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In vitro susceptibility studies of *Cryptococcus neoformans* isolated from patients with no clinical response to amphotericin B therapy

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The *in vitro* activities of three antifungal drugs alone and in combination were evaluated against five isolates of *Cryptococcus neoformans* using time–kill curves (TKC). The isolates were from AIDS patients who had either died or had failed to show a clinical response during amphotericin B (AMB) treatment. AMB, fluconazole (FCZ) and flucytosine (5FC), and combinations of the drugs (AMB plus 5FC, AMB plus rifampicin (RIF) and FCZ plus 5FC), were evaluated. With all five isolates AMB did not show fungicidal activity; instead, a persistent or tolerant effect was observed. Combinations of AMB plus 5FC and AMB plus RIF showed a clear synergic effect, except for one isolate tested with AMB plus RIF. In contrast, the FCZ plus 5FC combination did not inhibit growth of any isolate.

Introduction

Infections caused by *Cryptococcus neoformans* are an increasing problem in immunocompromised patients, particularly those with AIDS, in whom this organism is the fourth most common cause of life-threatening infection.[1] Approximately 90% of AIDS patients infected with *C. neoformans* develop meningitis.[1] In Buenos Aires city, Argentina, cryptococcal meningitis has been diagnosed in approximately 9% of patients with AIDS.[2]

Amphotericin B (AMB) and fluconazole (FCZ) are current acceptable therapies for cryptococcal meningitis. However, their effects remain suboptimal, and recurrence or treatment failure is still a problem. Recently, AMB plus flucytosine (5FC), for 2 weeks, followed by FCZ, was suggested as the treatment of choice.[3] Another combination therapy proposed has been FCZ plus 5FC, which seemed to be clinically useful in patients with meningitis[4] and in pulmonary cryptococcosis.[5] On the other hand, a synergic interaction between AMB and rifampicin (RIF) has been demonstrated *in vitro* with other fungi such as *Aspergillus*[6] and *Candida* spp.[7]

In a previous study, our group evaluated the *in vitro* activity of AMB against 16 isolates of *C. neoformans* obtained from AIDS patients with cryptococcal meningitis using time–kill curves (TKCs), and by determining MICs and minimal fungicidal concentrations (MFCs). In that study,[8] TKCs for AMB (1 mg/L) showed fungicidal activity against most of the isolates. Four isolates from patients who did not respond to conventional AMB therapy showed a persistent or tolerant effect. In spite of this, the MIC values obtained suggested that they were all susceptible.[8]

The aim of this study was to evaluate the interactive effects of combinations of drugs, namely, AMB plus 5FC, AMB plus RIF and FCZ plus 5FC, against five isolates of *C. neoformans* obtainedfrom patients who died or failed to respond to AMB therapy.

Materials and methods

Isolates

The five isolates of *C. neoformans* included in this study were selected from separate AIDS patients with a first episode of cryptococcal meningitis who did not respond to AMB therapy and whose isolates appeared tolerant in TKCs obtained using AMB (Figure 1a). One patient died at the beginning of the AMB therapy, having received a total dosage of 50 mg (isolate 399). Three patients died during administration of AMB therapy, the total dosage received in all these cases being ⩾500 mg. Isolates from

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these patients were assigned numbers 947, 1130 and 2672. In one patient, CSF culture still yielded *C. neoformans* (isolate 2294), despite the total dosage of AMB received being 750 mg.

Antifungal agents

The following antifungal agents were used in the study: AMB (Squibb, New Brunswick, NJ, USA), 5FC (Sigma Chemical Co., St Louis, MO, USA), FCZ (Pfizer S.A., Buenos Aires, Argentina) and RIF (Hoechst Marion Roussel, Buenos Aires, Argentina). The drugs were provided as powders of known potency. Stock solutions were prepared as follows: AMB and FCZ were dissolved in 100% dimethylsulphoxide (DMSO; Sigma Chemical Co.) at concentrations of 1 g/L and 10 g/L, respectively; 5FC was dissolved in sterile distilled water at a concentration of 10 g/L; and RIF in 4:6 (v/v) methanol–water at a concentration of 5 g/L. Stock solutions were stored at –70°C until needed.

Time–kill curves

Isolates were grown with shaking in RPMI 1640 (Sigma Chemical Co.) buffered with MOPS (Sigma Chemical Co.) to pH 7.0 for 18 h at 35ºC. Initial inocula were adjusted to 1 McFarland scale (*c*. 10^6 cfu/mL). One mL of these inocula was diluted 10-fold in 9 mL of MOPS-buffered RPMI containing the drugs to be tested, alone and in combination, at the following final concentrations: AMB at 1 mg/L, FCZ at 10 mg/L, 5FC at 10 mg/L and RIF at 5 mg/L. A control growth tube (10 mL of RPMI, pH 7.0) without drugs was included in all experiments. The tubes were incubated at 35ºC. Samples of 0.5 mL volume were removed from each of the tubes and subjected to serial 10-fold dilution at 0, 6, 12, 24, 48 and 72 h. From each of these serial 10-fold dilutions, 30 μL were plated on YM agar plates. After 72 h of incubation at 35ºC colony counts were determined. A ⩾99.9% reduction in the viable count compared with that seen at time zero was considered as the endpoint of the TKC. Tests were performed in duplicate.

Results

The TKC of the five isolates tested with AMB (1 mg/L) showed a low initial inhibition of growth. After 12 or 24 h of incubation, a reduction of <2 log was obtained. After this point, growth was resumed and at 48 or 72 h colony counts in the range 10^4–10^6 cfu/mL were detected (Figure 1a). None of the isolates was inhibited by 5FC (10 mg/L) (Figure 1b).

The combination of AMB plus 5FC showed synergic activity against all the isolates, particularly 399 and 947. The other three isolates showed a decrease of 1 or 2 log at 24 h, which was maintained at 48 h with fungicidal activity at 72 h (Figure 1b).

The TKC for RIF alone was similar to the control growth curves (Figure 1c). When the combination of AMB plus RIF was evaluated, a very marked synergic effect was noted with four isolates, which were killed at 6 h. For one isolate (1130) a killing effect was detected at 12 h with regrowth being observed at 24 h (Figure 1c).

FCZ, at a concentration of 10 mg/L, failed to kill any of the isolates (Figure 2a). The combination of FCZ plus 5FC did not show any variation from the curve obtained with FCZ alone.

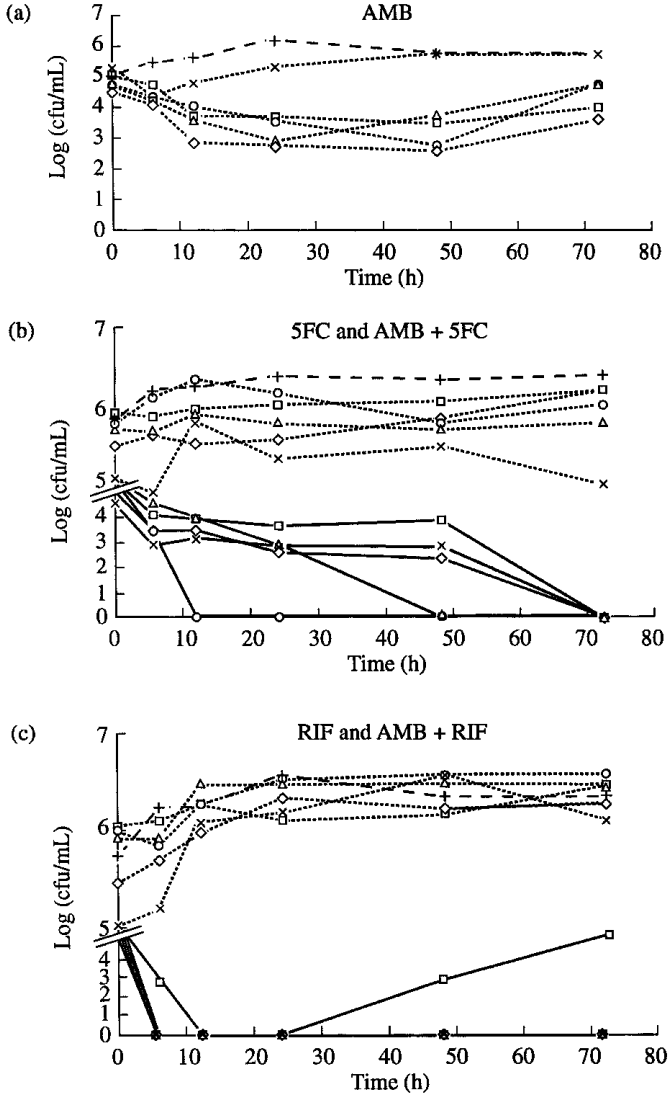

Figure 1. Time–kill curves of *C. neoformans* tested with amphotericin B (AMB) alone and in combination with flucytosine (5FC) and rifampicin (RIF): (a) for AMB; (b) for 5FC and the combination AMB plus 5FC; (c) for RIF and the combination AMB plus RIF. Control curves (dashed line), drug alone (dotted line) and combinations (continuous line) for isolates 947 (△), 2672 (◇), 399 (○), 1130 (□) and 2294 (×).

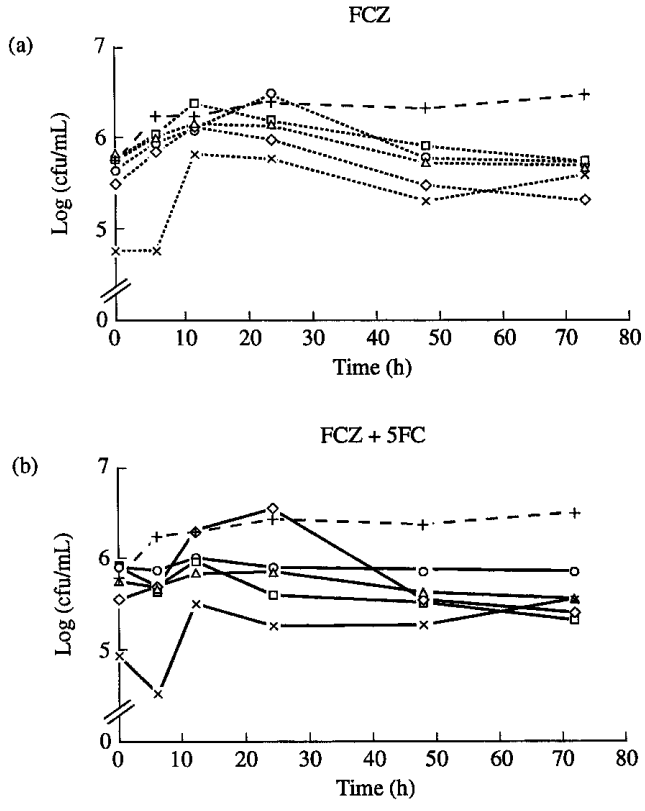

Figure 2. Time–kill curves of *C. neoformans* tested with fluconazole (FCZ) alone and in combination with flucytosine (5FC): (a) for FCZ; (b) for the combination FCZ plus 5FC. Control curves (dashed line), drug alone (dotted line) and combinations (continuous line) for isolates 947 (△), 2672 (◇), 399 (○), 1130 (□) and 2294 (×).

Discussion

Although AMB and FCZ are useful therapies, the treatment of cryptococcal meningitis in AIDS patients is still a problem with high failure rates sometimes noted. There are a number of studies[3,9] that have evaluated the possibility of more effective and less toxic alternatives. Combination therapy might be useful in these infections, involving immunocompromised hosts, in which enhanced drug activity is needed.[10]

This *in vitro* study was designed to evaluate different available combinations of drugs against *C. neoformans* isolated from patients who failed initial AMB therapy. To analyse possible synergic effects of the combinations, TKCs were used. This approach allows the estimation of microbicidal activity, which in several studies with bacteria was found to be a more accurate determinant of clinical outcome than a simple numerical MIC or MBC.[11]

Recently, Van der Horst *et al.*,[3] in a double-blind multicentre trial, determined that for initial treatment of AIDS-associated cryptococcal meningitis, the use of AMB plus 5FC was associated with an increased rate of CSF sterilization and decreased mortality at 2 weeks, as compared with AMB alone. Our *in vitro* results agree with this study, showing that the addition of a very low concentration of 5FC to AMB produced a synergic effect against tolerant isolates of *C. neoformans*. However, many patients cannot tolerate 5FC because of toxicity, primarily manifested as bone marrow suppression. Thus, alternative approaches would be highly desirable. Furthermore, because in the majority of cases therapy must be associated with the treatment of other infections, drug interactions occur and must be considered. In Argentina, tuberculosis is one of the most frequent infections in patients with AIDS. Therefore interaction between AMB and RIF (the first line agent for *Mycobacterium tuberculosis*) must be considered. Although RIF alone does not have antifungal activity, synergy with AMB has been previously demonstrated *in vitro* against *Candida* and *Aspergillus* spp.[6] In this preliminary study, AMB plus RIF showed encouraging results; however, it will be useful to evaluate this synergic effect *in vivo*, by evaluating patients with tuberculosis plus cryptococcosis, who are receiving both drugs.

Recently, two clinical trials suggested the use of a combination of FCZ plus 5FC for pulmonary cryptococcosis and cryptococcal meningitis therapy.[4,5] Our results did not show synergic interaction between these drugs against the isolates tested. However, it may be that synergy could not be detected at the low concentrations of drugs that were used. Further *in vitro* studies using higher concentrations for both drugs will be necessary to determine its usefulness.

Although more data are needed to evaluate the correlation between TKC and clinical outcome, these preliminary results suggest that for isolates tolerant to AMB, an alternative therapy could be considered.

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