Combination of Amphotericin B and Flucytosine against Neurotropic Species of Melanized Fungi Causing Primary Cerebral Phaeohyphomycosis


Primary central nervous system phaeohyphomycosis is a fatal fungal infection due mainly to the neurotropic melanized fungi Cladophialophora bantiana, Rhinocladiella mackenziei, and Exophiala dermatitidis. Despite the combination of surgery with antifungal treatment, the prognosis continues to be poor, with mortality rates ranging from 50 to 70%. Therefore, a search for a more-appropriate therapeutic approach is urgently needed. In this study, we therefore investigated the antifungal activity of amphotericin B and flucytosine against these species, the median fractional inhibitory concentration (FIC) indices for strains ranged from 0.25 to 0.38, indicating synergy. By use of Bliss independence analysis, a significant degree of synergy was confirmed for all strains, with the sum ΔE ranging from 90.2 to 698.61%. No antagonism was observed. These results indicate that amphotericin B, in combination with flucytosine, may have a role in the treatment of primary cerebral infections caused by melanized fungi belonging to the order Chaetothyriales. Further in vivo studies and clinical investigations to elucidate and confirm these observations are warranted.
sequencing of the internal transcribed spacer regions of ribosomal DNA (rDNA), as described previously (25, 26).

Stock cultures were grown on malt extract agar (MEA; Difco, Leeuwarden, The Netherlands) at 25°C for 1 to 3 weeks before the preparation of the inoculum. *Paecilomyces variotii* (ATCC 22319), *Candida parapsilosis* (ATCC 22019), and *Candida krusei* (ATCC 6258) were used as quality controls in all experiments.

**Preparation of the inoculum.** All isolates were subcultured on MEA at 25°C. Then conidial suspensions were harvested and were suspended in normal saline containing 0.025% Tween 20. Supernatants were adjusted spectrophotometrically to 530-nm wavelengths to optical densities (ODs) that ranged from 0.15 to 0.17 (68 to 71% transmission) for controls in all experiments.

**XTT dye** {2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide}, as described previously (28–30). XTT (Sigma-Aldrich, St. Louis, MO, USA) was initially dissolved in absolute ethanol at a concentration of 10 mg/ml and was subse- quently diluted to a concentration of 0.5 mg/ml. Menadione (Sigma-Aldrich) was initially dissolved in absolute ethanol at a concentration of 0.5 mg/ml. Menadione solution was added to each well, as described previously (30–32). Incubation was extended to 14 days. Subsequently, 50 l of medium and 50 l of the XTT-menadione solution but no inoculum. Percentages of fungal growth were calculated for each well by dividing the XTT conversion in each well by the XTT conversion in the drug-free growth control well. All experiments on each strain were performed using three independent replicates on different days.

**MIC determination.** The MICs of amphotericin B and flucytosine were determined as the lowest concentrations that completely inhibited growth relative to the growth in the drug-free well, as assessed by visual inspection. Because the MIC value for amphotericin B is considered the lowest drug concentration corresponding to <10% growth and the flucytosine MIC is the lowest drug concentration corresponding to 50% growth inhibition, 10%, 25%, and 50% growth endpoints were calculated as MIC endpoints for the amphotericin B–flucytosine combination (27).

**Definitions for drug interaction modeling.** In order to assess the nature of *in vitro* interactions between amphotericin B and flucytosine, the data obtained as described above were analyzed using two different models. These models were nonparametric approaches of the following two zero-interaction theories: the Loewe additivity (LA) and Bliss independence (BI) theories (33–36). The fractional inhibitory concentration (FIC) index is defined as follows: $\Sigma FIC = FICA + FICB = (\frac{MIC_A^{comb}}{MIC_A^{alone}} + (\frac{MIC_B^{comb}}{MIC_B^{alone}})$, where $MIC_A^{comb}$ and $MIC_A^{alone}$ are the MICs of the drugs A and B when acting alone, and $C_A^{comb}$ and $C_A^{alone}$ are the concentrations of the drugs A and B at the isoefective combina- tion, respectively (34). To determine the synergistic and antagonistic interactions among all $\Sigma FIC$s calculated for each isolate and replicate, the FIC index was determined as the $\Sigma FIC_{min}$ (the lowest $\Sigma FIC$) or the $\Sigma FIC_{max}$ (the highest $\Sigma FIC$) (34). Ten percent endpoints of fungal growth were used to assess pharmacodynamic interactions at different concentrations. Drug interactions were defined as synergistic if the FIC index was <0.5, as antagonistic if the FIC index was >4, and as noninteractive if the FIC index was between 0.5 and 4 (37).

The BI parameter was described by the equation $I_{BA} = I_A + I_B - (I_A \times I_B)$, where $I_{BA}$ is the predicted percentage of inhibition of a noninteractive theoretical combination, calculated with the experimental percentages of inhibition ($I_A$, $I_B$) of each drug acting alone (36). In the 3-dimensional plots, peaks above and below the zero plane indicate synergistic and antagonistic combinations, respectively, whereas the zero plane itself indicates no statistically significant interactions. The average sum of the three replicates of all Bliss interactions was used as a measure of the pharmacodynamic interactions for each strain. Drug interactions were considered synergistic if the FIC index was greater than zero (positive $\Delta E$), indiff erent if $\Delta E$ was zero, and antagonistic if $\Delta E$ was less than zero (negative $\Delta E$).

**Data analysis.** All data analyses were performed by using the GraphPad Prism software package (version 5.0 for Windows; GraphPad Software, San Diego, CA). The FIC and BI indices among the different groups were compared by analysis of variance (ANOVA) followed by a posttest

### Table 1: FIC indices based on 10% growth endpoints and Bliss independence results for melanized fungi causing primary cerebral infections

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Biosafety level</th>
<th>Strain no.</th>
<th>Source</th>
<th>Origin</th>
<th>MIC (µg/ml)$^a$</th>
<th>5-FC</th>
<th>FIC index</th>
<th>Sum $\Delta E$</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cladophialophora bantiana</em></td>
<td>3</td>
<td>CBS 101251</td>
<td>Human, brain abscess</td>
<td>USA</td>
<td>0.25</td>
<td>2</td>
<td>0.5</td>
<td>90.2</td>
<td>Synergy</td>
</tr>
<tr>
<td><em>Rhinocladiella mackenziei</em></td>
<td>3</td>
<td>CBS 65093</td>
<td>Human, brain abscess</td>
<td>Saudi Arabia</td>
<td>8</td>
<td>16</td>
<td>0.5</td>
<td>396.4</td>
<td>Synergy</td>
</tr>
<tr>
<td><em>Exophiala dermatitidis</em></td>
<td>2</td>
<td>CBS 120473</td>
<td>Human, brain abscess</td>
<td>USA</td>
<td>0.5</td>
<td>0.125</td>
<td>698.6</td>
<td>Synergy</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CBS 57976</td>
<td>Human, brain abscess</td>
<td>Japan</td>
<td>1</td>
<td>0.32</td>
<td>498.65</td>
<td>Synergy</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CBS 57876</td>
<td>Human, brain abscess</td>
<td>Japan</td>
<td>0.5</td>
<td>0.64</td>
<td>450.11</td>
<td>Synergy</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ AmB, amphotericin B; 5-FC, flucytosine.
Interaction surfaces obtained from response surface analysis of the Bliss independence no-interaction model for the in vitro combination of amphotericin B (AmB) and flucytosine (5-FC) against a Cladophialophora bantiana strain (CBS 102586) (AmB MIC, 0.25 μg/ml; 5-FC MIC, 2 μg/ml), a Rhinocladiella mackenziei strain (CBS 109634) (AmB MIC, 2 μg/ml; 5-FC MIC, 32 μg/ml), and an Exophiala dermatitidis strain (CBS 120473) (AmB MIC, 0.5 μg/ml; 5-FC MIC, 32 μg/ml). The x and y axes represent the efficacies of AmB and 5-FC, respectively. The z axis is ΔE, expressed as a percentage. The zero plane represents Bliss independent interactions, whereas values above the zero plane represent statistically significantly synergistic (positive ΔE) interactions. The magnitude of interactions is directly related to ΔE. The different tones in the 3-dimensional plots represent different percentile bands of synergy.
for linear trend. The correlation between the mean FIC indices and the sum $\Delta E$ was determined by Spearman’s correlation coefficient ($r$); a $P$ value of $\leq 0.05$ (by a two-tailed test) was considered significant.

RESULTS
The MICs for the isolates used in the current study and the results of the FIC index model are summarized in Table 1. For the combination of amphotericin B and flucytosine, the median MIC indices were 0.25 for C. bantiana ($\leq$ FIC ranging from 0.25 to 0.5), 0.38 for R. mackenziei ($\leq$ FIC ranging from 0.25 to 0.5), and 0.25 for E. dermatitidis ($\leq$ FIC ranging from 0.125 to 0.25), indicating synergy for all strains. In addition, a mean FIC value of $>4$ for all replicates was not obtained with any of the isolates tested, indicating that no antagonism was found.

Table 1 and Fig. 1 show the results of Bliss independence drug interaction analysis for the in vitro interactions of amphotericin B and flucytosine. The amphotericin B–flucytosine combination resulted in a synergistic interaction for all strains. The degree of synergy was highest among the E. dermatitidis strains (sum $\Delta E$, 450.11% to 698.61%), followed by R. mackenziei (sum $\Delta E$, 293.89% to 527.31%) and C. bantiana (sum $\Delta E$, 90.2% to 189.69%), respectively.

DISCUSSION
Overall, our results show that the amphotericin B–flucytosine combination has consistent synergistic effects against C. bantiana, R. mackenziei, and E. dermatitidis. The results of FIC analysis were supported by response surface analysis using the Bliss independence no-interaction model for the isolates tested. Both models were shown to correlate well with the in vivo results of combination therapy in experimental invasive fungal infections, such as invasive pulmonary aspergillosis (32, 38). Therefore, these results could help to support the combination of amphotericin B and flucytosine against infections caused by neurotropic species of melanized fungi. On the other hand, the Bliss independence theory was derived from the probability that two drugs do not interact with each other and therefore will act independently (38, 39).

C. bantiana causes severe infections, mainly in immunocompetent hosts worldwide, with a general preference for warm and humid climates. The species causes cerebral abscesses almost exclusively, with a high mortality rate (up to 70%) (1, 5, 7, 16). R. mackenziei causes cerebral infections mostly in debilitated patients, with a mortality rate of almost 100% if infections remain untreated; even in patients treated with surgery and antifungal therapy, mortality is almost 65%. This fungus is restricted to the Middle East, the Persian Gulf, Somalia, and Pakistan (2, 40, 41). E. dermatitidis is one of the most common clinically significant human pathogens in the black-yeast genus Exophiala, causing disseminated infection with a marked predilection for the central nervous system (CNS). Infections by this fungus are reported mainly from East Asia, although several cases in other geographical regions worldwide have been reported (42, 43). This fungus seems to be able to affect young, otherwise healthy patients (5, 42, 44, 45). E. dermatitidis cerebral infection is generally associated with a high mortality rate (about 50%) (17).

Evidence to support treatment choices for cerebral phaeohyphomycosis caused by these fungi is scarce at present, and patients have died in most cases despite a combination of surgery and antifungal therapy (2–4, 46). On the other hand, the use of a potent antifungal with increased efficacy does not guarantee the therapeutic outcome, since treatment failures might occur, possibly because of poor penetration into the CNS (47). Few studies have reported data on the efficacy of antifungal combination therapy against invasive fungal infections caused by neurotropic melanized fungi (12, 48, 49). Most studies investigating combinations of azoles with echinocandins or polyenes and/or combinations of echinocandins with polyenes have shown a synergistic or additive interaction in vitro and in vivo (12, 14, 48, 49). One study, using a murine model, tested double or triple combinations of amphotericin B, micafungin, voriconazole, flucytosine, and posaconazole in the treatment of disseminated infections caused by C. bantiana (12). Combination therapy with three of the drugs (posaconazole, micafungin, and flucytosine) appeared to be a promising option for the treatment of C. bantiana infections (12). In another study, Sun et al. investigated the in vitro interactions of the following combinations against E. dermatitidis strains: caspofungin with itraconazole, voriconazole, amphotericin B, or fluconazole; terbinafine with itraconazole; and fluconazole with amphotericin B (49). Combinations of caspofungin with voriconazole, amphotericin B, or itraconazole showed synergistic activity against E. dermatitidis (49).

Of note, combination therapy with amphotericin B and flucytosine is the recommended first-line treatment for disseminated cryptococcal meningitis, a fungal infection of the CNS, in both immunocompetent and immunosuppressed patients (22–24). Our results therefore suggest that a combination of amphotericin B and flucytosine may have a promising role in the treatment of primary cerebral phaeohyphomycosis due to neurotropic species of melanized fungi and possibly other emerging pathogens from this group of environmental fungi. In vivo studies and in vitro-in vivo correlation investigations to validate and confirm these observations are warranted.

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