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CLINICAL MICROBIOLOGY - SHORT COMMUNICATION

Anti-*Sporothrix* activity of ibuprofen combined with antifungal

Luana Pereira Borba-Santos[1] · Marcio Nucci[2] · Antonio Ferreira-Pereira[3] · Sonia Rozental[1]

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Abstract

The in vitro activity of ibuprofen, a nonsteroidal anti-inflammatory drug, was evaluated against *Sporothrix brasiliensis* and *S. schenckii*, either alone or in combination with amphotericin B, itraconazole, or terbinafine. The inhibitory activity of ibuprofen as a single agent was determined according to minimum inhibitory concentration (MIC) values, while the effect of ibuprofen combined with amphotericin B, itraconazole, or terbinafine was estimated by microdilution checkerboard methodology. The ultrastructural alterations of *S. schenckii* after exposure to the combination of ibuprofen and amphotericin B were evaluated by scanning electron microscopy (SEM) and flow cytometry analysis. As a single agent, ibuprofen inhibited *Sporothrix* growth with a MIC median of 256 μg/mL, while the MIC medians of ibuprofen in combination with antifungals were 16 μg/mL and 128 μg/mL. The MIC values of amphotericin B, itraconazole, and terbinafine were reduced when isolates were co-incubated with ibuprofen, mainly the polyene. The major alteration after treatment with the ibuprofen/amphotericin B combination was the increase in the presence of filamentous forms and high membrane damage with loss of plasma membrane integrity. In summary, we demonstrated that ibuprofen increases the in vitro activity of antifungals, mainly amphotericin B, against *S. brasiliensis* and *S. schenckii*. Future in vivo studies exploring combination therapy with ibuprofen and antifungals in animal models are needed to confirm its efficacy.

Keywords Ibuprofen · Amphotericin B · *Sporothrix schenckii* · *Sporothrix brasiliensis*

Introduction

The genus *Sporothrix* includes important human dimorphic fungi pathogens that cause sporotrichosis, a neglected endemic mycosis with worldwide distribution [1]. The pathogenic form of *Sporothrix* spp. is the yeast that is found in infected tissues of mammalian hosts. Sporotrichosis can be transmitted to humans through sapronotic or zoonotic routes. The sapronotic transmission route involves direct contact with contaminated soil and decomposing organic matter, where the fungus is found in the filamentous form (which converts to a yeast form in the mammalian host), while zoonotic transmission involves the inoculation of yeasts through scratches and/or bites from infected cats [2].

S. brasiliensis and *S. schenckii* are the most virulent species of the *Sporothrix* genus in the Americas [1]. In Brazil, *S. brasiliensis* is the most frequent etiological agent, followed by *S. schenckii*, which is the most common in Latin America overall [1]. The most usual clinical manifestation of sporotrichosis is the lymphocutaneous form (approximately 80% of patients exhibit this form), followed by the fixed cutaneous form [3]. However, extracutaneous forms and more severe forms can occur with cutaneous disseminated, pulmonary, osteoarticular, and neurological manifestations [3].

The first-line treatment against human sporotrichosis is itraconazole, which needs to be given for a prolonged period of time [4]. Terbinafine exhibits the best in vitro anti-*Sporothrix* activity and is effective in the treatment of cutaneous sporotrichosis; however, its effectiveness has not yet been demonstrated for other clinical forms [3]. In severe forms of sporotrichosis (pneumonia, meningitis, or disseminated disease), amphotericin B is the most common treatment option

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✉ Luana Pereira Borba-Santos
luanaborba@biof.ufrj.br

[1] Laboratório de Biologia Celular de Fungos, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Avenida Carlos Chagas Filho 373, Centro de Ciências da Saúde, Bloco G, Sala G0-044, Rio de Janeiro, RJ 21941-902, Brazil

[2] Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

[3] Instituto de Microbiologia Paulo de Goés, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

[4]. Lipid formulations of amphotericin B are less toxic but much more expensive, with a prohibitive cost for patients in developing countries, than deoxycholate amphotericin B, which presents cardiac and renal toxicity. Patients receiving amphotericin B are switched to oral itraconazole once the disease is under control [3].

Drug repurposing has the potential to yield effective treatment for fungal infections, as well as to speed and reduce the cost of antifungal development [5]. The nonsteroidal anti-inflammatory drug ibuprofen, commonly used as an antipyretic and analgesic medication, is a good example of a compound currently approved for clinical use that may have significant antifungal effect. In recent years, different studies have demonstrated that ibuprofen inhibits fungal growth and potentiates the activity of antifungal agents against some pathogenic fungi [6–11]. However, no studies to date have evaluated the antifungal activity of ibuprofen against *Sporothrix* species.

Herein, we evaluated the in vitro activity of ibuprofen against *S. brasiliensis* and *S. schenckii*, either alone or in combination with amphotericin B, itraconazole, or terbinafine.

Methods

Fungal isolates and culture conditions

Susceptibility to ibuprofen was evaluated in *S. schenckii* and *S. brasiliensis* (two reference isolates and five human clinical isolates of each species). Clinical strains used in this study were kindly provided by collaborating researchers and are deposited at the fungal culture collection of the Fungal Cell Biology Laboratory/Universidade Federal do Rio de Janeiro (Rio de Janeiro, Brazil). These data are registered at the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado, Brazil (SisGen, number ABF8BB7). Isolates were stored at − 20 °C in saline solution containing 10% glycerol and 10% glucose. Before experiments, the filamentous form was cultivated in Sabouraud broth (Difco, USA) at 36 °C for 7 days, with orbital shaking (at 150 rpm). Then, to obtain yeasts, filamentous fungi were inoculated into brain heart infusion broth (Difco, USA) supplemented with 2% glucose (pH 7.8) and cultivated at 36 °C, with orbital shaking for 7 days.

Drugs

Ibuprofen, amphotericin B, itraconazole, and terbinafine (Sigma Chemical Co., USA) stock solutions were diluted in dimethyl sulfoxide (DMSO) at 102.400 μg/mL (ibuprofen) and 1.600 μg/mL (amphotericin B, itraconazole, and terbinafine). Dilutions of compounds in RPMI 1640 medium (supplemented with 2% glucose and buffered to pH 7.2 using 0.165 M MOPS) were made fresh for each experiment.

Minimum inhibitory concentration test

To evaluate the inhibitory activity of ibuprofen alone against *Sporothrix* spp., minimum inhibitory concentration (MIC) values were determined according to the broth microdilution technique (document M27, by the Clinical and Laboratory Standards Institute) [12], with minor modification for use with *Sporothrix* spp. yeasts. MIC values were also determined for amphotericin B, itraconazole, and terbinafine. Briefly, serial two-fold dilutions of compounds were prepared in RPMI 1640 medium (supplemented with 2% glucose and buffered to pH 7.2 with 0.165 M MOPS) into flat-bottom 96-well microplates to obtain a final concentration ranging from 2 to 1.024 μg/mL ibuprofen and 0.03 to 16 μg/mL antifungal. Yeasts were added to each well at a final concentration of $0.5–1 \times 10^5$ CFU/mL. Microplates were incubated at 35 °C for 48 h in a 5% CO_2 chamber. Fungal growth was determined by visual inspection in an inverted light microscope and quantified by spectrophotometric readings at 492 nm in an EMax Plus plate reader (Molecular Devices, USA). Inhibition of fungal growth (I) relative to untreated controls was calculated according to the following equation: $I = 100 - (A \times 100/C)$, where A is the absorbance of treated wells and C is the absorbance of untreated wells. MIC was defined as the concentration that inhibited ≥ 50% of fungal growth relative to an untreated control. All results were representative of two independent experiments made in duplicate.

Combination test between ibuprofen and antifungal

To assess the effect of ibuprofen combined with amphotericin B, itraconazole, or terbinafine, the microdilution checkerboard methodology was performed [13]. Yeasts ($0.5–1.5 \times 10^5$ CFU/mL) were exposed to concentrations ranging from 16 to 1.024 μg/mL ibuprofen, 0.001 to 1 μg/mL amphotericin B, 0.001 to 8 μg/mL itraconazole, and 0.0001 to 1 μg/mL terbinafine. After 48 h at 35 °C (and 5% CO_2), MIC values for drugs as single agents and in combinations were determined. The effect of combinations was analyzed according to the fractional inhibitory concentration index (FICI), which is calculated as follows: $FICI = (MIC_{ibuprofen}$ in combination/$MIC_{ibuprofen}$ alone) + ($MIC_{antifungal}$ in combination/$MIC_{antifungal}$ alone) [13]. The following drug combination effects were considered: synergy if FICI ≤ 0.5, no interaction if FICI > 0.5–4, and antagonism if FICI > 4 [14]. The best ibuprofen and antifungal combinations were defined as the ones with the lowest FICI values. All results were representative of at least two independent experiments.

Scanning electron microscopy

To analyze the ultrastructural effects after exposure to ibuprofen/amphotericin B combination, a clinical *S. schenckii* isolate (Ss 110) was treated and visualized by scanning electron microscopy (SEM). Yeasts (1×10^5 CFU/mL) were exposed to 128 μg/mL ibuprofen and 0.001 μg/mL amphotericin B (alone and combined) for 48 h at 36 °C, with orbital agitation, in RPMI 1640 medium (supplemented with 2% glucose and buffered to pH 7.2 using 0.165 M MOPS). Untreated cultures were incubated under the same conditions as the treated samples. Untreated and treated cells were washed in PBS and fixed in 2.5% glutaraldehyde and 4% formaldehyde in 0.1 M cacodylate buffer for 40 min. Samples were washed in cacodylate buffer, adhered to poly-L-lysine-coated glass coverslips, dehydrated in a graded ethanol series, critical point-dried in CO_2, and coated with gold. Images were obtained in a FEI Quanta 250 SEM (FEI Company, USA). Images were processed using Photoshop software (Adobe, USA). Different cell types were counted (200 cells) and classified as isolated cell (yeast), budding yeast, and filamentous form (hyphae and pseudohyphae-like cells).

Flow cytometry analysis

To determine the cell effects in the Ss 110 isolate after exposure to the combination of ibuprofen and amphotericin B, cells were treated as described above and analyzed by flow cytometry. Untreated and treated cultures were filtered with double layer sterile gauze and washed in PBS. Cells (1×10^7) were incubated with 25 μM of 2′,7′-dichlorofluorescein diacetate (Sigma Chemical C_O., USA) or 20 μM of SYTOX™ Green (Thermo Fisher Scientific, USA) for 30 min at room temperature in the dark. Then, cells were washed in PBS, fixed in 2% formaldehyde in PBS, and washed again. Cells were analyzed in a BD Accuri™ C6 flow cytometer (BD Biosciences, USA) by counting 2000 events per sample, and data were analyzed using BD Accuri C6 software. Results are representative of three independent experiments. SYTOX™ Green does not cross intact membranes, while 2′,7′-dichlorofluorescein diacetate is used to quantify reactive oxygen species (ROS).

Results

Our data revealed that, as a single agent, ibuprofen inhibited *Sporothrix* growth with a MIC median of 256 μg/mL for both species (Table 1). For *S. brasiliensis*, the MIC medians of ibuprofen in combination with itraconazole, terbinafine, or amphotericin B were reduced to 16 μg/mL and 128 μg/mL, respectively (Table 1). Additionally, the MIC medians of terbinafine and amphotericin B were also reduced when combined with ibuprofen, as there was a two-fold decrement for amphotericin B and a four-fold decrement for terbinafine against *S. brasiliensis*. Distinctly, the MIC medians of itraconazole seem not to be affected when co-incubated with ibuprofen against *S. brasiliensis* (Table 1).

For *S. schenckii*, the decrements in the MIC medians were more prominent after co-incubation of ibuprofen and the antifungals. After co-incubation with ibuprofen, the MIC medians observed for amphotericin B were reduced 62-fold, while those for itraconazole and terbinafine were reduced two-fold (Table 1). According to the FICI interpretation, ibuprofen exhibited in vitro synergism with amphotericin B against two isolates and with itraconazole against five isolates (FICI ≤ 0.50). In addition, all tested isolates exhibited a reduction in the amphotericin B MIC after co-incubation with ibuprofen. Distinctly, combinations of ibuprofen and terbinafine did not exhibit synergism for *S. schenckii* (Table 1).

The Ss 110 isolate of *S. schenckii* presented a 125-fold reduction of the MIC value of amphotericin B when co-incubated with ibuprofen and was selected for further analysis by SEM and flow cytometry, aiming to determinate the antifungal effects. For these experiments, the Ss 110 isolate was exposed to 128 μg/mL ibuprofen plus 0.001 μg/mL amphotericin B, a combination that produced a 125-fold reduction of the amphotericin B dose able to inhibit fungal growth (Table 1).

SEM images revealed that, in all treatments, it was possible to observe a mix of yeast, budding cells, and filamentous forms (Fig. 1a–d). Yeast-hyphae conversion was occurring but in a different percentage in each situation (Fig. 1e). Untreated cultures accounted for 53% of single yeast cells, 29% of budding yeast, and 18% of filamentous forms. The use of ibuprofen alone did not interfere at the percentages of the different fungal morphologies (Fig. 1e), but the presence of conidia with altered structure could be observed (arrow in Fig. 1b). Treatment with amphotericin B led to an increase of single yeast (63%) and the appearance of amorphous cells (arrow Fig. 1c). In cultures treated with the combination of amphotericin B and ibuprofen, it was possible to observe the presence of a chlamydospore-like structure, which was not visualized in other samples (Fig. 1d). The combination treatment also led an increase in filamentous forms (25%).

The integrity of the fungal plasma membrane was also investigated. Our data revealed that the exposure of the *S. schenckii* Ss110 strain to the combination of ibuprofen and amphotericin B induced a loss of plasma membrane integrity, as demonstrated by increase of 2.9-fold in the SYTOX™ Green labeling. Ibuprofen and amphotericin B alone also induced an increase of loss of plasma membrane integrity around 2.5- and 2.1-fold, respectively (Table 2). ROS accumulation after treatment with ibuprofen and amphotericin B alone was also identified as leading to an increase of 1.4- and 1.6-fold in 2′,7′-dichlorofluorescein diacetate labeling,

Table 1 Antifungal activity of ibuprofen (IBP) alone and in combination with amphotericin B (AMB), itraconazole (ITC), or terbinafine (TRB) against *Sporothrix* isolates

Isolates	MIC[a] (μg/mL)				MIC in combination[b] (μg/mL)		
	IBP	AMB	ITC	TRB	IBP/AMB (Effect[c])	IBP/ITC (Effect[c])	IBP/TRB (Effect[c])
S. brasiliensis							
CBS 133006	128	0.03	0.06	0.125	64/0.001 (N)	16/0.015 (S)	64/0.004 (N)
CBS 132992	128	0.03	0.06	0.03	32/0.008 (N)	32/0.001 (S)	64/0.001(N)
B428	512	0.125	0.015	0.03	256/0.06 (N)	16/0.06 (A)	16/0.03 (N)
B758	512	0.5	0.03	0.008	256/0.125(N)	32/0.06 (N)	16/0.008 (N)
B972	512	0.25	0.5	0.03	256/0.06 (N)	16/1.00 (N)	16/0.06 (N)
HE06	256	0.125	0.06	0.008	128/0.06 (N)	16/0.5 (A)	16/0.008 (N)
Ss 56	128	0.06	0.125	0.03	32/0.002 (S)	64/0.001(N)	32/0.015 (N)
MIC median	256	0.125	0.06	0.03	*128/0.06*	*16/0.06*	*16/0.008*
S. schenckii							
ATCC 32286	256	0.25	0.03	0.015	128/0.002 (N)	64/0.001 (S)	128/0.001(N)
ATCC 16345	256	0.25	0.06	0.015	64/0.06 (S)	32/0.015 (S)	16/0.008 (N)
Ss 03	256	0.25	0.015	0.008	16/0.125 (N)	16/0.015 (N)	32/0.008(N)
Ss 22	256	0.125	0.06	0.125	128/0.001(N)	16/0.125(N)	128/0.008(N)
Ss 42	256	0.25	0.015	0.008	128/0.004 (N)	16/0.06(A)	128/0.001(N)
Ss 73	256	0.25	0.015	0.06	128/0.015(N)	16/0.015(N)	16/0.06(N)
Ss 110	256	0.125	0.03	0.008	128/0.001(N)	32/0.002(S)	128/0.001(N)
MIC median	256	0.25	0.03	0.015	*128/0.004*	*16/0.015*	*128/0.008*

[a] MIC, minimum inhibitory concentration, that reduced ≥ 50% of fungal growth relative to an untreated control, according to spectrophotometric readings. [b] Best combinations were defined as those with the lowest FICI values. FICI = ($MIC_{ibuprofen}$ in combination/$MIC_{ibuprofen}$ alone) + ($MIC_{antifungal}$ in combination/$MIC_{antifungal}$ alone). [c] Effects were considered synergistic (S) if FICI ≤ 0.50, no interaction (N) if FICI > 0.5–4, and antagonism (A) if FICI > 4

[d] MIC median, the median of MIC values

respectively. In contrast, treatment with the drug combinations decreased the production of ROS (Table 2).

Discussion

Although fungal infections are frequently observed nowadays, current therapeutic options against pathogenic fungi remain limited to a few therapeutic classes and drugs. This issue highlights the need to identify compounds/drugs with significant antifungal activity, especially against dimorphic fungi, which are responsible for numerous types of mycoses that may be particularly challenging to treat [15].

According to the drug repurposing concept, the activity of conventional antifungal agents could be enhanced when given together with drugs used for other clinical indications [5]. Here, we demonstrated that ibuprofen, an anti-inflammatory drug, increased the in vitro activity of antifungals, mainly amphotericin B, against *S. brasiliensis* and *S. schenckii*, the main etiological agents of sporotrichosis in Brazil and Latin America, respectively.

Ibuprofen alone showed a low in vitro antifungal activity against *S. brasiliensis* and *S. schenckii* (MIC median of 256 μg/mL). High MIC values for ibuprofen were previously reported for other fungal species such as *Cryptococcus neoformans* (MIC = 206 μg/mL), *Trichosporon asahii* (MIC = 500 μg/mL), *Pythium insidiosum* (MIC = 512 μg/mL), and *Candida* species (MIC = 1031 μg/mL) [7, 9–11].

Our results indicate that, although ibuprofen alone is not so effective against *S. brasiliensis* and *S. schenckii*, when combined with antifungal drugs, it was able to reduce the concentrations required to inhibit fungal growth in vitro. Previous studies have also reported that ibuprofen could increase the antifungal activity of amphotericin B, itraconazole, or terbinafine against other pathogenic fungi as *P. insidiosum*, *Fusarium solani*, and *T. asahii* [8, 9, 11].

Here, ibuprofen plus amphotericin B was the best combination tested against *S. schenckii* (Table 1). Although the combination treatment led to a slight reduction of the ibuprofen doses for most isolates (a reduction near 50%), it induced a great decrease in the dose of amphotericin B, up to 125-fold. Considering the high toxicity of amphotericin B, the side

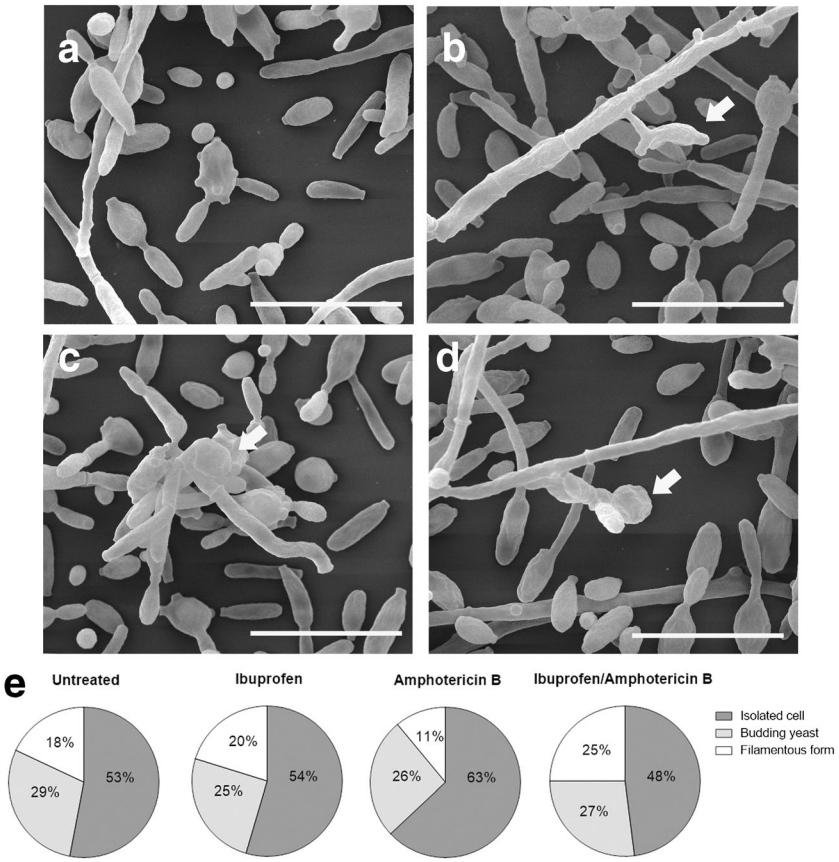

Fig. 1 *Sporothrix schenckii* alterations after exposure to the combination of ibuprofen and amphotericin B evaluated by scanning electron microscopy. The untreated culture exhibits yeasts with elongated shape, budding yeasts, and hyphae (**a**), while cultures treated with 128 μg/mL ibuprofen (**b**) or 0.001 μg/mL amphotericin B (**c**) show conidia with altered structure and amorphous cells (arrows). A chlamydospore-like structure was observed after exposure to the ibuprofen/amphotericin B combination (arrow in **d**), and filamentous forms were the most frequent after this treatment (**e**). Bars: 10 μm

effects caused in the patients that need to use it and that this effect could be repeated in vivo, this dose reduction could be a real gain for the treatment of patients.

Morphological analyses of clinical *S. schenckii* Ss 110 isolate by SEM showed that yeast-hyphae conversion occurs after 48 h of incubation, corresponding to approximately 20% of the cell population (Fig. 1e). At the beginning of the experiments, this morphology was lower than 10%, but after 48 h in the RPMI 1640, it doubled. A 25% increase in hyphae morphology was observed after treatment with the combination of ibuprofen and amphotericin B. It was also possible to observe the presence of a chlamydospore-like structure, which was not visualized in other samples, but its functions remain unclear.

The activity of ibuprofen against fungi was previously described to be related to the induction of plasma membrane damage and ROS accumulation, similar as described for amphotericin B [6, 10, 16]. Our cytometry results corroborate this information, showing that ibuprofen and amphotericin B alone caused the loss of membrane integrity and ROS accumulation in *S. schenckii* (Table 2). When combined, these drugs increased membrane damage, suggesting that the combinatory effect of ibuprofen and amphotericin B can be explained, in part, by an increase in cell permeability. It was also possible to observe a decrease in the ROS levels inside the cells after treatment with the combination that could correspond to inviable cells.

In Brazil, the number of hospitalizations and deaths due to sporotrichosis has increased over the last two decades [17]. Moreover, an increase in severe cases and atypical forms of sporotrichosis has also been reported [18]. Severe forms of sporotrichosis affect mainly immunocompromised individuals [3], who frequently present with comorbidities. Improvements in the efficacy of antifungals, mainly amphotericin B (the most common option for the treatment of more severe forms of the disease [3]), are extremely relevant. More importantly, no pharmacological interactions were described between ibuprofen and the antifungal drugs studied in this work. Our results highlight the importance of

Table 2 Evaluation of membrane integrity and reactive oxygen species (ROS) in *Sporothrix schenckii* after treatment with ibuprofen, amphotericin B, and their combination

Samples	Relative SYTOX™ Green labeling	Relative fluorescence intensity of 2′,7′-dichlorofluorescein diacetate
Untreated	1.0	1.0
Ibuprofen	2.5	1.4
Amphotericin B	2.1	1.6
Ibuprofen/amphotericin B	2.9	0.8

expanding studies about the anti-*Sporothrix* activity of non-steroidal anti-inflammatory drugs, which may improve the treatment of sporotrichosis in the future.

In summary, we demonstrated that ibuprofen increases the in vitro activity of antifungals, mainly amphotericin B, against *S. brasiliensis* and *S. schenckii*. Future in vivo studies exploring combination therapy with ibuprofen and antifungals in animal models are needed to confirm its efficacy.

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Compliance with ethical standards

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

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