Has a triple combination better activity than doubles against multiresistant fungi? Experimental *in vitro* evaluation

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Running title: Triple combination against multiresistant fungi

ABSTRACT

We have evaluated the *in vitro* interactions of amphotericin B, voriconazole and anidulafungin in double and triple combinations against four species of multiresistant fungi, i.e. *Fusarium solani, Lomentospora prolificans, Scopulariopsis brevicaulis* and *Scopulariopsis brumptii*. In general, amphotericin B combined with anidulafungin was the most synergistic, especially against *F. solani* (87.5%) when low concentrations of AMB were used i.e. 0.125 – 0.5 µg/mL. The less active combination was AMB+VRC, being the lowest percentage of synergy against *S. brevicaulis* (18.2%) and, in general, high concentrations of both antifungals were needed in order to obtain synergy. The triple combination was also highly synergistic against *F. solani* and *S. brevicaulis,* especially when the lowest concentrations of AMB i.e. were used, suggesting that the use of combined therapies would reduce the toxicity of the therapy. The triple combination was more effective than the double combinations in some cases, but not against all the strains, suggesting that the administration of three drugs is not always useful in the treatment of infections due to multiresistant fungi.

Keywords: antifungal resistance, antifungal combinations, *F. solani, L. prolificans, S. brevicaulis, S. brumptii, in vitro,* FICI

1. Introduction

Fungal opportunistic infections have increased over the past two decades as a result of the rising number of immunocompromised patients. Among the moulds, clinically important species of *Fusarium*, *Scedosporium* and *Scopulariopsis* are intrinsically resistant to antifungal drugs, including the most recent ones such as voriconazole (VRC), posaconazole (PSC) or echinocandins. The infections by multiresistant fungi have increased in recent years and the poor outcome of monotherapies together with the high mortality rates makes necessary to explore new therapies.

Fusarium solani species complex comprise hyaline moulds, widely found in nature that cause a broad spectrum of human infections. The most challenging and life-threatening disease is disseminated infection with an estimated mortality rate of up to 75%. Management of fusariosis has changed over the last decade, with an increasing use of VRC and combination therapies that have had a better outcome, although the mortality rate remains high [1].

Lomentospora prolificans (formerly *Scedosporium prolificans*) is a ubiquitous filamentous fungus able to produce disseminated disease. [2]. VRC is the preferred treatment, however, these infections are usually associated with poor outcomes and mortality rates of more than 75% despite the treatment [3].

Scopulariopsis are usually saprobic and commonly isolated from soil, air, plant debris and moist indoor environments [4]. *Scopulariopsis* is associated mainly with nail infections, but it occasionally causes cutaneous lesions following trauma or surgery, invasive diseases, and disseminated infections [5] in all types of patients. These are

almost invariably fatal, mainly due to the underlying conditions, and a high level of resistance of this fungus to conventional antifungal agents. Although *S. brevicaulis* is the most prevalent species, other species of the genus, like *S. brumptii* have been associated with human disease too [4].

In vitro studies have repeatedly shown that these species are resistant to almost all the current antifungal drugs [4,6]. VRC is recommended as the first-line treatment for fusariosis and scedosporiosis, but a treatment regimen has not been established for infections caused by *Scopulariopsis* spp. [7] because infections are rare. Most patients diagnosed with a fungal infection are usually treated first with amphotericin B (AMB), its lipid formulations or azoles with poor outcome. Combined therapy is considered to increase efficacy, minimize toxicity and lower the cost of the therapy by reducing the dosages of individual drugs.

The limited efficacy of the available antifungal drugs against these important fungal pathogens makes it crucial to find alternative therapies. For this reason, the objective of the present study was to investigate the *in vitro* interactions among AMB, AFG and VRC in double, as well as in triple combinations against relevant multiresistant fungi such as *F. solani, S. brevicaulis, S. brumptii* and *L. prolificans.* We chose three drugs belonging to some families of antifungals which have different mechanisms of action, hypothesizing that combinations might produce synergistic interactions against such pathogens.

2. Materials and methods

2.1. Drugs and strains

The *in vitro* activity of pure powders of AMB (Sigma Chemical Co. St. Louis, USA), VRC (Pfizer Inc., Madrid, Spain) and AFG (Pfizer Inc., Madrid, Spain), was tested alone, and in double and triple combinations against 38 fungal isolates, i.e. 11 *L. prolificans*, 8 *F. solani*, 11 *S. brevicaulis* and 8 *S. brumptii*. Strains belonging to the *F. solani* species complex were previously identified by amplification and sequencing of the nuclear ribosomal internal transcribed spacer (ITS) and translation elongation factor 1 α (EF-1α), those of *L. prolificans* were identified by sequencing the ITS and a fragment of the beta-tubulin gene (TUB) and the *Scopulariopsis* isolates were identified by sequencing the D1/D2 domains of the 28S rRNA gene and EF-1α. Three reference strains, *Candida krusei* ATCC 6258, *C. parapsilosis* ATCC 22019 and *Aspergillus fumigatus* ATCC MYA 3626, were included as quality controls. The isolates were grown at 30 ºC on potato dextrose agar (PDA) until sporulation occurred in the case of filamentous fungi i.e., from 7 to 10 days depending on the species. Inocula were obtained by flooding the plates with sterile saline and conidia were harvested with a sterile pipette. The suspensions were adjusted to the desired concentrations by haemocytometer counts and viability assessed by placing 10-fold dilutions onto PDA plates [11].

2.2. Antifungal activity assays

Single susceptibility testing of the isolates was carried out following the broth microdilution method according to the CLSI document M38-A2 [12]. After 48 h at 35 ºC, MICs of AMB and VRC were visually read with the aid of an inverted mirror and corresponded to the 100% of growth inhibition, while the MEC of AFG was read with

the aid of a stereomicroscope (x40 magnification) as the minimum concentration to produce an abnormal hyphal growth.

The activity of double combinations i.e, AMB + VRC, AMB + AFG and VRC + AFG, was tested by a 7x10 two-dimensional checkerboard with two-fold dilutions of each drug, as previously described [13]. For the combinations of VRC with either AMB or AFG, the final concentrations of the antifungal agents were 0.5 to 256 µg/mL for VRC (i.e. 10 twofold dilutions), 1 to 64 µg/mL for AMB (0.06 to 4 µg/mL against *F. solani*) (i.e. 7 twofold dilutions) and 2 to 128 µg/mL for AFG (i.e. 10 twofold dilutions). For the double combination of AMB plus AFG, the final concentrations of the antifungal agents were 0.125 to 64 µg/mL for AMB (0.016 to 8 µg/mL against *F. solani*) (i.e. 10 twofold dilutions) and 2 to 128 µg/mL for AFG (i.e. 10 twofold dilutions).

The concentrations of the drugs were selected on the basis of the previously determined MICs and MECs.

The triple combination was tested by a three-dimensional checkerboard technique, i.e. a 7x10 checkerboard with two-fold dilutions of AMB and VRC were set up as described above for the double combinations being AFG added to each well at constant final concentration i.e. 0.06, 0.25, 1 and 4 µg/mL.

For the combination AMB+VRC, 100% of growth inhibition or MIC-0 was chosen as endpoint. However, the most appropriate endpoint for echinocandins against moulds has been determined to be the MEC, which corresponds to MIC-2 (50% growth inhibition) [14]. Therefore, considering that the endpoint for the combined drugs must

be the same, in those combinations containing AFG i.e. AMB+AFG, VRC+AFG and AMB+VRC+AFG, we used the MIC-2.

The fractional inhibitory concentration indices (FICI) of the double combinations were calculated as follows: FICI = (MIC_{drugA} in combination/MIC_{drugA} alone) + (MIC_{drugB} in combination/MIC_{drugB} alone). For the triple combination, the third parameter MIC_{drugC} in combination/MIC_{drugC} alone, was added. Drug interactions were defined as synergistic if the lowest FICI was ≤ 0.5, no interaction (i.e., no interaction) if the lowest FICI was > 0.5 and ≤ 4, and antagonistic if the highest FICI was > 4 [15]. For the calculations, the high off-scale MICs were converted to the next highest concentration. Every isolate was assayed twice.

3. Results

3.1. Combinations against F. solani

Table S1 summarizes the MICs of AMB, AFG and VRC alone, the lowest FICIs and the corresponding MICs of the drugs in combination against the *F. solani* isolates. All the strains displayed remarkably high MICs for VRC (16 to > 256 μg/mL) and AFG (≥ 128 μg/mL) but, by contrast, they showed lower AMB MICs (1 to 8 μg/mL). All the double combinations showed a high percentage of synergy against this species, AFG plus VRC and AMB plus AFG being the most active (87.5%). AMB combined with VRC showed 62.5% of synergy, but concentrations of VRC ≥ 16 μg/mL together with 0.25 – 0.5 μg/mL of AMB were needed to achieve that. The triple combination showed 87.5% synergy. Antagonism was not observed in any case.

3.2. Combinations against L. prolificans

Results of the *in vitro* susceptibility testing of all interactions for every *L. prolificans* strain are given in supplementary material (Table S2). The highest percentage of synergy was observed for the combination AMB + AFG (72.7%), while the lowest was for the combination of AMB + VRC (45.5%), for which very high concentrations of AMB and VRC were needed to achieve the lowest FICI in some strains. For example, for strain FMR 9799, maximum synergy was observed when 4 μg/mL of AMB was combined with 16 μg/mL of VRC; however, these concentrations are not recommended due to their possible toxicity (Table S2).

When AFG was combined with VRC, synergistic interactions were found against 5 of the 11 *L. prolificans* isolates tested (54.5%). The interaction between the three drugs was synergistic for 7 of them (63.6%) and indifferent for 4 strains (36.4%) (Table 1).

In general, the most synergistic triple combination was with the lowest concentrations of AMB (1 µg/mL) (Table S2). The benefit of the triple combination over the double ones was clearly demonstrated in the strains FMR 6641, FMR 6721 and FMR 9798, since the synergistic effect in triple combinations was achieved at lower concentrations of each individual drug in comparison to the double ones. However, this benefit of the triple combination over the doubles was not so evident against strains FMR 9797 and FMR 9800, especially in comparison to the combination AMB+AFG, where concentrations for reaching the highest synergy were lower than those needed in the triple combination. Antagonism was not observed in any case.

3.3. Combinations against S. brevicaulis

The MICs of AMB, VRC and AFG against each strain of *S. brevicaulis*, and the lowest FICIs achieved with the double and triple combinations are summarized in Table S3. The double combination with the highest percentage of synergy was AFG + AMB (81.8%) (Table 1). AFG combined with VRC produced synergistic interactions against 8 of the 11 strains tested (72.7%) and indifference against 3 strains (27.3%) (Table 1). Interestingly, with this combination, antagonism was also observed against three strains of *S. brevicaulis* i.e. FMR 12246, FMR 12260 and FMR 12270, indicating that the same combination can give contrasting results depending on the concentrations used (data not shown) and as previously reported [13,16]. For these isolates, indifference was obtained with AFG at concentrations of 4 - 16 µg/mL, whereas antagonistic interactions were observed with higher concentrations of AFG (≥ 32 µg/mL) in combination with ≥ 2 µg/mL of VRC.

The triple combination was 81.8% synergistic, although it did not show any advantage over the AMB plus AFG in some cases. For example, against FMR 12258, the lowest FICI of the triple combination was achieved when AMB, VRC and AFG concentrations were 2, 4 and 4 µg/mL, respectively. However, the combination AMB + AFG was synergistic with 0.125 µg/mL of AMB plus 4 µg/mL of AFG, suggesting that the double combination is as effective as the triple.

3.4. Combinations against S. brumptii

Table S4 summarizes the MICs of the antifungal drugs and the FICIs of the combinations against the 8 *S. brumptii* strains. The double combination with the highest synergy was AMB plus AFG (62.5%), followed by AMB plus VRC (37.5%) and AFG plus VRC (37.5%). The triple combination was synergistic in 4 strains (50%), having, in most cases, the lowest FICIs with the lowest concentration of either AMB or VRC and being better than the double combinations (Table S4).

Overall, AMB plus AFG was the most synergistic combination against the four multiresistant species, being even better than the triple combination. On the contrary, the combination that showed less synergistic interactions was AMB plus VRC.

4. Discussion

The prevalence of infections caused by multiresistant fungi has increased in recent years and is becoming an important matter of concern due to their difficult management and poor outcome. Monotherapies usually fail in the treatment of these infections because of the limited range of activity of the current antifungals. Since antifungal compounds are not effective enough for most infections, the combination of surgery and antifungal drugs is a common choice [17]. A combination of two drugs is recommended for the treatment of some fungal diseases like cryptococcal meningoencephalitis [18] and there is little clinical experience with triple combinations, although they have sometimes been used as salvage therapy, as in some refractory aspergillosis [19–21]. Triple combinations might be useful for some multiresistant infections. In the present study, we chose four of the most resistant fungi, against which no standard therapy has been established. We found high synergy for the combinations tested, especially for AMB + AFG, which, in some cases, was better than the triple combination.

Against *F. solani*, in particular, synergy was high for all the combinations tested, i.e. nearly 90% for AFG + VRC, AMB + AFG and the triple combination, suggesting a potential role of these combinations in the treatment of fusariosis. This agrees with the results of a previous *in vitro* study that reported additive to synergistic interactions between AMB and VRC against *F. solani* isolates [22] and some degree of efficacy of the same combination in a murine model of disseminated infection by this fungus [23]. However, for most of the strains tested, synergy was achieved at concentrations of VRC that were not in the range of the levels achievable in serum, i.e. 16 or 32 µg/mL (Table S1).

AMB was synergistic with AFG in this study, and with CFG against the 82% of *F. solani* strains tested in another study [22]. The latter combination was reported to be effective in reducing fungal burden in a murine model of disseminated *F. solani* infection [24]. However, the combination of AMB with micafungin (MFG) did not show *in vivo* efficacy against this fungus, suggesting that the effect of the combinations of AMB with echinocandins seem to depend on which echinocandin is tested [23]. AFG, when used alone, has poor *in vitro* activity against *F. solani* but the combination of VRC with AFG or MFG has shown synergy *in vitro* and in murine models [23,25,26]. In the clinical setting, it is of note that the outcome of invasive fusariosis has significantly improved since the recent use of VRC and combined therapies [1]. Some clinical cases have reported favourable responses in patients with haematological malignancies treated with AMB or its lipid formulations plus CFG or VRC, highlighting the potential of these combinations [27].

The combined activity of antifungal agents against *L. prolificans* has rarely been evaluated. It has been demonstrated that VRC in combination with AMB or AFG showed synergy in a small percentage of strains while AMB plus AFG produced indifferent interactions in all the cases [28]. Our results are quite different since a high rate of synergy was observed for this latter combination while around 50% of synergy/indifference was obtained for the other double combinations assayed. The present results correlate better with Yustes and Guarro, who reported synergistic interactions between AMB and MFG against 14 of 17 (82%) *L. prolificans* strains tested [29]. Previously, only a triple antifungal combination had been tested *in vivo* against *L. prolificans* [30]. In that study, AMB, MFG and VRC were tested alone, in double and in triple combinations in a murine model of disseminated infection after

showing *in vitro* synergy only for the triple combination; however, the *in vivo* efficacy of that triple combination was worse than for MFG plus AMB or VRC [30]. Similar combinations have been used against *L. prolificans* infections with some therapeutic success with VRC plus CFG [31], a result that agrees with our *in vitro* interaction results, but not for the combinations VRC + LAMB and itraconazole (ITC) plus MFG [32]. Due to the small number of clinical cases, conclusions about the *in vitro* – *in vivo* correlation cannot be made.

Several antifungal combinations have been tested *in vitro* against *S. brevicaulis*, resulting in a high percentage of indifference for the combination AMB + VRC, and more than 50% of synergy for CFG + AMB [33], results that agree with ours. Combined antifungal therapy is often used for the treatment of *Scopulariopsis* infections with different outcomes, but combinations of more than two drugs are rarely used. An AMB lipid complex in combination with ITC and VRC plus CFG have shown *in vivo* synergy in patients with haematological malignancies [34,35], but other studies report therapeutic failure of the latter combination and for LAMB with CSP, VRC or MFG [36]. To our knowledge, only one triple combination has been tested *in vitro* against *Scopulariopsis* and *Microascus* species. PSC, CFG and TBF showed synergy against 100% of the *S. brevicaulis* strains [37]. In our case, the triple combination achieved 81.8% synergy against *S. brevicaulis* and a modest 50% in the case of *S. brumptii*. Animal studies are needed to prove the *in vivo* efficacy of these combinations.

In the present study, we used a checkerboard method that, although it has not been standardized for testing moulds, has the advantage of simplicity in performance and

306 interpretation. Some of our results are controversial in comparison to other *in vitro*
307 studies testing the same double combinations. This can be due to many models and
308 approaches having been described for testing *in vitro* drug interactions. It is also
309 known that, even when the same methodology is used for testing the *in vitro* activities
310 of drug combinations, variable conclusions might be made, depending on the way
311 data is analyzed and interpreted.
312
313 The triple combination has shown a high percentage of synergy against the four
314 species (50 – 87.5 %). Such synergistic effect was achieved in 81.4% of strains at
315 therapeutical concentrations of each individual drugs i.e., < 2 µg/mL of AMB and
316 VRC and ≤ 4 µg/mL in the case of AFG suggesting that the triple combination could
317 be used in the treatment of multiresistant infections, allowing the reduction of
318 amphotericin B and voriconazole doses, the antifungals that show more side effects
319 and toxicity.
320
321 In conclusion, our data demonstrates that powerful interactions are achievable with
322 AMB, VRC and AFG against clinically relevant multiresistant fungi and their
323 combinations show interesting results. We have also found that the triple combination
324 is not always better than a double one. Further animal studies are required to
325 demonstrate their possible efficacy.
326
327

Declarations

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing Interests: None.

Ethical Approval: Not required

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Supplementary data

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Table

Table 1. Median (range) MIC, MEC (µg/mL) and FICI values of the interaction of double and triple combinations against *F. solani, L. prolificans, S. brevicaulis* and *S. brumptii*.

Species	Single susceptibility[a]						Interaction assay[b]											
	MIC-0 median (range) (µg/ml)			MIC-2 median (range) (µg/ml)			AMB+VRC			AFG+VRC			AFG+AMB			AMB+VRC+AFG		
	AMB	VRC	AFG	AMB	VRC	AFG	FICI	Median (range) concentrations in synergistic combinations	No (%) of strains for which the combination showed synergism	FICI	Median (range) concentrations in synergistic combinations	No (%) of strains for which the combination showed synergism	FICI	Median (range) concentrations in synergistic combinations	No (%) of strains for which the combination showed synergism	FICI	Median (range) concentrations in synergistic combinations	No (%) of strains for which the combination showed synergism
F. solani (n = 8)	2 (1 – 8)	16 - >256	>128 (>128 - >128)	1 (0,5 – 2)	8 (4 – 8)	128 - >128	0.31 (0.2-1.5)	0.25 (0.125-0.5) / 16 (2-32)	5 (62.5)	0.22 (0.15 – 0.66)	16 (2-64) / 0.5 (0.5-2)	7 (87.5)	0.21 (0.11 – 0.52)	0.09 (0.016-0.25) / 8 (2-64)	7 (87.5)	0.42 (0.29 – 0.71)	0.125 (0.06-0.25) / 1 (0.5-2) / 1 (0.06-4)	7 (87.5)
L. prolificans (n = 11)	128 (4 – 128)	32 (32 – 64)	>128 (>128 - >128)	16 (2 – 64)	8 (4 – 16	8 (4 – 16)	0.52 (0.22 – 1.05)	6 (2-32) / 8 (2-16)	5 (45.5)	0.5 (0.32 – 1.13)	4 (2-4) / 2 (0.5-4)	6 (54.5)	0.35 (0.11 – 0.6)	0.75 (0.125-4) / 2 (2-8)	8 (72.7)	0.41 (0.22 – 0.92)	1 (0.5-2) / 2 (0.5-4) / 0.63 (0.06-4)	7 (63.6)
S. brevicaulis (n = 11)	32 (8 – 128)	64 (16 – 256)	>128 (>128 - >128)	8 (4 – 32)	16 (4 – 32)	8 - >128	0.58 (0.25 – 0.82)	4 (4-8) / 40 (8-64)	2 (18.2)	0.21 (0.05 – 1.13)	8 (2-64) / 1 (0.5-8)	8 (72.7)	0.28 (0.02 – 1.63)	0.75 (0.125-8) / 8 (2-32)	9 (81.8)	0.39 (0.24 – 0.82)	2 (1-4) / 3 (0.5-8) / 1 (0.06-4)	9 (81.8)
S. brumptii (n = 8)	16 (4 – 128)	16 (4 – 32)	>128 (>128 - >128)	8 (2 – 16)	4 (2 – 4)	16 (8 – 32)	0.49 (0.25 – 0.78)	2 (1-16) / 4 (2-8)	3 (37.5)	0.94 (0.32 – 1.59)	3 (2-4) / 0.75 (0.5-1)	3 (37.5)	0.44 (0.21 – 1.13)	0.5 (0.125-2) / 4 (2-8)	5 (62.5)	0.54 (0.32 – 0.91)	1 (1-2) / 0.75 (0.5-1) / 0.625 (0.06-4)	4 (50)

[a] MICs and MECs were determined following the recommendations of the document M38-A2 of CLSI [12]. [b] Interaction assay was performed as previously described [13]. AMB, amphotericin B; VRC, voriconazole; AFG, anidulafungin; FICI, fractional inhibitory concentration index.