

ORIGINAL ARTICLE

Effects of two 6-quinolinyl chalcones on the integrity of plasma membrane of *Paracoccidioides brasiliensis*

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Paracoccidioidomycosis is the most prevalent systemic mycosis in Latin America, yet few therapeutic options exist. Our aim was to search for new compounds with high efficacy, low toxicity, shorter treatment time and affordable cost. We studied two synthetic 6-quinolinyl chalcones, **3b** and **3e**, to determine their effects on VERO cells, antifungal activity, survival curve, interaction with other drugs and phenotypic effects against several isolates of *Paracoccidioides* spp. In this study, we verified that the compounds were not toxic, exhibited superior *in vitro* activity compared with that shown by trimethoprim-sulfamethoxazole, and after 5 days of treatment, decreased the fungal cell viability by approximately 70%. Additionally, no interactions were observed between the tested compounds and other drugs. We also found that these compounds induced morphological changes, such as shriveling of cells, fragmentation of the plasma membrane and cytoplasmic disorganization *in vitro*. The changes observed by microscopy assays corroborate the observation made with propidium iodide, where the number of cells stained with the compounds was higher than that observed after amphotericin B treatment. We observed an increase in the efflux of K⁺ and a loss of intracellular contents in cells treated with **3b** and **3e**, confirming their effects on fungal membranes. However, damage to the membrane was not associated with a decrease in membrane ergosterol levels. The experimental evidences showed no direct indications of cellular wall damage caused by these compounds. Thus, these results confirm the antifungal potential of **3b** and **3e** against *Paracoccidioides* spp. with possible action on the membrane.

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INTRODUCTION

Paracoccidioidomycosis (PCM) is caused by fungi of the *Paracoccidioides* genus, and is the most prevalent systemic mycosis in Latin America.¹ This is considered an important health problem in Brazil and has also been prevalent in Venezuela, Colombia, Ecuador and Argentina.^{2,3} In Latin America, the most susceptible population is low-income rural workers who have limited access to public or private health system.⁴

The most commonly used drugs for treating patients with PCM are sulfonamides, itraconazole (ITZ) and amphotericin B. Sulfonamides were the first class of drugs available for treating patients with PCM; however, long periods of treatment may be required (more than 2 years), and there is increasing concern about their toxicity, cost of treatment and unacceptable rates of noncompliance. Unfortunately, amphotericin B has also been associated with substantial toxicity.^{5,6}

Chalcones are α,β -unsaturated ketone (1,3-diphenyl-2-propen-1-one) derivatives and flavonoid precursors, and are composed of an important class of natural compounds with biological properties such as anti-inflammatory, antioxidant, antiviral, antifungal, antitumor, antimalarial, antileishmanial, analgesic and antituberculosis activity.^{7,8}

Chalcones, which have heterocyclic systems, have been the subject of many studies;^{9–11} however, not enough is known about 6-quinolinyl chalcones, specifically.

The 6-quinolinyl chalcones **3b** and **3e** were synthesized by Tavares *et al.*,¹² and were found to exhibit antifungal activity against *P. brasiliensis* (Pb18 isolate). With the aim of discovering novel drug leads that could result in better therapeutic agents against *Paracoccidioides* spp., we investigated the antifungal activity and the mechanisms of action of these compounds.

MATERIAL AND METHODS

Experimental compounds

The 6-quinolinyl chalcones **3b** and **3e** were provided by the Department of Chemistry, Federal University of Santa Catarina, Florianópolis, SC, Brazil (Table 1). These compounds were synthesized according to the methodology described by Tavares *et al.*¹²

Evaluation of the cytotoxic effects against VERO cells

The VERO (African green monkey kidney cells) lineage was used as a model of normal cells and was kindly provided by Dr Erna Kroon (Universidade Federal

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Table 1 Experimental compounds

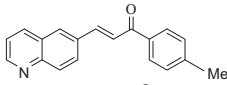
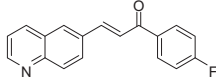
Substance	Structural formula	Molecular formula	Molecular weight	Log P-value
3b		C ₁₉ H ₁₅ NO	273, 33	5,36
3e		C ₁₈ H ₁₂ FNO	277, 29	4,93

Table 2 Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of 3b, 3e, itraconazole (ITZ) and trimethoprim-sulfamethoxazole (SMT) against 14 isolates of the *Paracoccidioides* genus

Isolate ^a	ITZ $\mu\text{g ml}^{-1}$		SMZ $\mu\text{g ml}^{-1}$		3b $\mu\text{g ml}^{-1}$		3e $\mu\text{g ml}^{-1}$	
	CIM	CFM	CIM	CFM	CIM	CFM	CIM	CFM
Pb18 (Pb7)	0.003	0.015	4.70	4.70	7.80	15.62	7.80	7.80
B339 (Pb1)	0.003	0.007	4.70	4.70	3.90	3.90	2.90	1.95
Mg5 (Pb11)	0.003	0.015	0.97	4.70	7.80	7.80	3.90	3.90
470 (Pb14)	0.009	0.015	14.10	37.50	3.90	15.62	7.80	31.25
9673 (Pb8)	0.007	0.031	2.30	9.37	3.90	3.90	3.90	3.90
Ap	0.007	0.007	75.00	75.00	7.80	15.62	7.80	31.25
Pinguim	0.005	0.015	4.70	9.37	3.90	7.80	3.30	3.90
1017 (Pb4)	0.070	0.250	150.00	300.00	23.40	62.50	2.60	3.90
608 (Pb3)	0.06	0.062	150.00	300.00	31.25	62.50	5.90	7.80
1925 (Pb2)	0.002	0.004	11.70	37.50	2.40	3.90	1.95	3.90
EPM83	0.007	0.015	150.00	150.00	2.60	3.90	4.60	3.90
Pb01	0.016	0.062	76.20	150.00	31.25	31.25	31.25	31.25
ED01	0.016	0.062	4.70	9.37	11.70	31.25	7.80	15.60
1578	0.008	0.031	4.70	9.37	15.62	31.25	10.40	31.25
^b MIC ₅₀ // MFC ₅₀	0.007	0.015	4.70	9.37	7.80	15.62	4.60	3.90
^b MIC ₉₀ // MFC ₉₀	0.016	0.062	150.00	300.00	31.25	62.50	10.40	31.25

^aBetween parentheses are names equivalent in Morais *et al.*³⁹

^bMIC₅₀// MFC₅₀ and MIC₉₀// MFC₉₀: MIC/MFC that inhibits/kills 50 and 90% of isolates of *Paracoccidioides* tested, respectively. (b) Survival curve of the isolate Pb18 of *P. brasiliensis* under treatment with substances 3b and 3e (at their MIC), expressed as the percentage of viable cells versus time.

de Minas Gerais, UFMG). This cell line was maintained in the logarithmic phase of growth in Dulbecco's modified Eagle's medium supplemented with 100 IU ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin enriched with 5% fetal bovine serum. The cells were maintained at 37 °C in a humidified incubator with 5% CO₂ and 95% air. The medium was changed twice weekly, and the cells were regularly examined and used until 20 passages.

The VERO cells at 1 × 10⁴ cells per well were pre-incubated in 96-well plates for 24 h at 37 °C to allow the cells to adapt, prior to the addition of the test compounds. All experimental compounds and ITZ (Sigma, St Louis, MO, USA) were dissolved in dimethylsulfoxide (DMSO) prior to dilution. The half-maximal IC₅₀ was determined over a range of concentrations (eight nonserial dilutions: from 0.8 to 500 $\mu\text{g ml}^{-1}$). The VERO cells were incubated with the compounds (3b and 3e) or 0.5% DMSO as a control in a 5% CO₂/95% air-humidified atmosphere at 37 °C for 48 h. Cell viability was estimated by measuring the rate of the mitochondrial reduction of tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Amresco, Solon, OH, USA).

Controls included drug-containing medium (background) and drug-free complete medium. Drug-free complete medium was used as a control (blank) and was treated in the same way as the drug-containing media. Results were expressed as a percentage of inhibition of cell viability compared with the 0.5%

DMSO control and were calculated as follows: % inhibition of cell viability (%) = 100 - (mean optical density (OD) treated - mean OD background) / (mean OD untreated culture, i.e. 0.5% DMSO - mean OD blank wells) × 100. Interactions between compounds and media were estimated based on variations between drug-containing media and drug-free media to avoid false positives or false negatives.¹³ All the samples were tested in triplicate in three independent experiments. The cytotoxicity of ITZ was evaluated under the same experimental conditions as the positive controls.

The IC₅₀ and minimum inhibitory concentration (MIC) data were used to calculate the selectivity index (SI) of each compound, as was done previously by Prottopopova *et al.*¹⁴ The SI was determined by the ratio of the IC₅₀ value in VERO cells to the IC₅₀ value in MIC and calculated as SI = IC₅₀/MIC.

Statistical analysis

The IC₅₀ value for cytotoxicity was determined using Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) software. Data were represented as median and 95% confidence interval. Statistical differences between the treatment and the control groups were evaluated using analysis of variance test followed by Bonferroni test ($P < 0.05$).

Fungus and inoculum

In this study, we used 11 isolates of *P. brasiliensis* (Pb18, B339, Mg5, 470, 9673, Ap, Penguin, 1017, 608, 1925 and EPM83), representing the phylogenetic species S1, PS2 and PS3 and three isolates of *P. lutzii* (Pb01, ED01 and 1578), all belonging to the Laboratory of Microorganism-Host Interaction, Institute of Biological Science, Universidade Federal de Minas Gerais (UFMG) (Table 2). The fungi cultures were maintained in chemically defined McVeigh & Morton (MMcM) medium and subcultures were performed after 7 days of growth at 37 °C. To obtain the inoculum, the cells of *Paracoccidioides* were aseptically collected with a bacteriological loop and suspended in 5 ml of 0.85% sterile saline. The suspension was homogenized by vortexing for approximately 10 s. The suspension containing larger aggregates was rested for decantation, and only the supernatant was collected. The transmittance was measured at a wavelength of 530 nm and then adjusted to 70% by spectrophotometry, equivalent to 1–5 × 10⁶ cells per ml.¹⁵ The resulting suspensions were diluted in MMcM broth medium (1:10) to obtain the final inoculum with 1–5 × 10⁵ cells per ml.¹⁶

Determination of MIC

A bioassay with 14 isolates of *Paracoccidioides* spp. was performed following the CLSI M27-A3 guidelines¹⁷ and modifications suggested by Nakai *et al.*¹⁸ and Johann *et al.*¹⁹ This assay was performed in chemically defined MMcM medium.²⁰ ITZ (Sigma) and trimethoprim/sulfamethoxazole (SMZ) (Belfar, Belo Horizonte, Brazil) were included as positive antifungal controls. The concentrations tested were: 0.48–250 $\mu\text{g ml}^{-1}$ for 3b and 3e, 0.0009–0.5 $\mu\text{g ml}^{-1}$ for ITZ and 1.17–600 $\mu\text{g ml}^{-1}$ for SMZ. Stock solutions were prepared in DMSO (Amresco, Solon, OH, USA) from which two-fold serial dilutions were prepared as described in the CLSI document M27-A3.¹⁷ The measurement of cell survival and proliferation rate was performed using an MTT assay, after 15 days of incubation at 37 °C.²¹ The reading was performed on a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA) by SoftmaxPro 5 software (Molecular Devices) with an absorbance of 570 nm. We reported the data of three independent experiments performed in duplicate.

Minimal fungicidal concentrations

The MFC of each compound tested was determined by streaking the entire content of each well that showed complete inhibition (100% inhibition) and from the last positive well (growth similar to that of the growth control well) and the growth control well onto the plates with yeast, peptone and dextrose (YPD) medium. The plates were incubated at 37 °C for 15 days. The MFC value was considered the lowest drug concentration at which no colonies were able to grow.²²

Time-kill curve procedures

The time-kill curve was performed with the isolate Pb18 of *P. brasiliensis*. This test determined the number of viable cells left in periodic incubation intervals as described by Klepser *et al.*²³ In this experiment, the time required by the antifungal drug to kill 100% of the yeast cells was evaluated. The yeast suspensions in the absence (control) or presence of experimental compounds were prepared in MMcM medium and distributed in microplates. The plates were incubated at 37 °C for 0, 24, 72, 120, 240 and 360 h. The results were determined using MTT assay, following the same protocol used during MIC assay. All the experiments were performed in duplicate.

Checkerboard microtiter test

Eight serial two-fold dilutions of **3b** and **3e** (125–0.97 µg ml⁻¹) and amphotericin B (Sigma) (2–0.015 µg ml⁻¹) were prepared using similar methodology as in the MIC test. The checkerboard was prepared in a microtiter plate for multiple combinations of two antimicrobial agents. Each row (*x*-axis) in the plate contained the same diluted concentration of the first antimicrobial compound, while the concentration in each subsequent row was half of this value. Similarly, each column (*y*-axis) in the plate contained the same diluted concentration of the second antimicrobial compound, while the concentration in each subsequent column was half of this value. The drug combination in which the growth was completely inhibited was considered as the effective MIC for the combination. This assay was performed only using isolate Pb18. One hundred microliters of yeast suspension, prepared using the same procedure as that of the inoculum of the MIC test, was added to each well and cultured for 15 days. Fractional inhibitory concentrations (FICs) were calculated as the MIC of the combination of drugs divided by the MIC of the drug alone. The FIC index (FICI) was calculated by adding both FICs, and interpreting in the following manner: values ≤0.5 indicate a synergistic effect, values within the >0.5–4.0 range indicate no interaction and values >4.0 indicate an antagonistic effect.²⁴ This experiment was also performed to determine the effect of **3b** and **3e** combined with SMZ (1.17–150 µg ml⁻¹) and ITZ (0.0009–0.125 µg ml⁻¹).

Phenotypic effects of compounds **3b** and **3e**

Ultrastructural analysis. Yeast cells (10⁶ cell per ml) of isolate Pb18 grown in MMcM broth containing sub-inhibitory concentrations of **3b** and **3e** were incubated at 37 °C for 15 days. The cells were then washed twice in phosphate-buffered saline (PBS), and initially fixed by immersing in a solution containing 2% (v/v) glutaraldehyde and 2% (w/v) PBS (pH 7.2) for 6 h at room temperature. The material was washed with PBS, and the yeast cells were postfixated in 1% osmium tetroxide (OsO₄) and 0.8% potassium ferricyanide in sodium cacodylate buffer for scanning electron microscopy. The cells were further washed to remove excess OsO₄ and dehydrated in a graded acetone series from 30 to 100% (v/v). Transmission electron microscopy followed by post fixation was carried out in 1% OsO₄, and the material was dehydrated in a graded acetone series from 30 to 100% (v/v) and embedded in Spurr resin. Semi-thin sections were stained with toluidine blue. Ultra-thin sections were stained with uranyl acetate and lead citrate²⁵ and analyzed using a DSM 950 scanning electron microscope (Zeiss, Oberkochen, West Germany) and EM 10 transmission electron microscope (Zeiss). This assay was performed in the Center for Acquisition and Image Processing (CAPI, ICB, UFMG, Belo Horizonte, Brazil).

Integrity membrane assay. Yeast grown to late log phase (usually 10⁷–10⁸ cells per ml) in RPMI medium containing sub-inhibitory concentrations of the compounds were used for the viability assay with the LIVE/DEAD Yeast

Viability Kit (Molecular Probes, Eugene, OR, USA), which contains the fluorophores propidium iodide (PI) and SYTO 9. Fifty microliters of the yeast culture was added to 1 ml of PBS and concentrated by centrifugation at 8g for 5 min in a microcentrifuge (MiniSpin, Eppendorf, Hamburg, Germany). Further, the supernatant was removed and the pellet was resuspended in 1 ml PBS. The prepared cells were stained according to the kit manufacturer's guidelines. The specimen was examined under a fluorescence microscope (Nikon Eclipse Ti, Tokyo, Japan) using the appropriate filter.^{26,27} The microscopic data shown in this work were obtained using a Nikon fluorescence microscope in the Image Acquisition and Processing Center (CAPI-ICB/UFMG). Yeast cells specifically stained with PI were analyzed using a BD FACSCalibur flow cytometry system (Becton Dickinson and Co., Mountain View, CA, USA), according to the manufacturer's kit instructions, and the results were represented as the median fluorescence intensity (arbitrary units of measurement) of the samples labeled with PI.

Extracellular leakage of potassium (K⁺). The K⁺ efflux measurement method previously described by Zhang and Takemoto²⁸ was used with modifications. The yeast *P. brasiliensis* isolate Pb18 was suspended in sterile 10 mM HEPES-NaOH buffer (pH 6.5) with 25 mmol l⁻¹ glucose. The washed cells were used to inoculate 10 ml of sterile buffer solution to obtain a final cell density of 10⁶ cells ml⁻¹. The yeast cells treated with compounds **3b** and **3e** (7.8–15.6 µg ml⁻¹) and amphotericin B (0.05 µg ml⁻¹) for 1, 3, 7, 10 or 15 days were incubated in a shaking incubator (Tecnal, TE-424, Brazil) at 37 °C with agitation at 120 r.p.m. Cell-free filtrates were obtained by individually filtering each suspension with 0.2 mm syringe filters (Jet Biofil, Guangzhou, China). The filtrates were subjected to atomic emission spectrometry using a flame emission spectrometer at 766.5 nm to determine the extracellular K⁺ concentrations using a standard calibration curve based on KCl solutions. The boiled (20 min) cell suspensions were used to estimate total cellular K⁺. The untreated cells were used as negative controls. K⁺ efflux was calculated as the percentage of total cellular K⁺ using the formula: K⁺ efflux (%) = ((extracellular K⁺ – extracellular K⁺ in negative control)/(total cellular K⁺)) × 100.

Release of cellular material. The methodology used to quantitate the loss of intracellular material, which has an absorbance at 260 nm, from yeast cells treated with **3b** and **3e** or amphotericin B was performed as described by Bennis *et al.*²⁹ with modifications. Aliquots (5 ml) were obtained from yeast suspension (10⁷ cell per ml) prepared in saline 0.85%. Two treatment groups were considered: (1) control cells suspended in saline; (2) cells treated for 48 h at concentrations equal to the MIC, 2 × MIC, 4 × MIC and 8 × MIC of **3b**, **3e** or amphotericin B in saline. After treatment, cells were centrifuged at 7 g for 5 min, and the absorbance of the supernatant at 260 nm was determined in an ultraviolet spectrophotometer (Hitachi U-1100, Lancashire, UK).

Ergosterol quantification. The ergosterol quantification in the fungal cell membrane was performed as described by Arthington-Skaggs *et al.*³⁰ with modifications. Approximately 50 mg of the fungal cell mass was transferred to polypropylene tubes. The yeast cells were then treated with **3b**, **3e**, ITZ or amphotericin B at a concentration equal to the MIC in YPD medium for 7 days under agitation at 37 °C. A growth control was also performed. After incubation, the tubes were centrifuged (Jouan, model BR4i) at 1643 g for 5 min at 4 °C, and the supernatant was removed. The cells were then washed with sterile distilled water and the wet weight of the cell pellet was determined. For the extraction of lipids, 3 ml of the ethanolic solution of 25% potassium hydroxide was added to each cell mass, followed by agitation for 1 min. The tubes were incubated in a water bath at 85 °C for 1 h and then kept at room temperature to cool. A mixture of 1 ml of sterile water and 3 ml of *n*-heptane was added, followed by agitation in a vortex for 3 min. The supernatant was removed, and the reading was taken in a spectrophotometer at 282 and 230 nm. A calibration curve with standard ergosterol (Sigma-Aldrich) was constructed and used to calculate the amount of ergosterol. The results were expressed as the percentage of ergosterol compared with the growth control.

Sorbitol assay. The sorbitol assay was performed with **3b** and **3e** only for the *P. brasiliensis* isolate Pb18 by the standard broth microdilution procedure described above in determination of MIC. Duplicate plates were prepared: one containing two-fold dilutions (from 250 to 1.95 µg ml⁻¹) of the compounds

and the other containing compounds along with 1 mol l^{-1} of sorbitol as an osmotic support. The MIC values were determined after 15 days of incubation at 37°C .³¹

Calcofluor assay. Calcofluor white or CFW (Sigma) was used to more accurately observe the defects in the cell wall. This compound is excited at a wavelength of 372 nm and emitted at 456 nm. In this assay, *P. brasiliensis* (Pb18 isolate) yeast cells ($10^6 \text{ cells ml}^{-1}$) were treated with subinhibitory concentrations of **3b** and **3e** ($3.9 \mu\text{g ml}^{-1}$). After the treatment, 1 ml of culture growing in the log phase was centrifuged for 5 min at 8 g and resuspended in 500 μl of water, to which 0.1% CFW was added.³² Cells were immediately observed by a fluorescence microscope using the appropriate filter. The microscopic data shown in this work were obtained using the Nikon fluorescence microscope in the Image Acquisition and Processing Center (CAPI-ICB/UFGM).

RESULTS

The experimental compounds **3b** and **3e** had no effect on the viability of the VERO cells at concentrations of up to $500 \mu\text{g ml}^{-1}$, which is reflected by the IC_{50} values greater than $500 \mu\text{g ml}^{-1}$ (Table 3). The SI values were calculated using the MIC value for *P. brasiliensis* Pb18 isolate and the toxicity results for VERO cells. The compounds showed high SI values (>64.1), and, therefore, were considered to be promising for follow-up tests.

Fourteen isolates of the genus *Paracoccidioides*, representing the three phylogenetic species S1, PS2 and PS3 and *P. lutzii* strains were susceptible to the two compounds tested. The average MIC values for the compounds **3b** and **3e** ranged from 2.4 to 31.25 and 1.95 to $31.25 \mu\text{g ml}^{-1}$, respectively. The MIC_{90} was 31.25 and $10.40 \mu\text{g ml}^{-1}$ for **3b** and **3e**, respectively. The MFC_{90} was $31.25 \mu\text{g ml}^{-1}$ for both the compounds tested (Table 2). The MIC_{90} values for ITZ and SMZ were 0.062 and $150 \mu\text{g ml}^{-1}$, respectively. The MFC_{90} value was 0.062 for ITZ and $300 \mu\text{g ml}^{-1}$ for SMZ.

The death curve of the *P. brasiliensis* Pb18 strain under treatment with **3b** and **3e** was determined at a concentration equal to the MIC value, and the compounds decreased the cell viability by 70% after 5 days of incubation (Figure 1b). No interaction was observed, as indicated by the average FICI values (ranging from 1.062 to 2.062), between the experimental compounds and amphotericin B, ITZ and SMZ, in the Pb18 isolate (Table 4).

The electron microscopy observations, evaluating the phenotypic effects of **3b** and **3e** in Pb18 isolate, showed that the yeast cells treated with **3b** at a subinhibitory concentration ($3.9 \mu\text{g ml}^{-1}$) resulted in wilted cells with abnormal morphology, and cells which had retracted internally or displayed ruptured membranes with disorganized cytoplasmic and digestive vacuoles (Figure 2e–h). Following **3e** treatment at a subinhibitory concentration ($3.9 \mu\text{g ml}^{-1}$), we found many withered cells with their membranes fragmented and cytoplasmic structure fully deteriorated (Figure 2i–l). However, in both treatments, the cell walls were still intact and cell shape was still maintained. These observations corroborate the results of the experiments with the osmotic sorbitol shield, in which no change in the MIC was observed, indicating no damage to the cell wall (data not shown). Similarly, assays with CFW showed no obvious differences between the cell wall

fluorescence patterns of untreated yeast cells and the cells treated with **3b** and **3e** (data not shown).

In order to gather further evidence of *P. brasiliensis* (Pb18) plasma membrane damage, the LIVE/DEAD Yeast Viability Kit FungaLight was used, which contained the solutions of our SYTO 9 green fluorescent nucleic acid stain, the red-fluorescent nucleic acid stain and PI. In this assay, we observed that cells treated with subinhibitory concentrations ($0.025 \mu\text{g ml}^{-1}$) of amphotericin B and stained with PI showed a fluorescence median intensity of 66.7, whereas in growing cells (control) the fluorescence median intensity was found to be only 7.7. The compounds **3b** and **3e**, both at a concentration of $3.9 \mu\text{g ml}^{-1}$, exhibited a fluorescence median intensity of approximately 93.9 and 94.7, respectively (Figure 3). Membrane evaluation showed an increased efflux of K^+ between 3 and 15 days of treatment with amphotericin B and experimental compounds compared with the untreated cells (Figure 4). Similarly, the release of cellular material suggests that the integrity of the fungal membrane was disrupted, as well as the cells treated with amphotericin B, exhibited an increased dose-dependent damage following experimental treatments. In addition, we observed a more significant loss of cell content in yeast cells treated with **3b** and **3e** at all concentrations tested compared with the cells treated with amphotericin B (Figure 5). However, the damage observed in the *P. brasiliensis* membrane may not be associated with the reduction of ergosterol in the membrane because we found similar levels of ergosterol in both control untreated and amphotericin B-treated cells. Only cells treated with itraconazole displayed significantly reduced levels of ergosterol, which occurs only in cells treated with drugs that act on the biosynthesis pathway of ergosterol (Figure 6).

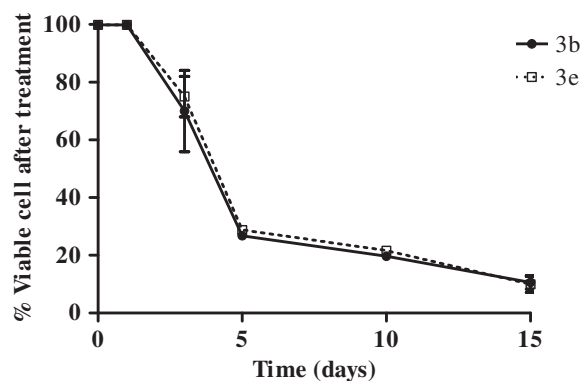


Figure 1 Survival curve of the isolate Pb18 of *P. brasiliensis* under treatment with substances **3b** and **3e** (at their minimal inhibitory concentration), expressed as the percentage of viable cells after treatment versus time.

Table 4 Fractional inhibitory concentration index (FICI) and interaction between compounds **3b** and **3e**, amphotericin B, itraconazole and trimethoprim–sulfamethoxazole against the isolate Pb18 of *P. brasiliensis*

	Mean FICI		Mean FICI	
	3b	Interaction	3e	Interaction
Amphotericin B	2.062	Ind. ^a	2.031	Ind.
Itraconazole	1.062	Ind.	1.100	Ind.
Trimethoprim–sulfamethoxazole	1.062	Ind.	1.062	Ind.

^aInd., indifferent.

Table 3 Cytotoxicity (IC_{50}) and selectivity index (SI) in VERO cells

	MIC (Pb18) ($\mu\text{g ml}^{-1}$)	$\text{IC}_{50}\text{VERO}$ ($\mu\text{g ml}^{-1}$)	SI ($\text{IC}_{50}/\text{MIC}$)
3b	7.8	>500	>64.1
3e	7.8	>500	>64.1
Itraconazole	0.003	>500	$>1.67 \times 10^5$

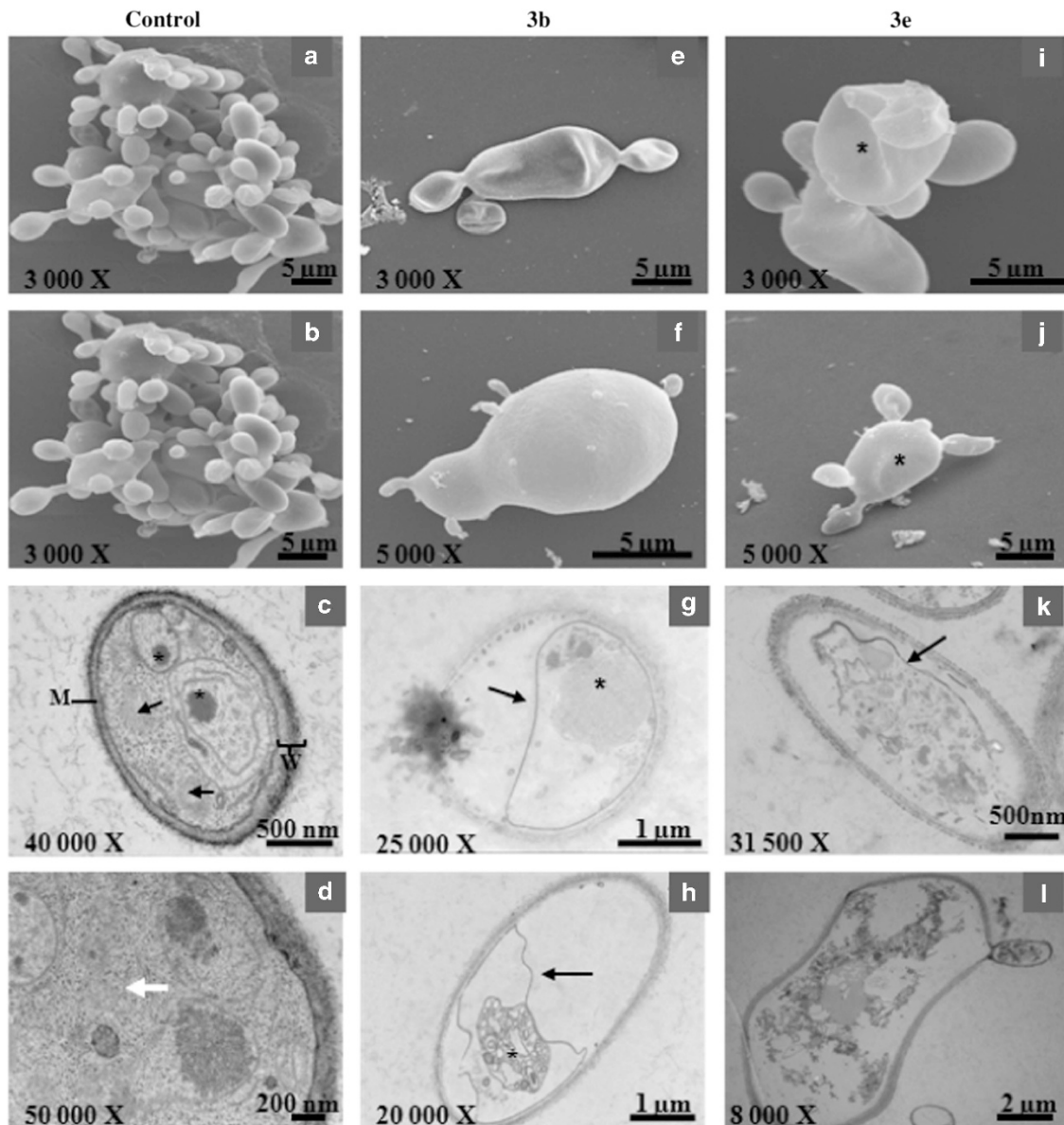
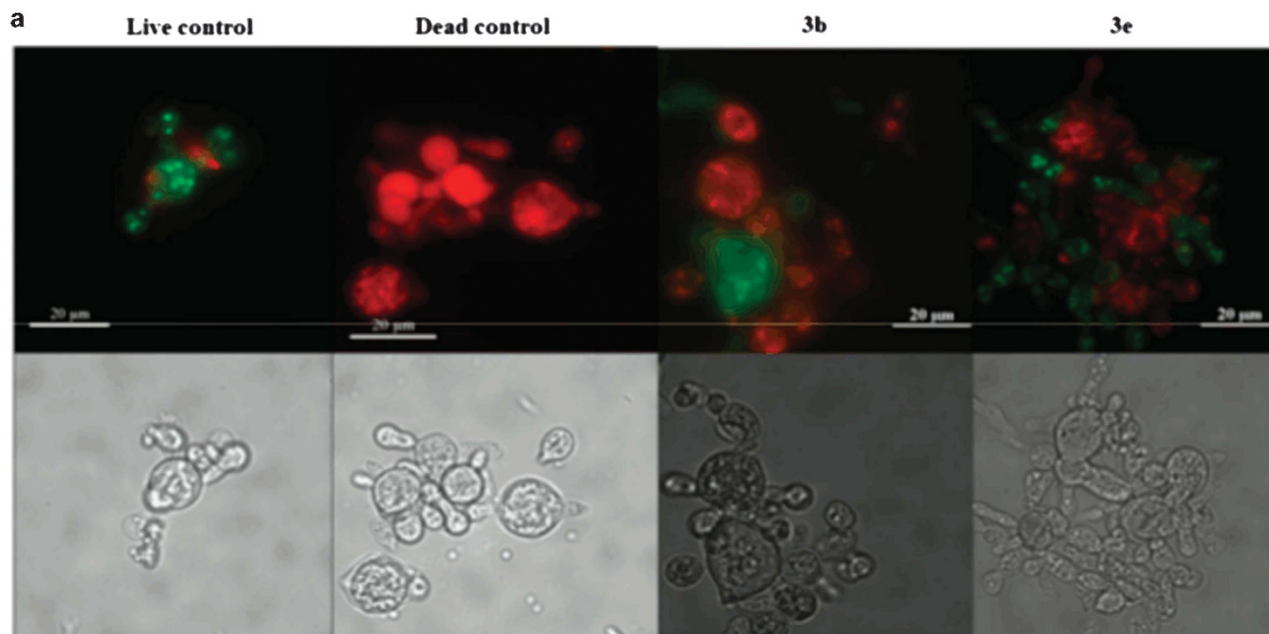


Figure 2 Yeasts of *Paracoccidioides brasiliensis* (Pb18) cultured in McVeigh & Morton medium with 15 days of growth. Yeasts were treated with the compounds **3b** and **3e** at subinhibitory concentrations ($3.9 \mu\text{g ml}^{-1}$) in medium McVeigh & Morton with 15 days of growth. (a, b) Control. (c) Control group with binucleated cells (asterisk), vacuoles (arrow), cytoplasmic membrane (M) and, externally, the cell wall (W) identified. (d) Granular cytoplasm (white arrow). (e) Withered cell. (f) Cell with differentiated morphology. (g) Membrane retracted (arrow) with condensed core (asterisk). (h) Cytoplasmic membrane retracted (arrow) with digestive vacuole (asterisk). (i) Withered cell (asterisk). (j) Withered cell (asterisk). (k) Cell with degraded plasma membrane (arrow) and cytoplasmic disorganization. (l) The mother cell and daughter cell with no plasma membrane and cytoplasmic disorganization. Scanning (a, b, e, f, i, j) and transmission (c, d, g, h, k, l) electron microscopy photography was used.

DISCUSSION

The present study aimed to identify new therapeutic options for PCM. The compounds **3b** and **3e**, derived from 6-quinoliny chalcones, were found to inhibit fungal growth, thereby killing them. In *in vitro* tests and were not cytotoxic to VERO cells. We showed that the genus *Paracoccidioides* is widely susceptible to these compounds, since different isolates that represent the phylogenetic species were equally susceptible to the action of these compounds. Comparing the *in vitro* antifungal activity of **3b** and **3e** with existing drugs used as controls, we found that ITZ was more active than the experimental compounds tested; however, SMZ showed a higher MIC value than the experimental compounds. The antifungal activity observed in the present

work with **3b** and **3e** is interesting because it is an intermediate between two drugs used for the treatment of PCM, and the most widely used by the Single Health System is SMZ.³ The antifungal activities of **3b** and **3e** were also observed by a death-time curve, which shows the kinetics of growth and action of these compounds; after 5 days of treatment, the cell viability was decreased by approximately 70%. Takahagi-Nakaira *et al.*³³ evaluated the death curve of isolated Pb18 treated with amphotericin B (concentration fungicide) and the findings were similar to those of the present study; all cells were killed within 100 h. However, no interactions were found between the experimental compounds (**3b** and **3e**), ITZ, SMZ or amphotericin B in *in vitro* tests.



b

Propidium Iodide (PI)	
	MFI*
Life control	7.7
Dead control	99.9
3b	93.9
3e	94.7

* MFI. Mean Fluorescence Intensity (arbitrary units of measurement).

Figure 3 Photomicrographs showing cells of *Paracoccidioides brasiliensis* (Pb18) grown on yeast extract peptone dextrose (YPD) broth at 37 °C and stained with LIVE/DEAD FungaLight Yeast Viability Kit, which contains solutions of our SYTO 9 green fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide (PI). (a) Yeasts treated with the compounds **3b** and **3e** (3.9 μg ml⁻¹) and amphotericin B (0.025 μg ml⁻¹) at subinhibitory concentrations in YPD broth with 15 days of growth. Scale bars = 20 μm. (b) Evaluation of median fluorescence intensity by flow cytometry of cells stained with PI.

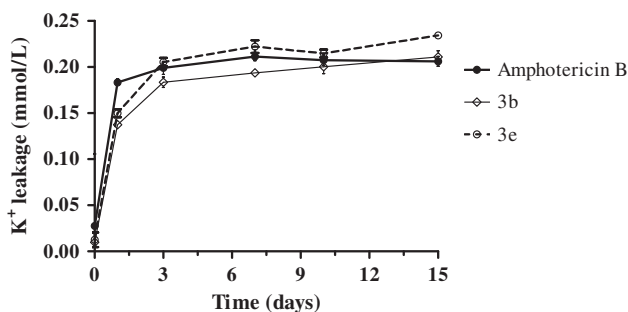


Figure 4 Effects of **3b**, **3e** and amphotericin B on the extracellular K⁺ leakage in *P. brasiliensis* (Pb18), using an inoculum of 1 × 10⁷ yeasts per ml. Curve time-dependent. Yeasts treated with the compounds **3b** and **3e** (15.6 and 7.8 μg ml⁻¹) and amphotericin B (0.05 μg ml⁻¹) with 15 days of growth.

In our assessment of the phenotypic effects caused by treatment with compounds **3b** and **3e**, we mainly looked at evidences of ultrastructural damage to yeast cells and the plasma membrane of *P. brasiliensis*. The compounds **3b** and **3e** caused fungal membrane

disorders, which suggests that the membrane is a direct or indirect target of these substances. In *Trichophyton mentagrophytes* treated with eficonazole, which blocks the ergosterol biosynthetic pathway, ultrastructural alterations, such as the withering of hyphae, accumulation of electron-dense granules between the membrane and the cell wall, degeneration of the organelles, and fragmentation of the plasma membrane, have been observed.³⁴ In the present work, we observed similar changes associated with the loss of cell membrane integrity; however, this activity was not due to direct effects on the ergosterol biosynthetic pathway. Our study found no evidence, suggesting the effects of **3b** and **3e** on the cell wall, since tests with sorbitol and CFW were negative.

In this work, fluorescence microscopy results indicated the possibility of drug action on the membrane leading to cell death. We used PI, which can only enter cells with significantly damaged membranes.³⁵ The percentage of cells with membrane damage was found to be higher following treatment with **3b** and **3e** than with amphotericin B. These results were further confirmed by flow cytometry to evaluate the median fluorescence intensity of the yeast cells labeled with PI, reinforcing the evidence for the ability of **3b** and **3e** to impair the integrity of *P. brasiliensis* (Pb18) membrane. Chee

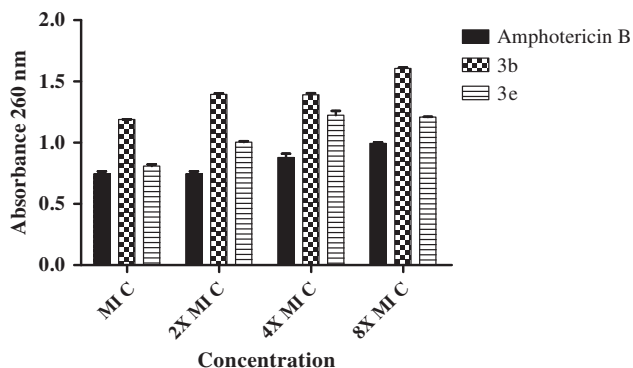


Figure 5 Effects of **3b**, **3e** and amphotericin B on the release of 260 nm-absorbing material in Pb18.

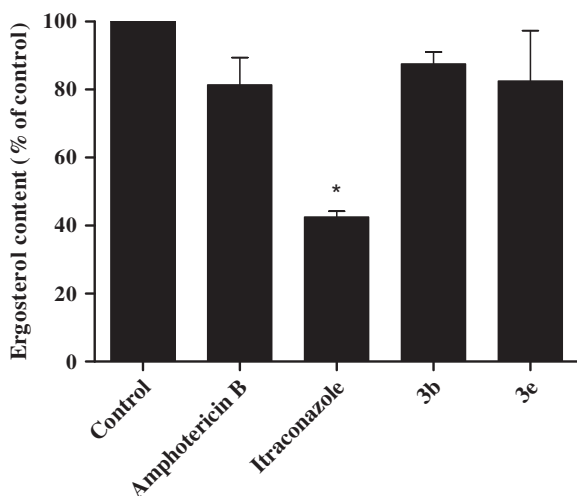


Figure 6 Percent ergosterol levels of Pb18 after 7 days of treatment with **3b**, **3e** amphotericin B and itraconazole at subinhibitory concentrations. *Statistically different ($P < 0.05$).

*et al.*³⁶ studied the action of limonene against *T. rubrum* by evaluating its effect on the viability of spores and hyphae that were stained with a Live/Dead viability kit and observed under a fluorescence microscope. The authors also considered their results with PI as evidence that limonene had some action on the fungal membrane.

Alterations to the cell surface have been associated with changes in cell permeability. These changes primarily occur in the cell wall and membrane before any changes can be detected inside the cell.³⁷ We used amphotericin B, an antifungal agent that affects the permeability of the cell membrane, as a positive control to cause cell rupture and fungal cell death.³⁵ We used scanning electron microscopy to observe changes on the cell surface, such as wilting and cell fragmentation of the cell membrane, which may be related to increased K^+ efflux and loss of cell components, reinforcing the hypothesis that **3b** and **3e** act on the fungal membrane. Previous studies^{35,38} showed that eugenol and cinnamaldehyde act on the membrane of *Candida albicans*, which is clearly evident based on the results obtained from K^+ release assays and intracellular materials that absorb at 260 nm.

CONCLUSION

In summary, our results highlight the great potential of compounds **3b** and **3e** against *Paracoccidioides* spp., which exhibit superior antifungal activity compared with SMZ, a drug widely used for the treatment of

PCM. In addition, these compounds showed evidence of a mechanism of action associated with the fungal membrane in the form of changes to the cell surface that lead to cell death.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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