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Synthesis and Antifungal Activity *In Vitro* of Isoniazid Derivatives against *Histoplasma capsulatum* var. *capsulatum*

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Histoplasmosis is a severe infection that affects millions of patients worldwide and is endemic in the Americas. Amphotericin B (AMB) and itraconazole are highly effective for the treatment of severe and milder forms of the disease, but AMB is toxic, and the bioavailability of itraconazole is erratic. Therefore, it is important to investigate new classes of drugs for histoplasmosis treatment. In this study, a series of nine isoniazid hydrazone derivatives were synthesized and evaluated for their antifungal activities *in vitro* against the dimorphic fungus *Histoplasma capsulatum* var. *capsulatum*. The drugs were tested by microdilution in accordance with CLSI guidelines. The compound *N*′-(1-phenylethylidene)isonicotinohydrazide had the lowest MIC range of all the compounds for the yeast and filamentous forms of *H. capsulatum*. The *in vitro* synergy of this compound with AMB against the planktonic and biofilm forms of *H. capsulatum* cells was assessed by the checkerboard method. The effects of this hydrazone on cellular ergosterol content and membrane integrity were also investigated. The study showed that the compound alone is able to reduce the ergosterol content of planktonic cells and can alter the membrane permeability of the fungus. Furthermore, the compound alone or in combination with AMB showed inhibitory effects against mature biofilms of *H. capsulatum*. *N*′-(1-Phenylethylidene)isonicotinohydrazide alone or combined with AMB might be of interest in the management of histoplasmosis.

Currently, one of the major drawbacks of treating systemic and opportunistic fungal infections is the narrow therapeutic arsenal available. In addition, severe side effects, toxicity, primary resistance of fungal species (1, 2), and therapeutic failures and/or refractoriness (3) are also considered important issues in the management of these infections (1, 4). In agents of severe infections, such as *Histoplasma capsulatum* (5), *Cryptococcus gattii* (6), and *Coccidioides* species (7), reduced susceptibility to antifungals has been detected worldwide. Therefore, many studies have been conducted to search for new antifungal drugs (8–10).

Histoplasmosis is a severe infection that affects millions of patients worldwide and is endemic in the Americas (4, 11). The disease is caused by *H. capsulatum* var. *capsulatum*, a soil-inhabiting fungal species that is usually found in association with bird or bat droppings (11). Respiratory and/or disseminated disease occurs after the inhalation of infectious microconidia, which convert to a yeast-like form within the lung parenchyma (11).

Nowadays, amphotericin B is the primary choice for induction therapy in moderate and severe cases of histoplasmosis, while long-term therapy with itraconazole is recommended for treating milder infections (11). Although both drugs are considered efficient, some disadvantages can arise during therapy: nephrotoxicity may occur in up to 33% of patients treated with amphotericin B (12), therapeutic levels of itraconazole may not be attained in the cerebrospinal fluid of some patients (11), and significantly reduced blood serum levels of itraconazole have been detected in HIV patients when this antifungal is coadministered with lopinavir and other protease inhibitors (13).

This study aimed to develop isoniazid-derived hydrazones and evaluate the inhibitory effects of these compounds against *H. capsulatum* var. *capsulatum*. Previous studies have shown the antifungal potential of antituberculosis drugs against the highly virulent pathogens *H. capsulatum* var. *capsulatum* and *Coccidioides posadasii* (10, 14). We also evaluated the *in vitro* synergism of one of these isoniazid analogs, *N*′-(1-phenylethylidene)isonicotinohydrazide [*N*′-(1-phenyl)], with amphotericin B in both the planktonic and biofilm forms of *H. capsulatum* var. *capsulatum* cells. The effects of this compound alone or in combination with amphotericin B on cellular ergosterol content and membrane integrity were also investigated.

MATERIALS AND METHODS

Microorganisms. A total of 25 strains of *H. capsulatum* var. *capsulatum* isolated in Brazil were included in the study. Most of the strains were recovered from AIDS patients with disseminated histoplasmosis. Two strains were isolated from pet cats presenting with cutaneous ulcers derived from disseminated disease. The isolates were identified by the visualization of yeast-like cells inside macrophages on direct Giemsa-stained smears, as well as by the macro- and microscopic characteristics of cultures in filamentous form. All tested isolates were maintained in potato

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dextrose agar at room temperature until testing (4). The strains belong to the fungal collection of the Specialized Medical Mycology Center (CEMM) of the Federal University of Ceará, Brazil. All procedures were performed inside a biosafety level 3 laboratory.

Synthesis of Schiff bases. The hydrazones were synthesized by a reaction between isoniazid (INH) and ketones, as described by Ilić et al. (15), with modifications, using the Schiff base method of preparation. The compounds were prepared with nine different ketones, one by a reaction of acetophenone, 2′-methoxyacetophenone, 3′-methoxyacetophenone, 4′-chloroacetophenone, 2′-nitroacetophenone, 3′-methylacetophenone, 4′-nitroacetophenone, 4′-bromoacetophenone, 2′-phenylacetophenone (0.003 mol each), and INH (0.006 mol) with 5.0 ml of ethanol. This mixture was sealed in a stainless steel reactor coated with Teflon and subjected to microwave radiation for 4 to 5 min. The purification process was performed by dissolving the impurities in ethyl alcohol and methyl alcohol at room temperature after successive washes, alternating with centrifugation, and the control reactions were performed by thin-layer chromatography (TLC) to verify the purity of the synthesized compounds. Tests were conducted with silica gel 60 (Φ, 63 to 200 μm; Carlo Erba, France) in silica 60 F254 plates (Merck, Germany) as the stationary phase. The mobile phase was formed by a mixture of acetate-hexane-methanol (7:1:2). The compounds were revealed after exposure to UV at two wavelengths (254 and 365 nm) and by spraying with vanillin acid solution, followed by heating at 150°C.

All the compounds formed were characterized structurally 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 (1H, 300 MHz; 13C, 75 MHz) using deuterated dimethyl sulfoxide (DMSO-d_6) as a solvent. All ketones used in this work were obtained from Aldrich and Sigma-Aldrich (Germany).

Antifungal susceptibility test of *H. capsulatum* planktonic cells. Cultures of *H. capsulatum* strains ($n = 18$) in filamentous form were grown on brain heart infusion (BHI) agar (BD Diagnostics, USA) at 28°C for 7 days. For yeast-like cells, strains ($n = 13$) randomly chosen from the set of clinical isolates were grown on BHI medium supplemented with 10% sheep blood at 35°C for 7 days (16, 17). Cultures of *H. capsulatum* were gently scraped with cotton swabs and transferred to about 4 ml of sterile saline solution. The suspensions were homogenized, read at 530 nm, and adjusted to 90 to 95% transmittance. Afterwards, the suspensions were diluted to 1:10 with RPMI 1640 medium (Sigma Chemical Co., USA) containing L-glutamine without sodium bicarbonate and buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma Chemical Co., USA) to obtain an inoculum of approximately 0.5×10^3 to 2.5×10^4 CFU · ml^{-1} (4).

The INH-derived molecules were weighed, dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Co., USA), and further diluted with RPMI medium to obtain the test concentrations. Stock solutions of amphotericin B (AMB) (Sigma Chemical Co., USA) and itraconazole (ITC) (Janssen Pharmaceutica, Belgium) were prepared in DMSO, as suggested by CLSI (18). Serial 2-fold dilutions were prepared with RPMI medium and stored at −20°C.

Susceptibility testing was performed by the broth microdilution protocol (18). Microtiter plates were incubated for 4 to 5 days or 7 days for the yeast-like and mold forms, respectively. All isolates were tested in duplicate. The controls were grown on RPMI medium buffered with MOPS without antimicrobials. As a quality control for each test, *Candida parapsilosis* strain ATCC 22019 was also included (18). The MICs of the hydrazones were based on a previous study with INH and were defined as the lowest drug concentration that caused 80% inhibition of visible fungal growth (10) For AMB, the MIC was defined as the lowest drug concentration that prevented any discernible growth (100% inhibition). The minimum fungicidal concentration (MFC) was defined as the lowest concentration that inhibited fungal growth completely after seeding 0.1 ml of the fungal suspension at concentrations above the MIC onto potato agar for 15 days at 35°C (10).

The INH-derived compounds were screened for antifungal activity against randomly chosen *H. capsulatum* strains ($n = 5$) in the yeast-like and filamentous forms. Further experimentation was performed only on the drugs that presented the lowest MIC values for both forms. INH derivatives were tested from 3.9 to 2,000 μg/ml, based on *H. capsulatum* susceptibility to INH; AMB and ITC were tested from 0.002 to 1.00 μg/ml and from 0.0001 to 0.0625 μg/ml, respectively (10).

Synergistic potential of *N*′-(1-phenylethylidene)isonicotinohydrazide, an isoniazid-derived hydrazone, and AMB by the checkerboard method. The *in vitro* synergism between *N*′-(1-phenylethylidene)isonicotinohydrazide [*N*′-(1-phenyl)] and AMB was evaluated in a checkerboard assay (19). Checkerboard synergy testing was performed in duplicate in microdilution assays to analyze both the yeast and filamentous forms. Combinations were formed with the isoniazid derivative plus AMB at the following concentrations: *N*′-(1-phenylethylidene)isonicotinohydrazide, 23.4 to 187.5 μg/ml, and AMB, 0.00098 to 0.00781 μg/ml. The MIC of each drug in combination (MIC_{syn}) was defined as the lowest concentration that caused 100% inhibition of visible fungal growth. Drug interactions were classified as synergistic, indifferent, or antagonistic according to the fractional inhibitory concentration index (FICI). The interaction was defined as synergistic if the FICI was ≤0.5, indifferent if it was >0.5 but <4.0, and antagonistic if it was >4.0 (20).

Effect of *N*′-(1-phenylethylidene)isonicotinohydrazide on ergosterol content of planktonic cells. Total sterols were extracted as described by Moran et al. (21), with modifications. Strains of *H. capsulatum* in yeast-like phase ($n = 8$) were grown on BHI medium supplemented with 10% sheep blood at 35°C for 7 days (16). After this period, a loopful of inoculum (0.5×10^3 to 2.5×10^4 CFU · ml^{-1}) was transferred to RPMI 1640 medium supplemented with *N*′-(1-phenylethylidene)isonicotinohydrazide at 2× MIC, MIC, and sub-MIC (MIC/2) (4). The tubes were incubated for 4 to 5 days at 35°C, and after this period, each cell suspension was adjusted to a 0.5 McFarland standard to obtain an inoculum of approximately 1.0 to 5.0×10^6 cells/ml, which was then centrifuged at 9,660 × *g* 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 in a bath. Following incubation, the tubes were allowed to cool, and total sterols were extracted by the addition of 0.6 ml of *n*-hexane and vigorous vortexing for 5 min. The tubes were centrifuged at 13,416 × *g* 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 (Sigma-Aldrich, Germany). The experiments were performed in duplicate. The results were compared with the ergosterol content of cells grown in RPMI 1640 medium without antifolate drugs, and itraconazole (MIC/2) was used as a positive control.

Effect of *N*′-(1-phenylethylidene)isonicotinohydrazide on cell membrane integrity. The effect of *N*′-(1-phenylethylidene)isonicotinohydrazide on cell membrane integrity was performed as suggested by Devi et al. (22), with adaptations. Susceptibility testing of *N*′-(1-phenylethylidene)isonicotinohydrazide, INH, and AMB against *H. capsulatum* in yeast-like form was conducted as described above. The cell contents of wells containing MIC/2 and a positive control without antimicrobials were harvested by centrifugation at 13,416 × *g* for 15 min. A volume of the supernatant was diluted 1:10 in distilled water, and the release of UV-absorbing molecules was analyzed by spectrometry at 260 nm for nucleic acids and at 280 nm for proteins.

Effect of *N*′-(1-phenyl) against mature *H. capsulatum* biofilms. *H. capsulatum* biofilms were formed as suggested by Pitangui et al. (23), with adaptations. Seven-day-old yeast-like cultures were suspended in sterile saline solution, and the suspensions were adjusted to 2.4×10^6 to 5.5×10^6 cells/ml in BHI broth. Afterward, 0.05 ml of inoculum aliquots was transferred to flat 96-well polystyrene plates and incubated at 35°C for 7 h

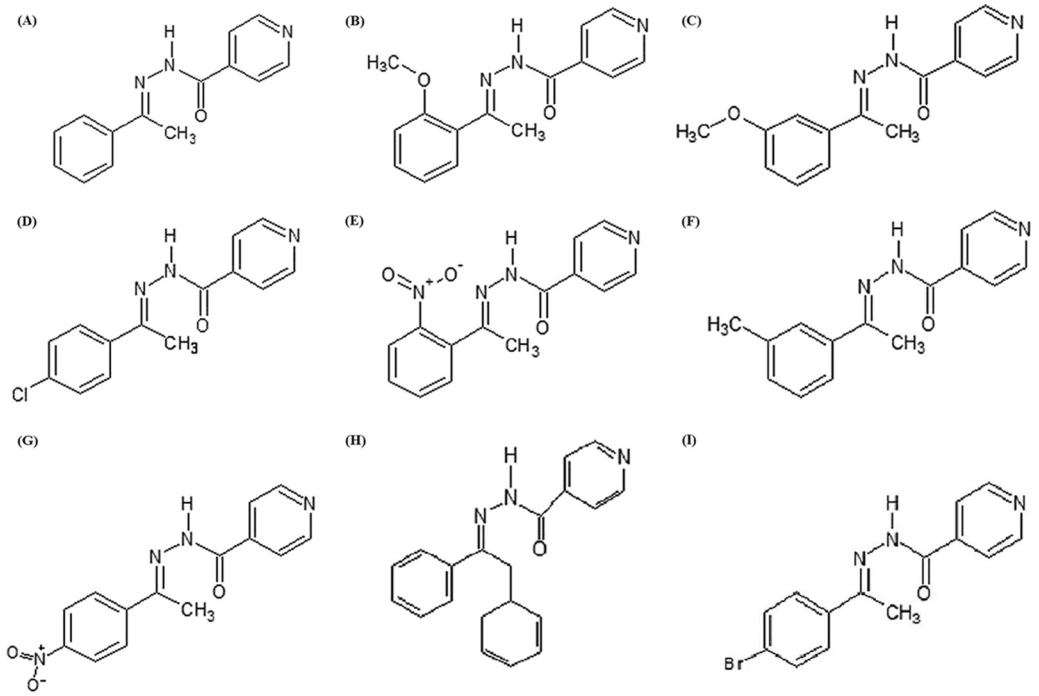

FIG 1 Structural formulas of synthetized isoniazid derivatives *N*′-[1-phenylethylidene]isonicotinohydrazide (A), *N*′-[1-(2-methoxyphenyl)ethylidene]isonicotinohydrazide (B), *N*′- [1-(3-methoxyphenyl)ethylidene]isonicotinohydrazide (C), *N*′- [1-(4-chlorophenyl)ethylidene]isonicotinohydrazide (D), *N*′- [1-(2-nitrophenyl)ethylidene]isonicotinohydrazide (E), *N*′-(1-*m*-tolylethylidene)isonicotinohydrazide (F), *N*′- [1-(4-nitrophenyl)ethylidene]isonicotinohydrazide (G), *N*′-(1,2-diphenylethylidene)-2-isonicotinohydrazide (H), and *N*′-[1-(4-bromophenyl)ethylidene]isonicotinohydrazide (I).

in a rotary shaker at 80 rpm for the preadhesion step. Next, the supernatants were removed and 0.2 ml of BHI broth was added to each well. The plates were further incubated for 72 h as described above, and subsequently, the wells were washed three times with phosphate-buffered saline (PBS) solution to remove nonadherent cells. Aliquots of 0.2 ml of BHI medium with antimicrobials were added to viable 72-h biofilms as follows: *N*′-(1-phenyl), 6,250 μg/ml to 97.6 μg/ml; AMB, 3.12 μg/ml to 0.048 μg/ml; and *N*′-(1-phenyl), 780 μg/ml to 11.7 μg/ml, plus AMB, 1.56 μg/ml to 0.023 μg/ml. Following incubation at 35°C for 72 h, the supernatant was aspirated, and an aliquot of 0.1 ml 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 0.2 ml of 33% acetic acid, and after 5 min at 25°C, the mixture was aspirated and read in a spectrophotometer at 550 nm (24). The tests were conducted in duplicate, and the controls were grown in medium without antimicrobials. The effect of *N*′-(1-phenyl) was compared with that of AMB, which is considered to be a strong inhibitor of fungal biofilms (25). Biofilm viability was monitored by the color change of resazurin solution (0.1 mg/ml in RPMI) after incubation at 37°C for ≥6 h (25).

Statistical analysis. The antimicrobial susceptibilities were compared using one-way analysis of variance (ANOVA) and Tukey's multiple comparison posttest. Differences between the treatments were evaluated for significance using the Wilcoxon signed-rank test. A P value of <0.05 was considered to be significant. The statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Nine isoniazid derivatives were synthesized (Fig. 1), with yields ranging from 11.2 to 82.1%. The biological activities of the compounds were investigated against strains of *H. capsulatum* and expressed in terms of the MIC in μg/ml. Table 1 shows the no-

TABLE 1 Antifungal activity of synthesized hydrazones against *H. capsulatum* var. *capsulatum* ($n = 5$) in yeast-like and filamentous forms

Compound	Molecular formula	Mol wt	MIC range (μg/ml) for:	
			Yeast form	Filamentous form
N′-[1-Phenylethylidene]isonicotinohydrazide	$C_{14}H_{13}N_3O$	239.3	7.8–250	15.6–250
N′-[1-(2-Methoxyphenyl)ethylidene]isonicotinohydrazide	$C_{15}H_{15}N_3O_2$	269.3	62.5–125	1,000–2,000
N′-[1-(3-Methoxyphenyl)ethylidene]isonicotinohydrazide	$C_{15}H_{15}N_3O_2$	269.3	31.25–125	2,000
N′-[1-(4-Chlorophenyl)ethylidene]isonicotinohydrazide	$C_{14}H_{12}ClN_3O$	273.7	125	125–500
N′-[1-(2-Nitrophenyl)ethylidene]isonicotinohydrazide	$C_{14}H_{12}N_4O_3$	284.3	31.25–125	250–500
N′-[1-*m*-Tolylethylidene]isonicotinohydrazide	$C_{15}H_{15}N_3O$	253.3	62.5–250	250–500
N′-[1-(4-Nitrophenyl)ethylidene]isonicotinohydrazide	$C_{14}H_{12}N_4O_3$	284.3	62.5–250	250–500
N′-(1,2-Diphenylethylidene)-2-isonicotinohydrazide	$C_{20}H_{17}N_3O$	315.4	62.5–250	1,000–2,000
N′-[1-(4-Bromophenyl)ethylidene]isonicotinohydrazide	$C_{14}H_{12}BrN_3O$	318.1	62.5–125	125–500

TABLE 2 MICs and MFCs of *N′*-(1-phenylethylidene)isonicotinohydrazide against *H. capsulatum* in yeast-like and filamentous forms ($n = 16$)

H. capsulatum strain	Data (μg/ml) by form of *H. capsulatum*			
	MIC		MFC	
	Yeast	Filamentous	Yeast	Filamentous
03-02-090	125	125	500	250
03-03-035	15.6	31.2	31.2	62.5
03-06-059	31.2	250	62.5	500
05-01-097	125	62.5	250	250
05-02-021	62.5	62.5	500	62.5
05-02-034	62.5	125	500	500
05-02-035	7.8	31.2	15.6	62.5
05-02-037	31.2	62.5	62.5	125
05-02-042	31.2	31.2	62.5	250
05-02-053	62.5	125	500	500
05-02-074	62.5	62.5	500	250
05-02-087	62.5	62.5	250	250
05-02-088	250	125	1,000	500
05-04-015	62.5	31.2	250	62.5
05-04-028	31.2	62.5	62.5	125
05-05-011	31.2	125	62.5	500

menclature, formulas, and molecular weights of the compounds synthesized. High-resolution mass spectrometry (HR-MS) confirmed the molecular weights and empirical formulas of the compounds.

All of the synthetized molecules were able to inhibit *H. capsulatum* growth in the yeast-like and filamentous forms. However, some isoniazid-derived compounds were effective only at high concentrations, as displayed in Table 1. Lower MIC values were obtained with the isoniazid-derived acetophenone *N′*-(1-phenylethylidene)isonicotinohydrazide for both yeast-like and filamentous forms, and for this reason, this drug was chosen for further experimentation.

The MICs of *N′*-(1-phenyl) ranged from 7.8 μg/ml to 250 μg/ml and from 31.2 μg/ml to 250 μg/ml for the yeast-like and filamentous forms, respectively. The MFC values ranged from 15.6 μg/ml to 1,000 μg/ml and from 62.5 μg/ml to 500 μg/ml for the yeast-like and filamentous forms, respectively (Table 2). No significant differences between the MIC ($P = 0.1808$) and MFC values ($P = 0.558$) for the phases were detected. The quality control *C. parapsilosis* ATCC 22019 showed MIC values in agreement with CLSI guidelines (26).

The compound *N′*-(1-phenyl) showed synergistic interactions with AMB against *H. capsulatum* in both the yeast-like and filamentous forms. When AMB and *N′*-(1-phenyl) were combined, the MIC_{syn} values were approximately 10 times lower than the MIC values. The hydrazone *N′*-(1-phenyl) plus AMB had a synergistic effect against 14 of 18 strains of *H. capsulatum* in filamentous form, with FICI values that ranged from 0.009 to 0.78 (Table 3). For the yeast-like form, the combination of *N′*-(1-phenyl) and AMB showed FICI values that ranged from 0.04 to 0.56. Only

TABLE 3 Synergistic *in vitro* activity of *N′*-(1-phenylethylidene)isonicotinohydrazide combined with AMB against *H. capsulatum* in yeast-like and filamentous forms ($n = 24$)

H. capsulatum strain	MIC (μg/ml) for drugs when:									
	Isolated				Combined					
	N′-(1-phenyl)[a]		AMB		*N′*-(1-phenyl)		AMB		FICI	
	Y	F	Y	F	Y	F	Y	F	Y	F
03-02-090	125	125	0.5	0.5	3.9	3.9	0.0078	0.0019	0.046	0.035
03-03-035	15.6	31.2	0.5	0.5	3.9	1.0	0.0078	0.0009	0.265	0.034
03-03-054	NT[b]	125	NT	0.5	NT	3.9	NT	0.0019	NT	0.035
03-03-055	62.5	NT	0.125	NT	7.8	NT	0.0156	NT	0.2496	NT
03-06-059	31.2	NT	0.125	NT	7.8	NT	0.0156	NT	0.3744	NT
05-01-096	NT	62.5	NT	0.5	NT	46.8	NT	0.0019	NT	0.753
05-01-097	NT	62.5	NT	0.25	NT	46.8	NT	0.0019	NT	0.752
05-02-018	NT	31.2	NT	0.0312	NT	1.0	NT	0.0009	NT	0.063
05-02-021	62.5	NT	1.0	NT	3.9	NT	0.0078	NT	0.0702	NT
05-02-034	62.5	31.2	0.5	0.5	3.9	1.0	0.0078	0.0009	0.078	0.034
05-02-035	NT	31.2	NT	0.125	NT	1.0	NT	0.0009	NT	0.039
05-02-042	NT	31.2	NT	0.0312	NT	23.4	NT	0.0009	NT	0.782
05-02-053	62.5	NT	0.5	NT	7.8	NT	0.0156	NT	0.156	NT
05-02-074	62.5	NT	0.5	NT	7.8	NT	0.0156	NT	0.156	NT
05-02-084	15.6	62.5	0.5	0.0312	1.95	23.4	0.0039	0.0009	0.132	0.406
05-02-085	NT	62.5	NT	0.5	NT	46.8	NT	0.0019	NT	0.753
05-02-086	NT	62.5	NT	0.5	NT	23.4	NT	0.0009	NT	0.376
05-02-087	62.5	62.5	1.0	1.0	3.9	3.9	0.00781	0.0019	0.070	0.064
05-02-088	250	125	0.5	0.5	7.8	23.4	0.0156	0.0009	0.062	0.189
05-02-091	NT	125	NT	1.0	NT	23.4	NT	0.0009	NT	0.188
05-03-040	NT	125	NT	0.5	NT	1.0	NT	0.0009	NT	0.009
05-04-015	62.5	31.2	0.0156	1.0	3.9	3.9	0.78	0.0019	0.563	0.126
05-04-028	NT	62.5	NT	0.5	NT	1.0	NT	0.0009	NT	0.017
05-05-011	31.2	NT	0.5	NT	7.8	NT	0.0156	NT	0.2808	NT

[a] Y, yeast-like; F, filamentous.
[b] NT, not tested.

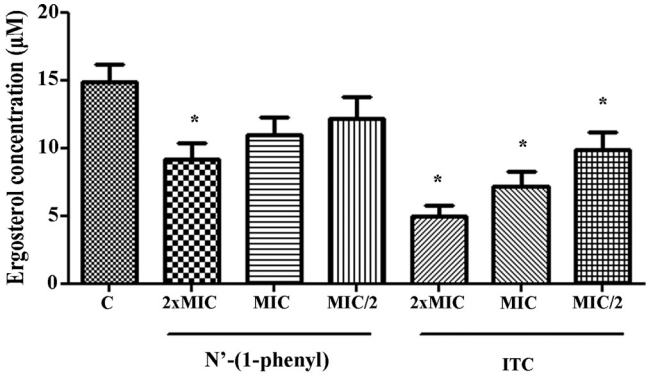

FIG 2 Ergosterol content of *H. capsulatum*. Cells in yeast-like phase were cultured in RPMI medium without antimicrobials as a control (C) or supplemented with *N*′-(1-phenyl) or itraconazole (ITC) at 2× MIC, MIC, and MIC/2. The experiments were conducted in duplicate, and the data are expressed as the means ± standard errors of the mean (SEM) ($n = 8$). The asterisks indicate statistically significant differences from controls ($P < 0.05$).

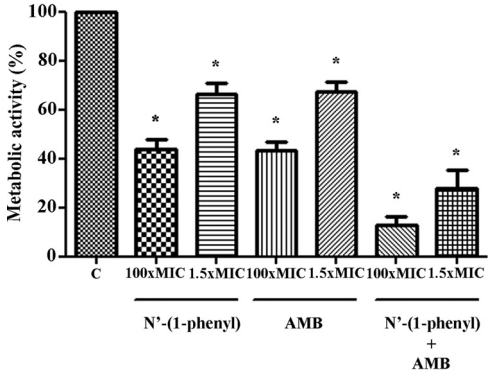

FIG 4 Metabolic activity of mature *H. capsulatum* biofilms after antimicrobial treatments. Yeast-like cells were grown in BHI broth medium for 72 h for biofilm formation and then treated with different concentrations of *N*′-(1-phenyl) or amphotericin B (AMB) alone or in combination. Controls (C) were formed by incubation in RPMI without antimicrobials. The results are presented as the percent reduction compared to the controls. The experiments were conducted in duplicate, and the data are expressed as the means ± SEM ($n = 6$). Asterisks indicate statistically significant differences from controls ($P < 0.05$).

one strain (*H. capsulatum* 05-04-015) showed an FICI of >0.5 (Table 3).

Effect of hydrazone *N*′-(1-phenyl) on ergosterol content of planktonic cells. The total ergosterol content was determined for eight strains grown in suprainhibitory (2× MIC), inhibitory (MIC), and subinhibitory (MIC/2) concentrations of the drug *N*′-(1-phenyl). A significant reduction in the ergosterol content was seen when the cells were incubated with *N*′-(1-phenyl) at 2× MIC. However, no significant reduction of ergosterol content was observed when cells were exposed to inhibitory and subinhibitory concentrations of this drug, in comparison with fungal growth in RPMI medium without antimicrobials (Fig. 2). Itraconazole (ITC) was used as a quality control for the same strains tested, leading to a significant decrease in the production of ergosterol for the concentrations 2× MIC, MIC, and MIC/2 (Fig. 2).

Effect of *N*′-(1-phenyl) on cell membrane integrity. Changes in membrane permeability were detected after treatment of *H. capsulatum* cells with *N*′-(1-phenyl) at MIC/2 (Fig. 3). The supernatant content of the yeast-like cultures showed absorbing components at 260 nm and 280 nm, suggesting leaking of nucleic acids and proteins, respectively, from the cell. Minor alterations in cell permeability were also induced by INH at MIC/2. AMB at MIC/2 was not able to induce an outflow of traceable material from the cells.

Effect of hydrazone *N*′-(1-phenyl) against *Histoplasma* biofilms. Mature biofilms of *H. capsulatum* were inhibited by approximately 50% after treatment with *N*′-(1-phenyl) at 100× MIC, 50× MIC, or 25× MIC indistinctly ($P < 0.05$). At 1.5× MIC, the inhibition was approximately 30%. Similar results were obtained with AMB. Better results were obtained with combinations formed by *N*′-(1-phenyl) plus AMB, which were able to reduce the mature biofilms in approximately 80% (Fig. 4).

DISCUSSION

Hydrazones are a class of organic compounds with the chemical structure $R_1R_2C{=}NNH_2$, formed by the replacement of the oxygen of the carbonyl group with the functional group -NNH_2. The azomethine proton (-NHN=CH-) formed is an important source for new drug development (9). In recent years, the hydrazones have received considerable attention for displaying a variety of biological activities, such as antituberculotic, antimicrobial (27), antitumor (28), analgesic (29), and anti-inflammatory (30) properties. Some of these compounds have been described as more efficient than standard antibiotics (27).

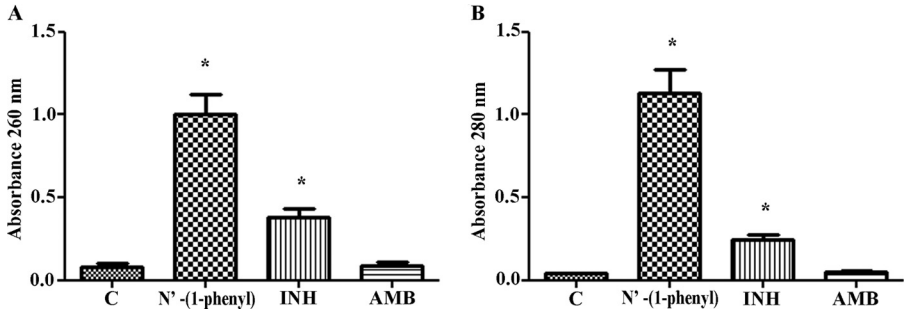

FIG 3 Absorbance of extracellular content of *H. capsulatum* cells at 260 nm (A) and 280 nm (B). Cells in yeast-like phase were incubated in RPMI 1640 medium without antimicrobials as a control (C), *N*′-(1-phenyl), isoniazid (INH), or amphotericin B (AMB) at MIC/2. The experiments were conducted in duplicate, and the data are expressed as the mean ± SEM ($n = 6$). Asterisks indicate statistically significant differences from controls ($P < 0.05$).

Considering the antifungal potential of INH previously described against the dimorphic pathogens *C. posadasii* (14) and *H. capsulatum* (10), in the present study, INH-derived hydrazones were evaluated against *H. capsulatum* in both yeast-like and filamentous forms. Synthetized hydrazone compounds showed antifungal activity, although all but one were effective at high concentrations only. The compound *N′*-(1-phenyl) was the most active among all the synthesized derivatives, presenting the lowest MIC values for both yeast-like and filamentous forms, so we chose it for further experimentation. Given the results, we investigated the antifungal activity of *N′*-(1-phenyl) in combination with the drug of choice, AMB, for the treatment of histoplasmosis. The data showed antifungal effects against *H. capsulatum* planktonic cells, being able to significantly reduce the MIC values of AMB *in vitro*.

In order to understand the cell target of *N′*-(1-phenyl), we investigated its effect on cell ergosterol content and membrane permeability. According to the results, *N′*-(1-phenyl) caused a reduction in the cell content of ergosterol in *H. capsulatum*, although to a lesser extent than that caused by itraconazole. Visbal et al. (31) also evaluated the antiproliferative effects of hydrazone analogues on the yeast-like phase of *Paracoccidioides brasiliensis* and observed growth inhibition of around 90%. The authors found that the hydrazone derivatives affect cell growth by inhibiting the synthesis of brassicasterol (predominant sterol) and ergosterol in the yeast-like phase. However, one of the compounds did not show an association with any blocking step in the biosynthesis of sterols, strongly suggesting different modes of action. The results here show that both *N′*-(1-phenyl) and INH are able to alter the membrane permeability of the fungus, making the cells more permeable to nucleic acids and proteins. The dispersion of intracellular components suggests that the crucial effect of *N′*-(1-phenyl) and INH on *H. capsulatum* may be the formation of pores in the plasma membrane. Leakage of cytoplasmic material is considered to be indicative of serious and irreversible damage to the cytoplasmic membrane (32). The release of intracellular content is a hallmark of damage and a loss of membrane integrity (22).

We also evaluated the antimicrobial potential of hydrazone against mature *H. capsulatum* biofilms. *N′*-(1-Phenyl) had considerable antibiofilm activity, similar to that of AMB. This result reinforces the antibiofilm potential of *N′*-(1-phenyl), once AMB is considered the most efficient drug against fungal biofilm (24). In addition, we found that combination of *N′*-(1-phenyl) with AMB enhanced the inhibitory activity of the antifungal drug against *H. capsulatum* biofilms, even at lower concentrations. Although details concerning the structure and dynamics of *H. capsulatum* biofilms *in vivo* are unknown, we believe that this antimicrobial combination might improve the management of infections caused by *H. capsulatum* biofilms.

Although there is no standard method for determining the inhibitory activity against fungal biofilms, many authors have chosen colorimetric methods based on metabolic activity measurement, of which the {2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide} XTT assay is the most commonly used. However, inconsistencies with this test have already been noted (33, 34). In a seminal study performed by Kuhn et al. (33) with *Candida* biofilms, it was clearly proven that different strains are able to metabolize XTT with different capabilities, making detailed standardization for each strain necessary. The authors also stated that the relationship between cell numbers and colorimetric signal may not be linear. In addition, some strains are able to retain the XTT formazan intracellularly, which might alter the final measurement. Besides these issues, a particular problem regarding biosafety should be noted when experiments are performed with biosafety level 3 (BSL3) microorganisms. In general, XTT assays require centrifugation of viable cells and further measurement of the absorbance of the supernatant. As centrifugation of living BL3 pathogens must be avoided due to the risk of infectious aerosol formation, we have chosen the crystal violet method, since it is an easy and straightforward technique, thus bypassing biosafety issues. Biofilm viability was monitored by the color change of resazurin solution (data not shown) in an attempt to corroborate the results obtained by the crystal violet method.

AIDS-related histoplasmosis is in most cases disseminated and highly lethal in the absence of an accurate diagnosis and appropriate antifungal therapy (35). Despite the excellent therapeutic effects of high doses of amphotericin B, studies have shown that mortality rates reach 50% in patients with AIDS and severe histoplasmosis, usually due to renal insufficiency as a result of aggressive use of amphotericin B. Although many patients respond favorably to treatment, the existence of other comorbidities worsens the prognosis and can be a life-threatening complication (36). For this reason, the search for new antifungal therapies is increasingly necessary.

Besides the ability to form biofilms, *H. capsulatum* utilizes a wide variety of highly effective virulence mechanisms, including temperature-regulated dimorphism and intracellular parasitism. The course of the related respiratory disease depends mainly on the capacity of the yeast *H. capsulatum* to survive and replicate within alveolar macrophages, the first line of cellular defense against *H. capsulatum* found in the host (23). In a preliminary experiment (data not shown), we investigated the ability of *N′*-(1-phenyl) to penetrate the macrophages and increase the death rate of the fungus in the phagosome. The results obtained suggest that the compound in combination with AMB is capable of restricting the intracellular growth of *H. capsulatum*, in concentrations 16 to 64 times smaller than those of *N′*-(1-phenyl) and AMB, respectively, compared to the MIC of those drugs tested singly. However, further experiments with a greater number of strains should be performed to revalidate the synergistic effect of these drugs against phagocytosed *H. capsulatum* cells.

The present study shows that *N′*-(1-phenyl), a hydrazone derivative, has antifungal potential against planktonic cells and biofilms of *H. capsulatum*. Further studies should be performed in order to evaluate the antifungal and anti-biofilm effects of *N′*-(1-phenyl) *in vivo*. The results obtained in this study highlight the importance of finding new molecular targets that can provide starting points for the rational design of new compounds with antifungal activity.

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