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Combination of amphotericin B and terbinafine against melanized fungi associated with chromoblastomycosis

Running title: Combination of antifungals against chromoblastomycosis

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ABSTRACT

Our *in vitro* studies showed that combination of amphotericin B and terbinafine had synergistic effects against the majority of melanized fungi associated with chromoblastomycosis (CBM) and similar infections, including *Cladophialophora carrionii, C. arxii, Exophiala dermatitidis, E. spinifera, Fonsecaea monophora, F. nubica, F. pedrosoi,* and *Phialophora verrucosa.* This combination could provide an option for treatment of severe or unresponsive cases of CBM, particularly in cases due to species of *Fonsecaea* and *Cladophialophora*.

KEY WORDS

Combination therapy, amphotericin B, terbinafine, melanized fungi, chromoblastomycosis

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TEXT

Chromoblastomycosis (CBM) is a serious fungal skin disease associated with significant morbidity (1). The disease is characterized histologically by muriform cells that cause chronic inflammation of the skin and subcutaneous tissues (2, 3). The infection leads to excessive proliferation of host tissue and formation of cauliflower-like eruptions on the skin, hyperkeratosis, or may exhibit intermediate forms, depending on the type of interaction between host and fungal cells (4, 5). Because of chronicity, the CBM lesions may also undergo neoplastic transformation leading to skin cancer (6). The chronic nature of infections seems to be due to inadequate innate recognition and subsequent failure to mount protective inflammatory responses (7).

The disease has worldwide distribution mainly in tropical and subtropical climates (8). Species of humid climates, particularly members of the genus *Fonsecaea* (*F. pedrosoi*, *F. monophora* and *F. nubica*), are prevalent agents of CBM (9). *Cladophialophora carrionii* is the predominant agent of the disease under arid, desert-like climatic conditions (10). Sporadic cases of CBM-like infections have also been reported by *Cladophialophora arxii* (11), *Exophiala dermatitidis* (12), *E. spinifera* (13), *Phialophora verrucosa* (14), and *Veronaea botryose* (15), although attribution to this disease category has not been confirmed.

CBM is extremely difficult to treat due to its recalcitrant nature, and there is no consensus regarding the treatment of choice (16). Based on open clinical studies and expert opinions, itraconazole is the first line recommended therapy for CBM (17), followed by terbinafine (18). However, infections by *F. pedrosoi* strains resistant to itraconazole have been reported (19). Cure rates with itraconazole and terbinafine monotherapy may range from 15 to 80 %, which on average is insufficient (20). When possible, the addition of physical therapeutic methods such as laser- and photodynamic therapy is recommended (21, 22), which is still associated with rather low cure and high refractory rates.

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Alternative therapeutic strategies employing newer antifungal agents and / or combination of drugs (23-26), might be promising to treat CBM more efficiently. In a recent study, we also demonstrated that amphotericin B in combination with flucytosine may have a role in the treatment of primary cerebral infections caused by other melanized fungi of the order *Chaetothyriales* (27). We therefore sought to investigate the *in vitro* antifungal activity of amphotericin B in combination with terbinafine against a collection of black fungi obtained from patients with CBM.

A collection of 46 isolates of melanized fungi associated with CBM or similar skin infections were studied, including: *C. carrionii* (n=10), *C. arxii* (n=1), *Exophiala dermatitidis* (n=9), *E. spinifera* (n=3), *Fonsecaea monophora* (n=7), *F. nubica* (n=5), *F. pedrosoi* (n=5), *Phialophora verrucosa* (n=3), and *Veronaea botryosa* (n=3). The identities of the organisms were confirmed by sequencing of the internal transcribed spacer regions of ribosomal DNA (rDNA), as described previously (28). All isolates were sub-cultured on MEA at 25°C. 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 all isolates, except *E. dermatitidis* that ranged from 0.09 to 0.13 (80 to 83% transmission), as described previously (27).

Amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) and terbinafine (Novartis, Arnhem, The Netherlands) were obtained as standard pure powders, and serial dilutions were prepared according to the Clinical and Laboratory Standards Institute (CLSI) broth microdilution guidelines (29). Antifungal susceptibility and drug interactions testing were performed by using the broth microdilution checkerboard (2-dimensional, 8-by-12) method (27). The final concentrations of the antifungal agents ranged from 0.125 to 8 mg/L for amphotericin B and 0.008 to 8 mg/L for terbinafine. To assess the nature of *in vitro* interactions between

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amphotericin B and terbinafine, the data obtained were analyzed using non-parametric approaches of the following two no (zero)-interaction theories: the Loewe additivity defined as fractional inhibitory concentration (FIC), and the Bliss independence (BI) parameter obtained from response surface analysis (30), as described previously (27). Drug interactions were defined as synergistic if the FIC index was <1, additive if the FIC index was = 1, and antagonistic if the FIC index was > 1 (31). The BI drug interactions were considered synergistic if $\Delta E > 0$ (positive ΔE), indifferent if $\Delta E=0$, or antagonistic if $\Delta E < 0$ (negative ΔE) (32). All experiments were performed in three independent replicates on different days. All data analyses were performed by using the software package GraphPad Prism, version 5.0, for Windows (GraphPad Software, San Diego, CA, USA). A P value of ≤ 0.05 was considered significant (two-tailed).

The mean MICs (and ranges) for amphotericin B across all isolates were 4.46 (0.125 to >8) mg/L, and 0.86 (0.16 to >8) for terbinafine, respectively (Table 1). For the amphotericin B and terbinafine combinations, the geometric mean FIC indices, in increasing order, were: 0.41 for *F. monophora* (∑FIC ranging 0.25 to 0.5), 0.5 for *E. spinifera* (∑FIC ranging 0.25 to 1), 0.63 for *E. dermatitidis* (∑FIC ranging 0.25 to 1), 0.7 for *C. carrionii* (∑FIC ranging 0.5 to 1), 0.72 for *P. verrucosa* (∑FIC ranging 0.5 to 1), 0.76 for *F. nubica* (∑FIC ranging 0.25 to 1), 0.76 for *F. pedrosoi* (∑FIC ranging 0.5 to 1), and 1 for *C. arxii* (n=1), which indicates synergy and additivity for these strains. However, antagonism was noted in *V. botryosa* isolates with a mean FIC value of 1.4 (∑FIC ranging 1 to 2).

The Bliss independence drug interaction analysis for the amphotericin B and terbinafine combination resulted in a synergistic interaction for 71.74% (33/46) of the strains tested. The degree of synergy was the highest among the *C. carrionii* strains (SUM ΔE 1546%), followed by *F. monophora* (SUM ΔE 1140%), *F. pedrosoi* (SUM ΔE 775%), *E. spinifera* (SUM ΔE 515%), *P. verrucosa* (SUM ΔE 481%), *E. dermatitidis* (SUM ΔE 449%), and *C. arxii* (SUM

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ΔE 90 %), respectively. The strongest synergistic interactions were found at amphotericin B and terbinafine concentrations range of 0.125 to 0.5 µg/ml and 0.008 to 0.5 µg/ml, respectively. Examples of Bliss independence 3-dimentional plots for the synergistic and antagonistic interaction of amphotericin B and flucytosine are shown in figure 1.

Overall, our results show that the amphotericin B and terbinafine combination has synergistic effects against majority of melanized fungi associated with CBM, including *C. carrionii*, *C. arxii*, *E. dermatitidis*, *E. spinifera*, *F. monophora*, *F. nubica*, *F. pedrosoi*, and *P. verrucosa.* The results of FIC analysis were supported by response surface analysis using Bliss independence no-interaction model for the isolates tested.

Terbinafine is one of most commonly used antifungal agents in treatment of patients with CBM (18), due to its high degree of effectiveness and tolerability. In an athymic murine model of CBM caused by *F. pedrosoi*, terbinafine, especially at the highest dose, was able to reduce the inflammatory response to the infection to levels similar to those with azoles (33), although total cure in patients with CBM remains difficult to achieve (26, 34). On the other hand, various formulations of amphotericin B have been developed and are now available in most countries (35). The compound is nevertheless not recommended as a first-line therapy in chronic infections because of its adverse effects, such as nephrotoxicity, neurotoxicity, hematological side-effects, and allergic reactions (36). However, use of combination therapy can reduce cost- and toxicity-related effects and may prevent the emergence of resistance (35). Combination therapy is also recommended in salvage therapy scenarios for patients with antifungal resistant and invasive refractory mycoses (37). Few studies have reported data on the efficacy of antifungal combination therapy in the treatment of severe and refractory CBM. Treatment with amphotericin B and subsequent combination of flucytosine and itraconazole was shown to be effective in a patient with a CBM-like infection caused by *P. verrucosa* (23). Combinations of itraconazole with flucytosine (24, 25), and itraconazole with terbinafine

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have also shown better efficacy than monotherapy for CBM caused by *F. pedrosoi* (26), and *F. monophora* (38). In general, however, also combination therapy still is inadequate, requiring long-term therapy at high doses, and treatment failure of CBM remains common. The *in vitro* results obtained in the present study confirmed that terbinafine is active against the majority of strains tested. Of the nine-species investigated, *Cladophialophora carrionii* and *Phialophora verrucosa* were more sensitive to terbinafine than species of *Fonsecaea* and *Exophiala*. The three species of *Fonsecaea* showed similar degrees of susceptibility. As in previous reports (39-41), in our study *E. spinifera* and *V. botryosa* were resistant to terbinafine and amphotericin B when used alone. Although synergistic interaction was found in a combination setting for *E. spinifera*, the combination of terbinafine and amphotericin B, exhibited indifferent interaction for tested isolates of *Veronaea botryosa*. In the current study, a wide range of amphotericin B MICs (0.125 to >8 mg/L) was observed for agents of CBM. *Exophoiala dermatitidis* and *P. verrucosa* were the species being relatively susceptible, which is in agreement with previous studies (27, 42). When terbinafine and amphotericin B were used in combination, the highest synergy was shown for *F. monophora*, and *E. spinifera*, followed by *E. dermatitidis*, *C. carrionii*, *F. nubica*, and *F. pedrosoi.* Our findings agree with those of Daboit *et al.* (43), demonstrating *in vitro* synergy between amphotericin B and terbinafine for *Fonsecaea* spp., *C. carrionii*, and *P. verrucosa.* Biancalana *et al.* (44), also reported 96.5% *in vitro* synergy between terbinafine and amphotericin B against clinical isolates obtained from cases of phaeohyphomycosis and CBM, including *F. pedrosoi*, *Curvularia* spp., *Exophiala jeanselmei*, *Alternaria alternata*, *Cladophialophora bantiana*, and *Bipolaris* spp. In contrast, Yu *et al.* (45), did not find interaction for this combination against agents of CBM.

Overall, the management of CBM is complicated and requires long-term antifungal therapy, surgery, thermotherapy, chemotherapy, or combinations of these (3). Importantly, the clinical

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experience with posaconazole and voriconazole is limited for CBM. However, the good *in vitro* activities and *in vivo* efficacies of these agents against dematiaceous fungi (46-48), together with the tolerance of the drug in long-term therapies, suggest that further studies are warranted to evaluate the potential use of these drugs for treatment of CBM.

Collectively, the present study demonstrated that the combination with terbinafine allows a significant reduction of amphotericin B MICs and could be an option for severe or unresponsive cases of CBM, particularly in cases due to *Fonsecaea* and *Cladophialophora* species, and in *E. spinifera*. Our results therefore suggest that a combination of amphotericin B and terbinafine may have a promising role in the treatment of CBM.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. The authors alone are responsible for the content and the writing of the paper.

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LEGENDS

Table 1. Minimum inhibitory concentration (MIC), Fractional inhibitory concentration (FIC) indices and Bliss independence results for the *in vitro* combination of amphotericin B (AmB) plus terbinafine (TBF) against melanized fungi associated with chromoblastomycosis.

FIGURE 1. Interaction surfaces obtained from response surface analysis of Bliss independence no-interaction model for *in vitro* combination of amphotericin B (AmB) plus terbinafine (TBF). The X- and Y- axis represent the efficacy of AmB and TBF, 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. The highest level of

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synergistic interactions was found between 0.25 µg/ml amphotericin B and terbinafine concentrations range of 0.008 to 0.5 µg/ml.

A. Synergistic interaction of AmB plus TBF against an *Exophiala spinifera* strain (CBS 194.61) (MIC AmB 0.5 µg/ml, MIC TBF >8 µg/ml). The mean ΔE ± standard error of the mean and sum ΔE were 5.36% ± 1.81% and 450.11%, respectively. The highest level of synergistic interactions was found between 0.25 µg/ml amphotericin B and terbinafine concentrations range of 0.008 to 0.5 µg/ml.

B. Antagonistic interaction of AmB plus TBF against a *Veronaea botryosa* strain (CBS 122826) (MIC AmB 1 µg/ml, MIC TBF 4 µg/ml). The mean ΔE ± standard error of the mean and sum ΔE were −3.23% ± 1.70% and −271.70%, respectively.

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Table 1. Minimum inhibitory concentration (MIC), Fractional inhibitory concentration (FIC) indices and Bliss independence results for the *in vitro* combination of amphotericin B (AmB) plus terbinafine (TBF) against melanized fungi associated with chromoblastomycosis.

	Fungal strains	Strain no.	Source	Origin	TBF (0.008 to 8)	AmB (0.125 to 8)	FIC index	Bliss independence index
					MIC (μg/ml)			
1	*Cladophialophora carrionii*	CBS 131844	Human, Chromoblastomycosis	China	0.031	1	0.75	111.3
2		CBS 131854	Human, Chromoblastomycosis	Madagascar	0.063	8	0.5	69.24
3		CBS 131833	Human, Chromoblastomycosis	China	1	8	1	-4.48
4		CBS 131847	Human, Chromoblastomycosis	China	0.031	4	0.5	410.73
5		CBS 160.54	Human, Chromoblastomycosis	Australia	0.031	1	0.75	-8.29
6		CBS 859.96	Dry plant debris	Venezuela, arid zone w of Coro	0.016	1	0.75	-40.42
7		CBS 863.96	Dry plant debris	Venezuela, arid zone w of Coro	0.016	2	0.75	6.37
8		CBS 131736	Soil	Venezuela, arid zone w of Coro	0.031	4	0.5	75.15
9		CBS 860.96	Dry plant debris	Venezuela, arid zone w of Coro	0.016	4	0.75	893.89
10		CBS 861.96	Dry plant debris	Venezuela, arid zone w of Coro	0.125	8	1	143.51
11	*Cladophialophora arxii*	CBS 102461	Human, Brain abscess	USA	0.5	4	1	90.2
12	*Exophiala. dermatitidis*	CBS 120542	Human or animal, Stool	Slovenia	0.5	4	0.5	138.26
13		CBS 120562	Human, Keratitis	USA	0.5	0.25	1	151.7
14		CBS 120473	Human, Brain	USA	0.25	0.5	1	-4736
15		CBS 424.67	Human, Chromoblastomycosis	Germany	0.5	0.125	1	-11.78
16		CBS 550.9	Human, Sputum, Cystic fibrosis	Germany	0.031	2	1	258.1
17		CBS 126590	Human, Sputum, Cystic fibrosis	Netherlands	0.5	1	0.25	89.2
18		CBS 120550	Steam Bath	Austria	0.5	2	0.5	133.05
19		CBS 120483	Flying fox's faeces	Thailand	0.25	4	0.25	346.03
20		CBS 109138	Hall of sauna complex	Netherlands	0.5	4	1	-43.35
21	*Exophiala spinifera*	CBS 899.68	Human, Nasal granuloma	USA	2	2	1	62.94
22		CBS 269.28	Human, Chromoblastomycosis	Unknown	0.5	8	0.5	64.9

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23		CBS 194.61	Human, Systemic mycosis	India	0.5	>8	0.25	450.11
24	***Fonsecaea monophora***	CBS 117236	Human, Brain abscess	USA	0.5	8	0.5	305
25		CBS 269.37	Unknown, Chromoblastomycosis	Unknown	0.25	8	0.5	209.7
26		CBS 117238	Unknown, Brain	England	5	8	0.25	49.7
27		CBS 122742	Human, Chromoblastomycosis	China	0.5	8	0.5	14.98
28		CBS 100430	Human, Brain	Africa	0.5	8	0.5	311.02
29		CBS 102229	Decaying vegetable	Brazil,, Parana, Piraquara	0.5	4	0.5	27.6
30		CBS 289.93	Animal, Lymph node, aspiration-biopsy	Netherlands	8	8	0.25	526.72
31	***Fonsecaea nubica***	CBS 277.29	Human, Chromoblastomycosis	Brazil	1	4	1	154.1
32		CBS 444.62	Human, Chromoblastomycosis	Suriname	0.5	8	1	-7.07
33		CBS 122733	Human, Chromoblastomycosis	China	0.25	4	1	-69.4
34		CBS 269.64	Human, Chromoblastomycosis	Cameroon	0.5	8	0.75	-79.6
35		CBS 125198	Human, Chromoblastomycosis	China	0.25	8	0.25	-223.17
36		CBS 127264	Human, Chromoblastomycosis	Mexico	1	4	1	-271.7
37	***Fonsecaea pedrosoi***	CBS 102247	Human, Chromoblastomycosis	Brazil,Parana	0.5	4	0.5	123.76
38		CBS 285.47	Human, Chromoblastomycosis	Puerto Rico	0.5	4	0.5	298.9
39		CBS 122739	Human, Chromoblastomycosis	Mexico	0.5	4	1	114.3
40		CBS 117910	Human, Chromoblastomycosis	Venezuela	0.5	4	1	297.27
41		CBS 671.66	Soil	Venezuela	0.5	2	1	64.1
42	***Phialophora verrucosa***	CBS 120349	Plant	China	0.5	4	1	123.76
43		CBS 262.93	Exudate from right hand (human or animal)	Germany	0.016	0.5	0.5	52941
44		CBS 115.89	Disseminated (human or animal)	Lybia	0.25	8	0.75	-47.99
45	***Veronaea botryosa***	CBS 122826	Railway tie treated with creosote for 20 years	Brazil	1	4	2	-271.7
46		CBS 121506	Cutaneous lesion, Wrist	Japan	>8	2	1	160.1

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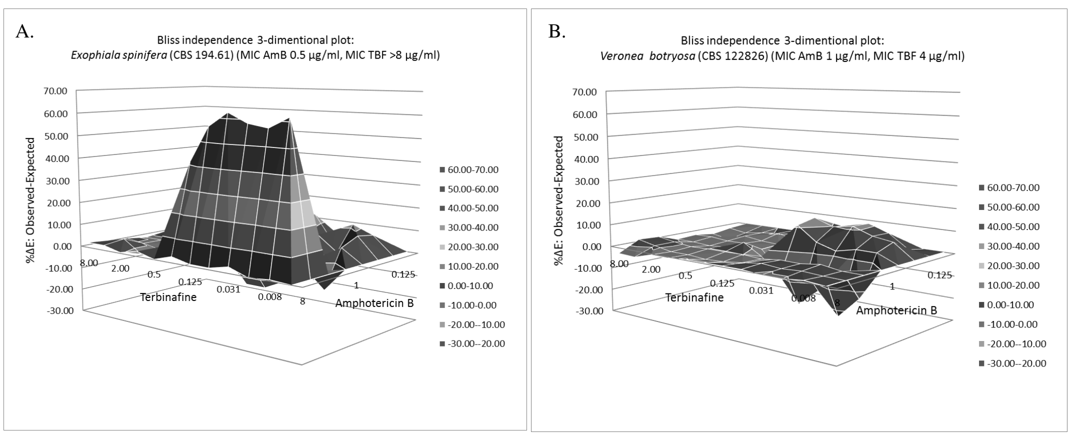

FIGURE 1. Interaction surfaces obtained from response surface analysis of Bliss independence no-interaction model for *in vitro* combination of amphotericin B (AmB) plus terbinafine (TBF). The X- and Y- axis represent the efficacy of AmB and TBF, respectively. The Z- axis is the ΔE in %. The 0-plane 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.

A. Synergistic interaction of AmB plus TBF against an *Exophiala spinifera* strain (CBS 194.61) (MIC AmB 0.5 µg/ml, MIC TBF >8 µg/ml). The mean ΔE ± standard error of the mean and sum ΔE were 5.36% ± 1.81% and 450.11%, respectively. The highest level of synergistic interactions was found between 0.25 µg/ml amphotericin B and terbinafine concentrations range of 0.008 to 0.5 µg/ml.

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