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Short communication

Ciprofloxacin shows synergism with classical antifungals against *Histoplasma capsulatum var. capsulatum* and *Coccidioides posadasii*

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Summary

This study evaluated the *in vitro* interaction between ciprofloxacin (CIP) and classical antifungals against *Histoplasma capsulatum* var. *capsulatum* in mycelial (n = 16) and yeast-like forms (n = 9) and *Coccidioides posadasii* in mycelial form (n = 16). This research was conducted through broth microdilution and macrodilution, according to Clinical Laboratory Standards Institute. Inocula were prepared to obtain from 0.5×10^3 to 2.5×10^4 cfu ml^{-1} for *H. capsulatum* and from 10^3 to 5×10^3 cfu ml^{-1} for *C. posadasii*. Initially, minimum inhibitory concentration (MIC) for each drug alone was determined. Then, these MICs were used as the highest concentration for each drug during combination assays. The procedures were performed in duplicate. For all combination assays, MICs were defined as the lowest concentration capable of inhibiting 80% of visible fungal growth, when compared to the drug-free control. Drug interaction was evaluated by paired sample *t*-Student test. The obtained data showed a significant MIC reduction for most tested combinations of CIP with antifungals, except for that of CIP and voriconazole against yeast-like *H. capsulatum*. This study brings potential alternatives for the treatment of histoplasmosis and coccidioidomycosis, raising the possibility of using CIP as an adjuvant antifungal therapy, providing perspectives to delineate *in vivo* studies.

Key words: Dimorphic fungi, ciprofloxacin, antifungal drugs, minimum inhibitory concentration reduction.

Introduction

Classical histoplasmosis and coccidioidomycosis are deep mycoses endemic to the American continent, especially in the United States, Mexico and Brazil, which are caused by the dimorphic fungi *Histoplasma capsulatum* var. *capsulatum* and *Coccidioides* spp. respectively. In Brazil, coccidioidomycosis is found only in the Northeast region, caused by the species *C. posadasii*, while histoplasmosis is diagnosed all over the country, with a mortality rate of 40%, when associated with AIDS.[1,2]

The treatment of these diseases consists of azoles in cases of mild to moderate symptoms and amphotericin B (AMB) in severe cases.[2,3] Although common antifungal therapies are efficient to treat these mycoses, refractory cases and relapses have been described in patients with disseminated disease.[2,4–6] Therefore, the pursuit of new therapeutic strategies against these pathogens has become more relevant.

Ciprofloxacin (CIP) is an antimicrobial drug of the fluoroquinolone group that inhibits the activity of the enzymes DNA gyrase and topoisomerase IV, which are

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essential for the replication and transcription of bacterial DNA. Studies found that quinolones may also act on topoisomerase II of fungi, potentially showing an antifungal effect.[7,8] Based on this context, this research evaluated the *in vitro* interaction between CIP and AMB, itraconazole (ITC), voriconazole (VRC) or caspofungin (CAS) against *H. capsulatum* and *C. posadasii*.

Materials and methods

For such, *H. capsulatum* isolates in mycelial (n = 16) and yeast-like forms (n = 9), and *C. posadasii* in mycelial form (n = 16) from the culture collection of the Specialized Medical Mycology Center of the Federal University of Ceará, were included in this study. The strains were handled within a class II biosafety cabinet, in a biosafety level three laboratory.

Stock solutions of AMB (Sigma Chemical Corporation, St. Louis, MO, USA), ITC (Janssen Pharmaceutica, Beerse, Belgium) and VRC (Pfizer Pharmaceuticals, New York, NY, USA) were diluted with dimethyl sulfoxide (DMSO) and CAS (Merck Sharp & Dohme, São Paulo, Brazil) was diluted with distilled water. CIP (Fresenius Kabi, São Paulo, Brazil) was used as water solution at 2000 μg ml^{-1}. All solutions were stored at −20 °C, until the moment of use. Serial dilutions of each antimicrobial agent were prepared with RPMI 1640 (Sigma Chemical Corporation), supplemented with L-glutamine, buffered at a pH of 7.0 with MOPS 165 mmol l^{-1} (Sigma Chemical Corporation).

Inocula of *H. capsulatum* in the mycelial form and *C. posadasii* were prepared after growing the strains in the mycelial phase for 7 days at 28 °C. Sterile saline was added to each culture tube, the mycelial surface was scraped with a swab and the content was transferred to a sterile tube, where the turbidity was adjusted through spectrophotometry, at 530 nm, to 95% of transmittance. As for the isolates of *H. capsulatum* in the yeast-like form, they were obtained through growth on Brain and Heart Infusion agar supplemented with sheep blood (10%), at 35 °C, and maintained through weekly passages. To prepare the fungal inoculum, a fragment of the colony of *H. capsulatum* in the yeast-like form was diluted in sterile saline and the turbidity was adjusted through spectrophotometry, as previously described. Afterwards, the fungal suspensions were diluted to obtain inocula ranging from 0.5×10^3 to 2.5×10^4 cfu ml^{-1} for *H. capsulatum* in both forms and from 10^3 to 5×10^3 cfu ml^{-1} for *C. posadasii* in the mycelial form.[3,9]

Susceptibility tests were carried out as described by Brilhante *et al.* [9] for *H. capsulatum* and Cordeiro *et al.* [3] for *C. posadasii*. Initially, minimum inhibitory concentration (MIC) for each drug alone was determined. MICs were defined as the lowest concentration that caused 100% of fungal growth inhibition for amphotericin B and 80% of inhibition for the other drugs. Then, these MICs were used as the highest concentration for each drug during combination assays. The concentration range for each drug when combined was CIP (0.488–250 μg ml^{-1}), AMB (0.000015–0.5 μg ml^{-1}), ITC (0.0000075–0.0312 μg ml^{-1}), VRC (0.00012–0.5 μg ml^{-1}) and CAS (0.00048–8 μg ml^{-1}) against *H. capsulatum* in mycelial form. Against *H. capsulatum* in yeast-like form, drug concentrations were CIP (0.122–250 μg ml^{-1}), AMB (0.00003–0.5 μg ml^{-1}), ITC (0.0000075–0.0312 μg ml^{-1}), VRC (0.0000038–0.0312 μg ml^{-1}) and CAS (0.00097–2 μg ml^{-1}). As for *C. posadasii* in mycelial form, the concentration ranges were CIP (3.125–50 μg ml^{-1}), AMB (0.0039–0.125 μg ml^{-1}), ITC (0.0078–0.5 μg ml^{-1}), VRC (0.0078–0.25 μg ml^{-1}) and CAS (1–32 μg ml^{-1}). The procedures were performed in duplicate. The results were read visually after 2 days of incubation at 35 °C for *C. posadasii* in mycelial form, 4 days for *H. capsulatum* in yeast-like form and 7 days for *H. capsulatum* in mycelial form.

The interaction between low concentrations of CIP (LowCIP) and AMB within the previously described concentration range was also evaluated,[10] using the following intervals of concentration for CIP: 0.0195–10 μg ml^{-1} against *H. capsulatum* and 0.625–10 μg ml^{-1} against *C. posadasii*, both in mycelial form.

For all combination assays, MICs were defined as the lowest concentration capable of inhibiting 80% of visible fungal growth, when compared to the drug-free control.[3,9] The effect of CIP on antifungal MICs was evaluated by paired sample t-Student test. The results were expressed as mean values and P-values lower than 0.05 were considered significant. In addition, drug interaction was evaluated by calculating the fractional inhibitory concentration index (FICI), which was classified as synergistic (FICI ≤ 0.5), indifferent (0.5 < FICI < 4) or antagonistic (FICI ≥ 4) for each combination tested against *H. capsulatum* and *C. posadasii*.[11] Afterwards, the obtained FICI values were compared through Wilcoxon test ($P < 0.05$). Four American Type Culture Collection (ATCC) type strains (*Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922) were included as drug control.

Results

Ciprofloxacin alone inhibited 8/9 strains of *H. capsulatum* in the yeast-like form (62.5 < MIC < 250 μg ml^{-1}),

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but it was ineffective against *H. capsulatum* and *C. posadasii* in the mycelial form. Thus, the highest CIP concentration tested against these fungi was also used as the highest concentration for the combination assays.

Statistical analyses revealed significant antifungal MIC reductions for all combinations of CIP with antifungal drugs against the strains of *C. posadasii* in the mycelial phase (CIP/AMB: $P = 0.0011$; CIP/ITC: $P = 0.0001$; CIP/VRC: $P = 0.0000$; CIP/CAS: $P = 0.0000$) and *H. capsulatum* in the mycelial phase (CIP/AMB: $P = 0.0006$; CIP/ITC: $P = 0.0000$; CIP/VRC: $P = 0.0000$; CIP/CAS: $P = 0.0001$). Significant antifungal MIC reductions were also observed against *H. capsulatum* in yeast-like phase (CIP/AMB: $P = 0.0499$; CIP/ITC: $P = 0.0016$; CIP/CAS: $P = 0.0063$), except for the combination of CIP and VRC ($P = 0.1720$) (Table 1).

For the combination of low ciprofloxacin concentrations with AMB, MICs for AMB were significantly smaller against the strains of *C. posadasii* ($P = 0.0000$), but not against those of *H. capsulatum* ($P = 0.1728$) (Table 1).

When evaluating the interaction between CIP and antifungal drugs, it was possible to verify that the main synergistic interactions were observed when combining CIP and ITC (14/16 strains; $0.02 \leq FICI \leq 0.37$), CIP and VRC (16/16 strains; $0.18 \leq FICI \leq 0.37$), or CIP and CAS (9/16 strains; $0.01 \leq FICI \leq 0.37$) against *H. capsulatum* in mycelial form, for which values of FICI were significantly smaller than those for CIP and AMB (6/16 strains; $0.18 \leq FICI \leq 0.37$) (ITC $P = 0.0073$; VRC $P = 0.0051$; CAS $P = 0.0458$). As for *C. posadasii*, synergistic interactions were observed for the combinations between CIP and ITC (13/16 strains; $0.09 \leq FICI \leq 0.37$), CIP and VRC (13/16 strains; $0.09 \leq FICI \leq 0.37$) or CIP and CAS (14/16 strains; $0.09 \leq FICI \leq 0.37$), whose FICI values were also significantly smaller than those for CIP and AMB (3/16 strains; FICI = 0.37) (ITC $P = 0.0088$; VRC $P = 0.0014$; CAS $P = 0.0073$). Even the combinations that were not synergistic exhibited significant MIC reductions, and no antagonistic interactions were observed.

Standard strains used as drug controls, showed MIC values within the range recommended by the Clinical Laboratory Standards Institute: *C. parapsilosis* ATCC 22019 (AMB: 1 μg ml^{-1}; ITC: 0.5 μg ml^{-1}; VRC: 0.0312 μg ml^{-1}; CAS: 0.5 μg ml^{-1}); *C. krusei* ATCC 6258 (AMB: 1 μg ml^{-1}; ITC: 0.5 μg ml^{-1}; VRC: 0.125 μg ml^{-1}; CAS: 0.25 μg ml^{-1}); *S. aureus* ATCC 29213 (CIP: 0.12 μg ml^{-1}) and *E. coli* ATCC 25922 (CIP: 0.004 μg ml^{-1}).

Table 1 Effects of the combination of ciprofloxacin and the antifungals amphotericin B, azoles and caspofungin on strains of *Histoplasma capsulatum* in mycelial and yeast-like forms, and *Coccidioides posadasii* in mycelia form.

		MIC – geometric mean (μg ml^{-1}) (isolated drugs)		MIC – geometric mean (μg ml^{-1}) (combined drugs)	
Strains	Drugs	Ciprofloxacin	Antifungal	Ciprofloxacin	Antifungal
Mycelial form					
Histoplasma capsulatum (16)[1]	CIP/AMB	>500	0.16	100.65	0.06
	LowCIP/AMB	>500	0.11	8.78	0.1
	CIP/ITC	>500	0.01	52.55	0.002
	CIP/VRC	>500	0.21	48.19	0.04
	CIP/CAS	>500	2.7	52.55	0.56
Coccidioides posadasii (16)	CIP/AMB	>100	0.1	29.73	0.06
	LowCIP/AMB	>100	0.1	2.19	0.02
	CIP/ITC	>100	0.15	11.97	0.03
	CIP/VRC	>100	0.14	10.97	0.03
	CIP/CAS	>100	28.1	10.51	5.9
Yeast-like form					
Histoplasma capsulatum (9)	CIP/AMB	198.42	0.11	107.15	0.06
	CIP/ITC	198.42	0.01	78.74	0.006
	CIP/VRC	198.42	0.006	135	0.004
	CIP/CAS	198.42	1.36	85.04	0.62

LowCIP, Low concentrations of ciprofloxacin; CIP, Ciprofloxacin; AMB, Amphotericin B; ITC, Itraconazole; VRC, Voriconazole; CAS, Caspofungin; MIC, Minimum inhibitory concentration, 100% of fungal growth inhibition for AMB and 80% of growth inhibition for the other drugs and for drug combinations.

[1]Number of tested strains.

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Discussion

Quinolones have been shown to improve *in vitro* effects of antifungal drugs against yeast and mould species.[8,12–14] In addition, the association of CIP and fluconazole was shown effective in treating invasive candidiasis and pulmonary mucormycosis in murine models.[15] However, the combination of CIP and classical antifungals has never been tested *in vitro* nor *in vivo* against dimorphic fungi.

Although typically not presenting antifungal activity, CIP could bind to fungal topoisomerase II,[10] possibly inhibiting DNA replication. This is only observed when CIP is associated with antifungals, probably because certain antimycotics alter fungal membrane permeability, increasing intracellular levels of this quinolone.[16] In addition, it has been suggested that CIP enhances the activity of azoles by overlapping substrate specificity of the ATP-binding cassette transporters (efflux pumps), which results in higher intracellular concentrations of these antifungal agents.[10] These facts could explain the effective interaction between CIP and azoles observed in this study. MICs of VRC alone against yeast-like form of *H. capsulatum* were already very low, suggesting that VRC molecules were not suffering the effects of efflux-pump activity, reaching the maximum intracellular concentration within the yeast cell. Hence, considering the possible effects of CIP on efflux pumps, the combination of CIP with VRC did not result in antifungal MIC reduction.

It has also been suggested that CIP may increase the susceptibility of (1.3)-β-D-glucan synthase to echinocandins,[10] which possibly explains the effective interaction between CIP and CAS against *H. capsulatum* and *C. posadasii*, even though echinocandins alone are not effective against the former.[2]

In this study, it was observed that the combination of CIP with AMB resulted in antifungal MIC reduction, corroborating the results of Sugar *et al.* [12] who showed that this drug combination provided significantly more protection to mice infected with *Candida albicans*, when compared to AMB or CIP alone. In addition, it was observed that LowCIP also significantly reduced the MIC of AMB against *C. posadasii*. This dose-dependent interaction between AMB and CIP has been demonstrated against *C. albicans* and *Aspergillus fumigatus.*[10] It has been shown that LowCIP seem to increase AMB-induced pore formation on fungal cell membranes, producing a synergistic effect, while high concentrations may produce antagonistic effects.[10]

In conclusion, the data from this study suggest new alternatives for the treatment of histoplasmosis and coccidioidomycosis, suggesting that CIP should behave as an adjuvant agent when added to the antifungal therapy, providing perspectives to delineate *in vivo* studies.

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