In Vitro Susceptibilities of Zygomycetes to Combinations of Antimicrobial Agents

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Combinations of antimicrobial agents were tested against 35 strains of zygomycetes. The interaction between amphotericin B and rifampin was synergistic or additive. Flucytosine alone was inactive and, upon combination with amphotericin B, synergy was not achieved. The combination of amphotericin B with terbinafine was synergistic for 20% of strains, and the interaction between terbinafine and voriconazole was synergistic for 44% of strains. Antagonism was not observed.

Zygomycosis is an aggressive infection occurring mostly in patients with diabetic ketoacidosis or neutropenia or in patients receiving corticosteroids (35). Despite antifungal therapy, mortality remains very high, particularly in the pulmonary and disseminated forms of the disease (21, 26, 42). Amphotericin B is the drug of choice for treatment, but its use is limited by its narrow therapeutic index (14). Few studies have been carried out to test antifungal combinations against zygomycetes in vitro (5, 41) or in animal models of zygomycosis (40). Combination of amphotericin B with rifampin has proven to be synergistic in vitro against yeasts (4, 8, 13, 27), dimorphic fungi (25, 36), Aspergillus (19, 24), and Rhizopus (5). Nevertheless, in animal models of fungal infections, results for this combination have been conflicting (10, 16). Interaction between amphotericin B and flucytosine has been shown to be additive or synergistic against yeasts in vitro and in vivo in animal models as well as in patients (34). With Aspergillus, indifferent-to-synergistic and in some instances antagonistic interactions have been reported for this combination (7, 19). Terbinafine is a sterol biosynthesis inhibitor that is primarily used for superficial mycoses, but its current applications are extending (33). Although the potential of this antifungal for zygomycosis is unknown, terbinafine exhibits low MICs against some zygomycete isolates (22). Combination of amphotericin B with terbinafine displayed synergistic interaction in Candida albicans (2) and in Aspergillus spp. (38), and this combination has been used successfully in patients for the treatment of aspergillosis (39) and zygomycosis (12, 31). Synergy between terbinafine and azoles has also been demonstrated in yeasts (2, 3, 15) and in filamentous fungi (28, 38, 43). The combination of terbinafine with voriconazole has rarely been investigated (38, 43).

The aim of this study was to investigate the in vitro interactions of amphotericin B with rifampin, flucytosine, and terbinafine as well as the interaction of terbinafine with voriconazole against zygomycetes.

A total of 35 isolates were tested. These comprised 15 Rhizopus spp. (8 R. oryzae and 7 R. microsporus) isolates, 10 Absidia corymbifera isolates, 6 Mucor spp. (3 M. hiemalis, 1 M. circinelloides, 1 M. racemosus, and 1 M. rouxii) isolates, 3 Rhizomucor spp. (2 R. pusillus and 1 R. miehei) isolates, and 1 Cunninghamella bertholletiae isolate. Candida krusei ATCC 6258 and Candida parapsilosis ATCC 22019 were included to ensure quality control. The drugs that were tested included voriconazole (Pfizer Central Research, Sandwich, United Kingdom), terbinafine (Novartis Pharma, Basel, Switzerland), flucytosine (ICN Pharmaceuticals, Zoetermeer, The Netherlands), amphotericin B (Bristol-Myers Squibb, Woerden, The Netherlands), and rifampin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). For the combination of terbinafine with either voriconazole or amphotericin B, a checkerboard with twofold dilutions of each drug was used. The final concentrations were 0.03 to 2 μg/ml for amphotericin B, 0.5 to 32 μg/ml for voriconazole, and either 0.004 to 2 μg/ml or 0.25 to 128 μg/ml for terbinafine, depending on the susceptibilities of the tested isolates. For the combination of amphotericin B with either rifampin or flucytosine, a limited checkerboard with twofold dilutions of amphotericin B and fourfold dilutions of the other drug was used. The final concentrations were 0.008 to 4 μg/ml for amphotericin B, 0.25 to 16 μg/ml for rifampin, and 8 to 128 μg/ml for flucytosine.

Drug combinations were tested using the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) proposed standard M38-P (29) modified for a broth microdilution checkerboard procedure. Spore suspensions were counted with a hemacytometer and then diluted into RPMI to a concentration of 2 × 10⁴ spores/ml (2× final concentration). Microplates were incubated at 37°C, and MICs...
were determined visually after 24 h of incubation. MIC determinations were done in duplicate, and results were within 1 log₂ dilution in 90% of the cases. Each well was given a numerical score from 4 (no reduction in growth) to 0 (absence of growth) according to the NCCLS guidelines (29). For the combination of amphotericin B with rifampin or flucytosine, MIC endpoints were defined as the lowest drug concentration (tested alone or in combination) which had a score of 0 (MIC-0). For the combination of terbinafine with voriconazole, MIC-2 was used as the endpoint for both drugs, alone or in combination. For the combination of terbinafine with amphotericin B, MIC-0 was used for amphotericin B alone and MIC-2 was used for terbinafine alone and for both drugs in combination. The fractional inhibitory concentrations (FICs) of both drugs used in combination were calculated and added to obtain the FIC indices (9). Drug interactions were defined as synergistic if the FIC index was ≤0.5, as additive if the FIC index was >0.5 and ≤1, indifferent if the FIC index was >1 and ≤4, and antagonistic if the FIC index was >4. The number of strains showing synergy within the different genera were compared by using Fisher’s exact test.

The geometric mean MIC and the MIC at which 90% of isolates were inhibited (MIC₀₉₀) of amphotericin B were 0.24 and 1 μg/ml, respectively. Only one strain (C. bertholletiae) showed a high amphotericin B MIC of 2 μg/ml. Growth of all isolates was not inhibited by rifampin (MIC > 16 μg/ml), with the exception of two Rhizomucor spp. isolates that exhibited a MIC of 16 μg/ml.

The interaction between amphotericin B and rifampin was synergistic in 69% of the cases and additive in 31% of the cases. Indifference and antagonism were not observed (highest FIC indices ranged from 0.5 to 2.02). For the synergistic interactions, the median concentration of amphotericin B in combination was 0.03 μg/ml (range, 0.008 to 0.25 μg/ml), and the median concentration of rifampin in combination was 4 μg/ml (range, 1 to 16 μg/ml). Synergism was observed for all the A. corymbifera isolates (P < 0.0028 compared with Rhizopus spp.), for 40% of the Rhizopus spp. isolates, and for most of the other species (Table 1).

Flucytosine alone was inactive against all the isolates (MIC > 128 μg/ml). Upon combination, synergy was not achieved. Additivity or indifference was observed for all the strains. Antagonism was not observed. Terbinafine MICs ranged from 0.015 to >128 μg/ml. The highest MICs of terbinafine were observed against R. oryzae and Mucor spp. For the combination between terbinafine and amphotericin B, synergistic interactions were observed for 20% of the strains and additive or indifferent interactions were noted for 80% of the strains. There were no antagonistic interactions. For the synergistic interactions, the median concentration of amphotericin B in combination was 0.125 μg/ml (range, 0.03 to 0.5 μg/ml) and the median concentration of terbinafine in combination was 0.25 μg/ml (range, 0.015 to 8 μg/ml). The percentage of isolates showing synergism was not significantly different between species (Table 1).

The geometric mean MIC and the MIC₀₉₀ of voriconazole were 7.8 and 32 μg/ml, respectively. The interaction between terbinafine and voriconazole was synergistic for 44% of the strains and additive or indifferent for 56% of the strains. Antagonism was not observed. For the synergistic interactions the median concentration of terbinafine in combination was 0.5 μg/ml (range, 0.015 to 16 μg/ml) and the median concentration of voriconazole in combination was 1 μg/ml (range, 0.5 to 8 μg/ml). Synergism was detected for 60% of the Rhizopus spp. isolates (Table 1). In contrast, no synergistic interaction was observed for A. corymbifera (P < 0.0028 compared with Rhizopus spp.; P < 0.0031 compared with other species).

Zygomycosis remains a very severe infection. The overall mortality of localized pulmonary zygomycosis is 65% (42), and it is >95% in disseminated forms of the disease (21, 42). Combination therapy is commonly used for difficult-to-treat bacterial and some fungal infections and could be a useful strategy in zygomycosis. In patients with zygomycosis, combination therapy with amphotericin B and rifampin (5, 6, 30) or amphotericin B and terbinafine (12, 31) has been reported. Nevertheless, it is not possible to draw conclusions from these anecdotal case reports. Few studies have been done to evaluate the potential of drug combinations against zygomycetes (5, 40, 41). In vitro data are limited to a study showing synergism between amphotericin B and rifampin in Rhizopus spp. (5). In a murine model of pulmonary mucormycosis due to R. oryzae, it was shown that combination of fluconazole with quinolones was effective (40). It has to be pointed out that positive interaction between these drugs was not demonstrated in vitro (41).

In the present study, we found synergistic interactions between amphotericin B and rifampin at clinically relevant concentrations of rifampin (32). Interestingly, there was a clear difference between different genera; a synergistic interaction was obtained for all the A. corymbifera strains compared to 40% of the Rhizopus spp. strains. Although different susceptibility testing methods and different definitions of synergism have been used, in vitro synergistic interaction has been usually documented for yeasts (4, 8, 13, 27, 37). For hyphomycetes,

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**TABLE 1. Summary of drug interaction for the four combinations tested**

<table>
<thead>
<tr>
<th>Genera (no. of strains)</th>
<th>AMB and RIF</th>
<th>AMB and 5FC</th>
<th>TER and AMB</th>
<th>TER and VRZ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Syn Add Ant</td>
<td>Syn Add Ant</td>
<td>Syn Add Ant</td>
<td>Syn Add Ant</td>
</tr>
<tr>
<td>Rhizopus spp. (15)</td>
<td>40 60 0</td>
<td>100 0 0</td>
<td>27 73 0</td>
<td>60 40 0</td>
</tr>
<tr>
<td>Absidia spp. (10)</td>
<td>100 0 0</td>
<td>100 0 0</td>
<td>10 90 0</td>
<td>0 100 0</td>
</tr>
<tr>
<td>Other (10)</td>
<td>80 20 0</td>
<td>100 0 0</td>
<td>20 80 0</td>
<td>67 33 0</td>
</tr>
<tr>
<td>All isolates (35)</td>
<td>69 33 0</td>
<td>100 0 0</td>
<td>20 80 0</td>
<td>44 56 0</td>
</tr>
</tbody>
</table>

* AMB, amphotericin B; RIF, rifampin; 5FC, 5-flucytosine; TER, terbinafine; VRZ, voriconazole. Syn, synergism (FIC index ≤ 0.5); Add, additivity or indifference (FIC index > 0.5 and ≤ 4); Ant, antagonism (FIC index > 4).
synergistic interactions were found in Aspergillus spp. (7, 19, 24) but not in Fusarium spp. (17). It has also been shown that rifampin acts synergistically with amphotericin B against dimorphic fungi in vitro (20, 25, 36). Nevertheless, in animal models results have been conflicting. Although potentiation of amphotericin B by rifampin has been demonstrated for the treatment of murine aspergillosis (1) or histoplasmosis and blastomycosis (23), the combination was not beneficial in the treatment of disseminated candidiasis in mice (10, 16). One reason for discrepancies between in vitro and in vivo results may be that FIC indices that are only just below 0.5 may not really be indicating very strong synergy. As zygomycosis remains a very difficult to treat infection, it is probably of interest to test this combination in animal models, particularly for Absidia infections.

We found synergistic interactions between amphotericin B and terbinafine for 20% of the strains, with a median concentration of terbinafine in combination of 0.25 μg/ml, which is within the range achievable in serum (11). In vitro studies have shown synergistic interactions between amphotericin B and terbinafine against C. albicans (2) and Aspergillus spp. (38). Moreover, this combination has been used in patients with zygomycosis (12, 31) and aspergillosis (39). Although combination between azoles and amphotericin B is considered potentially antagonistic, it is to be noticed that no antagonism was observed between terbinafine (an ergosterol biosynthesis inhibitor) and amphotericin B in the present study.

Although the first-line therapy for zygomycosis remains parenteral amphotericin B, we have tested the combination of voriconazole with terbinafine and found synergistic interaction between these two drugs in 44% of isolates. There was a significant difference between genera; synergism was not observed for A. corymbifera but was demonstrated for 60% of the Rhizopus spp. strains. Moreover, the median concentration of voriconazole in combination was 1 μg/ml, which is within the range achievable in serum (18). In vitro synergism has been demonstrated for the combination of terbinafine with either fluconazole or itraconazole against C. albicans (2, 3), Scedosporium prolificans (28), and Aspergillus spp. (38), and these combinations have also been used in humans. Few studies have tested the interaction of terbinafine with voriconazole. In two recent studies it has been shown that terbinafine in combination with voriconazole displayed potent synergies against C. albicans (43) and Aspergillus spp. (38).

In summary, the results of this study demonstrated that some antifungal combinations are synergistic in vitro against zygomycetes, with different results for Rhizopus spp. and A. corymbifera. Further studies in animal models of zygomycosis are necessary to confirm the clinical potential of these combinations.

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REFERENCES


