Combination of amphotericin B and flucytosine against neurotropic species of melanized fungi causing primary cerebral phaeohyphomycosis

Running title: Combination therapy for cerebral phaeohyphomycosis

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ABSTRACT

Primary central nervous system phaeohyphomycosis is a fatal fungal infection mainly due to the neurotropic melanized fungi *Cladophialophora bantiana*, *Rhinocladiella mackenziei* and *Exophiala dermatitidis.* Despite combination of surgery with antifungal treatment, the prognosis continues to be poor with mortality rates ranging from 50 to 70%. Therefore, search for more appropriate therapeutic approach is urgently needed.

Our *in vitro* studies showed that for the combination of amphotericin B and flucytosine against these species the median FIC indices of strains ranged from 0.25 to 0.38, which indicates synergy. Using 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 are warranted to further elucidate and confirm these observations.

KEY WORDS

Combination therapy, amphotericin B, flucytosine, melanized fungi, cerebral infection, phaeohyphomycosis

INTRODUCTION

Cerebral phaeohyphomycosis is a rare but frequently fatal fungal infection mainly due 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 are being encountered as causal agents of this infection, i.e. *C. modesta, E. asiatica, Fonsecaea monophora* and *F. pugnacius* (6, 7).

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

For treatment of cerebral phaeohyphomycosis, the optimal therapeutic regimen is not known. Therapy with amphotericin B alone (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 outcome (8, 11). Moreover, combination of a triazole plus an echinocandin and or flucytosine has shown better efficacy than monotherapy (12-15), but still 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 without treatment but the prognosis continues to be poor, with a case fatality rate up to 70% (11, 17-19).

Considering the poor clinical outcome, development of a more appropriate therapeutic approach is required. From a clinical perspective, amphotericin B plus 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 in patients with cerebral phaeohyphomycosis are scarce. In this study we therefore investigated *in vitro* antifungal activity of amphotericin B in combination with flucytosine against a collection of black fungi obtained from 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* originated from both human and animal brain abscesses were used (table 1). All strains were obtained from the reference collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands and handled under biosafety laboratory regulations (levels 2 and 3, accordingly). Identity of the organisms was confirmed by sequencing of the internal transcribed spacer regions of rDNA, as described previously (25, 26).

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

- **Preparation of inoculum**

All isolates were sub-cultured on MEA at 25°C. Then, conidial suspensions were harvested and 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 0.09 to 0.13 (80-83 % transmission) for *E. dermatitidis.*

- **Antifungal agents**

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

- **Susceptibility and drug interaction testing**

Antifungal susceptibility and drug interactions testing were performed by using the broth microdilution checkerboard (two-dimensional eight by- twelve) 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 concentrations of 0.5 mg/ml. Menadione (Sigma-Aldrich) was initially dissolved in absolute ethanol at a concentration of 10 mg/ml and subsequently added to the above-mentioned XTT solutions at concentrations of 6.25 µM for each solution. The final concentrations of the antifungal agents ranged from 0.125 to 8 mg/L for amphotericin B and 0.125 to 128 mg/liter for flucytosine. Aliquots of 50 µl of each drug at a concentration four times the targeted final concentration were dispensed in the wells of U-shaped 96-well microtiter plates (Costar, Corning, N.Y.). Trays were maintained for a period of less than 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 wells corresponding to a final concentration of 0.5 to 4 x 10^4 cfu/mL from each isolate. The microtiter plates were incubated at 35 to 37°C for 72 h. If no growth was observed or growth was inadequate, incubation was extended to 14 days. Subsequently, 50 µl of the above-mentioned XTT-menadione solutions were added to each well, as previously described (30-32). The microtitration plates were further incubated at 35 to 37°C for 2 h in order to allow conversion of XTT to its formazan derivative. XTT conversion was measured as optical density (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 XTT-menadione solution but no inoculum. Percentages of fungal growth were calculated for each well by dividing XTT conversion of each well by the XTT conversion of the drug-free growth control well. All experiments on each strain were performed using three independent replicates on different days.

- **MIC determination**

The MIC (minimum inhibitory concentration) of amphotericin B and flucytosine was defined as the lowest concentration that completely inhibited growth compared with that of the drug-free well as assessed by visual inspection. Because MIC corresponds to the lowest drug concentration corresponding to <10% growth for amphotericin B and 50% growth inhibition for flucytosine for flucytosine, for the amphotericin B-flucytosine combination, 10%, 25% and 50% growth endpoints were calculated as MIC end point respectively. (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 non-parametric approaches of the following two no (zero)-interaction theories: Loewe additivity (LA) and Bliss independence (BI) (33-36). The fractional inhibitory concentration (FIC) index is defined as follows: $\sum FIC = FICA + FICB = C_A^{comb}/MIC_A^{alone} + C_B^{comb}/MIC_B^{alone}$, where MIC_A^{alone} and MIC_B^{alone} are the MICs of the drugs A and B when acting alone and C_A^{comb} and C_B^{comb} are concentrations of the drugs A and B at the iso-effective combinations, respectively (34). To determine synergistic and antagonistic interactions among all ∑FICs calculated for each isolate and replicate, the FIC index was

168 determined as the $\sum FIC_{min}$ (the lowest ∑FIC) or the $\sum FIC_{max}$ (the highest ∑FIC) (34). 10%
169 endpoints of fungal growth were used to assess pharmacodynamic interactions at different
170 concentrations. Drug interactions were defined as synergic if the FIC index was <0.5,
171 antagonistic if the FIC index was > 4 and non-interactive between 0.5 and 4 (37).
172 The Bliss independence parameter (BI) was described by the equation: $I_{ind}=I_A+I_B - I_A \times I_B$;
173 where I_{ind} is the predicted percentage of inhibition of a non-interactive theoretical
174 combination, calculated with the experimental percentages of inhibition (I_A, I_B) of each drug
175 acting alone, respectively (36). In the three-dimensional plots, peaks above and below the
176 zero plane indicate synergistic and antagonistic combinations, respectively, whereas the zero
177 plane itself indicates no statistically significant interactions. The average sum of the three
178 replicates of all Bliss interactions was used as a measure of the pharmacodynamic
179 interactions for each strain. Drug interactions were considered synergistic if ΔE > 0 (positive
180 ΔE), indifference if ΔE=0, or antagonism if ΔE < 0 (negative ΔE).
181 - **Data analysis**
182 All data analyses were performed by using the software package GraphPad Prism, version
183 5.0, for Windows (GraphPad Software, San Diego, CA). The FICs and BI indices among the
184 different groups were compared with ANOVA followed by post-test for linear trend. The
185 correlation between the mean FIC indices and SUM ΔE was determined by Spearman's
186 correlation coefficient *r*; a *P* value of ≤ 0.05 was considered significant (two-tailed).
187
188 **RESULTS**
189 The MIC characteristics of the isolates used for the current study and results of the FIC index
190 model are summarized in Table 1. For the amphotericin B and flucytosine combinations, the
191 median FIC indices were 0.25 for *C. bantiana* (∑FIC ranging 0.25 to 0.5), 0.38 for *R.*

mackenziei (∑FIC ranging 0.25 to 0.5) and 0.25 for *E. dermatitidis* (∑FIC ranging 0.125 to 0.25), which indicates synergy for all strains. In addition, a mean FIC value of > 4 for all replicates was not noted in any of the isolates tested, indicating that no antagonism was found.

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

DISCUSSION

Overall, our results show that the amphotericin B and 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 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, their results could help to support 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 theory two drugs do not interact with each other and therefore will act independently in a probabilistic sense (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% in infections that remain untreated; even in patients treated with surgery and antifungal therapy, mortality is almost 65%. This fungus is restricted to the Middle East, Persian Gulf, Somalia and Pakistan (2, 41, 42). *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 CNS. Infections by this fungus are mainly reported from East-Asia, although several cases have been described in other geographical regions worldwide (43, 44). This fungus seems to be able to affect young, otherwise healthy patients (5, 43, 45, 46). *E. dermatitidis* cerebral infection is generally associated with a high mortality rate (about 50%) (17).

Evidence to support treatment choices in cerebral phaeohyphomycosis caused by these fungi at present is scarce and the patient has died in most cases despite combination of surgery and antifungal therapy (2-4, 40). On the other hand, using a potent antifungal with increased efficacy does not guarantee therapeutic outcome, since treatment failures might have occurred, possibly because of poor penetration into the central nervous system (CNS) (47).Few studies have reported data on the efficacy of antifungal combination therapy against invasive fungal infection caused by neurotropic melanized fungi (12, 48, 49). Most studies investigating the combinations of azoles with echinocandins or polyenes and or 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 the three drugs (posaconazole, micafungin and flucytosine) appeared to be a promising option for the

240 treatment of *C. bantiana* infections (12). In another study, Sun et al. investigated the *in vitro*
241 interactions of caspofungin with itraconazole, voriconazole, amphotericin B or fluconazole;
242 terbinafine with itraconazole; and fluconazole with amphotericin B, against *E. dermatitidis*
243 strains (49). The combinations of caspofungin with voriconazole, amphotericin B or
244 itraconazole showed synergic activity against *E. dermatitidis* (49).
245 Of note, combination therapy with amphotericin B and flucytosine is the recommended first-
246 line treatment for disseminated cryptococcal meningitis, which is a fungal infection of CNS,
247 in both immunocompetent and immunosuppressed patients (22-24). Our results therefore
248 suggest that a combination of amphotericin B and flucytosine may have a promising role in
249 the treatment of primary cerebral phaeohyphomycosis due to neurotropic species of
250 melanized fungi and possibly other emerging pathogens from this group of environmental
251 fungi. *In vivo* studies and *in vitro-in vivo* correlation investigations are warranted to validate
252 and confirm these observations.
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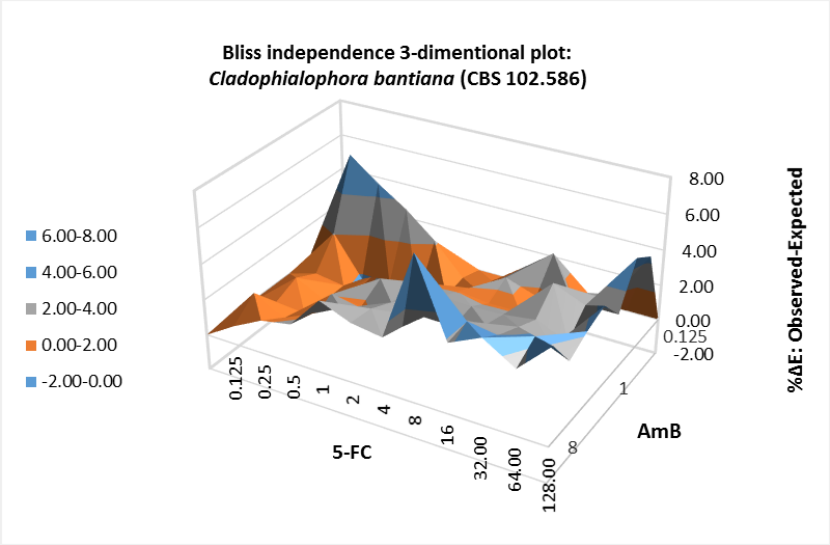
Fungal strains	Biosafety level	Strain no.	Source	Origin	AmB MIC (µg/ml)	5-FC MIC (µg/ml)	FIC index	SUM ΔE	Results
C. bantiana	3	CBS 101251	Human, brain abscess	USA	0.25	2	0.5	90.2	Synergy
C. bantiana		CBS 101158	Human, brain abscess	Japan	0.25	64	0.25	149.58	Synergy
C. bantiana		CBS 102586	Human, brain abscess	Brazil	0.25	2	0.25	189.69	Synergy
C. bantiana		CBS 984.96	Human, brain abscess	South Africa	0.5	4	0.5	140.16	Synergy
C. bantiana		CBS 100436	Cat, brain abscess	USA, California	0.5	2	0.25	114.23	Synergy
R. mackenziei	3	CBS 650.93	Human, brain abscess	Saudi arabia	8	16	0.5	396.42	Synergy
R. mackenziei		CBS 368.92	Human, brain abscess	Israel	8	16	0.25	527.31	Synergy
R. mackenziei		CBS 102589	Human, brain abscess	Egypt	8	32	0.25	293.89	Synergy
R. mackenziei		CBS 109634	Human, brain abscess	Oman	2	32	0.5	419.4	Synergy
E. dermatitidis	2	CBS 120473	Human, brain abscess	USA	0.5	32	0.125	698.61	Synergy
E. dermatitidis		CBS 579.76	Human, brain abscess	Japan	1	32	0.25	498.65	Synergy
E. dermatitidis		CBS 578.76	Human, brain abscess	Japan	0.5	64	0.25	450.11	Synergy

TABLE 1. FIC indices based on 10% growth endpoints and Bliss independence results for melanized fungi (*Cladophialophora bantiana*, *Rhinocladiella mackenziei and Exophiala dermatitidis*) causing primary cerebral infections.

A.



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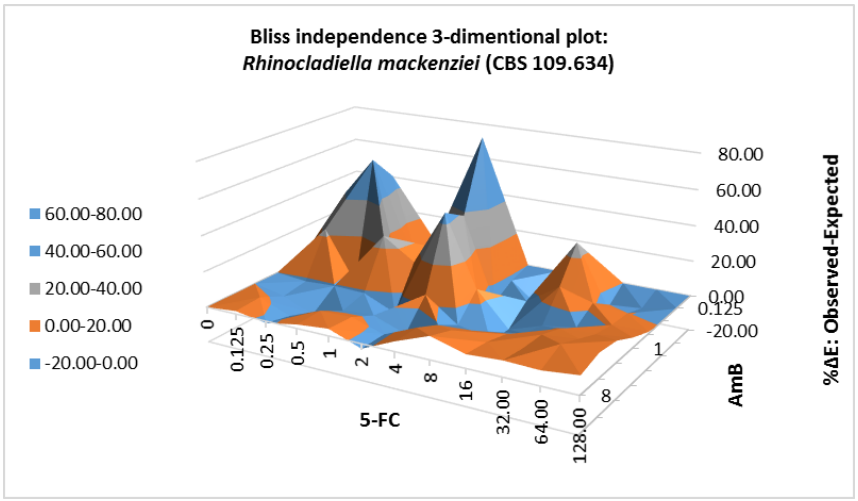
B.



C.

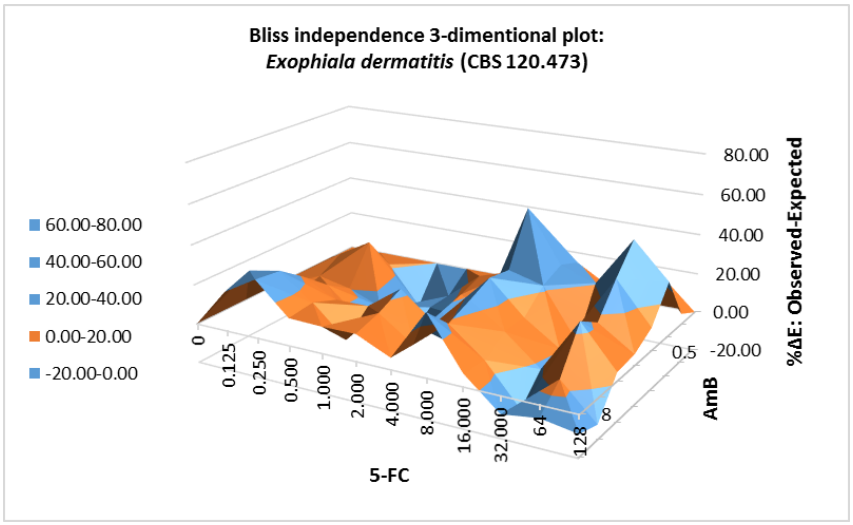


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FIGURE 1. Interaction surfaces obtained from response surface analysis of Bliss independence no-interaction model for *in vitro* combination of amphotericin B (AmB) plus flucytosine (5-FC) against a *Cladophialophora bantiana* strain (CBS 102.586) (MIC AmB 0.25 µg/ml, MIC 5-FC 2 µg/ml), a *Rhinocladiella mackenziei* strain (CBS 109.634) (MIC AmB 2 µg/ml, MIC 5-FC 32 µg/ml) and an *Exophiala dermatitis* (CBS 120.473) (MIC AmB 0.5 µg/ml, MIC 5-FC 32 µg/ml).

The X- and Y- axis represent the efficacy of AmB and 5-FC, respectively. The Z- axis is the ΔE in %. The 0-plane represents Bliss independent interactions whereas the volumes above the 0-plane represent statistically significantly synergistic (positive ΔE) interactions. The magnitude of interactions is directly related to ΔE. The different tones in three dimensional plots represent different percentile bands of synergy.