesis [20], are related to *Cryptococcus* biofilm formation.

The present study aimed to evaluate the inhibitory effect of the antimicrobial combinations sulfamethoxazole–trimethoprim (SMX/TMP) and sulfadiazine–pyrimethamine (SDZ/PYR) against planktonic cells and biofilms of *C. neoformans* and *C. gattii*. The effect of these drugs on the cellular ergosterol content and on the antifungal susceptibility of planktonic cells to amphotericin B was also investigated.

Materials and methods

Microorganisms

Strains of *C. neoformans* ($n=15$) and *C. gattii* ($n=15$) were obtained from environmental or clinical sources. The use of clinical strains was approved by the Ethics Committee of São José Hospital of Infectious Diseases (Fortaleza, Ceará, Brazil; Process 007-2009). The identification of *C. neoformans* and *C. gattii* was based on the following phenotypical characteristics: urease test on Christensen's urea agar (Difco Laboratories, England); phenoloxidase activity on birdseed agar (*Guizotia abyssinica*) supplemented with biphenyl (0.1 %); and chemotyping on CGB medium (L-canavanine, glycine, bromothymol blue), as previously described [21]. In addition, molecular characterization of each serotype was performed as described by Enache-Angoulvant et al. [22], through enzymatic restriction of the *CAP59* gene. Eight reference strains, obtained from Evandro Chagas Clinical Research Institute, Brazil (IPEC/FIOCRUZ), were used as controls: *C. neoformans* var. *grubii* WM 148 (serotype A, VNI/AFLP1), *C. neoformans* var. *grubii* WM 626 (serotype A, VNII/AFLP1A), *C. neoformans* WM 628 (serotype AD, VNIII/AFLP2), *C. neoformans* var. *neoformans* WM 629 (serotype D, VNIV/AFLP3), *C. gattii* WM 179 (serotype B, VGI/AFLP4), *C. gattii* WM 178 (serotype B, VGII/AFLP6), *C. gattii* WM 175 (serotype B, VGIII/AFLP5), and *C. gattii* WM 779 (serotype C, VGIV/AFLP7) [23].

Antimicrobial drugs

Stock solutions of SMX/TMP and SDZ/PYR were prepared in 10 % dimethyl sulfoxide (DMSO) and amphotericin B (AMB) was diluted in 100 % DMSO. Serial two-fold dilutions of SMX/TMP and SDZ/PYR were prepared with yeast nitrogen base (YNB) medium [22]. AMB was diluted in RPMI 1640 medium with L-glutamine and without sodium bicarbonate (Sigma Chemical Co., St. Louis, MO, USA), buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma Chemical Co.).

Inoculum preparation for susceptibility testing

Inocula of all tested isolates were prepared from 48-h-old cultures previously grown on potato dextrose agar at 35 °C. The colonies were suspended in 5 mL of sterile 0.9 % saline and the turbidity was adjusted to 0.5 on the McFarland scale. Afterwards, the suspension was diluted 1:100 and then 1:20 with YNB medium to obtain an inoculum of planktonic cells containing $0.5–2.5 \times 10^3$ cells/mL [24].

Antifungal susceptibility of *Cryptococcus* planktonic cells

Effect of SMX/TMP and SDZ/PYR on Cryptococcus growth

The effect of the combinations of SMX/TMP and SDZ/PYR against *C. neoformans* and *C. gattii* strains was investigated by broth microdilution, according to the Clinical and Laboratory Standards Institute (CLSI) document M27-A3 [25], except for the substitution of RPMI 1640 medium for YNB without PABA in this composition, because this component can interfere with the antifungal activity of sulpha drugs [24]. The final concentrations of each antimicrobial combination ranged as follows: 1.953 to 1,000 μg/mL for SMX and SDZ; 0.39 to 200 μg/mL for TMP; 0.098 to 50 μg/mL for PYR; and 0.015 to 8 μg/mL for AMB.

Susceptibility testing for planktonic cells was performed in 96-well microdilution plates at 37 °C for 48 h. All the isolates were tested in duplicate. For SMX/TMP and SDZ/PYR, the minimum inhibitory concentrations (MICs) were defined as the lowest drug concentration inhibiting growth by 80 % when compared to the control well [24, 26].

Effect of sub-inhibitory concentrations of SMX/TMP and SDZ/PYR on susceptibility to amphotericin B

The effect of SMX/TMP and SDZ/PYR on the susceptibility of *Cryptococcus* to AMB was also investigated. For this

purpose, strains of *C. neoformans* (n=7) and *C. gattii* (n=7) with previously known susceptibility patterns to AMB [27] were chosen.

A standardized inocula prepared as described above was incubated in YNB medium supplemented with SMX/TMP or SDZ/PYR at sub-inhibitory MIC (MIC/2) for 24 h at 37 °C in an orbital shaker at 150 rpm. Controls were grown in YNB medium without antimicrobials. After incubation, cultures were centrifuged at 2,000 rpm for 5 min. From the resulting pellet, an inoculum of $0.5–2.5\times10^3$ cells/mL was prepared in 5 mL of sterile 0.9 % saline and AMB MIC was determined by the CLSI M27-A3 broth microdilution method, using RPMI 1640 medium buffered to pH7.0 with 0.165 M MOPS [25]. The MIC for AMB was defined as the lowest concentration at which no growth was observed [25]. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6528 were used as quality control strains.

Effect of SMX/TMP and SDZ/PYR on ergosterol content of planktonic cells

Total sterols were extracted as described by Moran et al. [28], with modifications. Strains of *C. neoformans* (n=7) and *C. gattii* (n=7) were grown in potato dextrose agar for 48 h at 37 °C and, after this period, a loopful of inoculum was transferred to YNB supplemented with SMX/TMP or SDZ/PYR at sub-MIC (MIC/2) for each drug combination. The tubes were incubated for 48 h at 37 °C and then centrifuged at 12,000 rpm for 3 min. Cellular pellets were suspended in 0.5 mL of alcoholic KOH (3.945 g of KOH and 40 ml of sterile distilled water, brought to 100 ml with 100 % ethanol) and incubated for 1 h at 95 °C. Following incubation, the tubes were allowed to cool and total sterols were extracted by the addition of 600 μL of n-hexan and vigorous vortexing for 5 min. The tubes were centrifuged at 10,443 rpm for 1 min and the entire organic top layer was transferred to a new tube and mixed with 1 mL of n-hexane. Absorbance readings were performed at 295 nm. Ergosterol quantification was performed by comparison to the standard curve with ergosterol P.A. (Sigma-Aldrich, Germany). The experiments were performed in duplicate. The results were compared with the ergosterol content of cells grown in YNB without antifolate drugs; itraconazole (MIC/2) was used as a positive control.

Biofilm formation

For biofilm testing, inocula were prepared as described by Martinez et al. [9]. In brief, strains of *C. neoformans* (n=7) and *C. gattii* (n=7), randomly chosen from the set of clinical and environmental isolates, were grown in Sabouraud dextrose broth for 24 h at 30 °C in a rotary shaker at 150 rpm. After this period, the cells were collected by centrifugation and the pellet was washed two times with PBS. Suspensions were adjusted to 10^7 cells/mL in chemical medium (20 mg/mL thiamine, 30 mM glucose, 26 mM glycine, 20 mM $MgSO_4.7H_2O$, 58.8 mM KH_2PO_4) and then 100-µl inoculum aliquots were transferred to flat wells of 96-well polystyrene plates. The plates were incubated at 37 °C for 48 h and then the wells were washed three times with 0.05 % Tween 20 in Tris-buffered solution to remove non-adhered cells. Biofilm viability was monitored by the color change of resazurin solution (0.1 mg/mL in RPMI) after incubation at 37 °C for at least 6 h [29].

Effect of SMX/TMP and SDZ/PYR against mature Cryptococcus biofilms

The inhibitory activity of SMX/TMP and SDZ/PYR against *Cryptococcus* biofilms was evaluated according to Martinez et al. [9], with slight modifications. Aliquots of 200 μL of each antimicrobial combination at two different concentrations were added to viable 48-h biofilms as follows: SMX/SDZ at 5,000 mg/L–1,000 mg/L and 2,000 mg/L–400 mg/L; SDZ/TMP at 5,000 mg/L–250 mg/L and 2,000 mg/L–100 mg/L.

Following incubation at 37 °C for 48 h, the supernatant was aspirated and an aliquot of 100 μL of 0.3 % crystal violet was added to each well. After 5 min at 25 °C, the dye solution was aspirated and the wells were washed twice with sterile distilled water. The wells were filled with 200 μL of 100 % ethanol and, after 5 min at 25 °C, the mixture was aspirated and read in a spectrophotometer at 550 nm [29]. The tests were conducted in duplicate; controls were grown in medium without antimicrobials. The effect of antifolate drugs was compared with AMB, which is considered to be a strong inhibitor of *Cryptococcus* biofilms [14].

Effect of SMX/TMP and SDZ/PYR in preventing C. neoformans biofilm formation

In addition, the biofilm inhibition ability of SMX/TMP and SDZ/PYR were evaluated. Biofilm formation was conducted as previously described except for the addition of antifolates to the chemical medium at the following drug combinations: SMX/TMP at 5,000 mg/L–1,000 mg/L and 2,000 mg/L–400 mg/L; SDZ/PYR at 5,000 mg/L–250 mg/L and 2,000 mg/L–100 mg/L. Controls were grown in minimum medium without antimicrobials. Biofilm viability was monitored by resazurin metabolism and crystal violet dying [29], as described above.

Statistical analysis

The antimicrobial susceptibilities were compared using one-way analysis of variance (ANOVA) and Tukey's multiple comparison post-test. Differences between treatments were

evaluated for significance using the Wilcoxon signed-rank test. A p-value < 0.05 was considered to be significant. The statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

Results

Effect of antifolate drug combinations on *Cryptococcus* planktonic cells

The susceptibility profile of *Cryptococcus* planktonic cells to antifolate drugs are shown in Table 1. For *C. neoformans*, the MIC values ranged from 7.81 to 62.5 μg/mL for SMX and 1.56 to 12.5 μg/mL for TMP; 15.63 to 500 μg/mL for SDZ and 0.781 to 25 μg/mL for PYR. For *C. gattii*, the MIC values were higher ($p<0.001$) and ranged as follows: 62.5 to 250 μg/mL for SMX and 12.5 to 50 μg/mL for TMP; 125 to 500 μg/mL for SDZ and 6.25 to 25 μg/mL for PYR.

After pre-exposure to sub-MIC concentrations of SMX/TMP and SDZ/PYR, strains of *C. neoformans* and *C. gattii* showed a decrease in MIC values of AMB ($p<0.05$) (Table 2).

Effect of antifolate drug combinations on ergosterol content of planktonic cells

Sub-inhibitory concentrations of SMX/TMP and SDZ/PYR were also able to decrease the ergosterol content of *Cryptococcus* isolates ($p<0.05$) (Fig. 1). No significant differences in the mean amount of total ergosterol between *C. neoformans* and *C. gattii* grown in SMX/TMP or SDZ/PYR were observed.

Effect of antifolate drug combinations against *Cryptococcus* biofilms

The antifolate drugs were able to inhibit mature *Cryptococcus* biofilms by up to 90 % (Fig. 2). In addition, SMX/TMP and SDZ/PYR caused a reduction of approximately 80 % in the biofilm formation (Fig. 3) of both *C. neoformans* and *C. gattii*. There were no further statistically significant differences between antifolate drugs and AMB at any concentration ($p>0.05$) in both experiments.

Discussion

In recent years, several studies have shown the potential of antifungal drugs to inhibit folic acid synthesis in *Candida albicans* [30], *Paracoccidioides brasiliensis* [31], *Histoplasma capsulatum* [32], *Coccidioides posadasii* [26], and *Aspergillus* spp. [24]. Additionally, the combination SMX/

Table 1 Susceptibility profile of *Cryptococcus* planktonic cells to antifolate drugs

Strain	Species	MIC (μg/mL)	
		SMX/TMP	SDZ/PYR
03-02-062	*C. gattii*	125.0/25.0	500.0/25.0
03-02-069	*C. gattii*	125.0/25.0	250.0/12.5
03-02-070	*C. gattii*	62.5/12.5	125.0/6.25
03-02-071	*C. gattii*	62.5/12.5	250.0/12.5
03-02-073	*C. gattii*	250.0/50.0	500.0/25.0
05-03-028	*C. gattii*	125.0/25.0	250.0/12.5
05-03-029	*C. gattii*	125.0/25.0	250.0/12.5
05-03-030	*C. gattii*	62.5/12.5	125.0/6.25
05-03-031	*C. gattii*	125.0/25.0	125.0/6.25
05-03-032	*C. gattii*	125.0/25.0	250.0/12.5
05-03-033	*C. gattii*	125.0/25.0	125.0/6.25
03-02-074	*C. gattii*	125.0/25.0	250.0/6.25
05-02-080	*C. gattii*	125.0/25.0	250.0/12.5
05-03-037	*C. gattii*	250.0/25.0	500.0/6.25
05-02-082	*C. gattii*	125.0/25.0	250.0/12.5
Geometric mean		119.4/22.79	238.7/10.39
03-02-057	*C. neoformans*	15.625/3.125	62.5/3.125
03-02-061	*C. neoformans*	31.25/6.25	15.625/0.781
03-02-068	*C. neoformans*	62.5/12.5	125.0/6.25
03-02-072	*C. neoformans*	31.25/6.25	15.625/0.781
05-01-050	*C. neoformans*	31.25/6.25	62.5/3.125
03-02-060	*C. neoformans*	31.25/6.25	62.5/3.125
03-02-063	*C. neoformans*	62.5/12.5	125.0/6.25
03-02-064	*C. neoformans*	15.625/3.125	125.0/6.25
03-02-065	*C. neoformans*	31.25/6.25	62.5/3.125
03-02-066	*C. neoformans*	7.81/1.56	500.0/25.0
03-02-067	*C. neoformans*	15.625/3.12	62.5/3.125
03-02-075	*C. neoformans*	15.625/3.12	62.5/3.125
03-02-078	*C. neoformans*	31.25/6.25	125.0/6.25
03-02-080	*C. neoformans*	15.625/3.12	125.0/6.25
03-02-084	*C. neoformans*	15.625/1.56	62.5/3.125
Geometric mean		23.68/4.52	75.19/5.96
Reference strains			
WM148	*C. neoformans*	3.906/0.78	31.25/1.562
WM626	*C. neoformans*	3.906/0.78	31.25/1.562
WM628	*C. neoformans*	3.906/0.78	15.625/0.78
WM629	*C. neoformans*	7.81/1.56	31.25/1.562
WM179	*C. gattii*	250.0/50.0	250.0/12.5
WM178	*C. gattii*	125.0/25.0	250.0/12.5
WM161	*C. gattii*	125.0/25.0	250.0/12.5
WM779	*C. gattii*	125.0/25.0	250.0/12.5
Geometric mean		26.28/5.25	81.05/22.78

SMX sulfamethoxazole, *TMP* trimethoprim, *SDZ* sulfadiazine, *PYR* pyrimethamine

Table 2 Effect of pre-exposure to folate inhibitors on the antifungal susceptibility of *Cryptococcus* planktonic cells

Strain	Species	MIC (μg/mL)		
		AMB	SMX/TMP AMB[a]	SDZ/PYR AMB[a]
03-02-057	*C. neoformans*	1.25	0.3125	0.625
03-02-060	*C. neoformans*	0.25	0.125	0.125
03-02-063	*C. neoformans*	0.156	0.078	0.078
03-02-067	*C. neoformans*	1.25	0.3125	0.625
03-02-068	*C. neoformans*	1.25	0.156	0.3125
03-02-072	*C. neoformans*	0.5	0.0625	0.125
05-02-075	*C. neoformans*	1.25	0.3125	0.156
03-02-062	*C. gattii*	0.625	0.3125	0.312
03-02-069	*C. gattii*	1.25	0.156	0.3125
03-02-073	*C. gattii*	2.5	0.156	0.625
05-03-029	*C. gattii*	0.5	0.125	0.125
05-03-030	*C. gattii*	1.0	0.125	0.25
05-03-031	*C. gattii*	1.0	0.125	0.125
05-03-032	*C. gattii*	0.5	0.125	0.25

SMX sulfamethoxazole, *TMP* trimethoprim, *SDZ* sulfadiazine, *PYR* pyrimethamine, *AMB* amphotericin B

[a] Pre-exposure to folate inhibitors, followed by microdilution test to AMB

TMP has been used for years to treat paracoccidioidomycosis in Latin America [31], as well as for the treatment and prevention of *Pneumocystis jiroveci* pneumonia (PCP) in AIDS patients [33].

The present study shows that the combinations SMX/TMP and SDZ/PYR—in the same ratio in which they are administered in vivo (1:5)—have antifungal activity against *C. neoformans* and *C. gattii* planktonic cells and are also able to enhance AMB activity. The antifungal activity of sulfa drugs on *Cryptococcus* was previously demonstrated by Hanafy et al. [24], who investigated the inhibitory effect of nine different sulfa drugs against a small number of *Cryptococcus* strains and concluded that SMX showed promising results.

In this study, SMX was able to inhibit the growth of *C. neoformans* and *C. gattii* at concentrations of 6.23 μg/mL and 119.3 μg/mL, respectively, while Hanafy et al. [24] showed MIC values of up to 64 μg/mL for *C. neoformans* and >250 μg/mL for *C. gattii*. Possibly, this reduction in the MIC value is due to the synergistic effect of the drug with TMP. This study also shows that the SMX/TMP combination was more effective than SDZ/PYR.

Although the great majority of clinical and environmental isolates of *Cryptococcus* are sensitive to antifungals, the past decade has seen the emergence of resistant isolates in vitro to AMB and azoles [5, 6, 27]. In addition, the literature has reported the occurrence of strains of *C. gattii* with moderate resistance to FLC, ITC, and 5-fluorocytosine, as well as strains of *C. neoformans* with primary resistance to AMB, FLC, and voriconazole [5, 6, 34]. Given this scenario, researchers have attempted to find new antifungal drugs and to formulate strategies to increase the microbial sensitivity to antifungal agents for therapeutic use. This study demonstrates that pre-exposure to sub-MIC doses of antifolate drugs was able to increase the sensitivity of *Cryptococcus* to AMB. Discrete reduction (albeit statistically

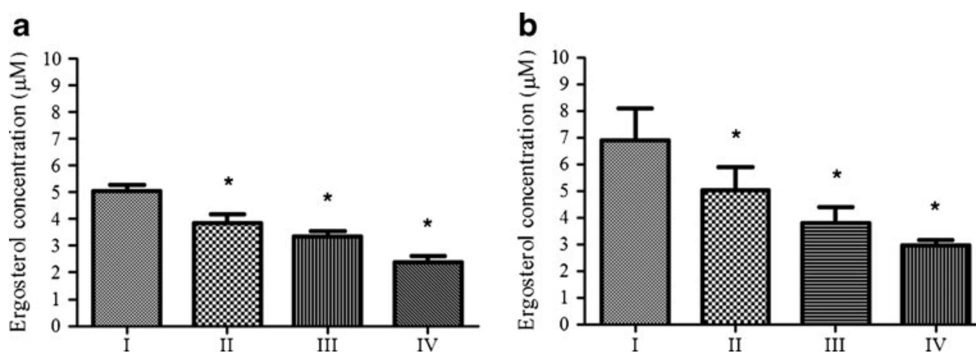

Fig. 1 Ergosterol content of *C. neoformans* (**a**) and *C. gattii* (**b**). Cells were cultured in YNB medium without antimicrobials as control (*I*) or supplemented with SMX/TMP MIC/2 (*II*), SDZ/PYR MIC/2 (*III*), or ITC MIC/2 (*IV*). The experiments were conducted in duplicate and the data are expressed as mean ± SEM (n=7). The *asterisks* indicate statistically significant differences from controls (p<0.05)

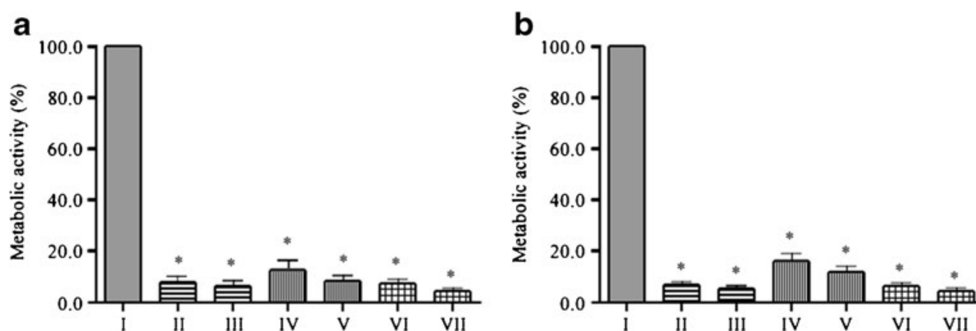

Fig. 2 Metabolic activity of *C. neoformans* (**a**) and *C. gattii* (**b**) biofilms when treated with different concentrations of antifolate drugs. Biofilms were grown in defined chemical medium without antimicrobials as controls (*I*) and, after 48 h, were tested against SMX/TMP at 2,000 mg/L–400 mg/L (*II*), SMX/TMP at 5,000 mg/L–1,000 mg/L (*III*), SDZ/PYR at 2,000 mg/L–100 mg/L (*IV*), SDZ/PYR at 5,000 mg/L–250 mg/L (*V*), AMB at 64 mg/L (*VI*), or AMB at 150 mg/L (*VII*). The results are represented as the percentage of reduction in comparison with controls. The experiments were conducted in duplicate and the data are expressed as mean ± SEM (n=7). The *asterisks* indicate statistically significant differences from controls (p<0.05)

relevant) of itraconazole and fluconazole MICs was also detected after pre-exposure to sub-MIC doses of SMX/TMP and SDZ/PYR (data not shown).

The data from this study show that the antifolate drugs cause a reduction in the cellular content of ergosterol in *C. neoformans* and *C. gattii* equivalent to that promoted by ITC, a drug that damages the synthesis of ergosterol. Navarro-Martínez et al. [30] suggested that, in *C. albicans*, folate inhibitors can inhibit S-adenosylmethionine (SAM), an enzyme cofactor which depends on sterol-C24-methyltransferase and that acts on the ergosterol biosynthetic pathway. It is possible that the reduction of ergosterol content in *Cryptococcus* occurs by a similar mechanism.

In this study, two important aspects of the anti-biofilm potential of folate inhibitor drugs were evaluated: the ability to prevent biofilm formation and the action of the mature biofilm. By employing an in vitro model with high reproducibility, as recommended by Martinez et al. [9], it was possible to demonstrate that SMX/TMP and SDZ/PYR have an anti-biofilm effect, acting on the two mechanisms investigated. Under the conditions tested, SMX/TMP and SDZ/PYR showed inhibitory activity similar to that of AMB at 64 mg/L, whose action on *Cryptococcus* biofilms has been previously described [14]. The results presented here are of great importance, given the high resistance of *Cryptococcus* biofilms to antifungal drugs for therapeutic use, such as FLC and voriconazole [14].

Although the antifolate combinations had shown a considerable anti-biofilm activity, we are aware that the concentrations tested in this study were above the therapeutic doses of each drug. However, it is possible that these concentrations may be attainable in catheters by way of intraluminal lock therapy.

Our results allow us to conclude that the combinations SMX/TMP and SDZ/PYR inhibit the growth of planktonic cells of *C. neoformans* and *C. gattii*, reduce their content of ergosterol, and increase their susceptibility to AMB. The drugs tested were able to prevent biofilm formation and also

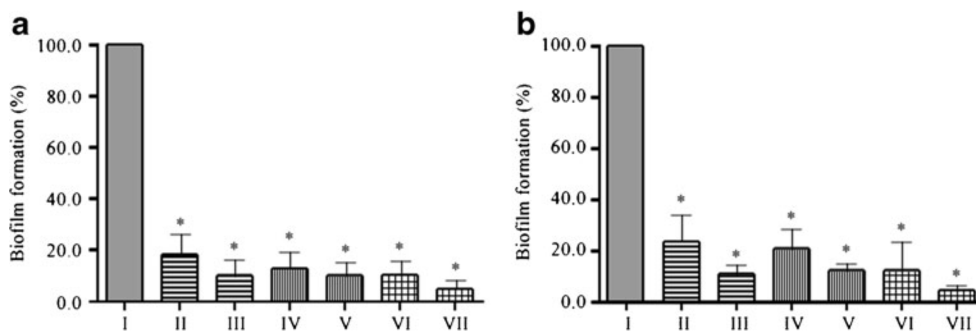

Fig. 3 Inhibition of biofilm formation in *C. neoformans* (**a**) and *C. gattii* (**b**). Biofilms were grown in defined chemical medium without antimicrobials as controls (*I*) or supplemented with SMX/TMP at 2,000 mg/L–400 mg/L (*II*), SMX/TMP at 5,000 mg/L–1,000 mg/L (*III*), SDZ/PYR at 2,000 mg/L–100 mg/L (*IV*), SDZ/PYR at 5,000 mg/L–250 mg/L (*V*), AMB at 64 mg/L (*VI*), or AMB at 150 mg/L (*VII*). The results are represented as the percentage of reduction in comparison with controls. The experiments were conducted in duplicate and the data are expressed as mean ± SEM (n=7). The *asterisks* indicate statistically significant differences from controls (p<0.05)

to significantly reduce the survival of cells in biofilm. Further studies in vivo are needed to confirm the usefulness of these results.

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Conflict of interest The authors declare that they have no conflict of interest.

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