In vitro evaluation of the type of interaction obtained by the combination of terbinafine and itraconazole, voriconazole or amphotericin B against dematiaceous molds

Running title: In vitro antifungal combination for dematiaceous molds

Fernanda Simas Corrêa Biancalana, Luzia Lyra and Angélica Zaninelli Schreiber.

Address reprint requests to: Dr. Angélica Zaninelli Schreiber
State University of Campinas-UNICAMP
School of Medical Sciences - Department of Clinical Pathology
CEP 13083-887 Campinas, São Paulo, Brazil.
Phone: 55-19-32893273; Fax: 55-19-35219434
E-mail: zaninele@fcm.unicamp.br

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Abstract

In vitro associations using the checkerboard microdilution method indicated lower MIC ranges and MIC median values for each drug in association than that obtained for each single drug. FIC results showed 100% of synergism in the association of terbinafine with voriconazole, 96.5% with amphotericin B and 75.9% with itraconazole. Drug combinations may be useful on treatments of dematiaceous molds infections as an alternative 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], depending always on the disease characteristics. Most reports consider azoles as the drug of choice for treatment [16,21,8,1,6], although terbinafine has been considered by some authors [13,16,1,22].

There are reports of good results with the association of terbinafine with voriconazole and itraconazole to patients [10,23]. There are some in vitro studies, most using the checkerboard method, that confirms these findings with lower MIC’s against a large variety of fungi, such as *Aspergillus* sp, *Candida* sp, *Mucorales* sp, *Pythium insidiosum*, *S. prolificans*, *Paecilomyces* sp, dermatophytes and zygomycetes [22,4,7,2,15,14].

There are few data of drug susceptibility *in vitro* of dematiaceous fungi [13,11,1,6,5,12,20] and fewer data on the use of combinations *in vitro* 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 and itraconazole, voriconazol or amphotericin B against dematiaceous molds.

Twenty-nine dematiaceous molds isolates of were studied: *Fonsecaea pedrosoi* (8), *Curvularia clavata* (1), *C. senegalensis* (1), *C. geniculata* (1), *C. lunata* (4), *Exophiala jeanselmei* (6), *Alternaria alternata* (5), *Cladophialophora bantiana* (1) and *Bipolaris* sp (2). All of them were clinical isolates obtained from phaeohyphomycosis, chromoblastomycosis and one case of meningitis, that were identified according to rotine classical methods (macro, micromorphology and some biochemical proofs).
Antifungal agents: terbinafine (Novartis-Pharma, Basel, Switzerland), itraconazole and amphotericin B (Sigma St. Louis, MO, USA) and voriconazole (Vfend, Pfizer, NC, USA). Terbinafine was dissolved in DMSO (dimethyl sulfoxide) and diluted into sterile distilled water. The other antifungal agents were dissolved and diluted into sterile distilled water. Antifungal dilutions ranged from 128.0 to 0.25 \( \mu \)g/ml for itraconazole and amphotericin B, from 32.77 \( \mu \)g/ml to 0.064 \( \mu \)g/ml for terbinafine and 2.048 to 0.002 \( \mu \)g/ml for voriconazole.

Individual MICs were determined following the microdilution method recommended by CLSI M38 A2. The MIC was defined as the lowest drug concentration that caused 100% of 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, Candida albicans ATCC 76615 and ATCC 90028 strains were used as quality control organisms.

Drug interactions were evaluated with the “checkerboard” microdilution design [18,7,2] that provided a matrix of all drug combinations in the required concentration assayed. Dilutions ranged from 8.2-0.004 \( \mu \)g/ml for terbinafine, 8.0-0.625 \( \mu \)g/ml for itraconazole, 32.0-0.25 \( \mu \)g/ml for amphotericin B and 2.048-0.008 \( \mu \)g/ml for voriconazole.

The interaction coefficient among drugs was quantitatively evaluated by means of the Fractional Inhibitory Concentration index (FIC), which was calculated by: FICI = [MIC A in combination/MIC A] + [MIC B in combination/MIC B]. Interaction was defined as synergistic if the FIC index was \( \leq \) 0.5, no interaction if 0.5 > FIC \( \leq \) 4.0 and antagonistic if FIC was > 4.0 as used for most recent studies [18,7,2].
MIC ranges, median values of isolated and combined drugs and FICI ranges are shown in table 1. Alone, voriconazole was the most active with MICs, ranging between 0.064 and 2.048 µg/ml (median 0.256 µg/ml), followed by itraconazole (MIC 0.05-8.0 µg/ml/median 0.25 µg/ml) and terbinafine (MIC 0.08-8.2 µg/ml/median 2.05 µg/ml). Amphotericin B had the higher MIC ranges alone, between 0.25 and 32.0 µg/ml (median 4.0 µg/ml), with 96.5% of isolates showing resistance (≥2.0 µg/ml), confirming its low activity against dematiaceous molds [17,1,23,3].

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

Good results were expected for 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 α demetylase action.

Our findings agree with previous reports that show in vitro synergy between azoles and terbinafine for Zygomycota, Fonsecaea pedrosi, Cladophialophora carrioni, Phialophora verrucosa, Scedosporium, Pythium and Aspergillus species [22,7,2,14,19]. Other authors also reported good interactions between terbinafine and amphotercin B against Zygomycota [7]. However, Yu et al reported indifference to this combination.
against some dematiaceous fungi causing chromolastomycosis [22], as well as Guarro et al against Paecilomyces spp [15]. Studies with Aspergillus spp showed antagonism for all isolates [4,10,9].

Drastical reduction of amphotericin B MICs by the addiction 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 terbinafine and itraconazole [17]. Zhang et al. showed also good results treating two cases of relapse of chromoblastomycosis using this same combination [23].

We speculated if 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 molds 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, spite of the reduced sample size 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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Table 1 – Minimal inhibitory concentrations of terbinafine isolated and combined with itraconazole, amphotericin B and voriconazole and calculated interactions for the 29 clinical isolates of dematiaceous molds included in the study.

<table>
<thead>
<tr>
<th>Species</th>
<th>MIC single drug (x10^{-3} mg/ml)</th>
<th>Combination MIC (x10^{-3} mg/ml)</th>
<th>FICI</th>
<th>Combination MIC (x10^{-3} mg/ml)</th>
<th>FICI</th>
<th>Combination MIC (x10^{-3} mg/ml)</th>
<th>FICI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TB</td>
<td>AMB</td>
<td>TB</td>
<td>AMB</td>
<td>TB ITZ</td>
<td>TB</td>
<td>AMB</td>
</tr>
<tr>
<td>F. pedrosoi (n=6)</td>
<td>0.256-2.0</td>
<td>0.00025-0.125</td>
<td>0.005-</td>
<td>0.256-0.25-</td>
<td>0.0025-0.0625</td>
<td>0.004-</td>
<td>0.256-0.125-</td>
</tr>
<tr>
<td>C. clavata (n=1)</td>
<td>2.05-0.5</td>
<td>0.008-0.032</td>
<td>0.0068</td>
<td>2.05-0.25</td>
<td>0.008-0.0625</td>
<td>0.00625</td>
<td>0.008-0.25</td>
</tr>
<tr>
<td>C. mangiferae (n=1)</td>
<td>4.10-2.0</td>
<td>0.008-0.0625</td>
<td>0.033</td>
<td>4.10-0.5</td>
<td>0.008-0.0625</td>
<td>0.0125</td>
<td>4.10-0.5</td>
</tr>
<tr>
<td>C. guadalupensis (n=1)</td>
<td>4.10-0.25</td>
<td>0.002-0.0625</td>
<td>0.25</td>
<td>4.10-0.125</td>
<td>0.004-0.0625</td>
<td>0.506</td>
<td>4.10-0.512</td>
</tr>
<tr>
<td>C. lunata (n=1)</td>
<td>2.05-0.2</td>
<td>0.008-0.0625</td>
<td>0.251</td>
<td>2.05-0.25</td>
<td>0.008-0.0625</td>
<td>0.02-</td>
<td>0.08-0.064</td>
</tr>
<tr>
<td>L. ramorum (n=1)</td>
<td>4.10-0.125</td>
<td>0.002-0.0625</td>
<td>0.004</td>
<td>4.10-0.125</td>
<td>0.002-0.0625</td>
<td>0.002-</td>
<td>0.008-0.064</td>
</tr>
<tr>
<td>A. alternata (n=1)</td>
<td>4.10-0.4</td>
<td>0.004-1.0</td>
<td>0.032</td>
<td>4.10-0.10</td>
<td>0.004-0.25</td>
<td>0.5065</td>
<td>4.10-0.125</td>
</tr>
<tr>
<td>Bipolaris sp (n=2)</td>
<td>4.10-0.625</td>
<td>0.008-0.0625</td>
<td>0.25</td>
<td>4.10-0.512</td>
<td>0.004-0.0625</td>
<td>0.251</td>
<td>4.10-0.256</td>
</tr>
<tr>
<td>C. bantiana (n=1)</td>
<td>8.20-32.0</td>
<td>0.008-0.0625</td>
<td>0.003</td>
<td>8.20-0.25</td>
<td>0.008-0.0625</td>
<td>0.251</td>
<td>8.20-0.2048</td>
</tr>
</tbody>
</table>

MIC: Minimal inhibitory concentrations; TB: Terbinafine; ITZ: Itraconazole; AMB: Amphotericin B; VOR: Voriconazole; FICI: Fractional inhibitory concentration index; for most isolates indicated Sinergism except for: (*) only one isolate showed indifference; (++): four isolates showed indifference; (+): 3.5% of isolates showed indifference; (•): 3.5% of isolates showed indifference; (••): 24.1% of isolates showed indifference.
References


