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Synergistic Effect of Antituberculosis Drugs and Azoles *In Vitro* against *Histoplasma capsulatum* var. *capsulatum*▽

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This study evaluated *in vitro* interactions of antituberculosis drugs and triazoles against *Histoplasma capsulatum*. Nine drug combinations, each including an antituberculosis drug (isoniazid, pyrazinamide, or ethambutol) plus a triazole (itraconazole, fluconazole, or voriconazole), were tested against both growth forms of *H. capsulatum*. Stronger synergistic interactions were seen in isoniazid or pyrazinamide plus triazoles for the mold form and ethambutol plus voriconazole for the yeast-like form. Further studies should evaluate these combinations *in vivo*.

Previously we demonstrated the inhibitory effect of some antituberculosis drugs alone or combined with antifungals against the pathogen *Coccidioides posadasii* (5, 6). Stronger synergistic interactions were seen in the combinations including ethambutol (ETB) plus triazoles as well as pyrazinamide (PZA) plus itraconazole (ITR). Based on these results, the purpose of this study was to investigate the effect of these combinations against the dimorphic pathogen *Histoplasma capsulatum* var. *capsulatum*—the etiological agent of American histoplasmosis, regarded as the most frequent systemic fungal infection worldwide (1, 7) and an important opportunistic infection among AIDS patients (7, 8, 13, 14). The emergence of resistance to fluconazole (FLC) as a cause of failure during treatment of histoplasmosis in patients with AIDS indicates a need for studies seeking new therapeutic options for this mycosis (13).

A total of 18 strains of *Histoplasma capsulatum* var. *capsulatum* (henceforth called *H. capsulatum*) isolated in Brazil were included in the study. Most of the strains were recovered from AIDS patients with histoplasmosis and were isolated from bone marrow puncture ($n = 10$) and peripheral blood ($n = 6$). Two strains isolated from cutaneous ulcers of domestic felines with disseminated histoplasmosis were also tested. 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.

For macrobroth testing, inoculum preparation was carried out as described by Li et al. (9) with minor modifications. First, the *H. capsulatum* strains were grown on brain heart infusion agar (BHI; BD Diagnostics) at 28°C for 7 days. Sterile 0.9% saline was added to the agar slant, and the cultures were gently scraped with cotton swabs. The suspensions were read at 530 nm and adjusted to 90 to 95% transmittance. The suspension was then diluted 1:10 with RPMI 1640 medium (Sigma Chemical Co.) containing L-glutamine and without sodium bicarbonate and buffered to pH 7.0 with 0.165 M MOPS (morpholinepropanesulfonic acid; Sigma Chemical Co.) to obtain an inoculum of approximately 0.5×10^3 to 2.5×10^4 CFU · ml^{-1}. Inoculum preparation for microbroth tests was performed as described by Brilhante et al (3). The suspensions were diluted with RPMI medium buffered to pH 7.0 with MOPS to obtain an inoculum of approximately 0.5×10^3 to 2.5×10^4 CFU · ml^{-1}. The density of each inoculum was checked by quantitative colony counts on Sabouraud dextrose agar (9).

Stock solutions of itraconazole (ITR) (Janssen Pharmaceutica, Belgium) and voriconazole (VRZ) (Pfizer Pharmaceuticals) were prepared in dimethyl sulfoxide (DMSO; Sigma Chemical Co.). Fluconazole (FLC) (Pfizer Pharmaceuticals) was prepared in distilled water (CLSI standard published in 2002). Ethambutol (ETB) (Iquego, Brazil) and isoniazid (INH) and pyrazinamide (PZA) (Lafepe, Brazil) were prepared in DMSO (5). Serial 2-fold dilutions of each antimicrobial agent were prepared with RPMI medium.

First the strains were tested against each drug alone to determine the MIC (4). The drug concentrations tested ranged as follows: ITC, 0.0001 to 0.0625 μg/ml; FLC, 0.9765 to 500 μg/ml; VRZ, 0.00195 to 1.00 μg/ml; ETB, 195 to 12,500 μg/ml; INH, 18 to 300 μg/ml; and PZA, 195 to 6,250 μg/ml (6, 9, 10).

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TABLE 1. MICs and MFCs for antifungals and antituberculosis drugs against *H. capsulatum* strains in filamentous and yeast-like forms[a]

Antimicrobial drug	MFC range (μg/ml)		MIC range (μg/ml)		MIC (geometric mean;μg/ml)	
	F	Y	F	Y	F	Y
ITC	0.0078–0.0625	0.0312–0.0625	0.0039–0.0312	0.0039–0.0312	0.017	0.017
FLC	31.25–250	31.25–62.5	15.625–62.5	3.9–31.25	38.37	7.8
VRZ	31.25–250	0.0312–0.125	0.0156–0.25	0.0078–0.0312	0.16	0.022
ETB	3,130–12,500	1,560	1,560–6,250	390–1,560	3,880	1,240
INH	80–300	>450	40–300	75–150	130	126
PZA	780–6,250	>2,340	550–3,130	190–780	1,700	540

[a] Abbreviations: F, filamentous; Y, yeast-like; ITC, itraconazole; FLC, fluconazole; VRZ, voriconazole; EMB, ethambutol; INH, isoniazid; PZA, pyrazinamide.

Broth macrodilution tests based on the CLSI reference document M38-A2 (4) were used to determine the MIC for *H. capsulatum* mold form. The results were read visually, and MIC endpoints were determined after 7 days of incubation at 35°C. Broth microdilution assays were performed for *H. capsulatum* yeast-like form as described by Nakai et al. (10), and the readings were conducted after 4 days. All tests were repeated at least twice, and every fungal strain was tested in duplicate. MICs of the azoles and antituberculosis drugs were defined as the lowest drug concentration that caused 80% inhibition of visible fungal growth (5, 6, 9, 10). The minimum fungicidal concentration (MFC) was determined as described by Li et al. (9), with minor modifications. The lowest concentration that inhibited fungal growth completely was determined after seeding 100 μl of the fungal suspension at concentrations above the MIC onto BHI agar for 15 days at 35°C. Quality control strains *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were included in each batch of tests. In addition, a *Coccidioides posadasii* strain (CEMM 01-6-085), previously tested against the antituberculosis drugs (5), was included.

Checkerboard synergy testing was performed in duplicate in macrodilution and microdilution assays for analysis of filamentous and yeast forms, respectively. Positive growth controls were performed in RPMI medium without antimicrobials. Combinations included ETB plus ITC, ETB plus FLC, ETB plus VRZ, INH plus ITC, INH plus FLC, INH plus VRZ, PZA plus ITC, PZA plus FLC, and PZA plus VRZ. Combinations were formed with each drug at the following concentrations: ITC, 0004875 to 0.0078 μg/ml; FLC, 0.487 to 31.25 μg/ml; VRZ, 0.0039 to 0.125 μg/ml; ETB, 98 to 1,560 μg/ml; INH, 4.6 to 75 μg/ml; and PZA, 24 to 780 μg/ml. The MIC of each drug in combination was defined as the lowest concentration that caused 80% inhibition of visible fungal growth (6). 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 >0.5 but <4.0, and antagonistic if >4.0 (11). The responses to each drug combination in filamentous and yeast-like forms were compared by Student's *t* test. Antifungal interaction with each antituberculosis drug was evaluated by analysis of variance (ANOVA) in both growth phases. A P value of <0.05 was considered significant in both tests.

All *H. capsulatum* strains were inhibited by the azoles. However, antituberculosis drugs were effective only at high concentrations, as displayed in Table 1. All antimicrobial combinations proved to be synergistic against at least a remarkable number of *H. capsulatum* strains (Table 2). According to Student's *t* test, which was employed to compare the responses to each drug combination, it was detected that the following drug combinations presented the lowest FICI values in the mold form: INH plus ITR ($P = 0.0002$), INH plus FLC ($P = 0.0017$), INH plus VRZ ($P = 0.0002$), and PZA plus VRZ ($P = 0.0462$). Regarding the yeast-like form, the ETB plus VRZ combination showed the lowest FICI value ($P = 0.0013$). Analysis of variance showed that PZA plus ITR ($P = 0.0001$) and INH plus ITR ($P = 0.006$) showed the highest FICI values (means) for mold and yeast-like forms, respectively. Lower MFC values were detected when drugs were tested in combination. Regarding antituberculosis drugs, lower MFC values were seen with combinations formed by VRZ in both filamentous and yeast-like forms.

For many years, chronic cavitary histoplasmosis was misdiagnosed as tuberculosis and patients suffering from this mycosis received empirical antituberculosis therapy without success (12). Our results make us believe that although ETB, INH, and PZA have a slight effect on *H. capsulatum* growth *in vitro*, the concentration necessary for fungal killing is much higher than the peak plasma concentration of each drug. In the present study, when the antituberculosis drugs were combined with azoles, synergistic interactions were observed. As a result, lower doses of each drug were necessary for fungal inhibition. Fungal growth was inhibited by INH plus FLC and by PZA plus azoles at concentrations near peak plasma levels of these antituberculosis drugs (2).

Previous studies have shown that combinations formed by ETB and triazoles and by PZA and ITC were the best against *C. posadasii* (6). The results here show that combinations formed by ETB with triazoles have a better inhibitory effect against *H. capsulatum*. This preliminary study reveals worthwhile results and suggests that these antituberculosis drugs may act on molecular targets expressed mainly by dimorphic fungi. It is well known that antituberculosis drugs act on specific targets in mycobacterial metabolism. Isoniazid, for instance, acts by inhibition of both the proteins enoyl-acyl reductase and β-ketoacyl acyl carrier protein (ACP)-synthase I, resulting in inhibition of mycolic acid biosynthesis (15). Therefore, we suppose that isoniazid may also act on enzymes of *H. capsulatum* fatty acid synthase complex, such as 3-oxoacyl synthase, which, in turn, may hamper the biosynthesis of lipids from plasmatic membrane. For the other antituberculosis drugs evaluated in this study,

TABLE 2. MFC, MIC, FICI, and interaction effects for combinations of antituberculosis drugs and antifungals against *H. capsulatum* strains in filamentous and yeast-like forms[a]

Combination	MFC range (μg/ml)				MIC range (μg/ml)				MIC geometric mean (μg/ml)				FICI range		No. of strains showing synergism/total no.	
	Antituberculosis		Antifungal		Antituberculosis		Antifungal		Antituberculosis		Antifungal					
	F	Y	F	Y	F	Y	F	Y	F	Y	F	Y	F	Y	F	Y
ETB + ITC	1,560	>1,170	0.0078	>0.009	98–780	195–390	0.0005–0.004	0.001–0.003	287	310	0.0014	0.003	0.062–0.374	0.25–0.50	18/18	9/9
ETB + FLC	390–1,560	390	7.8–31.25	3.9	390–780	49–390	7.8–15.6	0.975–7.8	437	84	8.42	1.67	0.186–0.624	0.15–0.49	17/18	9/9
ETB + VRZ	>4,680	>147	>0.375	>0.0234	196–1,560	49	0.0019–0.125	0.0078	636	49	0.055	0.008	0.031–0.75	0.28–0.31	10/18	5/5
INH + ITC	75	47	0.008	0.0078	9–37	18–47	0.0001–0.004	0.001–0.008	18	27	0.0019	0.004	0.124–0.375	0.25–0.50	18/18	8/8
INH + FLC	9.3–19	47	3.9–7.8	1.95	5–19	23–47	1.95–7.8	0.975–1.95	12	30	4.91	1.26	0.124–0.374	0.28–0.56	18/18	6/8
INH + VRZ	37.5–75	93	0.038–0.063	0.031	9–38	23	0.015–0.063	0.008	14.8	23	0.025	0.0078	0.124–0.5	0.40	18/18	5/5
PZA + ITC	390–780	>585	0.004–0.008	>0.003	196–780	24–195	0.0009–0.008	0.0002–0.001	276.5	97	0.0026	0.001	0.188–0.75	0.03–0.54	14/18	6/7
PZA + FLC	390–780	>585	15.6–31.2	>11.7	98–390	24–195	3.9–7.8	0.49–3.9	174.5	58	6.434	1.16	0.125–0.5	0.15–0.38	18/18	8/8
PZA + VRZ	98–390	390	0.015–0.062	0.0625	49–390	24–49	0.0078–0.006	0.004–0.008	161.6	39	0.026	0.006	0.094–0.376	0.12–0.5	18/18	6/6

[a] Abbreviations: F, filamentous; Y, yeast-like; ETB, ethambutol; INH, isoniazid; PZA, pyrazinamide; ITC, itraconazole; FLC, fluconazole; VRZ, voriconazole.

we believe that binding sites analogous to those in mycobacteria may be found in the fungal mitochondria, resulting in inhibitory effect. Further investigations are required to evaluate these hypotheses, as they could lead to discovery of promising antifungal targets.

Because of the pharmacological properties of pyrazinamide, we supposed that this drug may cause stronger fungal inhibition *in vitro* at pH 5.0. We had even run a test with RPMI buffered at pH 5.0, but unfortunately, the *H. capsulatum* strains showed poor growth at this pH, making antifungal testing not feasible. Although some experts have performed susceptibility tests with *H. capsulatum* strains at 37°C (13) or 25°C (10), we observed that *H. capsulatum* strains tested in this study showed optimum growth rate at 35°C. When cultured in high-nutrient media at 37°C, some cells set up a budding process and formed yeast-like structures. Therefore, susceptibility tests were performed at 35°C.

The results in this study add further evidence to the antifungal potential of associations formed by antituberculosis drugs and azoles. Further studies should be performed in order to determine the molecular mechanisms related to the antifungal activity observed, as well the therapeutic and/or prophylactic potential of these combinations *in vivo* against *H. capsulatum*.

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