Antifungal pharmacodynamic characteristics of fluconazole and amphotericin B against Cryptococcus neoformans

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The activity of fluconazole and amphotericin B against three isolates of Cryptococcus neoformans was evaluated, with fluconazole and amphotericin B MICs of 2.0–4.0 mg/L and 1.0 mg/L respectively, using time–kill curve methods. Fluconazole was fungistatic against all isolates tested (<99.9% decrease in cfu from initial inoculum). The fungistatic activity of fluconazole was not enhanced by increasing the concentration of antifungal in solution. In contrast, amphotericin B was markedly fungicidal (≥ 99.9% decrease in cfu from initial inoculum). Both the rate and the extent of amphotericin B activity were enhanced when drug concentration was increased.

Introduction

Meningitis secondary to Cryptococcus neoformans is the most common life-threatening fungal infection among AIDS patients and, when untreated, is uniformly fatal among this patient population. When seemingly appropriate antifungal therapy is given, mortality associated with cryptococcal meningitis remains high (5.5–20%).1,2 Although the combination of amphotericin B and flucytosine is often the regimen of choice, fluconazole with or without flucytosine is also an effective treatment.1, 3 There remains a considerable amount of disagreement regarding optimal dosing of these agents in this setting. Doses of 0.3–1.0 mg/kg/day and 200–1000 mg/day have been employed for amphotericin B and fluconazole, respectively.1–6 Understanding antifungal pharmacodynamics may help determine optimal dosing. We sought to characterize the relationships between concentrations of fluconazole and amphotericin B and the rate and extent of antifungal activity against C. neoformans in vitro.

Materials and methods

Antifungal agents

Stock solutions of fluconazole (Pfizer, New York, NY, USA) and amphotericin B (Sigma Chemical Co., St Louis, MO, USA) were prepared using RPMI 1640 medium (Sigma) buffered to a pH of 7.0 with 0.165 M morpholine propanesulfonic acid (MOPS) buffer (Sigma) as solvent.

Dimethylsulphoxide (DMSO) was used to aid the solubilization of amphotericin B. DMSO comprised <1% of the total test solution volume. Growth curves were conducted with DMSO at concentrations equal to those present in test solutions to verify that it did not inhibit the growth of test isolates. Stock solutions were stored at −70°C until needed.

Test isolates

Three clinical isolates of C. neoformans (625.012, 682.027 and 853.033) were selected for testing. They were obtained from the Department of Pathology, Medical Mycology Division, The University of Iowa College of Medicine, Iowa City, IA, USA.

Antifungal susceptibility testing

The MICs of fluconazole and amphotericin B against each test isolate were determined according to NCCLS guidelines.7 Candida parapsilosis (ATCC 22019) and Candida krusei (ATCC 6258) served as quality control isolates. RPMI 1640 medium buffered to a pH of 7.0 with MOPS buffer served as growth medium. Fungal suspensions were prepared by touching four or five colonies from a 24–48 h culture plate and suspending fungi in 5 mL of sterile 0.9% saline. Fungal suspensions were standardized to a 0.5 McFarland turbidity standard, then diluted 1 in 1000 with growth medium to yield an initial inoculum of approximately 1 × 10^3 to 5 × 10^3 cfu/mL. One hundred
microlitres of the diluted suspension were added to each well of a microtitre tray containing 100 μL of antifungal at a concentration equal to twice the final concentration. The trays were incubated at 35°C for 72 h in a moist, dark chamber, then MICs were recorded. The fluconazole MIC was defined as the lowest concentration of drug that resulted in an 80% reduction of fungal growth compared with control. The amphotericin B MIC was defined as the lowest concentration of drug that completely inhibited visible growth. MIC determinations were performed in duplicate.

Antifungal carryover determination

Before the kill curve studies were started, the effect of solubilized antifungal on colony count determinations was evaluated. A fungal suspension was prepared with the test isolate to yield an inoculum of approximately 5 × 10^3 cfu/mL. One hundred microlitres of this suspension was added to 900 μL of sterile water or sterile water plus either fluconazole or amphotericin B at concentrations equal to multiples of the MIC ranging from 0.125 × MIC to 16 × MIC. Immediately following addition of the fungal suspension, a sample was removed and plated on potato dextrose agar plates (Remel, Lenexa, KS, USA) for colony counting, by direct plating of either 10 μL or 30 μL of test solution. Following 48 h incubation at 35°C, the number of cfu was determined. Tests were conducted in quintuplicate.

Time–kill curve procedures

Time–kill studies were performed as described previously. Before testing, fungi were subcultured twice on potato dextrose agar plates. Colonies from a 24–48 h culture were suspended in approximately 9 mL of sterile water and adjusted to a 0.5 McFarland turbidity standard. One millilitre of the adjusted fungal suspension was then added to either RPMI medium (control) or a solution of RPMI plus an appropriate amount of antifungal stock solution. These procedures resulted in a starting inoculum of approximately 1 × 10^5 to 5 × 10^5 cfu/mL and antifungal concentrations equal to 0.5, 1, 2, 4 and 8 × MIC for fluconazole and 0.125, 0.25, 0.5, 1, 2, 4, 8 and 16 × MIC for amphotericin B. Test solutions were placed on a shaker and incubated with agitation at 35°C. At predetermined times, 100 μL samples were obtained from each solution, serially diluted ten-fold, and a 10 μL sample plated on potato dextrose agar for colony counting. When it was expected that there would be <1000 cfu/mL, a 10 μL sample was removed from the test solution and plated without dilution. The lower limit of detection of these methods was 100 cfu/mL. Colony counts were recorded following incubation at 35°C for 48–72 h. Kill curve experiments were performed in duplicate.

A nalysis

For antifungal carryover studies, mean colony count data for each agent at each multiple of the MIC tested were compared with the control. Significant antifungal carryover was defined as a reduction in mean cfu/mL of >25% compared with the control. Mean colony count data (log_{10} cfu/mL) from time–kill studies were plotted as a function of time for each isolate and used to compare the rate and extent of antifungal activity at the various concentrations. The time required to achieve a 99.9% reduction from the initial inoculum was determined for each isolate at the various antifungal concentrations tested. The difference in log_{10} cfu/mL between control and experimental samples (expressed as a percent of control) after 6 h was plotted against the multiple of the antifungal MIC. The concentration that resulted in 50% of the maximum effect (EC_{50}) was calculated for each regimen–isolate combination using an inhibitory sigmoid model (WinNonlin, Scientific Consulting, Inc.).

R esults

Antifungal susceptibility

Fluconazole MICs were 2 mg/L (strain 853.033) or 4 mg/L (strains 625.012 and 682.027). The MIC of amphotericin B was 1 mg/L for all three of the strains tested. All of the strains were considered susceptible to fluconazole and amphotericin B.

Antifungal carryover

A ntifungal carryover was not observed at any of the fluconazole concentrations with either sampling method. Similarly, when a 10 μL sample was taken from solutions containing amphotericin B and plated directly for colony count determination, no antifungal carryover was observed. In contrast, a significant carryover effect was noted for isolates 625.012 (27.9% reduction) and 853.033 (46.8% reduction) at an amphotericin B concentration equal to 16 × MIC when a sampling volume of 30 μL was used. Based upon these findings, 10 μL was used as the maximum sampling volume for direct sample plating during time–kill studies.

Time–kill curves

A representative plot of log_{10} cfu/mL against time for fluconazole and amphotericin B is presented in the Figure. Fungistatic activity ( <99.9% decrease in cfu/mL from the initial inoculum) was observed with fluconazole against all three isolates. The rate and extent of antifungal activity were not affected by altering the concentration of fluconazole in solution.

In contrast, rapid fungicidal activity ( >99.9% decrease
in cfu/mL from the initial inoculum) was noted for each of the cryptococcal isolates when tested against amphotericin B. For strains 682.027 and 853.033, fungistatic activity was noted with concentrations of $0.25 \times \text{MIC}$. As the concentration of amphotericin B was increased above $0.25 \times \text{MIC}$, rapid fungicidal activity was observed for both isolates. Similarly, for strain 625.012, fungistatic activity was produced by a concentration equal to $0.125 \times \text{MIC}$ of amphotericin B, whereas rapid fungicidal activity was observed with concentrations of $>0.25 \times \text{MIC}$. For each of the test isolates, the rate of fungicidal activity increased as the concentration of amphotericin B in solution was increased. The time for each isolate to achieve a 99.9% reduction in cfu/mL from the initial inoculum in the presence of amphotericin B is presented in the Table.

The activity of amphotericin B against C. neoformans generally increased as the amphotericin B concentration increased. EC$_{50}$s of 0.21, 0.42 and 0.36 mg/L were calculated for amphotericin B against isolates 625.012, 682.027 and 853.033, respectively. Since the subinhibitory effects of fluconazole were not adequately characterized, similar dose–response curves were not constructed for fluconazole. However, the fungistatic activity exhibited by fluconazole appeared to be independent of the concentration of drug in solution, at least over the multiples of the MIC tested (0.5–8 $\times \text{MIC}$).

**Discussion**

In this study, we report the antifungal characteristics of fluconazole and amphotericin B against three clinical isolates of C. neoformans using in-vitro time–kill curve methods. Fluconazole exhibited fungistatic activity at each of the multiples of the MIC tested. Maximum activity was observed at $0.5 \times \text{MIC}$ and increasing the concentration of fluconazole in solution did not enhance the rate or extent of antifungal activity. Therefore, the activity of fluconazole against C. neoformans seems to be relatively independent of the drug concentration in solution. In contrast, both the rate and extent of fungicidal activity of amphotericin B improved as the concentration of drug in solution was increased. This concentration-dependent effect was most pronounced in terms of the rate of fungicidal activity.

Since this study was conducted in vitro, direct extra-
In general, mortality was similar. Similar findings were reported by Menichetti 1,2,4–6 and Saag and colleagues 2. These clinical findings may be explained by the concentration-dependent activity of amphotericin B. Furthermore, the results reported by Saag and colleagues using 200 mg of fluconazole daily are similar to those reported by Larsen and colleagues in two studies using 400 mg daily. 4,10 Similar findings were reported by Menichetti et al. 3, who observed a mortality rate of 18.2% among 113 patients treated with fluconazole 800–1000 mg daily. These in-vivo findings appear to support our in-vitro data which describe concentration-independent activity for fluconazole against C. neoformans.

Various dosing regimens for amphotericin B have been evaluated for the treatment of cryptococcal meningitis in a number of clinical trials. 1,2,4–6 In general, mortality was lower in studies where higher doses of amphotericin B were used. The rate of mortality decreased from 14% (9/63 patients) with a dose of ≥0.3 mg/kg daily of amphotericin B (no flucytosine) 4 to 5.5% with an amphotericin B dose of 0.7 mg/kg (10/179 with flucytosine and 11/202 without flucytosine) 5 and 3% (1/31 patients) with an amphotericin B dose of 1 mg/kg plus flucytosine 100–150 mg/kg daily. 5 These clinical findings may be explained by the concentration-dependent activity of amphotericin B against C. neoformans which we have described in vitro. Improved outcomes observed with the higher doses of amphotericin B may be linked to a more rapid rate of fungal eradication resulting from increased drug concentrations at the site of infection.

We have described the pharmacodynamic characteristics of fluconazole and amphotericin B against C. neoformans. Fluconazole exhibited concentration-independent fungistatic activity whereas amphotericin B displayed concentration-dependent fungicidal activity. These data should be considered when formulating dosing regimens for fluconazole and amphotericin B in the treatment of cryptococcal meningitis.

### Acknowledgements

These results were presented at the 34th Annual Meeting of the Infectious Diseases Society of America, New Orleans, LA, in September 1996 (Abstract no. 77). This work was supported by a grant from Pfizer, Inc.

### References


**C. neoformans** time–kill curves


Received 4 April 1997; returned 12 June 1997; revised 22 July 1997; accepted 28 October 1997