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In vitro activity of amphotericin B and itraconazole in combination with flucytosine, sulfadiazine and quinolones against *Exophiala spinifera*

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The combined effects of antifungal and antibiotic drugs against *Exophiala spinifera* were evaluated *in vitro* by the chequer-board method, calculated as a fractional inhibitory concentration (FIC) index. Amphotericin B was combined with flucytosine and ciprofloxacin, whereas itraconazole was combined with ciprofloxacin, levofloxacin, lomefloxacin and sulfadiazine. Synergic effects were observed for the combinations of itraconazole with ciprofloxacin and levofloxacin, and amphotericin B with ciprofloxacin and flucytosine. No antagonism was observed for any combination tested.

Keywords: *Exophiala,* black yeasts, quinolones, drug combinations

Black yeast-like fungi are increasingly being recognized as potential human pathogens. Clinical pictures include either subcutaneous or systemic mycetoma, chromoblastomycosis and phaeohyphomycosis.[1]

As model species we selected *Exophiala spinifera*. Although this is a very rare agent of disease in humans, it is one of the most aggressive species of black yeast, potentially causing disseminated infections with fatal outcome in children and adolescents. In adults, in contrast, infections are mostly localized or subcutaneous lesions.[2]

The optimal therapy for black yeast infections is controversial. Surgical excision, antifungal drug monotherapy or drug combinations have been used, but prolonged treatment is needed because relapses often occur. Consequently, new approaches to treatment are overdue.

Quinolones are potent inhibitors of DNA gyrase and are extensively used in clinical practice for bacterial infections.[3] Sulphonamides act as competitive antagonists of *p*-aminobenzoic acid (PABA), which is an integral component of the structure of folic acid. Decreased folic acid synthesis results in a decrease in nucleotides with subsequent growth inhibition.[4] Both types of drug have favourable pharmacokinetic profiles in cerebrospinal fluid and bone.[4]

In one report, a nodule on a finger was treated with surgical excision and three antifungals, but after a month another lesion appeared in the left arm with no healing of the finger lesion. In this case, treatment with co-trimoxazole was initiated on diagnosis of a *Nocardia* superinfection, and after 11 days both lesions had improved. This suggests that sulphonamides might be effective in treating this infection.[5]

Quinolones have a broad spectrum of activity as inhibitors of DNA gyrase, type 2 topoisomerase, which is present in prokaryotes and eukaryotes. The presence of high levels of topoisomerases I and II has been reported in pathogenic fungi.[6] Although they are inapplicable as sole antifungal agents, quinolones augment the activity of amphotericin B and azoles.[7,8] For example, in a murine model study of candidiasis, similar survival rates were demonstrated in mice treated with fluconazole alone 80 mg/kg/day and in those treated with fluconazole (40 mg/kg/day) and ciprofloxacin.[8]

The aim of the present study was to investigate the *in vitro* activity of quinolones and sulfadiazine, either alone or in combination with amphotericin B or itraconazole, as well as

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Table 1. Numbers, MIC data and source of the strains tested [2,10]

		MIC (mg/L)							
CBS	Other reference	AMB	ITZ	5-FC	CIP	LOM	LEV	SDZ	Source
425.92		1	0.031	4	>16	>12	>20	>160	heated apple juice
236.93		0.5	0.031	8	>16	>12	>20	>160	apple juice
194.61		1	0.063	16	>16	>12	>20	>160	disseminated
899.68	ATCC 18218	0.25	0.125	8	>16	>12	>20	>160	nasal granuloma
	dH 11328	0.5	0.031	32	>16	>12	>20	>160	skin
	dH 11326	2	0.063	4	>16	>12	>20	>160	skin
356.83		1	0.125	4	>16	>12	>20	>160	skin
	dH 12309	2	0.031	16	>16	>12	>20	>160	chromoblastomycosis
	dH 11327	1	0.125	2	>16	>12	>20	>160	head lesion
269.28		1	0.063	2	>16	>12	>20	>160	skin

ATCC, American Type Culture Collection, Manassas, VA, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; dH, G. S. de Hoog private collection; AMB, amphotericin B; ITZ, itraconazole; 5-FC, flucytosine; CIP, ciprofloxacin; LOM, lomefloxacin; LEV, levofloxacin; SDZ, sulfadiazine.

the combination of amphotericin B and flucytosine against *E. spinifera* strains.

Thus, for this study eight clinical and two environmental well-documented isolates of *E. spinifera* were used (Table 1). Drugs tested were amphotericin B (Bristol-Myers Squibb, Woerden, The Netherlands), itraconazole (Janssen-Cilag, Beerse, Belgium), flucytosine (ICN Pharma BV, Zoetermeer, The Netherlands), ciprofloxacin (Bayer AG, Leverkusen, Germany), lomefloxacin (Searle Nederland, Maarssen, The Netherlands), levofloxacin (Hoechst Pharma, Amsterdam, The Netherlands) and sulfadiazine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). All drugs were dissolved in dimethylsulphoxide (DMSO), with the exception of flucytosine and ciprofloxacin, which were dissolved in water.

Two-fold serial dilutions of the drugs were made in RPMI-1640 medium (Gibco BRL, Woerden, The Netherlands) to obtain final concentrations that ranged from: 0.015 to 8 mg/L for amphotericin B; 0.02 to 1 mg/L for itraconazole; 0.25 to 128 mg/L for flucytosine; 0.125 to 8 mg/L for ciprofloxacin; 0.09 to 6 mg/L for lomefloxacin; 0.15 to 10 mg/L for levofloxacin; and 2.5 to 160 mg/L for sulfadiazine. RPMI-1640 medium (with L-glutamine, without bicarbonate) was buffered to pH 7.0 with 0.165 M MOPS (Sigma-Aldrich, Steinheim, Germany).

Tests were performed in 96-well flat-bottom microtitration plates (Corning, Ames, MA, USA), which were kept at –70°C until the day of testing. The MIC of each drug alone was tested by a broth microdilution method, according to NCCLS guidelines (M38-P)[9] that provide for testing of polyenes, flucytosine and azole antifungal agents against filamentous fungi. Conidial suspensions were diluted in RPMI-1640 to obtain twice the desired inoculum concentration. A drug-free well containing 0.01% DMSO in the medium served as the growth control for the drugs dissolved in this solvent. Plates were incubated at 35°C for 72 h. The MICs were determined by spectrophotometry. The relative optical densities (ODs) for each well based on measurements at 405 nm were calculated (as a percentage) based on the following equation: [(OD of drug-containing well – background OD)/(OD of drug-free well – background OD)] × 100%. The MIC of each drug alone was defined as the lowest concentration of the drug that showed at least 95% reduction of growth compared with that of the growth control (MIC-0) for amphotericin B, quinolones and sulfadiazine, and for flucytosine and itraconazole as the lowest concentration of the drug that showed 50% reduction of growth compared with that of the growth control (MIC-2).

A two-dimensional, two-agent broth microdilution chequerboard method was used to study the interaction between the drugs. For all the combinations tested, MIC endpoints considered were MIC-0 when amphotericin B was combined and MIC-2 when itraconazole was combined with the corresponding drug. The FICs of both drugs used in combination were calculated and added to obtain the FIC indices. The FIC index was calculated as follows: (MIC of drug A + drug B/MIC of drug A) + (MIC of drug A + drug B/MIC of drug B). Drug interactions were defined as synergic if the FIC index was ≤0.5, antagonistic if the FIC index was >4 and non-interactive between 0.5 and 4.

The MIC of amphotericin B ranged from 0.25 to 2 mg/L; that of itraconazole from 0.031 to 0.125 mg/L; that of flucyto-

Table 2. Amphotericin B and itraconazole in combination with quinolones, flucytosine or sulfadiazine

	Amphotericin B				Itraconazole							
	ciprofloxacin		flucytosine		ciprofloxacin		levofloxacin		lomefloxacin		sulfadiazine	
Strains[2,10]	FICI	INT	FICI	INT	FICI	INT	FICI	INT	FICI	INT	FICI	INT
356.83	ND	ND	0.37	SYN	1.06	NI	1.00	NI	0.75	NI	1.00	NI
899.68	0.18	SYN	ND	ND	0.28	SYN	0.25	SYN	0.75	NI	0.31	SYN
11326	0.37	SYN	0.31	SYN	0.078	SYN	0.015	SYN	0.09	SYN	1.00	NI
194.61	1.00	NI	0.37	SYN	0.5	SYN	0.13	SYN	0.09	SYN	0.13	SYN
11327	0.25	SYN	0.75	NI	0.31	SYN	0.25	SYN	1.00	NI	0.13	SYN
425.92	1.00	NI	0.75	NI	1.00	NI	1.00	NI	0.62	NI	1.00	NI
269.28	0.25	SYN	0.37	SYN	0.09	SYN	0.02	SYN	0.09	SYN	0.13	SYN
12309	0.51	NI	0.75	NI	0.62	NI	0.13	SYN	0.75	NI	ND	ND
236.93	0.5	SYN	0.31	SYN	0.5	SYN	0.07	SYN	0.13	SYN	0.13	SYN
11328	0.75	NI	0.75	NI	0.62	NI	1.00	NI	1.5	NI	ND	ND

FICI, fractional inhibitory concentration index; SYN, synergic; NI, no interaction; INT, interpretation; ND, not determined.

sine from 2 to 32 mg/L. For sulfadiazine and quinolones no activity was found with the drug alone (Table 1). Amphotericin B combined with flucytosine showed a synergic effect for five strains and no interaction for four. For amphotericin B plus ciprofloxacin, a synergic effect was observed against five strains and no interaction for two (Table 2).

Itraconazole combined with levofloxacin, lomefloxacin and ciprofloxacin showed synergic activity against seven, four and six strains, respectively. No interaction when combined with itraconazole was observed with six strains for lomefloxacin, four strains for ciprofloxacin and three strains for levofloxacin. When itraconazole was combined with sulfadiazine, synergic activity was observed against five strains and no interaction against three strains (Table 2). No antagonistic effect was observed in any combination.

Only very few *in vitro* and animal studies have been carried out with this group of fungi. One study, on a central nervous system phaeohyphomycosis in mice infected with three different genera of black fungi. *Ochroconis constricta* showed good correlation *in vitro–in vivo* with amphotericin B, although high doses of amphotericin B were needed. For *Cladophialophora bantiana*, flucytosine was the most effective, but no single drug achieved full recovery. This drug was also the most effective against *Exophiala dermatitidis*.[11]

Quinolones and sulfadiazine were selected for this study because of their favourable distribution in the body and their ability to enhance antifungal activity when used in combination. Flucytosine was selected as a classic drug that possesses activity against black fungi. Our study indicates that for some isolates, quinolones augmented the activity either of itraconazole or amphotericin B when combined, since synergic effects were sometimes observed and antagonism not demonstrated for any combination tested. The concentrations tested can be achieved *in vivo*. Enhanced activity of antifungal agents combined with these classes of antimicrobials has also been found for *Candida* species, although occasionally discrepancies were observed between *in vitro* and *in vivo* data.[8,10]

In our study, we found synergic interaction when sulfadiazine were combined with itraconazole. No effect was observed in *E. spinifera* when the drug was used alone. The blood levels that can be reached *in vivo* (30–60 mg/L)[4] exceed the MIC of the combination of sulfadiazine and itraconazole. Synergic effects between sulphonamides and azoles were also found in *Candida* in which synergy was observed between ketoconazole and co-trimoxazole.[13]

In summary, the results presented here strongly suggest that quinolones or sulphonamides enhance the antifungal activity of drugs currently used for some isolates of *E. spinifera*. This provides potential alternative therapeutic options in infections from dematiaceous fungi. More investigations are needed to confirm these observations.

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