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Does a triple combination have better activity than double combinations against multiresistant fungi? Experimental in vitro evaluation

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ABSTRACT

In this study, the in vitro interactions of amphotericin B (AmB), voriconazole (VRC) and anidulafungin (AFG) in double and triple combinations against four species of multiresistant fungi (*Fusarium solani*, *Lomentospora prolificans*, *Scopulariopsis brevicaulis* and *Scopulariopsis brumptii*) were evaluated. In general, AmB combined with AFG was the most synergistic, especially against *F. solani* (7/8; 87.5%) when low concentrations of AmB were used, i.e. 0.125–0.5 μg/mL. The least active combination was AmB + VRC, with the lowest percentage of synergy against *S. brevicaulis* (2/11; 18.2%) and, in general, high concentrations of both antifungals were needed to achieve synergy. The triple combination was also highly synergistic against *F. solani* and *S. brevicaulis*, especially when the lowest concentrations of AmB were used, suggesting that use of combined therapies would reduce the toxicity of therapy. The triple combination was more effective than the double combinations in some cases, but not against all strains, suggesting that administration of three drugs is not always useful in the treatment of infections due to multiresistant fungi.

1. Introduction

Opportunistic fungal 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 antifungals such as voriconazole (VRC), posaconazole and echinocandins. Infections caused by multiresistant fungi have increased in recent years and the poor outcome of monotherapies together with high mortality rates makes it necessary to explore new therapies.

Fusarium solani species complex comprises 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 increasing use of VRC and combination therapies that have led to better outcomes, 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 >75% despite treatment [3].

Scopulariopsis spp. are usually saprobic and are commonly isolated from soil, air, plant debris and moist indoor environments [4]. *Scopulariopsis* is mainly associated 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 underlying patient conditions as well as the high level of resistance of this fungus to conventional antifungal agents. Although *Scopulariopsis brevicaulis* is the most prevalent species, other species of the genus, such as *Scopulariopsis brumptii*, have also been associated with human disease [4].

In vitro studies have repeatedly shown that these species are resistant to almost all of 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] as these infections are rare. Most patients diagnosed with a fungal infection are treated first with amphotericin B (AmB), its lipid formulations or azoles, with poor outcomes. Combined therapy is considered to increase efficacy, to minimise toxicity and to lower the cost of 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

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therapies. For this reason, the objective of the present study was to investigate the in vitro interactions among AmB, anidulafungin (AFG) and VRC in double and triple combinations against relevant multiresistant fungi, including *F. solani*, *S. brevicaulis*, *S. brumptii* and *L. prolificans*. Three drugs belonging to families of antifungals with different mechanisms of action were chosen, hypothesising that combinations might produce synergistic interactions against such pathogens.

2. Materials and methods

2.1. Drugs and strains

The in vitro activity of pure powder of AmB (Sigma Chemical Co., St Louis, MO), VRC (Pfizer Inc., Madrid, Spain) and AFG (Pfizer Inc.) was tested alone and in double and triple combinations against 38 fungal isolates, comprising 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 alpha (EF-1α). *L. prolificans* isolates were identified by sequencing the ITS and a fragment of the β-tubulin gene, and *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, *Candida parapsilosis* ATCC 22019 and *Aspergillus fumigatus* ATCC MYA 3626) were included as quality controls.

Isolates were grown at 30 °C 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 then harvested with a sterile pipette. The suspensions were adjusted to the desired concentrations by haemocytometer counts and the viability was assessed by placing 10-fold dilutions onto PDA plates [8].

2.2. Antifungal activity assays

Single susceptibility testing of the isolates was carried out by the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [9]. After 48 h at 35 °C, minimum inhibitory concentrations (MICs) of AmB and VRC were visually read with the aid of an inverted mirror and corresponded to 100% growth inhibition (MIC-0), whilst the minimum effective concentration (MEC) of AFG was read with the aid of a stereomicroscope (40× magnification) as the minimum concentration producing abnormal hyphal growth.

The activities of double combinations (i.e. AmB + VRC, AmB + AFG and VRC + AFG) were tested in a 7 × 10 two-dimensional chequerboard with two-fold dilutions of each drug as previously described [10]. For the combinations of VRC with either AmB or AFG, the final concentrations of the antifungal agents were 0.5–256 μg/mL for VRC (i.e. 10 two-fold dilutions), 1–64 μg/mL for AmB (0.06–4 μg/mL against *F. solani*) (i.e. 7 two-fold dilutions) and 2–128 μg/mL for AFG (i.e. 7 two-fold dilutions). For the double combination of AmB + AFG, the final concentrations of the antifungal agents were 0.125–64 μg/mL for AmB (0.016–8 μg/mL against *F. solani*) (i.e. 10 two-fold dilutions) and 2–128 μg/mL for AFG (i.e. 7 two-fold 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 chequerboard technique. A 7 × 10 chequerboard with two-fold dilutions of AmB and VRC was set up as described above for the double combinations, with AFG added to each well at a constant final concentration (i.e. 0.06, 0.25, 1 and 4 μg/mL).

For the combination AmB + VRC, 100% growth inhibition (MIC-0) was chosen as the 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) [11]. 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, the MIC-2 was used.

The fractional inhibitory concentration indices (FICIs) of the double combinations were calculated as follows: FICI = ($MIC_{drug\ A}$ in combination/$MIC_{drug\ A}$ alone) + ($MIC_{drug\ B}$ in combination/$MIC_{drug\ B}$ alone). For the triple combination, the third parameter, $MIC_{drug\ C}$ in combination/$MIC_{drug\ C}$ alone, was added. Drug interactions were defined as synergistic if the lowest FICI was ≤0.5, no interaction if the lowest FICI was >0.5 and ≤4, and antagonistic if the highest FICI was >4 [12]. For the calculations, high off-scale MICs were converted to the next highest concentration. Every isolate was assayed twice.

3. Results

3.1. Antimicrobial combinations against F. solani

Supplementary Table S1 summarises 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 of the strains displayed remarkably high MICs for VRC (16 μg/mL to >256 μg/mL) and AFG (≥128 μg/mL) but, by contrast, they showed lower AmB MICs (1–8 μg/mL). All of the double combinations showed a high percentage of synergy against this species, with AFG + VRC and AmB + AFG being the most active (87.5% synergy). AmB + VRC showed 62.5% synergy, but VRC concentrations of ≥16 μg/mL together with 0.25–0.5 μg/mL AmB were needed to achieve this. The triple combination showed 87.5% synergy. Antagonism was not observed in any case.

3.2. Antimicrobial combinations against L. prolificans

Results of the in vitro susceptibility testing of all interactions for every *L. prolificans* strain are given in Supplementary Table S2. The highest percentage of synergy was observed for the combination AmB + AFG (72.7%), whilst 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 AmB was combined with 16 μg/mL VRC; however, these concentrations are not recommended due to their possible toxicity (Supplementary Table S2).

When AFG was combined with VRC, synergistic interactions were found against 6 (54.5%) of the 11 *L. prolificans* isolates tested. The interaction between the three drugs was synergistic for 7 strains (63.6%) and was 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) (Supplementary Table S2). The benefit of the triple combination over the double combinations was clearly demonstrated in 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 with double combinations. However, this benefit of the triple combination over the double combinations was not so evident against strains FMR 9797 and FMR 9800, especially in comparison with the combination AmB + AFG, where concentrations reaching the highest synergy were lower than those needed in the triple combination. Antagonism was not observed in any case.

3.3. Antimicrobial combinations against S. brevicaulis

The MICs of AmB, VRC and AFG against each strain of *S. brevicaulis* as well as the lowest FICIs achieved with the double and triple