In Vitro Evaluation of Various Antifungal Agents Alone and in Combination by Using an Automatic Turbidimetric System Combined with Viable Count Determinations

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A new method combining automatic turbidimetry and sequential viable count determinations was developed to evaluate the in vitro activity of various antifungal agents alone and in combination against three clinical isolates of Candida spp. (two Candida albicans and one C. tropicalis) at two inocula (10⁶ and 10⁷ CFU/ml). Specific parameters were derived from the time-kill curves: the maximal rate of killing, the lowest biomass, and the overnight biomass. Their intra-assay and between-assay reproducibilities were high, with respective standard deviations of 0.4 and 0.25 to 1.4 log CFU/ml. Amphotericin B alone showed a linear relationship between rate of killing or lowest biomass and the log of concentration from 0.03 to 4 mg/liter that was similar for the three strains tested. 5-Fluorocytosine (flucytosine) alone showed a dose-related reduction of overnight biomass for concentrations up to 8 mg/liter with no further increase at higher concentrations for one strain of C. albicans and a paradoxical decrease for one strain of C. tropicalis. Ketoconazole alone was found to be only fungistatic with no increased activity at concentrations up to 16 mg/liter. Amphotericin B plus flucytosine interacted synergistically in 46 to 60% of the combinations tested against C. tropicalis depending on the initial inoculum. Indifference was observed for the two strains of C. albicans. Amphotericin B or flucytosine plus ketoconazole was usually indifferent against the three tested strains.

There is a lack of standardization of the methods used to evaluate in vitro antifungal activity. The results are highly dependent upon the medium, inoculum size, and temperature (3, 21, 23). Recently, a multicenter study (2) showed that the broth dilution procedure recommended by Shadomy and Espinel-Ingroff (22) was reproducible in each laboratory, whereas interlaboratory precision was poor. Published studies (7, 8) that use turbidimetry measurement are encouraging, and there is a suggestion that this technique be proposed to standardize the in vitro susceptibility testing of yeasts. Moreover, the in vitro evaluation of combinations of various compounds also shows a lack of standardization as far as the definition of synergism, indifference, or antagonism is concerned. Therefore, comparison of the results obtained by different investigators is often difficult. Nevertheless, the value of antifungal combinations has been widely studied to improve the prognosis of life-threatening infections and has been recently reviewed (19).

The aim of this study was to develop a reliable method to evaluate systematically in the in vitro activity of various antifungal drugs, alone or in combinations, against Candida species, using standardized parameters derived from continuous optical density measurements (turbidimetric system) and viable count determinations (time-kill curves).

MATERIALS AND METHODS

All tests were performed in a Bonet-Maury biophotometer (ISA-Biologie, Paris, France) with six quartz cuvettes of 8 ml each. Constant mixing was provided in each cuvette by a magnetic barrel moved by a magnetic stirrer. Optical transmission through each cuvette was automatically measured every 2 min and recorded on a chart (percent transmission versus time in hours).

The following clinical isolates were studied: two strains of Candida albicans (strains 1 and 2) and one strain of C. tropicalis. These three strains were isolated from fungemic cancer patients hospitalized at the Institut Jules Bordet. Stock cultures were maintained on Sabouraud slants (Difco Laboratories, Belgium) in screw-capped tubes at room temperature. Fresh Sabouraud agar plates were inoculated from the stock cultures and incubated for 24 h at 30°C. Four colonies were transferred in a tube containing 1 ml of yeast nitrogen base (YNB) broth (Difco, Belgium) plus 1% glucose and homogenized. This suspension was used to inoculate the test cuvette with 1 or 10 drops, according to the target inoculum chosen for each experiment. The optical transmission was set at 100% and incubation was started at 30°C. The antifungal agents were added at a transmission of 85%, which corresponded to the exponential phase of growth, which occurred usually after 1 to 2 h of incubation, considered time zero. A sample of 0.1 ml was obtained from the first cuvette for viable count determination prior to the addition of antifungal agent to control the initial inoculum. At this time the cuvettes inoculated with 1 drop had reached 2 × 10⁵ CFU/ml (range, 1 × 10⁵ to 3 × 10⁵ CFU/ml), and those inoculated with 10 drops had reached 2 × 10⁶ CFU/ml (1 × 10⁶ to 3.2 × 10⁶). Each hour during the first 5 h and after overnight incubation, a 0.1-ml sample was obtained from each cuvette for viable count determination. This was done by sequential 10-fold dilutions in distilled water and by plating each dilution on Sabouraud agar plates with a calibrated loop (0.01 ml). The medium used in the cuvettes was YNB broth (Difco) plus 1% glucose and 0.15% asparagine.

The following compounds were tested: amphotericin B deoxycholate (Fungizone; Squibb, Belgium), final concentration of 0.03 to 4 mg/liter; 5-fluorocytosine (flucytosine; Roche, Belgium); 0.25 to 32 mg/liter; ketoconazole (Janssen Pharmaceutica, Belgium), 1 to 16 mg/liter. All possible
combinations of these drugs in pairs were also tested within the range of concentrations previously mentioned, using the two previously described inocula (2 × 10^5 and 2 × 10^6 CFU/ml). For each experiment studying the in vitro activity of a single agent, five cuvettes contained various concentrations of the tested agent and one cuvette was the control without antifungal agent. For evaluation of the in vitro activity of combinations, one cuvette was the drug-free control, one cuvette contained a fixed concentration of drug A, two cuvettes contained two different concentrations of drug B, and two cuvettes contained the two different combinations of drug A with drug B.

**Preparation of stock solutions.** Stock solutions were prepared according to the recommendations of the manufacturer and stored at −80°C until used. Amphotericin B was dissolved in distilled water at a concentration of 5,000 mg/liter, flucytosine was obtained already diluted as intravenous infusion containers and further diluted to obtain a concentration of 10,000 mg/liter, and ketoconazole was dissolved in dimethyl sulfoxide at a concentration of 5,000 mg/liter.

**Time-kill curves.** The following parameters were derived from the killing curves: the maximal rate of killing (RK), expressed in log CFU per milliliter per hour, was defined as the highest reduction in viable count per hour; the lowest biomass reached (LB), expressed in log CFU per milliliter, was defined as the highest reduction in the viable count during the experiment and was obtained by subtracting the lowest viable count observed from the initial inoculum size; the overnight biomass (OB), expressed in log CFU per milliliter, was defined as the viable count observed after overnight incubation.

Synergy was reached when one of the following criteria was achieved: the maximal RK of the combination was ≥0.5 log CFU/ml per h greater than the corresponding value of the most active drug used alone; the LB obtained with the combination was lower (≥1 log CFU/ml) than the corresponding LB obtained with the most active drug used alone; the OB obtained with the combination was lower (≥1 log CFU/ml) than the corresponding OB obtained with the most active drug used alone.

Antagonism was defined as a similar quantitative decrease in activity measured by the same parameters.

All other situations were considered indifference.

**Reproducibility.** The intra-assay reproducibility of the viable count determination was assessed by sampling 20 times a cuvette prepared as described above to reach an inoculum of 2 × 10^5 CFU of *C. albicans* strain 1 per ml.

For each combination tested, control cuvettes containing each antifungal agent at every tested concentration were included simultaneously. The results for all concentrations of each agent tested alone (24 experiments) were submitted to statistical analysis, and this constituted the between-assay reproducibility.

Twelve combinations were tested in duplicate, including six different combinations of amphotericin B plus flucytosine, three combinations of amphotericin B plus ketoconazole, and three combinations of flucytosine plus ketoconazole. Most combinations were tested in unbuffered YNB, but six different combinations of amphotericin B plus flucytosine were also tested simultaneously in buffered YNB (21) to evaluate the pH effect.

**RESULTS**

**Reproducibility.** The study of intra-assay reproducibility of the viable count determination gave the following results: the mean inoculum was 2.5 × 10^5 CFU/ml, the range was 1.6
to $4.3 \times 10^5$ CFU/ml, and the standard deviation was $0.4 \times 10^5$ CFU/ml.

The between-assay reproducibility (over 24 experiments) of the measure of the maximal RK varied with the concentration of amphotericin B: at 0.06 mg/liter the standard deviation was $0.35 \log$ CFU/ml per h whereas at 4 mg/liter it ranged from 0.4 to 1.1 log CFU/ml per h. The between-assay reproducibility of the measure of the LB also depended on the concentration of amphotericin B and varied from 0.4 to 1.4 log CFU/ml per h. The between-assay reproducibility of the measure of the OB for flucytosine varied from 0.25 to 0.6 log CFU/ml. Twelve combinations tested in duplicate provided a similar pattern of interactions between each pair of the tested antifungal combinations when synergy, indifference, and antagonism were defined as previously described criteria. No discordance occurred in the interpretation of the combinations tested in buffered or unbuffered YNB. Figure 1 shows such a comparison between buffered and unbuffered YNB and the pH evolution in the control cuvette without antifungal agents.

Activity of a single agent. The effects observed with amphotericin B, flucytosine, or ketoconazole alone are summarized in Fig. 2 for *C. albicans* strain 1. The results obtained for the two other strains were similar (Fig. 3 and 4).

Amphotericin B. For amphotericin B, the latency to the first optical effect was about 0.5 h and did not change when the drug's concentration was increased. The optical curves showed a typical decrease in optical density accompanying a rapid killing of the yeast cells as shown on the corresponding time-kill curves. Both the optical and killing curves indicated a dose-related activity when measured by either the latency to regrowth (in hours) derived from the optical curve or the parameters derived from the killing curve (maximal RK, LB, or OB). This relationship between dose and activity was also confirmed for the three tested strains by plotting the maximal RK and the LB against amphotericin B log concentration (Fig. 5). The linear correlations obtained within the range of tested concentrations were highly statistically significant (Pearson coefficient of linear correlation, 0.95; $P < 0.001$). Inoculum dependency was low: a decrease of 0.5 log CFU/ml per h occurred in the maximal RK for each tested concentration by increasing the inoculum from $2 \times 10^4$ to $2 \times 10^6$ CFU/ml, resulting in a loss of about 0.5 log CFU/ml in the LB.

Flucytosine. The types of curves observed for flucytosine alone were different from those obtained for amphotericin B alone (Fig. 2 and 3). As can be seen on the optical curves, the growth was similarly affected by the various concentrations of flucytosine tested. The delay to regrowth appeared to depend on the concentration of flucytosine. The killing curves (Fig. 2 and 3) demonstrated a fungicidal effect occurring later than the one observed with amphotericin B. Figure 6 shows the relationship between the LB and the concentration of flucytosine within the range of 0.12 to 64 mg/liter. The LB was linearly dependent on the concentration of flucytosine only for low concentrations (<4 mg/liter). At higher concentrations (4 to 64 mg/liter) the LB was no longer affected by the concentration of flucytosine for the strain of *C. tropicalis* or was paradoxically affected for the two other strains of *C. albicans*. Inoculum dependency was higher...
than that observed for amphotericin B since a loss of 1 log CFU/ml occurred in the LB by increasing the inoculum from $2 \times 10^5$ to $2 \times 10^6$ CFU/ml.

**Ketoconazole.** Optical and killing curves failed to demonstrate an effect of ketoconazole at high inoculum for all tested concentrations (Fig. 2 and 3). However, ketoconazole affected the optical and killing curves at the low inoculum tested ($10^5$ CFU/ml) (Fig. 7). Similar results were obtained for the three tested strains.

**Combinations of antifungal drugs.** (i) Amphotericin B plus flucytosine. By using the three parameters derived from the killing curves, synergy was observed by at least one criterion in 45.8% (22 of 48) (inoculum, $2 \times 10^6$ CFU/ml) and 60.4% (29 of 48) (inoculum, $2 \times 10^5$ CFU/ml) of the combinations

**FIG. 3.** In vitro activities of amphotericin B, flucytosine (5-fluorocytosine), and ketoconazole in liquid YNB against *C. tropicalis*, measured by continuous turbidimetry recording or sequential viable count determinations. Concentrations are in milligrams per liter. Arrow indicates addition of the antifungal agent.

**FIG. 4.** In vitro activity of amphotericin B in liquid YNB against *C. albicans* 2, measured by continuous turbidimetry recording or sequential viable count determinations. Concentrations are in milligrams per liter. Arrow indicates addition of the antifungal agent.
against *C. tropicalis*. Antagonism was observed in 16.6% (8 of 48) of the combinations (inoculum, $2 \times 10^6$ CFU/ml) and in 25% (12 of 48) of the combinations tested at high inoculum ($2 \times 10^6$ CFU/ml). Antagonism was mostly observed when the maximal RK was considered: 16.6% (8 of 48) and 20.8% (10 of 48), respectively, for low and high inocula. Antagonism was less often demonstrated when evaluated by the other parameters: respectively, 4.1% (2 of 48) and 8.3% (4 of 48) for the OB, 2% (1 of 48) and 6.2% (3 of 48) for the LB. The effect of the combination of amphotericin B and flucytosine was related to the characteristic effect on the killing curve of each drug tested alone. Within the combination, amphotericin B mainly affected the RK (which occurred early), while flucytosine mainly affected the OB. The interval of concentration of amphotericin B, for which synergy was more likely to occur, was narrow; synergy was seldom observed for combinations containing low (<0.25 mg/liter) or high (>1 mg/liter) concentrations of amphotericin B (Fig. 8).

(ii) Other combinations. The combination of amphotericin B with ketoconazole was usually indifferent; however, antagonism could be observed whenever the amphotericin B concentration was $\geq$0.25 mg/liter (Fig. 8). Similarly, the combination of flucytosine and ketoconazole was usually indifferent; however, antagonism could also be observed whenever the ketoconazole concentration was $>$2 mg/liter. This was observed for the three strains tested.

DISCUSSION

The in vitro study of antifungal activity remains a major area of controversy, and discordance among several investigators must be related to the methodology. The use of an automated turbidimetric system has marked advantages over conventional methods performed either in broth (micro- or macromethod) or in agar, for the following reasons: control of the growth phase (the antifungal agents can be added during either exponential growth or stationary phase); control of the inoculum by simultaneous measure of the viable count; constant shaking to provide homogeneous contact between yeast cells and active agents, permitting a homogeneous sampling during the incubation. In a multicuvet system it is also possible to compare simultaneously different concentrations of such agent or combinations of drugs in strictly controlled conditions. The volume of broth (8 ml) used here allowed us to study a large population of cells.

The reproducibility of the system was high as exemplified...
by the small standard deviations shown in Fig. 5 and 6. The cutoff values in the maximal RK, the LB, and the OB between indifference and synergy or antagonism were chosen according to the intra-assay reproducibility of the viable count determination and the between-assay reproducibility of these parameters measured in cuvettes evaluating a single agent. The cutoff value for the LB and OB used here were smaller than what is usually recommended for the investigation of antibiotic combinations against bacteria; however, the conventional 2-log difference has not been supported by similar reproducibility studies with yeasts.

Amphotericin B showed a dose-activity relationship. The linearity of the relation should permit use of the checkerboard method to assess the interaction between amphotericin B and other drugs, providing that these drugs show the same property. However, whether efficacy of amphotericin B correlates with the dose remains controversial (1, 5, 16, 24, 26). Failures and relapses are numerous despite amphotericin B therapy. Preliminary data from our patients suggest a lack of fungicidal activity in the sera of patients treated with conventional regimens of amphotericin B (F. Meunier-Carpentier, A. Coune, J. P. Sculier, N. Collette, C. Heymans, C. Brassinne, C. Laduron, and J. Klastersky, Program Abstr. 25th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 305, p. 146, 1985). The major problem in transposing an in vitro generated dose-activity relationship to an in vivo situation is the toxicity of amphotericin B, precluding its use at higher doses. Recently, it has been suggested that incorporation of amphotericin B into liposomes might be one way of administering amphotericin B at high doses without increasing its side effects (13; Meunier-Carpentier et al., 25th ICAAC), and this approach, using unilamellar liposomes, has already shown an enhanced in vitro fungicidal activity of amphotericin B against yeasts (F. Meunier-Carpentier, J. Taterman, C. Brassinne, C. Laduron, and A. Coune, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1030, p. 273, 1984).

The correlation between in vitro susceptibility in broth of C. albicans isolates to flucytosine and the in vivo response to therapy was confirmed in an experimental murine candidiasis (25). In the present study, flucytosine was found to be much less rapidly fungicidal than amphotericin B; it was nevertheless possible to construct a dose-activity relationship. For one strain (C. albicans 1) a maximal effect was reached at 4 mg/liter followed by a plateau even when the concentration was raised to 64 mg/liter; the other strain (C. tropicalis) showed a paradoxical effect. In this situation, the checkerboard methodology to assess the activity of combinations would be inappropriate.

Ketoconazole showed only fungistatic activity, and no dose-activity relationship could be generated in our automated turbidimetric system.

The system we have used here provides the opportunity to evaluate quantitatively drug-activity alone or in combina-

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**FIG. 6.** Dose-activity relationship of flucytosine on the LB. This parameter was derived from the killing curves. Each point represents the mean of 24 experiments. Bars represent standard deviation. 5 Fluorocytosine, Flucytosine.

**FIG. 7.** In vitro activity of ketoconazole against C. albicans 1 at a low initial inoculum (10⁵ CFU/ml).
The use of parameters such as the maximal RK or the LB permits the study of intra- and interassay reproducibility, which is not the case with other methods. The interaction between amphotericin B and flucytosine was found complex, since certain combinations were synergistic while others were antagonistic or indifferent. Hamilton and Elliott (10) have shown an in vitro and in vivo synergistic interaction between amphotericin B and flucytosine against *Cryptococcus neoformans*. Medoff et al (17, 18) have found a synergistic interaction between amphotericin B and flucytosine against one strain each of *C. albicans* and *C. tropicalis* and a single concentration of amphotericin B (0.2 and 0.78 mg/liter, respectively) and flucytosine (100 mg/liter). It was suggested that amphotericin B increases the penetration of flucytosine through the fungal cytoplasmic membrane.

The combination of amphotericin B and ketoconazole was occasionally synergistic when amphotericin B was in the sub-MIC range of concentration. However, for higher concentrations of amphotericin B, antagonism was usually observed. Ketoconazole (14, 27) is an inhibitor of sterol synthesis, and amphotericin B activity is probably mediated by interaction with membrane ergosterol (4, 11, 26). On this basis, antagonism was to be expected. Odds (20), using different methods and original definitions for MIC, synergy, indifference, additivity, and antagonism, reported that amphotericin B plus ketoconazole affected synergistically 4 of 11 strains while affecting antagonistically 1 of 11 strains. Unfortunately, the concentrations of each antifungal agent at which synergy could occur were not provided. We have previously shown that the combination of amphotericin B with rifampin was only occasionally synergistic (28), using the same methodology. Synergy was only documented for one of three studied strains, and the occurrence of synergy significantly depended on the ratio of concentrations of amphotericin B and rifampin. Synergy was more likely to occur in Sabouraud than in YNB medium. It is thought that amphotericin B increases the permeability of the fungal cell to rifampin, allowing it to inhibit the DNA-primed RNA polymerase (12, 15). However, in two animal models of disseminated candidiasis, rifampin did not enhance the cure rate obtained with amphotericin B (6, 9). Therefore, the clinical relevance of these in vitro observations as well as the use of this combination in the treatment of patients remain controversial.

Although the approach used here may be more cumbersome than the traditional methods, its ultimate value should be determined by its correlation with experimental models of yeast infections in animals and clinical outcome in patients with severe *Candida* spp. infections.

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