Antifungal Pharmacodynamic Characteristics of Fluconazole and Amphotericin B Tested against Candida albicans

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Time-kill curves were determined for three isolates of Candida albicans tested against fluconazole and amphotericin B at multiples of the MIC. Fluconazole produced fungistatic activity, with concentration-related growth effects observed over a narrow range of concentrations. Amphotericin B exhibited fungicidal activity, with enhancement of activity over a broader range of concentrations.

The numbers of infections secondary to pathogenic fungi have steadily increased over the past 30 years. Factors contributing to the increased incidence of fungal infections include overuse of broad-spectrum antimicrobial agents, prolonged survival of patients with immune system defects (e.g., diabetics, organ transplant recipients, and oncology patients), and the human immunodeficiency virus. Infection caused by Candida species now ranks fourth among the most common nosocomial pathogens (1). Although the frequency of fungal infections continues to rise, our knowledge regarding the dynamics of antifungals remains in its infant stages. In contrast, over a similar time frame, the characterization of antibacterial pharmacodynamic properties and the study of bacterial-antimicrobial interactions have led to significant alterations in our approach to the utilization of agents such as the β-lactams and aminoglycosides. Therefore, we conducted time-kill studies with fluconazole and amphotericin B against Candida albicans in an effort to characterize the relationships between concentration of these agents and antifungal activity.

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Antifungal agents. Fluconazole (Pfizer Inc., New York, N.Y.) and amphotericin B (Sigma Chemical Company, St. Louis, Mo.) were utilized for the time-kill procedures. A stock solution of each antifungal was prepared utilizing RPMI 1640 medium (Sigma) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma) as solvent. Dimethyl sulfoxide (DMSO) was used to aid the solubilization of amphotericin B. The final concentration of DMSO was such that the concentration in test solutions comprised less than 1% of the total solution composition. Growth curves were conducted with DMSO at concentrations equal to those present in test solutions to verify the lack of an inhibitory effect on the growth of test isolates. Stock solutions were divided into 7-ml aliquots and stored at −70°C until needed for testing.

Test isolates. Fungi selected for testing included two American Type Culture Collection (ATCC) strains, ATCC 90028 and ATCC 90029, and one clinical isolate, OY31.5, of C. albicans. Isolates were obtained from the organism collection of the Special Microbiology Laboratory, Department of Pathology, University of Iowa College of Medicine.

Antifungal susceptibility testing. The MICS of fluconazole and amphotericin B against each test isolate were determined according to the methods proposed by the National Committee for Clinical Laboratory Standards (4). Two ATCC strains, Candida parapsilosis ATCC 22019 and Candida krusei ATCC 6258, served as quality control isolates. MIC analyses were conducted with an inoculum of approximately 0.5 × 10^3 to 2.5 × 10^3 CFU/ml. RPMI 1640 medium buffered to pH 7.0 with MOPS buffer served as growth medium. Briefly, a fungal suspension was prepared and adjusted to a 0.5 McFarland turbidity standard (approximately 1 × 10^6 to 5 × 10^6 CFU/ml). A 1:1000 dilution was performed to yield a starting inoculum of 1 × 10^3 to 5 × 10^3 CFU/ml. One hundred microliters of the fungal suspension was added to each well of a microdilution tray containing 100 μl of the antifungal at a concentration equal to two times the final concentration. The trays were incubated at 35°C in moist, dark chambers. MICs were recorded following 48 h of incubation. The MIC of fluconazole was defined as the concentration which resulted in an 80% reduction of fungal growth compared to the control. The MIC of amphotericin B was defined as the concentration which resulted in complete visual inhibition of growth. Susceptibility tests were repeated on three separate occasions.

Antifungal carryover determination. Prior to initiation of the kill curve studies, assessment of the effect of solubilized antifungal on fungal colony count determinations was made. A fungal suspension was prepared with isolate ATCC 90028 to yield an inoculum of approximately 5 × 10^3 CFU/ml. One hundred microliters 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 times the MIC (0.125 × MIC) to 32 × MIC. Immediately following the addition of the fungal suspension to the aqueous solution, an aliquot was removed and plated on potato dextrose agar plates for colony count determination. Four methods were analyzed for colony count determination: direct plating of 10, 30, or 100 μl of test solution or dilution of 30 μl of test solution in 10 ml of sterile water followed by vacuum filtration utilizing a 0.45-μm-pore-size filter and placement of the filter onto a potato dextrose agar plate. Following 24 to 48 h of incubation at 35°C, the numbers of CFU were determined. Tests were conducted in quintuplicates.
Time-kill curve procedures. Isolates were stored in sterile water at room temperature until their use. Prior to testing, fungi were subcultured twice on potato dextrose agar plates. Three to five colonies from a 24- to 48-h growth plate were suspended in approximately 9 ml of sterile water. The fungal suspension was adjusted according to spectrophotometric methods to a 0.5 McFarland turbidity standard (approximately $1 \times 10^5$ to $5 \times 10^6$ CFU/ml). One milliliter of the adjusted fungal suspension was then added to 9 ml of either RPMI 1640 medium buffered with MOPS buffer, control, or a solution of growth medium plus an appropriate amount of antifungal stock solution. This resulted in a 1:10 dilution of the fungal suspension and yielded a starting inoculum of approximately $1 \times 10^5$ to $5 \times 10^6$ CFU/ml. The resulting fluconazole concentrations in test solutions were equal to 0.5, 1, 2, 4, 8, 16, and 32 times the MICs for test isolates. For amphotericin B, the resulting concentrations were equal to 0.125, 0.25, 0.5, 1, 2, 4, 8, and 16 times the MIC. Test solutions were placed on a shaker and incubated at 35°C. At predetermined time points (0, 6, 12, 24, 36, and 48 h for fluconazole; 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, and 24 h for amphotericin B) following the introduction of the test isolate into the system, 100-μl aliquots were removed from each test solution. Tenfold serial dilutions were performed on samples, and a 10-μl aliquot from each dilution was streaked on a potato dextrose agar plate for colony count determination. For amphotericin B, when the colony count was expected to be less than 1,000 CFU/ml and the concentration was $<8 \times \text{MIC}$, a 10-μl sample was taken directly from the test solutions and plated on a potato dextrose agar plate without dilution. Additionally, when a colony count of less than 1,000 CFU/ml was expected regardless of the antifungal concentration, 30 μl of test solution was diluted in 10 ml of sterile water and filtered through a 0.45-μm-pore-size filter. The filter was then placed on a potato dextrose agar plate. These procedures were selected because they were found to eliminate the antifungal carryover effect noted with amphotericin B. The lower limit of fungal quantitation according to these methods is 33 CFU/ml. Following incubation at 35°C for 48 h, the number of CFU on each plate was determined. Kill curve experiments were run in duplicate.

Analysis. For antifungal carryover studies, colony count results for each concentration were compared with the control. Antifungal carryover was considered significant if test results demonstrated a $>25\%$ decrease in the number of CFU compared to the control (5). A representative plot of the percent difference in the log$_{10}$ of the numbers of CFU/milliliter from kill curve replicates were plotted versus time and used for visual comparisons of the rate and extent of antifungal activity. The time to achieve a 99.9% reduction from the initial inoculum was determined for each isolate (5). Dose-response plots depicting the difference in the log$_{10}$ of the numbers of CFU/milliliter between control samples and experimental samples (expressed as a percent of control) versus antifungal concentration at 24 h were constructed, and the 50% effective concentration (EC$_{50}$) was determined for each agent by utilizing an inhibitory sigmoid model (WinNonlin, Scientific Consulting, Inc.). Additional dose-response curves were constructed for amphotericin B at 8 h.

Antifungal susceptibility results. The median MICs of fluconazole for test isolates ranged from $\leq 0.125 \, \mu g/ml$ to 0.25 μg/ml for ATCC 90028 and ATCC 90029. The median MICs of amphotericin B observed were 0.5 μg/ml for ATCC 90029 and OY31.5 and 1.0 μg/ml for ATCC 90028. All of the test strains were considered to be susceptible to fluconazole and amphotericin B (6, 7). Quality control results were consistently within acceptable ranges (4).

Antifungal carryover results. No antifungal carryover was observed with fluconazole by any of the sampling methods. All samples were within 25% of the mean control colony count. In contrast, significant antifungal carryover was noted with amphotericin B. Samples of 100 μl yielded significant carryover ($>25\%$ deviation in mean colony counts from control) at concentrations of $\geq 1 \times \text{MIC}$. When sampling volumes of 10 and 30 μl were evaluated, a carryover effect was observed at concentrations of $\geq 16 \times \text{MIC}$ and $\geq 8 \times \text{MIC}$, respectively. According to the filtering method, the residual inhibitory effect exerted by amphotericin B was eliminated. Colony counts from filtered samples were found to be within 25% of both filtered and unfiltered controls.

Time-kill curves. Plots of the log$_{10}$ of the numbers of CFU/milliliter versus time for fluconazole and amphotericin B are presented in Fig. 1. Fluconazole exhibited fungistatic (<99.9% decrease in the log$_{10}$ of the number of CFU/milliliter compared with starting inoculum) activity against each of the test isolates; however, three distinct effects on growth were observed. At fluconazole concentrations equal to 0.5×MIC, no inhibitory effect was observed and resultant curves were nearly identical to those for the control. At concentrations ranging from 1×MIC (ATCC 90028 and ATCC 90029) to 2×MIC (OY31.5), slight growth was observed; however, growth was less than that observed with the control. Lastly, at concentrations of $>2 \times \text{MIC}$, a concentration-independent fungistatic effect was observed. The EC$_{50}$ values for ATCC 90028, ATCC 90029, and OY 31.5 were determined to be 0.95, 0.87, and 1.74, respectively. Exposure to fluconazole did not produce a 99.9% reduction in the log$_{10}$ of the number of CFU/milliliter against test isolates at any of the multiples of the MIC tested. In contrast, marked concentration-dependent fungicidal (>99.9% decrease in the log$_{10}$ of the number of CFU/milliliter compared with starting inoculum) activity was noted with amphotericin B. Although the rate and extent of fungicidal activity varied among test isolates, both markers of fungicidal activity increased with increasing concentrations of amphotericin B. Against isolates ATCC 90028 and OY31.5, a distinct concentration can easily be identified below which static activity is observed and above which fungicidal activity is exhibited. Visually, this concentration was determined to be 0.5×MIC for both isolates. This corresponds closely with the EC$_{50}$ values calculated for isolates ATCC 90028 (EC$_{50}$ = 0.59) and OY31.5 (EC$_{50}$ = 0.43). Isolate ATCC 90029 exhibited a slightly different concentration-to-activity relationship. Against this isolate, the rate of fungicidal activity was lower than those observed for the other fungi. As a result, it is more difficult to visually determine the transitional concentration; however, at 24 h static activity is observed at concentrations of $\leq 2 \times \text{MIC}$ and fungicidal activity is observed for concentrations of $>4 \times \text{MIC}$. The EC$_{50}$ for ATCC 90029 was determined to be 0.93. In general, the time required to achieve a 99.9% reduction in the log$_{10}$ of the number of CFU/milliliter decreased as the concentration of amphotericin B in the test solution increased. A representative plot of the percent difference in the log$_{10}$ of the number CFU/milliliter versus the multiples of the MICs of both fluconazole and amphotericin B is presented in Fig. 2. Additionally, dose-response curves for amphotericin B against isolate ATCC 90028 at various time points are presented to illustrate the relationship between the concentration of amphotericin B in solution and the fungicidal effect over time.

Discussion. The activity exhibited by an antimicrobial is described by a sigmoidal dose-response curve. On this curve of concentration versus activity, a segment of the curve can be identified which characterizes the transition between levels of activity (e.g., negligible to significant). If the range of concen-
trations over which this transition occurs is small, we commonly refer to these agents as exhibiting concentration-independent activity. Conversely, if the range of concentrations over which the transition of activity is observed is large, an agent is labeled as demonstrating concentration-dependent activity. In this study, we have described the concentration-response characteristics for fluconazole and amphotericin B tested against three isolates of *C. albicans*. Although enhancement of activity was observed with both agents over a range of concentrations, the range of concentrations over which improved antifungal activity was noted with fluconazole was relatively small, i.e., 0.5 × MIC to 2 × MIC. Maximal inhibitory effects were observed at concentrations equal to the MIC for one isolate (ATCC 90028) and 2 × MIC for two isolates (ATCC 90029 and OY31.5).
and 24 (E) dose-response curves for fluconazole (E) and amphotericin B (C) following 24 h of drug exposure; (B) dose-response curves for C. albicans ATCC 90028 against amphotericin B at 8 (A) and 24 (C) h of drug exposure.

90029 and OY31.5). The rate of activity noted with fluconazole, i.e., the slope of the time-kill curve, was not influenced by concentration once the maximal fungistatic concentration was surpassed. These antifungal characteristics are reminiscent of antibacterial agents such as the β-lactams, which exhibit similar dynamic characteristics. In contrast, improvement in the antifungal activity of amphotericin B was observed over a wider range of concentrations. For each of the isolates, discernible improvement in the extent of fungicidal activity was noted as the amount of drug in solution was increased. This effect may be highlighted by evaluating dose-response plots for amphotericin B at early time points before the extent of activity is maximized (Fig. 2). Similarly, the rate of activity for each of the isolates improved as the concentration of amphotericin B in solution increased. We have reported similar killing dynamics for these agents against clinical isolates of Cryptococcus neoformans (3).

The differences observed between dose and antifungal activity relationships for fluconazole and amphotericin B should prompt us to evaluate our approach to the utilization of these agents. The data presented here suggest that the optimal response with fluconazole against C. albicans would be observed when fluconazole concentrations at the site of infection exceed 2×MIC. Furthermore, fluconazole concentrations of >4×MIC do not appear to be associated with additional antifungal activity, at least in vitro. It is important to stress that it is the concentration of fluconazole at the site of infection, not the concentration in serum, that is the important antifungal activity. However, given the reasonably good correlation between fluconazole concentrations in serum and in fluid and/or tissue, concentrations in serum can be mathematically linked to the amount of drug at the site of infection (2). Although fluconazole distributes widely throughout the body, it may be necessary to administer relatively high doses of fluconazole, 400 to 800 mg, to maintain continuous effective concentrations at the site of infection.

Our data demonstrate that the rate and extent of fungicidal activity exhibited by amphotericin B in vitro are enhanced by increasing the concentration of drug present in solution. Although numerous factors influence the activity of an agent in vivo, our data suggest that the antifungal activity of amphotericin B may be maximized by the administration of large doses in an effort to optimize the concentration-to-MIC ratio.

The characterization of the pharmacodynamic properties of agents such as the β-lactams and aminoglycosides has had a significant impact on our utilization of these agents. We have described similar pharmacodynamic characteristics for fluconazole and amphotericin B in vitro. Consideration should be given to the in vivo study of optimization of fluconazole and amphotericin B dosing regimens based on the antifungal pharmacodynamic properties described for these agents.

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REFERENCES