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

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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. Our *in vitro* studies showed that with the combination 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.

Cerebral phaeohyphomycosis is a rare but frequently fatal fungal infection due mainly to neurotropic black fungi belonging to the ascomycete order *Chaetothyriales*: *Cladophialophora bantiana*, *Rhinocladiella mackenziei*, and *Exophiala dermatitidis* (1–5). Other opportunistic pathogens from this group of environmental fungi (i.e., *Cladophialophora modesta*, *Exophiala asiatica*, *Fonsecaea monophora*, and *Fonsecaea pugnacius*) are being encountered as causal agents of this infection (6, 7).

The infection may occur in immunosuppressed patients following the inhalation of conidia; however, a high proportion of primary cerebral infections are reported in apparently immunocompetent individuals without any obvious predisposing factors (2, 4). If the infection remains untreated, mortality can be as high as 100% within weeks, months, or years (4).

The optimal therapeutic regimen for the treatment of cerebral phaeohyphomycosis is not known. Therapy with amphotericin B alone (a standard or lipid preparation) may not be adequate (8–10), while *in vivo* studies and single cases suggest that voriconazole and posaconazole may provide better outcomes (8, 11). Moreover, combinations of a triazole with an echinocandin and/or flucytosine have shown better efficacy than monotherapy (12–15), but the results are not yet conclusive. When possible, complete surgical removal of brain lesions combined with systemic antifungal therapy is recommended (13, 16). For those who are treated, mortality is lower than for those without treatment, but the prognosis continues to be poor, with a case fatality rate as high as 70% (11, 17–19).

Considering the poor clinical outcomes, the development of a more appropriate therapeutic approach is required. From a clinical perspective, the combination of amphotericin B and flucytosine is generally associated with improved survival among patients with systemic fungal infections (20, 21), including cryptococcal meningitis (22–24). However, data on the clinical use of this combination for patients with cerebral phaeohyphomycosis are scarce. In this study, we therefore investigated the *in vitro* antifungal activity of amphotericin B in combination with flucytosine against a collection of black fungi obtained from patients with primary brain infections.

MATERIALS AND METHODS

Fungal isolates. A collection of 12 clinical isolates consisting of 5 strains of *C. bantiana*, 4 strains of *R. mackenziei*, and 3 strains of *E. dermatitidis* originating from both human and animal brain abscesses were used (see Table 1). All strains were obtained from the reference collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands, and were handled under biosafety laboratory regulations (for biosafety levels 2 and 3, as appropriate). The identities of the organisms were confirmed by

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

Fungal species	Biosafety level	Strain no.	Source	Origin	MIC (μg/ml)[a]		FIC index	Sum ΔE	Result
					AmB	5-FC			
Cladophialophora bantiana	3	CBS 101251	Human, brain abscess	USA	0.25	2	0.5	90.2	Synergy
		CBS 101158	Human, brain abscess	Japan	0.25	64	0.25	149.58	Synergy
		CBS 102586	Human, brain abscess	Brazil	0.25	2	0.25	189.69	Synergy
		CBS 98496	Human, brain abscess	South Africa	0.5	4	0.5	140.16	Synergy
		CBS 100436	Cat, brain abscess	California, USA	0.5	2	0.25	114.23	Synergy
Rhinocladiella mackenziei	3	CBS 65093	Human, brain abscess	Saudi Arabia	8	16	0.5	396.42	Synergy
		CBS 36892	Human, brain abscess	Israel	8	16	0.25	527.31	Synergy
		CBS 102589	Human, brain abscess	Egypt	8	32	0.25	293.89	Synergy
		CBS 109634	Human, brain abscess	Oman	2	32	0.5	419.4	Synergy
Exophiala dermatitidis	2	CBS 120473	Human, brain abscess	USA	0.5	32	0.125	698.61	Synergy
		CBS 57976	Human, brain abscess	Japan	1	32	0.25	498.65	Synergy
		CBS 57876	Human, brain abscess	Japan	0.5	64	0.25	450.11	Synergy

[a] AmB, amphotericin B; 5-FC, flucytosine.

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 at 530-nm wavelengths to optical densities (ODs) that ranged from 0.15 to 0.17 (68 to 71% transmission) for *C. bantiana* and *R. mackenziei* and from 0.09 to 0.13 (80 to 83% transmission) for *E. dermatitidis*.

Antifungal agents. Amphotericin B and flucytosine (Sigma-Aldrich, St. Louis, MO, USA) were obtained as standard pure powders, and serial dilutions were prepared according to the Clinical and Laboratory Standards Institute (CLSI) broth microdilution guidelines (27).

Susceptibility and drug interaction testing. Antifungal susceptibility testing and drug interaction testing were performed by using the broth microdilution checkerboard (2-dimensional, 8-by-12) method, utilizing 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) was dissolved in normal saline at a concentration of 0.5 mg/ml. Menadione (Sigma-Aldrich) was initially dissolved in absolute ethanol at a concentration of 10 mg/ml and was subsequently added to the XTT solutions at a concentration of 6.25 μM for each solution. The final concentrations of the antifungal agents ranged from 0.125 to 8 mg/liter for amphotericin B and from 0.125 to 128 mg/liter for flucytosine. Aliquots (50 μl) of each drug at a concentration four times the targeted final concentration were dispensed into the wells of U-shaped 96-well microtiter plates (Costar, Corning, NY). Trays were maintained for a period of <1 month at −70°C until the day of testing. After the microtitration trays were defrosted, 100 μl of the inoculum was added to each well, corresponding to a final concentration of 0.5×10^4 to 4×10^4 CFU/ml for each isolate. The microtiter plates were incubated at 35 to 37°C for 72 h. If no growth was observed, or if growth was inadequate, the incubation was extended to 14 days. Subsequently, 50 μl of the XTT-menadione solution was added to each well, as described previously (30–32). The microtitration plates were further incubated at 35 to 37°C for 2 h in order to allow the conversion of XTT to its formazan derivative. XTT conversion was measured as the OD with a microtitration plate spectrophotometric reader (Anthos HTIII; Anthos Labtec Instruments, Salzburg, Austria) at 450 nm/630 nm (30–32). For each well, the XTT conversion was calculated after subtraction of the background OD, which was the OD of a simultaneously incubated well with 200 μl of medium and 50 μl 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 defined 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: $\sum\text{FIC} = \text{FICA} + \text{FICB} = (C_A^{\text{comb}}/\text{MIC}_A^{\text{alone}}) + (C_B^{\text{comb}}/\text{MIC}_B^{\text{alone}})$, where $\text{MIC}_A^{\text{alone}}$ and $\text{MIC}_B^{\text{alone}}$ are the MICs of the drugs A and B when acting alone, and C_A^{comb} and C_B^{comb} are the concentrations of the drugs A and B at the isoeffective combinations, respectively (34). To determine the synergistic and antagonistic interactions among all ∑FICs calculated for each isolate and replicate, the FIC index was determined as the $\sum\text{FIC}_{\text{min}}$ (the lowest ∑FIC) or the $\sum\text{FIC}_{\text{max}}$ (the highest ∑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 $I_{\text{ind}} = I_A + I_B - (I_A \times I_B)$, where I_{ind} 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 ΔE was greater than zero (positive ΔE), indifferent if ΔE was zero, and antagonistic if ΔE was less than zero (negative ΔE).

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

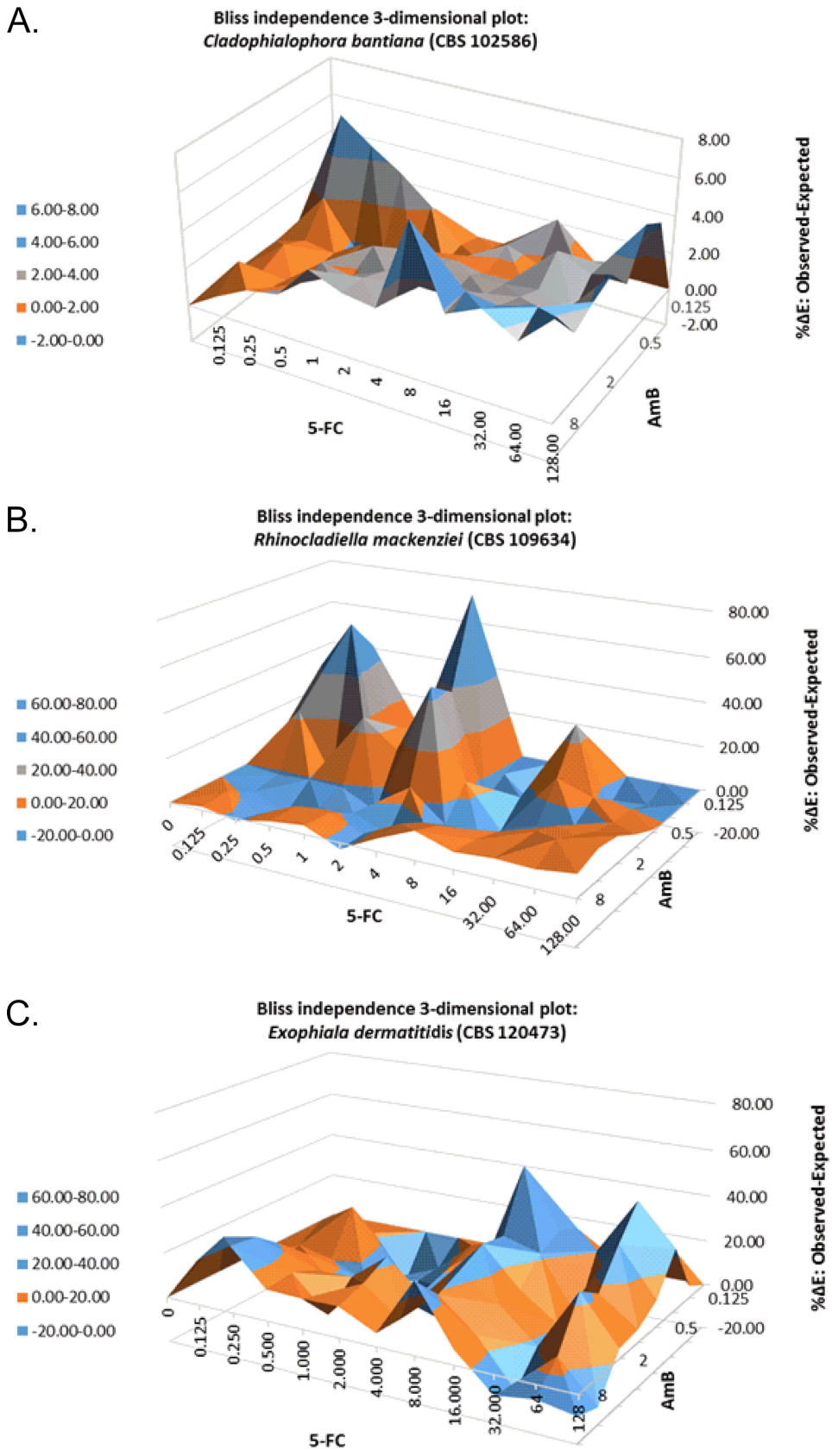

FIG 1 Interaction surfaces obtained from response surface analysis of the Bliss independence no-interaction model for the *in vitro* combination of amphotericin B (AmB) and flucytosine (5-FC) against a *Cladophialophora bantiana* strain (CBS 102586) (AmB MIC, 0.25 μg/ml; 5-FC MIC, 2 μg/ml), a *Rhinocladiella 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 ΔE was determined by Spearman's correlation coefficient (*r*); a *P* value of ≤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 FIC indices were 0.25 for *C. bantiana* (∑FIC ranging from 0.25 to 0.5), 0.38 for *R. mackenziei* (∑FIC ranging from 0.25 to 0.5), and 0.25 for *E. dermatitidis* (∑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 ΔE, 450.11% to 698.61%), followed by *R. mackenziei* (sum ΔE, 293.89% to 527.31%) and *C. bantiana* (sum ΔE, 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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