

## *In Vitro* Evaluation of the Type of Interaction Obtained by the Combination of Terbinafine and Itraconazole, Voriconazole, or Amphotericin B against Dematiaceous Molds<sup>∇</sup>

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***In vitro* associations using the checkerboard microdilution method indicated lower MIC ranges and MIC median values for each drug (terbinafine, itraconazole, voriconazole, and amphotericin B) in association than those obtained for each single drug. Fractional inhibitory concentration index (FIC) results showed 100% synergism in the association of terbinafine with voriconazole, 96.5% in the association of terbinafine with amphotericin B, and 75.9% in the association of terbinafine with itraconazole. Drug combinations may be useful for treatment of dematiaceous mold infections as an alternative treatment to enhance the effectiveness of each drug.**

Dematiaceous fungi have been increasingly recognized as important pathogens, especially in immunocompromised patients (13), although there is little experience in the treatment of these infections. The selection of antifungal drugs, duration of therapy, and therapeutic doses are not yet well established (11), always depending on the disease characteristics. Most reports consider azoles as the drugs of choice for treatment (1, 6, 8, 16, 21), although terbinafine has been considered by some authors (1, 13, 16, 22).

There are reports of good results with the association of terbinafine with voriconazole and itraconazole in patient treatment (10, 23). There are some *in vitro* studies, most using the checkerboard method, that confirm these findings, with lower MICs against a large variety of fungi, such as *Aspergillus* spp., *Candida* spp., *Mucorales* spp., *Pythium insidiosum*, *Scedosporium prolificans*, *Paecilomyces* spp., dermatophytes, and zygomycetes (2, 3, 7, 14, 15, 22).

There are few data on the *in vitro* drug susceptibility of dematiaceous fungi (1, 5, 6, 11, 12, 13, 20) and fewer data on the use of *in vitro* combinations of antifungal agents for these fungi (22).

The aim of this study was to investigate the *in vitro* interaction obtained by the combination of terbinafine with itraconazole, voriconazole, or amphotericin B against dematiaceous molds.

Isolates of 29 dematiaceous molds were studied: *Fonsecaea pedrosoi* (8 isolates), *Curvularia clavata* (1 isolate), *Curvularia senegalensis* (1 isolate), *Curvularia geniculata* (1 isolate), *Curvularia lunata* (4 isolates), *Exophiala jeanselmei* (6 isolates), *Alternaria alternata* (5 isolates), *Cladophialophora bantiana* (1 isolate), and a *Bipolaris* sp. (2 isolates). All of them were

clinical isolates obtained from cases of phaeohyphomycosis and chromoblastomycosis and one case of meningitis that were identified according to routine classical methods (macromorphology, micromorphology, and some biochemical proofs).

The antifungal agents used were terbinafine (Novartis-Pharma, Basel, Switzerland), itraconazole and amphotericin B (Sigma, St. Louis, MO), and voriconazole (Vfend; Pfizer, NC). Terbinafine was dissolved in dimethyl sulfoxide (DMSO) and diluted in sterile distilled water. The other antifungal agents were dissolved and diluted in sterile distilled water. Antifungal dilutions ranged from 128.0 to 0.25  $\mu\text{g/ml}$  for itraconazole and amphotericin B, 32.77  $\mu\text{g/ml}$  to 0.064  $\mu\text{g/ml}$  for terbinafine, and 2.048 to 0.002  $\mu\text{g/ml}$  for voriconazole.

Individual MICs were determined following the microdilution method recommended by CLSI M38 A2 [2a]. The MIC was defined as the lowest drug concentration that caused 100% inhibition of visible fungal growth. Tests were performed in duplicate and repeated if the difference between duplicates was higher than two dilutions. *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6852, and *Candida albicans* ATCC 76615 and ATCC 90028 strains were used as quality control organisms.

Drug interactions were evaluated with the “checkerboard” microdilution design (2, 7, 18), which provided a matrix of all drug combinations in the required concentration assayed. Dilutions ranged from 8.2 to 0.004  $\mu\text{g/ml}$  for terbinafine, 8.0 to 0.625  $\mu\text{g/ml}$  for itraconazole, 32.0 to 0.25  $\mu\text{g/ml}$  for amphotericin B, and 2.048 to 0.008  $\mu\text{g/ml}$  for voriconazole. The interaction coefficient among drugs was quantitatively evaluated by means of the fractional inhibitory concentration index (FIC), which was calculated as follows:  $\text{FIC} = (\text{MIC A in combination}/\text{MIC A}) + (\text{MIC B in combination}/\text{MIC B})$ . Interaction was defined as synergistic if the FIC was  $\leq 0.5$ , no interaction if the FIC was  $> 0.5$  and  $\leq 4.0$ , and antagonistic if the FIC was  $> 4.0$ , as used for most recent studies (2, 7, 18).

MIC ranges, median values of isolated and combined drugs, and FIC ranges are shown in Table 1. Alone, voriconazole was the most active, with MICs ranging between 0.064 and 2.048

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TABLE 1. MICs of terbinafine alone and combined with itraconazole, amphotericin B, and voriconazole and calculated interactions for 29 clinical isolates of dematiaceous molds<sup>a</sup>

Species (no. of isolates)	MIC (10 <sup>-3</sup> mg/ml)			MIC (10 <sup>-3</sup> mg/ml)			MIC (10 <sup>-3</sup> mg/ml)			FIC				
	Single drug		Combination	Single drug		Combination	Single drug		Combination					
	TB	AMB	TB	TB	ITZ	TB	TB	VCZ	TB		VCZ			
<i>Fonsecaea pedrosoi</i> (6)	0.256-4.10	2.0-32	0.00025-0.002	0.125-0.25	0.005-0.126	0.256-4.10	0.25-0.5	0.00025-0.004	>0.0625	0.127-0.251	0.064-0.128	0.00025-0.002	0.001-0.008	0.018-0.0645
<i>Curvularia clavata</i> (1)	2.05	0.5	0.008	0.032	0.068	2.05	0.25	0.008	0.0625	0.254	0.512	0.008	0.016	0.035
<i>Curvularia senegalensis</i> (1)	4.10	2.0	0.008	0.0625	0.033	4.10	0.5	0.008	0.0625	0.127	0.256	0.008	0.016	0.0645
<i>Curvularia genticulata</i> (1)	4.10	0.25	0.002	0.0625	0.25	4.10	0.125	0.004	0.0625	0.50 <sup>b</sup>	0.512	0.004	0.004	0.009
<i>Curvularia lunata</i> (4)	0.008-2.05	2.0-8.0	0.002-0.008	0.032-0.0625	0.008-0.281	0.008-2.05	0.25-8.0	0.002-0.008	0.0625-0.25	0.002-0.5 <sup>b</sup>	0.064-0.256	0.002-0.008	0.016	0.0656-0.5
<i>Exophiala jeanselmei</i> (6)	0.512-1.02	1.0-16	0.001-0.002	0.125-1.0	0.033-0.127	1.02	0.25-8.0	0.002-1.0	0.0625	0.033-1.07 <sup>b</sup>	1.02	0.064-0.512	0.001-0.002	0.017-0.0645
<i>Alternaria alternata</i> (5)	0.016-4.10	1.0-4.0	0.002-0.004	0.0625-1.0	0.063-0.512 <sup>b</sup>	0.016-4.10	0.125-1.0	0.002-0.004	0.0625-0.25	0.063-2.0 <sup>c</sup>	0.016-4.10	0.256-1.024	0.002-0.004	0.008-0.128
<i>Bipolaris</i> sp. (2)	1.02	4.0	0.008	0.0625	0.023	1.02	2.0	0.008	0.0625	0.039	1.02	0.256	0.008	0.008-0.016
<i>Cladophialophora bantiana</i> (1)	8.20	32.0	0.008	0.0625	0.003	8.20	0.25	0.008	0.0625	0.251	8.20	2.048	0.008	0.016
MIC range for all isolates (29)	0.008-4.10	0.25-32	0.00025-0.008	0.032-1.0	0.005-0.502 <sup>d</sup>	0.016-8.20	0.05-8.0	0.00025-1.0	0.0625-0.25	0.002-1.07 <sup>e</sup>	0.016-8.2	0.064-2.048	0.00025-0.008	0.001-0.128
Median values	2.05	4.0	0.002	0.125	0.033	2.05	0.25	0.004	0.0625	0.251	2.05	0.256	0.004	0.008

<sup>a</sup> TB, terbinafine; ITZ, itraconazole; AMB, amphotericin B; VOR, voriconazole; FIC, fractional inhibitory concentration index. The FIC indicated synergism for most isolates unless otherwise noted.

<sup>b</sup> Only one isolate showed no interaction.

<sup>c</sup> Four isolates showed no interaction.

<sup>d</sup> 3.5% of isolates showed no interaction.

<sup>e</sup> 24.1% of isolates showed no interaction.

µg/ml (median, 0.256 µg/ml), followed by itraconazole (MIC, 0.05 to 8.0 µg/ml; median, 0.25 µg/ml) and terbinafine (MIC, 0.08 to 8.2 µg/ml; median, 2.05 µg/ml). Amphotericin B had the highest MIC ranges alone, between 0.25 and 32.0 µg/ml (median, 4.0 µg/ml), with 96.5% of isolates showing resistance ( $\geq 2.0$  µg/ml), confirming its low activity against dematiaceous molds (1, 4, 17, 23).

In the combination test, the efficacy of each antifungal seemed to rise, with lower MIC ranges: 0.001 to 0.128 µg/ml (median, 0.008 µg/ml) for voriconazole, 0.0625 to 0.25 µg/ml (median, 0.0625 µg/ml) for itraconazole, 0.00025 to 0.008 µg/ml (median, 0.002 µg/ml) for terbinafine, and 0.032 to 1.0 µg/ml (median, 0.125 µg/ml) for amphotericin B. Our results indicated 100% synergism between terbinafine and voriconazole, 96.5% synergism between amphotericin B and terbinafine, and 75.9% synergism between terbinafine and itraconazole. No cases of antagonism were observed. The same results were obtained for the first and second replicate, confirming the reproducibility of the methods used in this study.

Good results were expected for the interaction between terbinafine and azoles. These drugs act at different points of the pathway of ergosterol biosynthesis, with terbinafine inhibiting squalene epoxidation and the azoles inhibiting 14 $\alpha$ -demethylase action.

Our findings agree with those of previous reports that show *in vitro* synergy between azoles and terbinafine for *Zygomycota*, *Fonsecaea pedrosoi*, *Cladophialophora carrioni*, *Phialophora verrucosa*, and *Scedosporium*, *Pythium*, and *Aspergillus* species (2, 7, 14, 19, 22). Other authors also reported good interactions between terbinafine and amphotericin B against *Zygomycota* (7). However, Yu et al. reported no interaction for this combination against some dematiaceous fungi causing chromoblastomycosis (22), as did Ortoneda et al. for activity against *Paeecilomyces* spp. (15). Studies with *Aspergillus* spp. showed antagonism for all isolates (3, 9, 10).

A drastic reduction of amphotericin B MICs by the addition of terbinafine can be a sign that a combination of terbinafine with amphotericin B could be useful in the treatment of invasive infections caused by dematiaceous molds.

Revankar related good results for chromoblastomycosis with the combination of terbinafine and itraconazole (17). Zhang et al. also showed good results when treating two cases of relapse of chromoblastomycosis using this same combination (23).

We speculated that a previous or concomitant treatment with amphotericin B or terbinafine could result in an increase in cell permeability and consequently lower MICs.

Drug combination may be a useful approach for treatment of dematiaceous mold infections, as it has been demonstrated for other difficult-to-treat fungal infections like cryptococcal meningitis. It can be an alternative to enhance the effectiveness of each drug and achieve efficacy using lower dosages.

In conclusion, despite the small sample sizes for some evaluated species, the findings of the present study are very encouraging, showing only synergistic or indifferent effects and no antagonistic interactions. Further studies are warranted to elucidate the clinical potential applications of these data.

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