Microbial Pathogenesis 98 (2016) 1–5

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Synthesis and *in vitro* antifungal activity of isoniazid-derived hydrazones against *Coccidioides posadasii*

Rossana de Aguiar Cordeiro [a, *], Charlline Vládia Silva de Melo [a], Francisca Jakelyne de Farias Marques [a], Rosana Serpa [a], Antônio José de Jesus Evangelista [a], Erica Pacheco Caetano [a], Jair Mafezoli [b], Maria da Conceição Ferreira de Oliveira [b], Marcos Reinaldo da Silva [b], Tereza de Jesus Pinheiro Gomes Bandeira [a, c], José Luciano Bezerra Moreira [a], Raimunda Sâmia Nogueira Brilhante [a], Marcos Fábio Gadelha Rocha [a, d], José Júlio Costa Sidrim [a]

[a] Specialized Medical Mycology Center, Federal University of Ceará, Fortaleza, Ceará, Brazil
[b] Department of Organic and Inorganic Chemistry, Federal University of Ceará, Fortaleza, Ceará, Brazil
[c] Christus College, School of Medicine, Fortaleza, Ceará, Brazil
[d] Graduate Program in Veterinary Science, State University of Ceará, Fortaleza, Ceará, Brazil

ARTICLE INFO

Article history:
Received 11 March 2016
Received in revised form
4 May 2016
Accepted 17 June 2016
Available online 19 June 2016

Keywords:
Coccidioides
Hydrazones
Isoniazid
Antifungal susceptibility
Synergism

ABSTRACT

Coccidioidomycosis is a potentially severe infection caused by dimorphic fungi *Coccidioides immitis* and *Coccidioides posadasii*. Although guidelines are well established, refractory disease is a matter of concern in the clinical management of coccidioidomycosis. In the present study three isoniazid-derived hydrazones *N′*-[(*E*)-1-(4-methoxyphenyl)ethylidene]pyridine-4-carbohydrazide, *N′*-[(*E*)-1-(4-methylphenyl) ethylidene]pyridine-4-carbohydrazide, and *N′*-[(*E*)-1-(phenyl)ethylidene]pyridine-4-carbohydrazide were synthesized and evaluated for antifungal activity against *C. posadasii*. Susceptibility assays were performed by macrodilution testing. Interactions between the hydrazones and amphotericin B or itraconazole were evaluated by the checkerboard method. We also investigated the impairment of such compounds on cell ergosterol and membrane integrity. The synthesized molecules were able to inhibit *C. posadasii in vitro* with MIC values that ranged from 25 to 400 μg/mL. Drug interactions between synthesized molecules and amphotericin B proved synergistic for the majority of tested isolates; regarding itraconazole, synergism was observed only when strains were tested against *N′*-[(*E*)-1-(phenyl) ethylidene]pyridine-4-carbohydrazide. Reduction of cellular ergosterol was observed when strains were challenged with the hydrazones alone or combined with antifungals. Only *N′*-[(*E*)-1-(4-methylphenyl) ethylidene]pyridine-4-carbohydrazide altered membrane permeability of *C. posadasii* cells. Isoniazid-derived hydrazones were able to inhibit *C. posadasii* cells causing reduction of ergosterol content and alterations in the permeability of cell membrane. This study confirms the antifungal potential of hydrazones against pathogenic fungi.

1. Introduction

Coccidioidomycosis is a deep-seated disease that occurs exclusively in arid and semiarid areas of the Americas. The disease is caused by *Coccidioides immitis* and *Coccidioides posadasii*, which are considered the most virulent among the pathogenic fungi [1,2]. Approximately 60% of infected individuals are asymptomatic, while the remaining ones can develop acute or chronic pneumonia. Disseminated coccidioidomycosis including meningeal involvement can affect 1–5% of infected individuals and most frequently occurs in immunocompromised patients [2,3].

The treatment of coccidioidomycosis basically depends on the severity of the disease and the patient's individual risk factors [4].

* Corresponding author. Federal University of Ceará, Campus do Porangabussu, Rua Cel. Nunes de Melo, 1315, Rodolfo Teófilo, CEP: 60430-275, Fortaleza, Ceará, Brazil.
E-mail address: rossanacordeiro@ufc.br (R.A. Cordeiro).

http://dx.doi.org/10.1016/j.micpath.2016.06.022
0882-4010/

In general, amphotericin B is reserved for severe cases, whereas azole derivatives, especially fluconazole and itraconazole, are used as consolidation therapy or as drug of choice for treatment of non-complicated pulmonary disease [4]. Although guidelines are well established and the antifungals available are effective against *Coccidioides* spp., refractory disease is a matter of concern in the clinical management of patients with severe coccidioidomycosis [4–7]. In addition, amphotericin B cytotoxicity and drug-drug interactions between azoles and other coadministered agents are described as important issues in antifungal therapy [8,9].

Previously, we have shown that the hydrazone molecule *N′*-[(*E*)-1-(phenyl)ethylidene]pyridine-4-carbohydrazide has antifungal activity against *Histoplasma capsulatum* var. *capsulatum* [10]. In the present study we have developed isoniazid-derived hydrazones and evaluated their antifungal activity against another dimorphic fungal species, *C. posadasii*.

2. Materials and methods

2.1. Microorganisms

A total of 12 strains of *C. posadasii* isolated in Brazil were included in this study. Clinical strains (n = 10) were recovered from patients with acute or chronic pulmonary disease; environmental strains (n = 2) were isolated from soil samples in endemic areas. Isolates were identified by macro and micromorphological analysis, reactivation spherules in animal model [11], immunodiffusion test with IDCF antigen (Immy Immunodiagnostics, USA) and PCR [12]. The strains were obtained from the fungal collection of the Specialized Medical Mycology Center (CEMM, Federal University of Ceará, Fortaleza, Brazil). All procedures were performed within a class II biological safety cabinet in a biosafety level 3 laboratory.

2.2. Synthesis of Schiff bases

The hydrazones were synthesized by a reaction between isoniazid (INH) and ketones, as described by de Aguiar Cordeiro et al. [10]. The compounds were prepared with three ketones (Aldrich and Sigma-Aldrich, Germany), one by reaction: acetophenone, 4′-methoxyacetophenone and 4′-methylacetophenone (0.003 mol each), and INH (0.006 mol) with 5.0 mL of ethanol. Compounds were characterized by high-resolution mass spectrometry (HR-MS) with a liquid chromatography MS-ion trap-time of flight (LCMS-IT-TOF) spectrometer (Shimadzu) and by nuclear magnetic resonance (NMR) with a Bruker Avance model X-ray diffraction (XRD) 500 spectrometer (^{1}H, 300 MHz; ^{13}C, 75 MHz) using deuterated chloroform ($CDCl_3$) and dimethyl sulfoxide (DMSO-d_6) as solvents.

2.3. Cytotoxicity assays

The cytotoxicity activity of the synthesized hydrazones was evaluated against human cell lines NCIH358M (bronchoalveolar lung carcinoma), PC-3M (metastatic prostate carcinoma), OVCAR-8 (ovarian cell carcinoma), and HB4a (breast epithelial cells), according to Berrigde et al. [13]. Cells were grown in RPMI-1640 medium with 10% fetal bovine serum, 2 mM of glutamine, 100 μg/mL of streptomycin and 100 U/mL of penicillin, and incubated at 37 °C with 5% CO_2 atmosphere. For experiments, cells (1.0×10^5 cells/well) were plated in 96-well microplates and then hydrazones (250 μg/mL in DMSO) were added to each well. Plates were incubated for 72 h at 37 °C in a 5% CO_2 atmosphere. Control groups received the same amount of sterile DMSO. At the end of incubation period, the plates were centrifuged and the supernatant was removed. A volume of 150 μL of fresh medium containing 0.5 mg/mL of MTT was added to each well and the plates were reincubated for 3 h as described above. Formazan was dissolved in 150 μL of DMSO and the absorbance was measured using a multiplate reader at 595 nm.

2.4. Inoculum preparation for antifungal susceptibility testing

C. posadasii strains were grown on potato agar and incubated for 7 days at 25–28 °C. Inoculum preparation as described elsewhere [14]. Tests were conducted with an inoculum of approximately $1–5 \times 10^3$ colony-forming units/mL in RPMI 1640 medium buffered to pH 7.0 with 0.165 M MOPS (Sigma Chemical Co., USA) [14].

2.5. In vitro susceptibility testing

INH and INH-derived hydrazones were evaluated as described by de Aguiar Cordeiro et al. [10]. Serial two-fold dilutions of each drug were prepared with RPMI medium and stored at −20 °C. *C. posadasii* strains (n = 12) were tested against each drug alone to determine the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC). Drug concentrations ranged as follows: INH-derivatives, 3.9–2000 μg/mL [10]; AMB, 0.007–4.0 μg/mL; ITC, 0.015–8.0 μg/mL; INH, 15-4000 μg/mL [14]. Broth macrodilution testing was performed according to the CLSI guidelines [15]. All procedures were performed in duplicate and the results were read visually. As internal quality control for each test, *Candida parapsilosis* ATCC 22019 and *Histoplasma capsulatum* CEMM 03-02-090 were also included. Both strains were previously tested against INH-derived drugs (Cordeiro et al., 2014). MIC endpoints were determined after 48 h at 35 °C. For hydrazones, MIC was defined as the lowest drug concentration that caused 80% inhibition of visible fungal growth [10]. Regarding antifungals, MIC was considered as the lowest drug concentration that caused 100 and 80% inhibition of visible fungal growth for AMB and ITC, respectively [16]. The hydrazones' MFC values were defined as the lowest drug concentration at which there was no fungal growth, after subculturing 0.1 mL of the cellular suspension at concentrations above the MIC [10].

2.6. Checkerboard macrodilution assay

Fungal strains were tested against six drug combinations formed by a hydrazone plus AMB or ITC, in a checkerboard assay [17]. Combinations were formed with each hydrazone at concentrations that ranged from 12.5 to 800 μg/mL or 0.0625–1.0 μg/mL for AMB or ITC. The MIC of each drug in combination was defined as the lowest concentration that caused 100% inhibition of fungal growth. Drug interactions were classified according to the fractional inhibitory concentration index – FICI [17]. Experiments were performed in duplicate in macrodilution assays.

2.7. Effect of INH-derived hydrazones on ergosterol content of *C. posadasii*

Total sterol quantification was performed as described by Moran et al. [18], with adjustments. Strains of *C. posadasii* (n = 12) previously grown on potato agar at 35 °C for 7 days were transferred to RPMI 1640 medium supplemented with INH-derived hydrazones at 2xMIC, MIC and MIC/2 concentrations. Drug combinations formed by the synthesized hydrazones and other antifungals at MIC and MIC/2 concentrations were also tested. Cultures were incubated for 2 days at 35 °C, after which the density of each fungal suspension was adjusted to 0.5 McFarland scale and then centrifuged at 9660*g* for 3 min. Cellular pellets were treated with 20% alcoholic KOH and total sterols were extracted with *n*-hexane. Absorbance readings of organic phase of each sample were performed at 295 nm.

Ergosterol quantification was performed by comparison with the standard curve obtained with commercial ergosterol (Sigma-Aldrich, Germany). All procedures were performed in duplicate. Controls were formed by fungal growth in RPMI 1640 medium without antimicrobials. Cultures treated with ITC at 2xMIC, MIC and MIC/2 concentrations were also included as controls.

2.8. Effect of INH-derived hydrazones on cell membrane permeability

The effect of INH-derived hydrazones on cell membrane permeability was analyzed as suggested by de Aguiar Cordeiro et al. [10]. After susceptibility testing with hydrazones alone, the cell content of each tube at MIC and MIC/2 concentrations was harvested by centrifugation at 13,416*g* for 15 min. Drug combinations formed by hydrazones and antifungals at MIC and MIC/2 concentrations were also tested. The experiments were conducted in duplicate; controls were formed by fungal cultures grown in RPMI medium without antimicrobials or supplemented with INH or AMB at both MIC and MIC/2 concentrations.

2.9. Statistical analysis

Results were evaluated by one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. A p-value < 0.05 was considered significant in all analyses. The statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Hydrazone synthesis

A total of three INH-derived hydrazones were synthesized, with yields that ranged from 20.6 to 42.6%. Structural formula of each compound is shown in Fig. 1. The molecular weights and empirical formulas of each compound were confirmed by high-resolution mass spectrometry (HR-MS). The hydrazones did not display cytotoxicity activity at 250 μg/mL.

3.2. Antifungal susceptibility

All the synthesized molecules were able to inhibit *C. posadasii* growth *in vitro*. Higher MICs were obtained with *p*MeOPhEINH and *p*MePhEINH, with values ranging from 400 to 100 μg/mL for both compounds. For PhEINH, MICs ranged from 25 to 100 μg/mL. Further details regarding antifungal activity of each compound are shown in Table 1. *C. posadasii* strains were also tested against antifungals and INH as quality control and showed the following MIC values (geometric mean): 0.105 μg/mL and 0.177 μg/mL for AMB and ITC, respectively, and 891.9 μg/mL for INH. *C. parapsilosis* ATCC 22019 showed MIC values in agreement CLSI guidance[15].

Drug interactions between synthesized hydrazones and AMB proved synergistic to the majority of tested isolates, as follows: 7/12 for *p*MeOPhEINH and 11/12 for *p*MePhEINH and PhEINH. Regarding ITC, synergism was observed only when strains were tested against PhEINH (11/12). Indifferent interactions were detected for combinations formed by *p*MeOPhEINH plus ITC and *p*MePhEINH plus ITC (0/12). Details regarding MIC values and FICI of synergistic combinations are displayed in Table 2.

3.3. Effect of INH-derived hydrazones on ergosterol content

Significant reduction of cellular ergosterol was observed when strains were challenged with the synthesized hydrazones at 2xMIC, MIC and MIC/2 concentrations ($p < 0.05$), as shown in Table 3. PhEINH was able to reduce cellular ergosterol content more efficiently than *p*MeOPhEINH and *p*MePhEINH for each tested concentration ($p < 0.05$).

Significant decrease of fungal ergosterol content was also observed when strains were incubated with hydrazones combined with antifungals ($p < 0.05$), as shown in Table 3. However, reduction in fungal ergosterol content was better with antifungals alone than combined with hydrazones ($p < 0.05$).

3.4. Effect of INH-derived hydrazones on cell membrane integrity

Among the synthesized compounds, only *p*MePhEINH at both MIC and MIC/2 concentrations was able to induce alterations of membrane permeability of *C. posadasii* cells ($p < 0.05$) (Table 4). Membrane integrity was also disturbed by INH at both MIC and MIC/2 concentrations; AMB was not able to induce the leakage of proteins or nucleic acids from fungal cells. *p*MePhEINH was more efficient alone than when combined with AMB or ITC ($p < 0.05$). There were no differences in the supernatant absorbing components when cells were treated with *p*MeOPhEINH and PhEINH alone or combined with AMB or ITC ($p > 0.05$), as shown in Table 4.

4. Discussion

In recent years, several studies have focused on the development of antifungal drugs [19–22]. Among such compounds,

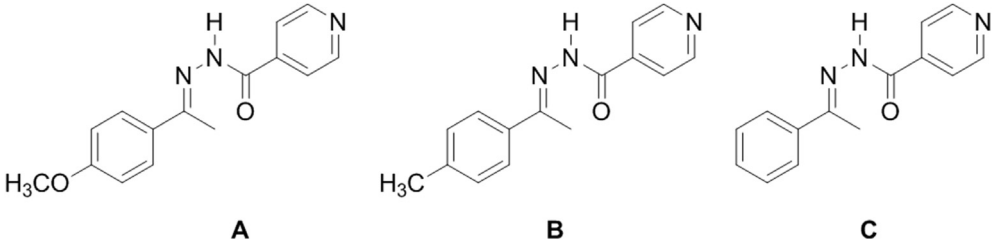

Fig. 1. Structural formula of synthesized isoniazid derivatives *N*′-[(*E*)-1-(4-methoxyphenyl)ethylidene]pyridine-4-carbohydrazide (A), *N*′-[(*E*)-1-(4-methylphenyl)ethylidene]pyridine-4-carbohydrazide (B) and *N*′-[(*E*)-1-(phenyl)ethylidene]pyridine-4-carbohydrazide (C). A. Solid white. **Melting Point**: 189–191.3 °C. **RMN ^{1}H** (300 MHz, DMSO-d_6): 1.37 (s, 3H, CH_3); 2.83 (s, 3H, OCH_3); 6.02 (d, 2H, *J* 5 Hz); 6.75–6.76 (m, 1H); 6.82–6.87 (m, 4H); 7.72 (d, 1H, *J* 3.5 Hz) and 7.78 (d, 1H, *J* 2.7). **RMN ^{13}C** (75.7 MHz, DMSO-d_6): 15.1 (CH_3); 55.7 (OCH_3); 114.2–161.1 (CAr + C_{Pyr}); 162.6 (C=N); 164.3 (C=O). **I. R. (KBr)** *v* 3170; 1650; 1538; 1254; 1268 and 634 cm^{-1}. HRMS [ESI$^+$, m/z] calc. for $C_{15}H_{15}N_3O_2[Na^+]$ 292.1056, found 292.1074. B. Solid white. **Melting Point**: 203.7–204.8 °C.**RMN ^{1}H** (300 MHz, $CDCl_3$): 2.34 (s l, 6H, 2 CH_3); 7.14–7.51 (m, 3H, aromatic); 7.68–7.73 (m, 3H, aromatic); 8.72 (s l, 2H, pyridinyl) and 9.8 (s, 1H, pyridinyl). **RMN ^{13}C** (75.7 MHz, $CDCl_3$): 12.8 (CH_3); 21.9 (CH_3); 120.7–149.6 (11 C_{Ar} + C_{Pyr}); 150.6 (C=N) and 168.9 (C=O). **I. R. (KBr)** *v* 3188; 1663; 1391, 1302 and 638 cm^{-1}. HRMS [ESI$^+$, m/z] calc. for $C_{15}H_{15}N_3O[Na^+]$ 276.1107, found 276.1139. C. Solid cristaline. **Melting Point**: 170.7–171.6 °C. **RMN ^{1}H** (300 MHz, $CDCl_3$): 2.37 (s, 3H, CH_3); 7.36 (s l, 3H, aromatic); 7.6–7.81 (m, 4H, aromatic); 8.73 (s l, 2H, pyridinyl) and 10.8 (s, 1H, pyridinyl). **RMN ^{13}C** (75.7 MHz, $CDCl_3$): 13.0 (CH_3); 121–149.7 (11 C_{Ar} + C_{Pyr}); 150.7 (C=N) and 169.3 (C=O). **I. R. (KBr)** *v* 3173; 1683; 1657; 1649; 1537; 1282 and 777 cm^{-1}. HRMS [ESI$^+$, m/z] calc. for $C_{14}H_{13}N_3O[Na^+]$ 262.0951, found 262.0966.

Table 1
Antifungal activity of synthesized hydrazones against *Coccidioides posadasii* strains (n = 12).

Compound	Molecular formula	Mol wt	MIC range	MIC (GM)	MFC range	MFC (GM)
N'-[(*E*)-1-(4-methoxyphenyl)ethylidene]pyridine-4-carbohydrazide (*p*MeOPhEINH)	$C_{15}H_{15}N_3O_2$	269.3	100–400	224.4	200–800	448.9
N'-[(*E*)-1-(4-methylphenyl) ethylidene]pyridine-4-carbohydrazide (*p*MePhEINH)	$C_{15}H_{15}N_3O$	253.3	100–400	211.8	200–800	475.6
N'-[(*E*)-1-(phenyl)ethylidene]pyridine-4-carbohydrazide (PhEINH)	$C_{14}H_{13}N_3O$	239.3	25–100	50.0	200–400	224.4

MIC: minimum inhibitory concentration; MFC: minimum fungicide concentration; GM: geometric mean.

Table 2
Minimum inhibitory concentrations (MIC) range, MIC geometric mean, fractional inhibitory concentration index (FICI) range and interaction effects for eight combinations of hydrazones and antifungal drugs against *Coccidioides posadasii* strains (n = 12).

Combination	MIC range (μg/mL)		MIC geometric mean		FICI range	Synergism
	Hydrazone	Antifungal	Hydrazone	Antifungal		
*p*MeOPhEINH + AMB	12.5–100	0.031–0.0625	31.5	0.031	0.281–1.125	7/12
*p*MePhEINH + AMB	25–100	0.0009–0.003	42	42	0.140–0.562	11/12
PhEINH + AMB	12.5–50	0.0009–0.001	10.5	10.5	0.128–2.015	11/12
*p*MeOPhEINH + ITC	200–400	0.125–0.5	317.5	0.250	0.521–2	0/12
*p*MePhEINH + ITC	200–400	0.125–1	317.5	0.315	0.625–1	0/12
PhEINH + ITC	3.125–12.5	0.0004–0.5	7	0.004	0.0624–2	11/12

Table 3
Ergosterol content of *Coccidioides posadasii* cells after growth in RPMI medium supplemented with hydrazones alone or combined with antifungals. Results were compared with ergosterol content of cells in RPMI medium without antimicrobials as control or supplemented with ITC and are expressed as mean ± SEM.

Treatment	Concentration of ergosterol (μM)		
	2xMIC	MIC	MIC/2
*p*MeOPhEINH	16.47 ± 1.3^{§,a}	19.86 ± 1.1^{§,a}	22.19 ± 1,0^{§,a}
*p*MePhEINH	16.26 ± 1.13^{§,a}	19.76 ± 0.94^{§,a}	23.34 ± 0.52^{§,a}
PhEINH	11.32 ± 0.96^{§,b}	13.02 ± 0.77^{§,b}	17.24 ± 1.3^{§,b}
*p*MeOPhEINH + AMB	10.48 ± 0.26^{§,b}	14.42 ± 0.32^{§,b}	18.37 ± 0.42^{§,b}
*p*MePhEINH + AMB	11.76 ± 0.32^{§,ab}	13.47 ± 0.15^{§,b}	13.94 ± 0.24^{§,b}
PhEINH + AMB	9.96 ± 0.27^{§,b}	11.93 ± 0.54^{§,b}	19.16 ± 0.32^{§,b}
*p*MeOPhEINH + ITC	10.06 ± 0.39^{§,b}	15.33 ± 1.3^{§,a}	18.59 ± 0.91^{§,a}
*p*MePhEINH + ITC	14.74 ± 0.78^{§,b}	17.16 ± 0.13^{§,a}	17.66 ± 0.07^{§,a}
PhEINH + ITC	10.48 ± 0.25^{§,b}	13.31 ± 0.25^{§,b}	14.34 ± 0.16^{§,b}
ITC	5.24 ± 0.5^{§,c}	10.58 ± 0.45^{§,c}	15.2 ± 0.4^{§,b}
Control 29.18 ± 0.49			

ITC: itraconazole; AMB: amphotericin B.
$^{§}p < 0.05$ vs. control; Values in the same column followed by the same superscript letters are not significantly different from each other ($p > 0.05$).

organic compounds with the structure $R_1R_2C = NNH_2$, formed by the substitution of the carbonyl group with the functional group $-NNH_2$. Former studies have attested to the antifungal potential of these compounds against *Candida* [27–30], *Histoplasma capsulatum* [10] and *Cladosporium cladosporioides* [31]. In addition, hydrazones also display many pharmacological properties such as anti-inflammatory, analgesic, anticancer, antiplatelet and anticonvulsant, besides broad antimicrobial activity [25].

In the present study *C. posadasii* strains were inhibited *in vitro* by the synthesized hydrazones *p*MeOPhEINH, *p*MePhEINH and PhEINH. The lowest MIC values were obtained with PhEINH – a phenyl hydrazone molecule. Interestingly, the presence of electron donating groups $-CH_3$ or $-OCH_3$ reduced the antifungal activity of those compounds.

In a previous study, we showed the antifungal potential of PhEINH against *H. capsulatum*, another dimorphic fungi. MICs ranged from 31.2 to 250 μg/mL for both filamentous and yeast forms [10]. The results presented here show that *C. posadasii* strains in the filamentous growth phase are more susceptible to PhEINH,

Table 4
Absorbance of extracellular content of *Coccidioides posadasii* cells at 260 nm and 280 nm. Cells were incubated in RPMI medium supplemented with hydrazones alone or combined with antifungals. Results were compared with growing cells in medium without antimicrobials as control or supplemented with AMB or ITC and are expressed as mean ± SEM.

Treatment	260 nm		280 nm	
	MIC	MIC/2	MIC	MIC/2
*p*MeOPhEINH	0.472 ± 0.04^{a}	0.274 ± 0.05^{a}	0.386 ± 0.06^{a}	0.414 ± 0.05^{a}
*p*MePhEINH	1.055 ± 0.24^{§,b}	0.7808 ± 0.23^{§,b}	0.924 ± 0.195^{§,b}	0.705 ± 0.13^{§,b}
PhEINH	0.455 ± 0.03^{a}	0.304 ± 0.04^{a}	0.448 ± 0.04^{a}	0.285 ± 0.03^{a}
*p*MeOPhEINH + AMB	0.388 ± 0.01^{a}	0.387 ± 0.01^{a}	0.389 ± 0.02^{a}	0.425 ± 0.02^{a}
*p*MePhEINH + AMB	0.371 ± 0.01^{a}	0.329 ± 0.01^{a}	0.433 ± 0.01^{a}	0.368 ± 0.009^{a}
PhEINH + AMB	0.134 ± 0.003^{a}	0.146 ± 0.003^{a}	0.216 ± 0.007^{a}	0.175 ± 0.01^{a}
*p*MeOPhEINH + ITC	0.686 ± 0.01^{a}	0.320 ± 0.01^{a}	0.373 ± 0.03^{a}	0.331 ± 0.06^{a}
*p*MePhEINH + ITC	0.384 ± 0.008^{a}	0.329 ± 0.01^{a}	0.433 ± 0.01^{a}	0.288 ± 0.04^{a}
PhEINH + ITC	0.134 ± 0.003^{a}	0.1321 ± 0.005^{a}	0.150 ± 0.002^{a}	0.127 ± 0.004^{a}
AMB	0.176 ± 0.04^{a}	0.153 ± 0.02^{a}	0.176 ± 0.04^{a}	0.198 ± 0.04^{a}
INH	2.293 ± 0.22^{§,c}	1.61 ± 0.22^{§,c}	2.293 ± 0.22^{§,c}	1.237 ± 0.129^{§,c}
Control	0.114 ± 0.03		0.089 ± 0.006	

ITC: itraconazole; AMB: amphotericin B; INH: isoniazid.
$^{§}p < 0.05$ vs. control; Values in the same column followed by the same superscript letters are not significantly different from each other ($p > 0.05$).

hydrazones have gained attention as potential molecules for drug design [23–26]. Hydrazones, related to ketones and aldehydes, are

presenting MICs that ranged from 25 to 100 μg/mL. All synthesized hydrazones were able to interact synergistically with AMB,

reducing the MIC values of this antifungal *in vitro*. Strong synergism between PhEINH and AMB against *Histoplasma capsulatum* has been previously demonstrated [10]. However, although synergism between hydrazone molecules and triazole has been described [32], in the present study only PhEINH was able to interact synergistically with ITC.

According to the results obtained in the present study, the synthesized hydrazones at supra MIC, MIC or infra MIC concentrations were able to reduce the cell content of ergosterol in *C. posadasii*, although to a lesser magnitude than ITC. Previous studies have demonstrated that hydrazones are able to inhibit of ergosterol biosynthesis of pathogenic fungi [10,33,34], as well as the parasite *Trypanosoma cruzi* [35]. However it is important to emphasize that although combinations formed by the synthesized hydrazones and antifungals were able to reduce cell ergosterol in *C. posadasii*, they were not more efficient than ITC alone.

Among the synthesized hydrazones, unexpectedly only *p*MePhEINH altered the cell membrane permeability of the fungus, causing the release of 280 nm and 260 nm-absorbing molecules. However, this effect was not increased when *p*MePhEINH was combined with antifungals. Recently it was demonstrated that hydrazones are able to alter membrane permeability of *H. capsulatum* [10] and *Candida* spp. [34]. We suppose that *p*MeOPhEINH and PhEINH cause minor damage to *C. posadasii* cell membrane, allowing the escape of low weight compounds such as ionic particles. Further experiments should be performed to validate this hypothesis.

In conclusion, the present study showed that nontoxic INH-derived hydrazones were able to inhibit *C. posadasii* cells *in vitro*, causing reduction of ergosterol content and alterations in the permeability of fungal membranes. The obtained results reinforce the antifungal potential of PhEINH against dimorphic fungi. Further experimentation should evaluate the effect of these compounds during experimental infection with *C. posadasii*.

Conflict of interest

None to declare.

Acknowledgements

This work was funded by the National Council for Scientific and Technological Development (CNPq; Brazil; Process numbers: 562296/2010-7; 504189/2012-3; 474563/2013-8; 307606/2013-9; 443167/2014-1) and by the Coordination for the Improvement of Higher Education Personnel (CAPES; Brazil; Process numbers: PNPD 2103/2009; AE1-0052-000650100/11).

References

[1] D.M. Dixon, *Coccidioides immitis* as a select agent of bioterrorism, J. Appl. Microbiol. 91 (4) (2001) 602–605.
[2] O. Welsh, L. Vera-Cabrera, A. Rendon, et al., Coccidioidomycosis, Clin. Dermatol. 30 (6) (2012) 573–591.
[3] R.A. Cox, D.M. Maggee, Coccidioidomycosis: host response and vaccine development, Clin. Microbiol. Rev. 17 (4) (2004) 804–839.
[4] J.N. Galgiani, N.M. Ampel, J.E. Blair, et al., Coccidioidomycosis, Clin. Infect. Dis. 41 (9) (2005) 1217–1223.
[5] M.M. Kim, H.R. Vikram, S. Kusne, et al., Treatment of refractory coccidioidomycosis with voriconazole or posaconazole, Clin. Infect. Dis. 53 (11) (2011) 1060–1066.
[6] R. Schein, J. Homans, R.A. Larsen, M. Neely, Posaconazole for chronic refractory coccidioidal meningitis, Clin. Infect. Dis. 53 (12) (2011) 1252–1254.
[7] E.R. Levy, J.M. McCarty, A.L. Shane, P.S. Weintrub, Treatment of pediatric refractory coccidioidomycosis with combination voriconazole and caspofungin: a retrospective case series, Clin. Infect. Dis. 56 (11) (2013) 1573–1578.
[8] R.J. Brüggemann, J.W. Alffenaar, N.M. Blijlevens, E.M. Billaud, J.G. Kosterink, P.E. Verweij, D.M. Burger, Clinical relevance of the pharmacokinetic interactions of azole antifungal drugs with other coadministered agents, Clin. Infect. Dis. 48 (10) (2009) 1441–1458.
[9] R. Laniado-Laborín, M.N. Cabrales-Vargas, Amphotericin B: side effects and toxicity, Rev. Iberoam. Micol. 26 (4) (2009) 223–227.
[10] R. de Aguiar Cordeiro, F.J. de Farias Marques, R. de Aguiar Cordeiro, et al., Synthesis and antifungal activity *in vitro* of isoniazid derivatives against *Histoplasma capsulatum* var. *capsulatum*, Antimicrob. Agents Chemother. 58 (5) (2014) 2504–2511.
[11] R.A. Cordeiro, R.S. Brilhante, M.F. Rocha, et al., Phenotypic characterization and ecological features of *Coccidioides* spp. from Northeast Brazil, Med. Mycol. 44 (7) (2006) 631–639.
[12] T. Umeyama, A. Sano, K. Kamei, et al., Novel approach to designing primers for identification and distinction of the human pathogenic fungi *Coccidioides immitis* and *Coccidioides posadasii* by PCR amplification, J. Clin. Microbiol. 44 (5) (2006) 1859–1862.
[13] M.V. Berridge, A.S. Tan, K.D. McCoy, R. Wang, The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts, Biochemica 4 (1996) 14–19.
[14] Cordeiro RdeA, R.S. Brilhante, M.F. Rocha, et al., In vitro synergistic effects of antituberculous drugs plus antifungals against *Coccidioides posadasii*, Int. J. Antimicrob. Agents 34 (3) (2009) 278–280.
[15] CLSI, Clinical and Laboratory Standards Institute, Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi, second ed., 2008 approved standard M38–A2.
[16] R.S.N. Brilhante, E.P. Caetano, J.J.C. Sidrim, et al., Ciprofloxacin shows synergism with classical antifungals against *Histoplasma capsulatum* var. *capsulatum* and *Coccidioides posadasii*, Mycoses 56 (3) (2012) 397–401.
[17] F.C. Odds, Synergy, antagonism, and what the chequerboard puts between them, J. Antimicrob. Chemother. 52 (1) (2003) 1.
[18] G.P. Moran, E. Pinjon, D.C. Coleman, D.J. Sullivan, Analysis of drug resistance in pathogenic fungi, in: K. Kavanagh (Ed.), Medical Mycology: Cellular and Molecular Techniques, John Wiley & Sons, Ltd., Hoboken, NJ, 2007, pp. 93–113.
[19] L. Ostrosky-Zeichner, A. Casadevall, J.N. Galgiani, et al., An insight into the antifungal pipeline: selected new molecules and beyond, Nat. Rev. Drug Discov. 9 (9) (2010) 719–727.
[20] A. Burger-Kentischer, D. Finkelmeier, P. Keller, et al., A screening assay based on host-pathogen interaction models identifies a set of novel antifungal benzimidazole derivatives, Antimicrob. Agents Chemother. 55 (10) (2011) 4789–4801.
[21] J.A. Paiva, Pereira JM New antifungal antibiotics, Curr. Opin. Infect. Dis. 26 (2) (2013) 168–174.
[22] R. Calderone, N. Sun, F. Gay-Andrieu, et al., Antifungal drug discovery: the process and outcomes, Future Microbiol. 9 (6) (2014) 791–805.
[23] G. Uppal, S. Bala, S. Kamboj, M. Saini, Therapeutic review exploring antimicrobial potential of hydrazones as promising lead, Der Pharma Chem. 3 (2011) 250–268.
[24] I. Ali, W.A. Wani, A. Khan, Synthesis and synergistic antifungal activities of a pyrazoline based ligand and its copper(II) and nickel(II) complexes with conventional antifungals, Microb. Pathog. 53 (2) (2012) 66–73.
[25] R. Narang, B. Narasimhan, S. Sharma, A review on biological activities and chemical synthesis of hydrazide derivatives, Curr. Med. Chem. 19 (2012) 569–612.
[26] G. Verma, A. Marella, M. Shaquiquzzaman, et al., A review exploring biological activities of hydrazones, J. Pharm. Bioallied Sci. 6 (2014) 69–80.
[27] Y. Ozkay, Y. Tunali, H. Karaca, I. Isikdag, Antimicrobial activity and a SAR study of some novel benzimidazole derivatives bearing hydrazone moiety, Eur. J. Med. Chem. 45 (2010) 3293–3298.
[28] M.D. Altıntop, A. Özdemir, G. Turan-Zitouni, et al., Synthesis and *in vitro* evaluation of new nitro-substituted thiazolyl hydrazone derivatives as anticandidal and anticancer agents, Molecules 19 (2014) 14809–14820.
[29] G.L. Backes, D.M. Neumann, B.S. Jursic, Synthesis and antifungal activity of substituted salicylaldehyde hydrazones, hydrazides and sulfohydrazides, Bioorg. Med. Chem. 22 (2014) 4629–4636.
[30] D.M. Neumann, A. Cammarata, G. Backes, et al. Synthesis and antifungal activity of substituted 2,4,6-pyrimidinetrione carbaldehyde hydrazones. Bioorg. Med. Chem. 22: 813–826.
[31] D.C. Reis, A.A. Despaigne, J.G. Da Silva, et al., Structural studies and investigation on the activity of imidazole-derived thiosemicarbazones and hydrazones against crop-related fungi, Molecules 18 (2013) 12645–12662.
[32] J. Vivas, P. Alvarado, G. Visbal, et al., *In vitro* synergisms among hydrazones, ajoeno and posaconazole against *Cryptococcus* spp, Investig. Clin. 52 (2011) 312–322.
[33] G. Visbal, G. San-Blas, A. Maldonado, et al., Synthesis, in vitro antifungal activity and mechanism of action of four sterol hydrazone analogues against the dimorphic fungus Paracoccidioides brasiliensis, Steroids 76 (2011) 1069–1081.
[34] M.A. Malik, S.A. Al-Thabaiti, M.A. Malik, Synthesis, structure optimization and antifungal screening of novel tetrazole ring bearing acyl-hydrazones, Int. J. Mol. Sci. 13 (2012) 10880–10898.
[35] M.E. Caputto, A. Ciccarelli, F. Frank, et al., Synthesis and biological evaluation of some novel 1-indanone thiazolylhydrazone derivatives as anti-*Trypanosoma cruzi* agents, Eur. J. Med. Chem. 55 (2012) 155–163.