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Short communication

In vitro synergistic effects of antituberculous drugs plus antifungals against *Coccidioides posadasii*

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ABSTRACT

The aim of the present study was to evaluate the in vitro interactions of antituberculous drugs (ATDs) with antifungals against *Coccidioides posadasii*. Eighteen drug combinations, formed by an ATD (isoniazid, pyrazinamide or ethambutol) plus an antifungal (amphotericin B, ketoconazole, itraconazole, fluconazole, voriconazole or caspofungin), were tested using the checkerboard method. All the antimicrobial combinations inhibited *C. posadasii* strains and synergistic interactions were observed for 10 combinations. Antagonism between the tested drugs was not observed. Stronger synergistic interactions were seen in the combinations formed by triazoles plus ethambutol as well as itraconazole plus pyrazinamide. Further studies in animal models are needed to confirm the usefulness of these combinations.
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1. Introduction

Coccidioidomycosis is a deep-seated infection caused by the ascomycete soil-dwelling fungi *Coccidioides immitis* and *Coccidioides posadasii* [1]. The disease occurs in the Americas, with well-registered areas of endemicity in southwestern USA, Mexico, Venezuela, Argentina and Brazil [2,3]. Following inhalation of arthroconidia-laden dust, ca. 35–40% of symptomatic patients present pulmonary disease, which may range from 'flu-like' to progressive pneumonia [2]. Disseminated disease may arise in ca. 1–5% of patients with primary coccidioidomycosis, even without any clinical or radiographic evidence of previous pulmonary infection [2].

Coccidioidomycosis treatment depends on the severity of infection, the presence of disseminated disease and individual risk factors [4]. Current therapeutic options rely on amphotericin B, ketoconazole, fluconazole and itraconazole [4]. New antifungal drugs, such as voriconazole [5], posaconazole [6] and caspofungin [7], have been tested mainly on disseminated disease. Although present antifungal therapies are effective against coccidioidomycosis, refractory infections and relapses in patients with disseminated disease have been described [6]. This scenario has led to further research for new antifungal agents against *Coccidioides* spp.

We previously demonstrated that antituberculous drugs (ATDs) have a mild inhibitory effect in vitro against *C. posadasii* strains, probably by acting on fungal mitochondria [8]. Based on this, the purpose of this study was to investigate whether the inhibitory effect of these drugs against *C. posadasii* may be enhanced by association with antifungals.

2. Materials and methods

2.1. Fungal cultures

This study included 15 isolates of *C. posadasii*, comprising 12 clinical strains isolated from sputum or bronchoalveolar lavage and 3 strains from soil. The cultures belong to the Specialized Medical Mycology Center's fungal collection (CEMM, Federal University of Ceará, Fortaleza, Brazil). Routine identification procedures for each strain included mycological analysis and polymerase chain reaction (PCR) [8]. Strain manipulations were performed within a class II biological safety cabinet in a biosafety level 3 laboratory.

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2.2. Antimicrobial drugs

Stock solutions of amphotericin (AMB) (Sigma Chemical Co., St Louis, MO), ketoconazole (KTC), itraconazole (ITC) (Janssen Pharmaceutica, Beerse, Belgium) and voriconazole (VRZ) (Pfizer Pharmaceuticals, New York, NY) were prepared in dimethyl sulphoxide (DMSO); fluconazole (FLC) and caspofungin (CAS) (Merck Sharp & Dohme, Sao Paulo, Brazil) were prepared in distilled water. Stock solutions of isoniazid (INH), pyrazinamide (PZA) and ethambutol (ETB) (Ministério da Saúde, Brazil) were prepared in DMSO. All solutions were stored at −80 °C until use. Serial two-fold dilutions of each antimicrobial agent were prepared with RPMI 1640 medium (Sigma Chemical Co.) with L-glutamine and without sodium bicarbonate and were buffered to pH 7.0 with 0.165 M MOPS (Sigma Chemical Co.) [8].

2.3. Inoculum preparation for antifungal susceptibility testing

Prior to antimicrobial testing, *C. posadasii* strains were taken from storage in 0.9% saline at 4 °C, subcultured onto Sabouraud glucose agar (Difco, Detroit, MI) and incubated for 10 days at 25 °C for viability and purity evaluations. For inoculum preparation, sterile saline was added to each agar slant and the cultures were gently scraped with cotton swabs. The suspension was transferred to a sterile tube, allowed to settle for 5 min and then the upper homogeneous supernatant was read at 530 nm and adjusted to 95% transmittance. The suspensions containing arthroconidia and hyphae fragments were diluted 1:10 with RPMI 1640 medium to obtain an inoculum of $1–5 \times 10^3$ colony-forming units/mL [8].

2.4. In vitro susceptibility testing

Antifungal assays were performed according to Clinical and Laboratory Standards Institute guidelines [9]. First, the strains were tested against each drug alone to determine the minimum inhibitory concentration (MIC). The ranges of drug concentrations tested were as follows: AMB, 0.007–4.0 μg/mL; KTC, 0.009–5.0 μg/mL; ITC, 0.015–8.0 μg/mL; FLC, 0.048–25.0 μg/mL; VRZ, 0.031–4.0 μg/mL; CAS, 0.25–128.0 μg/mL; INH, 0.015–4.0 mg/mL; PZA, 1.562–25.0 mg/mL; and ETB, 0.039–10.0 mg/mL. For the macrodilution assay, sterile, plastic, screw-cap tubes containing 0.1 mL of the antimicrobial drug combination were inoculated with 0.9 mL of suspension of each fungal isolate. The procedures were repeated at least twice and each fungal strain was tested in duplicate. The results were read visually and MIC endpoints were determined after intervals of 2 days of incubation at 35 °C. MICs of CAS and azoles were defined as the lowest drug concentration that caused 80% inhibition of visible fungal growth. For AMB, the MIC corresponded to the lowest concentration of drug at which there was no fungal growth [9]. Regarding ATDs, the MIC was defined as the lowest drug concentration that caused 80% inhibition of visible fungal growth [8].

2.5. Checkerboard macrodilution assay

After MIC definition for each drug alone, the strains were tested against 18 drug combinations formed by an ATD plus an antifungal, according to the method described above. For double combinations, the MIC of each drug alone was considered as the higher concentration, except for PZA, which did not inhibit *C. posadasii* strains at 25.0 mg/mL. Combinations were formed by each drug at the following concentrations: AMB, 0.007–0.116 μg/mL; KTC, 0.013–0.22 μg/mL; ITC, 0.009–0.153 μg/mL; FLC, 0.277–4.44 μg/mL; VRZ, 0.007–0.125 μg/mL; CAS, 2.0–32.0 μg/mL; INH, 0.015–0.25 mg/mL; PZA, 0.048–12.5 mg/mL; and ETB, 0.038–0.62 mg/mL. The MIC of each drug in combination was defined as the lowest concentration that caused 80% inhibition of visible fungal growth. Drug interactions were classified as synergistic, indifferent or antagonistic according to the fractional inhibitory concentration index (FICI) [10], which is defined as the sum of the fractional inhibitory concentration of each drug, which in turn is the MIC of each drug when used in combination divided by the MIC of the same drug when used alone. The interaction was defined as synergistic if the FICI was ≤0.5, indifferent at FICI >0.5–4.0 and antagonistic at an FICI >4.0 [10].

2.6. Statistical analysis

The study was conducted utilising descriptive variable analysis. The Mann–Whitney *U*-test was used for analysis of synergistic

Table 1
Minimum inhibitory concentrations (MICs), fractional inhibitory concentration index (FICI) range and interaction effects for combinations of antifungals (μg/mL) and antituberculous drugs (mg/mL) against 15 *Coccidioides posadasii* strains.

Combination	MIC range (drugs in combination)		Geometric mean MIC (drugs in combination)		FICI range	Result	No. (%) of strains showing synergism
	Antifungal	ATD	Antifungal	ATD			
AMB + INH	0.03	0.06	0.03	0.06	0.48–0.49	S	15 (100)
AMB + PZA	0.03–0.06	6.25–12.50	0.03	6.54	0.48–0.90	I	14 (93.3)
AMB + ETB	0.01–0.03	0.08–0.16	0.03	0.15	0.24–0.50	S	15 (100)
KTC + INH	0.02–0.22	0.03–0.25	0.06	0.07	0.30–1.65	I	3 (20.0)
KTC + PZA	0.03–0.06	3.12–6.25	0.05	5.97	0.25–0.57	I	1 (6.7)
KTC + ETB	0.01–0.06	0.04–0.16	0.03	0.08	0.24–0.80	S	13 (86.7)
ITC + INH	0.02–0.04	0.03–0.06	0.03	0.05	0.13–0.55	S	10 (66.7)
ITC + PZA	0.01–0.04	1.56–6.25	0.02	2.98	0.01–0.49	S	15 (100)
ITC + ETB	0.01–0.04	0.04–0.16	0.01	0.06	0.08–0.55	S	14 (93.3)
FLC + INH	1.11–>4.44	0.06–> 0.25	3.04	0.17	0.42–2.42	I	1 (6.7)
FLC + PZA	0.28–1.11	1.56–6.25	0.77	4.32	0.11–0.65	S	13 (86.7)
FLC + ETB	0.28–0.55	0.04–0.08	0.40	0.06	0.10–0.30	S	15 (100)
VRZ + INH	0.03–0.12	0.06–0.25	0.07	0.14	0.49–2.00	I	2 (13.3)
VRZ + PZA	0.03	6.25	0.03	6.25	0.49	S	15 (100)
VRZ + ETB	0.01–0.02	0.04–0.08	0.01	0.05	0.12–0.24	S	15 (100)
CAS + INH	8.00–16.00	0.06–0.12	12.12	0.09	0.60–1.20	I	0 (0)
CAS + PZA	8.00–16.00	6.25–12.25	12.12	9.47	1.00–1.50	I	0 (0)
CAS + ETB	8.00–32.00	0.15–0.32	16.00	0.31	0.59–2.00	I	0 (0)

ATD: antituberculous drug; S: synergism; I: indifference; AMB: amphotericin B; INH: isoniazid; PZA: pyrazinamide; ETB: ethambutol; KTC: ketoconazole; ITC: itraconazole; FLC: fluconazole; VRZ: voriconazole; CAS: caspofungin.

combinations. Results were expressed as the mean and a *P*-value of <0.05 was considered significant.

3. Results

All the strains were susceptible to the antifungals tested alone. The 48-h geometric mean MIC for each antifungal was as follows: AMB, 0.12 μg/mL; KTC, 0.14 μg/mL; ITC, 0.15 μg/mL; FLC, 4.43 μg/mL; VRZ, 0.12 μg/mL; and CAS, 22.62 μg/mL. Regarding the ATDs, only PZA was unable to inhibit *C. posadasii* strains in vitro. The 48-h geometric mean MIC for the ATDs was 0.25 mg/mL for INH and 0.62 mg/mL for ETB.

All the antimicrobial combinations tested inhibited *C. posadasii* strains. However, synergistic interactions were observed in 10 drug combinations (Table 1). Among the synergistic combinations, stronger interactions were seen with triazoles (ITC, FLC or VRZ) plus ETB ($P < 0.0002$), which presented the lowest FICI values. MIC values of each drug in these combinations were reduced more than 10 times compared with that of each drug alone. Indifferent combinations were formed by: AMB plus PZA; KTC plus INH or PZA; FLC or VRZ plus INH; and CAS plus INH, ETB or PZA. Antagonism between the tested drugs was not observed.

4. Discussion

Coccidioidal infections may result in a chronic relapsing disease that presents a challenge to the available therapy [6,7]. Thus, searches for new therapeutic choices for coccidioidomycosis infection have been conducted by combining antifungals [11], testing broad-spectrum antimicrobials [12] or using ATDs [8].

In this study, synergistic interactions were found between: AMB plus INH; KET plus ETB; and ITC, FLC or VRZ plus ETB or PZA. The synergistic potential of antifungals with INH, ETB and PZA has not been investigated previously. As a consequence, pharmacokinetic interactions of these drug combinations are unknown. INH is a bactericidal prodrug that acts by inhibiting cell wall mycolic acid synthesis in *Mycobacterium tuberculosis*. Following drug activation by mycobacterial catalase–peroxidase, several reactive oxygen species and reactive organic radicals are generated that impair DNA, lipid, carbohydrate and NAD metabolism [13]. We suppose that this unspecific damage may also occur in the fungal cell. The synergic associations between AMB or ITC plus INH need further investigation to understand their mechanisms of action.

It is noteworthy that although PZA alone has no effect against *C. posadasii*, as previously demonstrated [8], synergistic interactions between PZA with triazoles were detected in this study. We believe that low doses of these antifungals altered the fungi membrane integrity, allowing PZA to enter the cell and reach its molecular targets. As PZA alters membrane energy metabolism in *M. tuberculosis*, we suppose that the inhibitory effect detected on *C. posadasii* cells may be a result of mitochondrial damage [8].

ETB inhibits the biosynthesis of cell wall arabinogalactans in mycobacteria [13]. Analogous binding sites to this drug may be found in the fungal mitochondria [8]. Another expected mechanism of action of ETB may be related to the inhibition of galactose-derived molecules such as galactomannans of the *C. posadasii* cell wall. Surprisingly, in this study ETB showed a strong synergistic interaction with all triazoles tested. The results presented here open a wide field of research regarding the antifungal potential of EMB against many other pathogenic fungi.

Among the ATDs, it is well known that rifampicin has the potential to form synergistic combinations with antifungals in vitro [14]. However, in vivo studies have shown that rifampicin can reduce plasma levels of KTC, FLC, ITC, VRZ, posaconazole and CAS [15]; for this reason, rifampicin was not tested in the present study.

In summary, the results of this study demonstrated that among the synergistic drug combinations, those formed by triazoles plus ETB and by ITC plus PZA presented better antifungal potential against *C. posadasii* strains. Further studies in animal models are needed to confirm the usefulness of these drug combinations.

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Ethical approval: Not required.

References

[1] Fisher MC, Koenig GL, White TJ, Taylor JW. Molecular and phenotypic description of *Coccidioides posadasii* sp. nov., previously recognized as the non-California population of *Coccidioides immitis*. Mycologia 2002;94:73–84.
[2] Cox RA, Magee DM. Coccidioidomycosis: host response and vaccine development. Clin Microbiol Rev 2004;17:804–39.
[3] Cordeiro RA, Brilhante RSN, Rocha MFG, Bandeira SP, Fechine MA, Camargo ZP, et al. Twelve years of coccidioidomycosis in Ceará State. Northeast Brazil: epidemiologic and diagnostic aspects. Diagn Microbiol Infect Dis 2008, doi:10.1016/j.diagmicrobio.2008.09.016.
[4] DiCaudo DJ. Coccidioidomycosis: a review and update. J Am Acad Dermatol 2006;55:929–42; quiz 943–5.
[5] Cortez KJ, Walsh TJ, Bennett JE. Successful treatment of coccidioidal meningitis with voriconazole. Clin Infect Dis 2003;36:1619–22.
[6] Stevens DA, Rendon A, Gaona-Flores V, Catanzaro A, Anstead GM, Pedicone L, et al. Posaconazole therapy for chronic refractory coccidioidomycosis. Chest 2007;132:952–8.
[7] Antony S. Use of the echinocandins (caspofungin) in the treatment of disseminated coccidioidomycosis in a renal transplant recipient. Clin Infect Dis 2004;239:879–80.
[8] de Aquiar Cordeiro R, Brilhante RS, Rocha MF, Fechine MA, Camargo ZP, Sidrim JJ. In vitro inhibitory effect of antituberculosis drugs on clinical and environmental strains of *Coccidioides posadasii*. J Antimicrob Chemother 2006;58:575–9.
[9] National Committee for Clinical Laboratory Standards. *Reference method for broth dilution antifungal susceptibility testing of filamentous fungi*. Approved standard M38-A. Wayne, PA: NCCLS; 2002.
[10] Odds FC. Synergy, antagonism, and what the chequerboard puts between them. J Antimicrob Chemother 2003;52:1.
[11] Antony SJ, Jurczyk P, Brumble L. Successful use of combination antifungal therapy in the treatment of coccidioides meningitis. J Natl Med Assoc 2006;98:940–2.
[12] Shubitz LF, Galgiani JN, Tian ZQ, Zhong Z, Timmermans P, Katz L. Efficacy of ambruticin analogs in a murine model of coccidioidomycosis. Antimicrob Agents Chemother 2006;50:3467–9.
[13] Zhang Y. The magic bullets and tuberculosis drug targets. Annu Rev Pharmacol Toxicol 2005;45:529–64.
[14] El-Azizi M. Enhancement of the in vitro activity of amphotericin B against the biofilms of non-*albicans Candida* spp. by rifampicin and doxycycline. J Med Microbiol 2007;56:645–9.
[15] Baciewicz AM, Chrisman CR, Finch CK, Self TH. Update on rifampin and rifabutin drug interactions. Am J Med Sci 2008;335:126–36.