In Vitro Interaction of Flucytosine with Conventional and New Antifungals against *Cryptococcus neoformans* Clinical Isolates

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Combinations of flucytosine with conventional and new antifungals were evaluated in vitro against 30 clinical isolates of *Cryptococcus neoformans*. Synergy determined by checkerboard analysis was observed with combinations of fluconazole, itraconazole, voriconazole, amphotericin B, and caspofungin with flucytosine against 77, 60, 80, 77, and 67% of the isolates, respectively. Antagonism was never observed. Killing curves showed indifferent interactions between triazoles and flucytosine and synergy between amphotericin B and flucytosine. The final inoculum in each well was 1.0 × 10³ to 5.0 × 10³ CFU/ml. Trays were incubated at 35°C for 72 h, and MICs were recorded spectrophotometrically. Susceptibility tests were run in duplicate. MICs of a drug alone or in combination were defined as the minimum concentration that inhibited by 50% (or 95% for AMB alone) the growth of the organism compared to the level of growth of the control. Additionally, the minimum concentrations that inhibited growth by 95% (MIC₉₅) were also recorded for all drugs. The FIC of each drug used in combination was calculated and added to obtain the FICI (8). The interactions were defined as synergistic if the lowest FICI was ≤0.5, additive if the FICI was >0.5 and ≤4, and antagonistic if the highest FICI was >4.

Isolate 27 was selected for time-kill studies. The starting inoculum was 1.0 × 10⁵ CFU/ml in a final volume of 20 ml of RPMI 1640. Test suspensions were incubated at 35°C with shaking. At predetermined time points, aliquots were removed, diluted, and plated on Sabouraud agar plates in duplicate. CFU were enumerated after incubation at 35°C for 48 h. The limit of detection was 20 CFU/ml. Trays were incubated at 35°C for 72 h, and MICs were recorded spectrophotometrically. Susceptibility tests were performed in duplicate.

Duplicate MICs of 5FC, FCZ, ITZ, VRZ, AMB, and CAS alone were within ±1 log₂ dilution for 94, 87, 96, 100, 100, and 100% of the isolates, respectively.

5FC MICs ranged from 0.5 to ≥32 μg/ml, with a geometric mean MIC between 3.12 and 5.16 μg/ml. For isolates 7, 14, and 23, the MICs of 5FC were ≥32 μg/ml, and these isolates were considered to be resistant to this drug (16).

**TABLE 1. Summary of the interactions between antifungal drugs for clinical isolates of *C. neoformans***

<table>
<thead>
<tr>
<th>Mode of interaction</th>
<th>% of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 30)</td>
</tr>
<tr>
<td>5FC + FCZ</td>
<td>77</td>
</tr>
<tr>
<td>5FC + ITZ</td>
<td>23</td>
</tr>
<tr>
<td>5FC + VRZ</td>
<td>0</td>
</tr>
<tr>
<td>5FC + AMB</td>
<td>23</td>
</tr>
<tr>
<td>5FC + CAS</td>
<td>23</td>
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</tbody>
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Infections caused by *Cryptococcus neoformans* have become a major problem, especially for patients with AIDS. With the availability of highly active antiretroviral therapy in Western countries, the incidence of cryptococcosis has decreased in AIDS patients (15) but is still associated with high mortality. Amphotericin B (AMB), fluconazole (FCZ), and flucytosine (5FC) are the most commonly used antifungals to treat cryptococcosis (7, 24). New azoles, like voriconazole (VRZ), have potent in vitro activity against *C. neoformans* (20). Caspofungin (CAS), a new antifungal with broad-spectrum activity, showed poor activity against *C. neoformans* in vitro (3) and in vivo (1), but it has been shown to improve in the in vitro activity of AMB or FCZ (9). The potential role of these new drugs used in combination for the management of *C. neoformans* infections remains to be determined.

The aim of the present study was to evaluate the activity of 5FC in combination with conventional or new antifungals against the same panel of clinical isolates of *C. neoformans*. Antifungal interactions were tested by a broth microdilution technique, performed according to the National Committee for Clinical Laboratory Standards M27-A document (16), modified for checkerboard studies. Interpretation was made by calculation of fractional inhibitory concentration indices (FICI). Time-kill studies were also performed (8).

Thirty clinical isolates of *C. neoformans* from our private collection were studied. Two quality control strains (16) were included in each series of experiments.

FCZ (Pfizer Ltd., Sandwich, United Kingdom), itraconazole (ITZ) (Janssen Pharmaceutica, Beerse, Belgium), VRZ (Pfizer), AMB (Sigma Chemical Co., St. Louis, Mo.), and CAS (Merck and Co., Rahway, N.J.) were all tested in combination with 5FC (Sigma). The final concentrations were 32 to 0.06 μg/ml for 5FC, 32 to 0.5 μg/ml for CAS, 1 to 0.015 μg/ml for ITZ, 0.125 to 0.009 μg/ml for VRZ, and 2 to 0.03 μg/ml for AMB. For FCZ, final concentrations were 32 to 0.5 μg/ml or 2 to 0.03 μg/ml, depending on the susceptibilities of the strains.

The final inoculum in each well was 1.0 × 10⁵ to 5.0 × 10⁵ CFU/ml. Trays were incubated at 35°C for 72 h, and MICs were recorded spectrophotometrically. Susceptibility tests were run in duplicate. MICs of a drug alone or in combination were defined as the minimum concentration that inhibited 50% (or 95% for AMB alone) the growth of the organism compared to the level of growth of the control. Additionally, the minimum concentrations that inhibited growth by 95% (MIC₉₅) were also recorded for all drugs. The FIC of each drug used in combination was calculated and added to obtain the FICI (8). The interactions were defined as synergistic if the lowest FICI was ≤0.5, additive if the FICI was >0.5 and ≤4, and antagonistic if the highest FICI was >4.

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5FC MICs ranged from 0.5 to ≥32 μg/ml, with a geometric mean MIC between 3.12 and 5.16 μg/ml. For isolates 7, 14, and 23, the MICs of 5FC were ≥32 μg/ml, and these isolates were considered to be resistant to this drug (16).
MICs ranged from 0.25 to 8, 0.03 to 0.5, 0.004 to 0.12, and 0.25 to 1 \( \mu \)g/ml, with geometric mean MICs of FCZ, ITZ, VRZ, and AMB of 1.95, 0.11, 0.023, and 0.62 \( \mu \)g/ml, respectively. For synergistic interactions, the concentrations of 5FC, FCZ, ITZ, VRZ, and AMB in combination were four, eight, eight, and four times the MIC\(_{95}\) as determined by the broth microdilution technique. For antagonistic interactions, the concentrations of 5FC, FCZ, ITZ, VRZ, and AMB in combination were four, eight, eight, and four times the MIC\(_{95}\) as determined by the broth microdilution technique. The limit of detection was 1.3 log\(_{10}\) CFU per milliliter.

**Table 1** gives a summary of the interactions of the different antifungals with 5FC. The combinations of 5FC with each of the other antifungals showed synergism in more than 60% of the cases. Antagonism was never observed. With 5FC combinations with FCZ, ITZ, VRZ, and AMB, synergistic interactions were observed with two of the 5FC-resistant isolates and...
additivity was noted with the third isolate. With the CAS-5FC combination, all three additivity was noted with the third isolate. With the CAS-5FC combination, all three isolates were obtained with all three isolates. When MIC<sub>50</sub> were considered, antifungal interactions were mostly additive for all combinations (data not shown). Synergistic interactions were observed with ITZ (3%) and CAS (13%) but not with FCZ, VRZ, and AMB.

Figure 1A shows the results of the time-kill studies of the azoles. None of the three triazoles showed fungicidal activity when they were used alone. An increase in the number of CFU per milliliter with 5FC alone was observed at 48 and 72 h of incubation due to the growth of 5FC-resistant colonies. Combinations of triazoles with 5FC were neither fungicidal nor synergistic.

As shown in Fig. 1B, AMB alone was fungicidal and killing was dose dependent. At concentrations of at least one times the MIC, the addition of 5FC did not result in a higher rate of killing. For AMB alone at a concentration of one-half the MIC, the addition of 5FC did not result in a higher rate of killing. For AMB alone at a concentration of one-half the MIC, the addition of 5FC did not result in a higher rate of killing. For AMB alone at a concentration of one-half the MIC, the addition of 5FC did not result in a higher rate of killing. For AMB alone at a concentration of one-half the MIC, the addition of 5FC did not result in a higher rate of killing. For AMB alone at a concentration of one-half the MIC, the addition of 5FC did not result in a higher rate of killing. For AMB alone at a concentration of one-half the MIC, the addition of 5FC did not result in a higher rate of killing. For AMB alone at a concentration of one-half the MIC, the addition of 5FC did not result in a higher rate of killing. For AMB alone at a concentration of one-half the MIC, the addition of 5FC did not result in a higher rate of killing. For AMB alone at a concentration of one-half the MIC, the addition of 5FC did not result in a higher rate of killing. For AMB alone at a concentration of one-half the MIC, the addition of 5FC did not result in a higher rate of killing. For AMB alone at a concentration of one-half the MIC, the addition of 5FC did not result in a higher rate of killing.

Our results showed the same trends of interactions for all five combinations, even against 5FC-resistant strains. AMB in combination with 5FC is currently the gold standard for the treatment of cryptococcosis, as was suggested for in vitro and animal models (21, 22) and demonstrated for patients (4, 26). By time-kill methodology, synergy between AMB and 5FC can be detected only at 72 h, in agreement with the results of previous studies (11, 23).

For the combination of azoles with 5FC, we found 60 to 80% of synergy by checkerboard studies, but our time-kill experiments showed no significant interaction among azoles and 5FC, as was reported by others (23). Discrepancies between results obtained by these two techniques are not unusual for fungistatic agents. A checkerboard analysis provides only static inhibitory data at one time point. In contrast, time-kill curves explore dynamic fungicidal activity over time. A previous study reported synergistic interactions between FCZ and 5FC by using checkerboard methodology (17). Murine models generally showed synergy between these two drugs (5, 6, 13, 18), while a rabbit model did not (10). In patients, a combination of FCZ and 5FC was more effective than FCZ alone (14). In a recent in vitro study, the combination of ITZ with 5FC was shown to be synergistic for 63% of the strains while antagonism was not observed (2), in agreement with our results. In animal studies, indifferent-to-synergistic interactions were found (21), but there have been no trials with humans.

Although VRZ is very active in vitro against C. neoformans (26), it is not marketed for the treatment of cryptococcosis. We found synergy between VRZ and 5FC in the checkerboard study with very low MICs alone and in combination.

Preliminary studies evaluating CAS alone showed no activity against C. neoformans (1, 3), but synergy has been reported between CAS and either AMB or FCZ in vitro (9). Here we found high MICs of CAS alone and synergy when it was used in combination with 5FC. In more than 50% of the cases the MICs of CAS in combination were in the range of achievable levels in serum (25). Nevertheless, it has to be pointed out that the high level of binding of drugs to proteins or other pharmacokinetics properties are not taken into account by in vitro tests. The potential of combination between an echinocandin and 5FC in cryptococcosis remains to be determined. In the present study, three strains were resistant to 5FC. Interestingly, interactions between 5FC and other antifungals for these strains were comparable to those obtained with susceptible strains. Although 5FC use has been advocated to be questionable when in vitro 5FC resistance is found (12), our results suggest that a combination therapy including 5FC might also be beneficial for the management of cryptococcal infections due to 5FC-resistant isolates. Nevertheless, the ability to overcome 5FC resistance may be correlated with its mechanism of resistance. If the resistance is related to a defect of intracellular penetration, the resistance may be overcome by using a drug such as AMB that favors the cellular uptake of 5FC. On the other hand, if resistance is related to a lack of deaminase, combination with a permeabilizing drug is not likely to be effective.

In conclusion, all tested combinations of 5FC with triazoles or CAS were mostly synergistic and comparable to the AMB-5FC combination. Precise relevance of these in vitro results has now to be evaluated with animal models of cryptococcosis.

**REFERENCES**


