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In Vitro Determination of Optimal Antifungal Combinations against *Cryptococcus neoformans* and *Candida albicans*

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There is currently no rapid, reliable, and reproducible in vitro technique to describe the growth-inhibitory interactions of antifungal drug combinations over a wide range of drug concentrations. We have developed a microdilution plate assay that was used to determine optimal drug combinations and concentrations of one-, two-, and three-drug regimens of amphotericin B (AmphB), fluconazole (FLU), and 5-fluorocytosine (5FC) for growth inhibition of three isolates each of *Cryptococcus neoformans* and *Candida albicans*. These growth inhibition data were then used in a multifactorial design technique to (i) generate contour and surface response plots to aid visual interpretation and (ii) develop mathematical equations describing the growth responses of the fungi to a wide range of antifungal concentrations and ratios. Our data indicated that (i) antifungal drug-drug interactions affecting yeast growth are complex functions of the drugs used in combination, their absolute concentrations, and also their relative (proportional) concentrations; (ii) AmphB-FLU combinations had additive effects against *C. albicans* over wide concentration ranges for each agent but were indifferent (i.e., were less than additive) in their inhibitory effect on *C. neoformans*; (iii) other two-drug combinations (FLU-5FC or AmphB-5FC) had indifferent effects on the growth of both fungi; and (iv) three-drug combinations (AmphB-FLU-5FC) showed an additive inhibitory effect on the growth of both *C. albicans* and *C. neoformans*. The finding that no antagonism was observed in combinations employing AmphB and FLU in this in vitro model is of critical importance since it argues against the current theoretical concept, based on the individual drug's mode of action, of antagonism between these two drugs. These microdilution techniques provide a method to determine rational regimens of antifungal agents in multidrug combinations for future testing to correlate in vitro activity with in vivo response. The use of this approach has made the evaluation of complex antifungal drug-drug interactions possible and provided important new information to the evolving field of antifungal drug combination.

The incidence of severe fungal infections in humans has increased steadily over the past 2 decades, particularly among postoperative and immunocompromised patients (2). There is currently no single antifungal agent combining low toxicity with proven clinical efficacy against a broad spectrum of fungi. Although amphotericin B (AmphB) is the standard therapy for most systemic fungal infections, its renal toxicity limits its use (21). The azole antifungal agents (most notably, fluconazole [FLU]) are much less toxic than AmphB and have been useful in the prevention of systemic fungal infections in bone marrow transplant patients (9) and suppression of mucosal candidiasis and cryptococcal meningitis in AIDS patients (18). However, frequent therapeutic failures and emergence of azole resistance have limited their utility. These problems underline the need for new therapeutic approaches for fungal infections (1). One approach for circumventing these problems is to use combined drug treatments. Antifungal agents in combination can, in certain cases, exhibit improved efficacy, a broader spectrum of action, and a reduced duration of therapy (17).

Clinicians currently have little information on which to base decisions regarding appropriate doses and combinations of antifungal agents for treating patients with life-threatening fungal infections. The combination most studied is AmphB and 5-fluorocytosine (5FC) in the inhibition of *Candida albicans* (10, 15, 22) and *Cryptococcus neoformans* (3, 13). Only a limited number of in vivo studies have examined the efficacy of antifungal drug combinations (17). Recently, the combination of FLU and 5FC has been studied for the treatment of cryptococcal meningitis (1, 11).

For rational design of antifungal drug combinations for potential treatment of invasive mycoses, it is imperative to have a simple, rapid, reliable in vitro methodology that is predictive of in vivo outcome. Traditional methods of antifungal drug susceptibility testing define the MIC and minimal fungicidal concentration. Recently, we described a microdilution method that defines inhibitory concentrations over wide concentration ranges (7). In the current study, we used this assay method, combined with a multifactorial design technique, to define the levels of inhibition of both *C. albicans* and *C. neoformans* by AmphB, FLU, and 5FC alone and in two- or three-drug combinations. The methods employed are relatively simple, rapid, and amenable to automation.

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MATERIALS AND METHODS

Antifungal agents. FLU (lot Z6 109-92000-11; 100% FLU when assayed as supplied with the Pfizer Quality Control Reference Standard) was kindly provided by Roerig/Pfizer Pharmaceuticals (New York, N.Y.) and prepared as a stock solution in sterile distilled water at 10 mg/ml. AmphB was purchased from Bristol-Myers Squibb (Princeton, N.J.) as a lyophilized powder in vials containing

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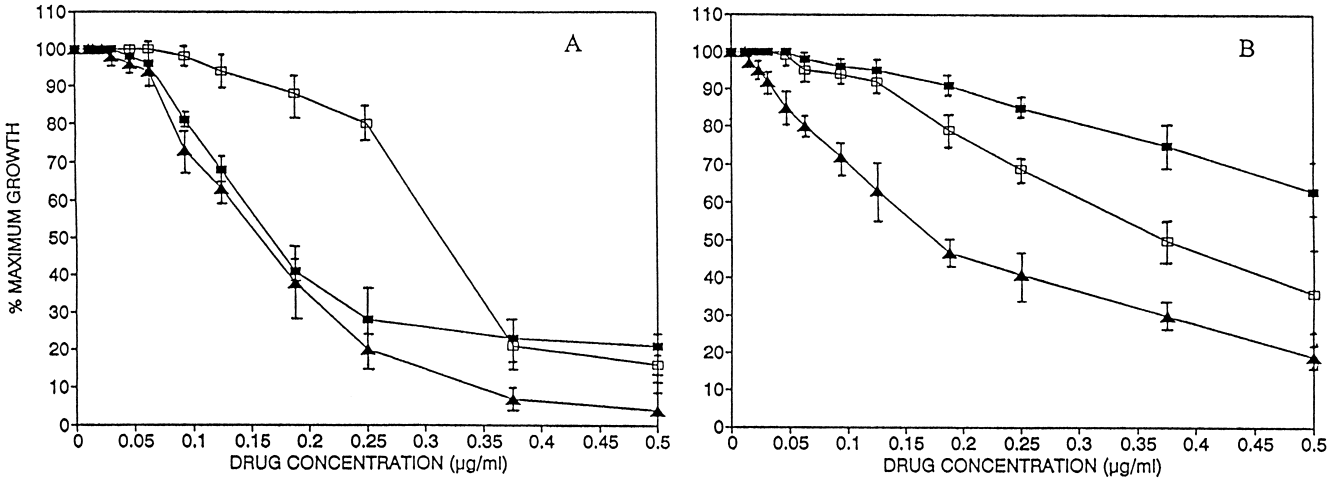

FIG. 1. Inhibition of *C. neoformans* ATCC 36556 and *C. albicans* ATCC 36082 by AmphB-FLU combinations. (A) *C. neoformans* inhibition by FLU (□), AmphB (■), and equal amounts of FLU and AmphB (▲). (B) *C. albicans* inhibition by FLU (■), AmphB (□), and equal amounts of FLU and AmphB (▲).

50 mg of AmphB and 41 mg of sodium deoxycholate buffered with 25.2 mg of sodium phosphate. The powder was reconstituted in distilled water by following the manufacturer's instructions. AmphB stock solutions were tested against *C. albicans* ATCC 36082, our laboratory standard isolate, to check for biological activity and ensure consistency between different batches. 5FC (minimum of 99% purity as assayed by thin-layer chromatography with the Sigma Quality Control Reference Standard) was obtained from Sigma Chemical Co. (St. Louis, Mo.), and solutions were prepared in accordance with the manufacturer's recommendations. All drugs were stored at −70°C as stock solutions until the day of the experiment, when they were thawed and diluted in the appropriate medium. All solutions were protected from light during the experiment.

Organisms. *C. albicans* ATCC 36082 and *C. neoformans* ATCC 36556 were obtained from the American Type Culture Collection (ATCC), Rockville, Md. Two clinical isolates of *C. albicans* (CA15 and CA17) and *C. neoformans* (CN1623 and CN1624) were also examined. Clinical isolates were obtained from the Clinical Microbiology Laboratories at Harbor-UCLA Medical Center, Torrance, Calif. The clinical isolates were used to examine whether the in vitro results obtained with the ATCC isolates were strain dependent. The organisms were maintained at 4°C on Sabouraud dextrose agar slants (Difco Laboratories, Detroit, Mich.) and subcultured routinely.

Susceptibility testing. The MICs of AmphB, FLU, and 5FC for inhibition of *C. albicans* and *C. neoformans* were determined by a modification of the microdilution technique described elsewhere (7). Antifungal agents were diluted in either RPMI 1640 medium for *C. albicans* or yeast nitrogen base medium supplemented with 0.5% glucose for *C. neoformans* to provide a stock solution of each antifungal agent. The RPMI 1640 medium was obtained from Gibco Laboratories (Grand Island, N.Y.), and the yeast nitrogen base medium was from Difco. Both media were buffered to pH 7.0 with 0.05 M morpholinepropanesulfonic acid. Drug-free medium was dispensed into all wells of microdilution assay plates. For tests with ATCC strains, 10 twofold dilutions of antifungal stock solutions were made by addition to wells 2 through 11. Concentrations used to determine the MICs of the three drugs were 60, 30, 15, 7.5, 3.75, 1.88, 0.94, 0.47, 0.23, and 0.12 μg/ml. Well 1 served as a sterility control and blank for the spectrophotometric assays; well 12 served as a growth control. Ten microliters of a fungal cell suspension was added to each well of the microdilution plates to achieve a final inoculum of approximately 10^4 CFU/ml. Fungal inocula were prepared by inoculating 10 ml of fresh yeast nitrogen base broth (Difco) with 10^6 yeast cells, obtained from colonies grown on agar media, and incubating the mixture at 35°C for 24 to 48 h. Cells were washed and suspended in normal saline, and their numbers were standardized by counting in a hemocytometer. Inoculated plates were incubated for either 24 h (*C. albicans*) or 48 h (*C. neoformans*) at 35°C (7). Yeast cell growth was determined by measurement of optical density at 420 nm in each well with an automatic microplate reader (Dupont Instruments, Wilmington, Del.) after agitation of the plates to form a homogeneous cell suspension for either 3 min (for *C. albicans*) or 15 s (for *C. neoformans*) with a vortex shaker (Vortex-Genie 2 Mixer; Scientific Industries, Inc., Bohemia, N.Y.). The MIC end point was defined as the lowest drug concentration exhibiting a 50% (or greater) reduction in optical density at 420 nm compared with the growth in the control well. The media and incubation conditions employed in this study parallel those proposed in the NCCLS reference method for antifungal susceptibility testing (16).

Tests with clinical isolates used fewer sample wells. Guided by the inhibition results obtained for the ATCC strains, fourfold dilutions were employed to test the inhibition of the clinical isolates by various combinations. The concentration ranges used were 0 to 1.5 μg/ml for AmphB and FLU and 0 to 0.5 μg/ml for 5FC. This reduced the total number of combined drug concentrations analyzed for clinical isolates from 1,080 to 616. The use of fewer datum points resulted in time and material savings, as well as sufficient datum points for use in describing growth responses to various drug combinations.

Drug inhibition analysis. Selection of the range of concentrations and ratios of antifungal agents for combination testing was based on the MIC determined for the individual antifungal agent against *C. albicans* and *C. neoformans* strains. The range for each test drug in the combination included concentrations both above and below the individual MIC. Microdilution assays of ATCC strains were run in duplicate at each concentration. The assays were repeated a minimum of twice on separate days, and the means were used in the analysis. Clinical isolates of *C. albicans* and *C. neoformans* were tested in duplicate. In this study, combinations showing greater than additive inhibitory effects on growth but less than synergistic effects are defined as having additive effects, while combinations showing less than additive inhibitory effects on growth are defined as having an indifferent effect.

Analysis of data. Analyses of antifungal drug concentrations and interactions affecting fungal growth were performed by using multifactorial design models as reported previously (5, 8). With the microdilution method and automated plate reading, it was possible to obtain several hundred datum points for describing growth responses to a wide range of antifungal concentrations and ratios. Sta-

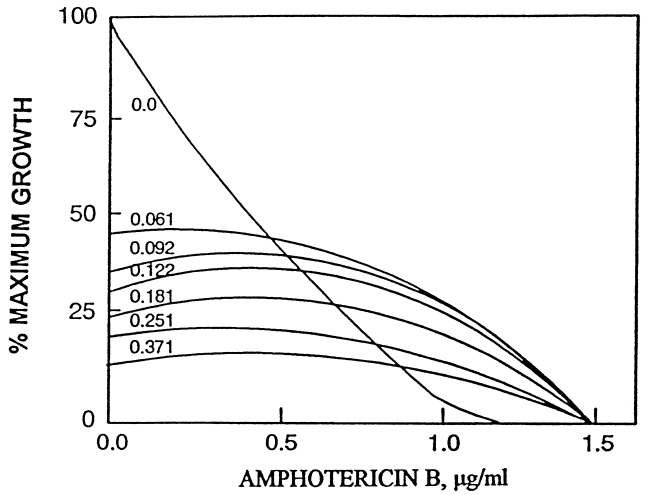

FIG. 2. Inhibition of *C. albicans* growth by AmphB in the presence of different levels of 5FC. The numbers on each curve represent the concentrations of 5FC in micrograms per milliliter. Each value indicates the percent growth change when candidal growth in the presence of AmphB alone is compared with growth in AmphB plus 5FC.

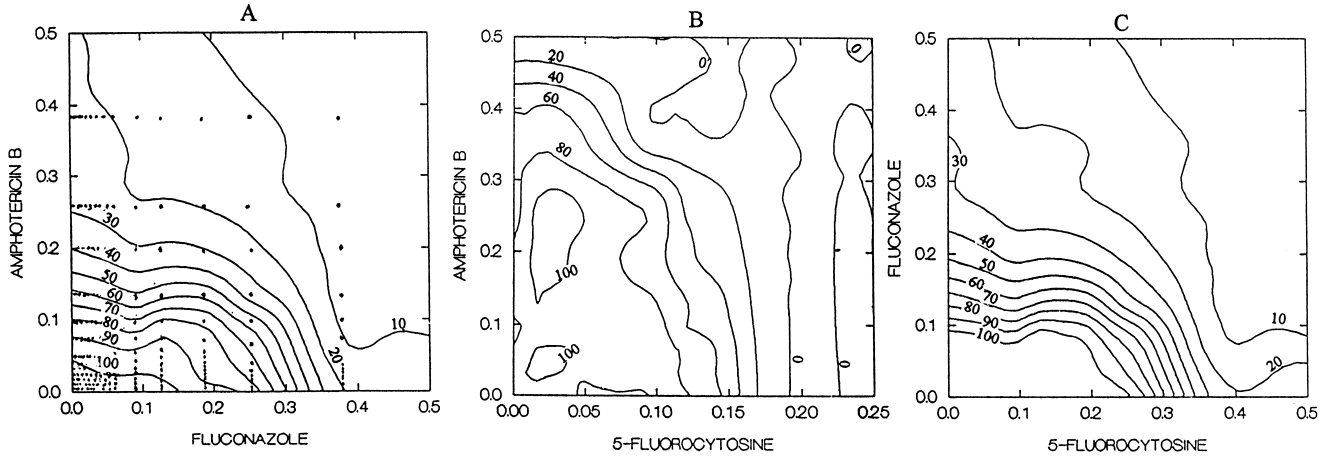

FIG. 3. Contour plots showing percentages of maximum growth of *C. neoformans* in the presence of (micrograms per milliliter) AmphB plus FLU (A), AmphB plus 5FC (B), and FLU plus 5FC (C). The numbers on the contours indicate percent growth corresponding to the concentrations of each drug pair. The points on the contour plane of panel A indicate the concentrations of AmphB and FLU used to develop the contour plots. Growth data at these points (in duplicate) were used to generate the contours. Equations describing the best fit to the *C. neoformans* ATCC 36556 growth data: AmphB-FLU (A), Growth = 100 − 240(FLU) − 101(AmphB) + 106 (FLU)(AmphB) + 159(FLU)^2; AmphB-5FC (B), Growth = 103 − 109(5FC) − 328(AmphB) + 160(5FC)(AmphB) + 223(5FC)^2; FLU-5FC (C), Growth = 108 − 215(FLU) − 205(5FC) + 123(FLU)(5FC) + 123(FLU)^2 + 107(5FC)^2.

tistical analyses, graphic representations, and fits of data to polynomial models were achieved with the Systat program (Systat Inc., Evanston, Ill.). The data were used to generate contour and surface response plots to aid visual interpretation and to develop mathematical equations with coefficients providing the best empirical fit of the experimental data. We employed a polynomial model to describe fungal growth response to the presence of antifungal agents. Other models could be used, e.g., the three-dimensional analytical method described by Prichard et al. (20). With the large number of datum points used in this study, a polynomial function provided a good fit for the data. Typical polynomial equations describing results from studies with three drugs, D, E, and F, in combination have the following general form: Growth = $C_0 + C_1$ [D] + C_2 [E] + C_3 [F] + C_4 [D][E] + C_5 [D][F] + C_6 [E][F] + C_7 [D][E][F] + C_8 [D]^2 + C_9 [E]^2 + C_{10} [F]^2 + etc., where C_n represents the values of the n coefficients that indicate the magnitude of the effect of drug concentrations on growth. D, E, and F are the concentrations of the three drugs in micrograms per milliliter. The coefficients define the magnitudes of individual and interactive effects of the drugs. The polynomial equations can be used to quantify growth at any selected drug combination. However, inspection of contour shape provides the best intuitive interpretation of the magnitude of a drug interaction.

RESULTS

Antifungal susceptibilities. The MICs of AmphB, FLU, and 5FC for *C. neoformans* ATCC 36556 were 0.47, 3.75, and 0.94 μg/ml, respectively. The MICs of AmphB, FLU, and 5FC for *C. albicans* ATCC 36082 were 0.23, 1.88, and <0.12 μg/ml. The MICs for the clinical isolates of *C. albicans* were similar to the values above. The MIC for *C. neoformans* isolate CN 1623 was also similar to those reported above, but isolate CN 1624 differed in that it was less susceptible to AmphB (no inhibition was noted at levels as high as 1.9 μg/ml).

Growth inhibition by two-drug combinations. Figure 1A (*C. neoformans*) and B (*C. albicans*) shows fungal growth inhibition of the ATCC strains by AmphB and FLU, singly and in combinations, in which the masses (and, therefore, the molarities) of the two antifungal agents were maintained in constant ratios. Figure 1A shows that inhibition of *C. neoformans* by the combination of AmphB and FLU was greater than inhibition by either drug alone. Figure 1B shows that inhibition of *C. albicans* by a combination of these same drugs was also greater than the inhibition by either drug alone. Inhibition of *C. albicans* growth by AmphB is presented in Fig. 2. The individual curves show the effects of various concentrations of 5FC on growth inhibition by AmphB. The curves plotted are each based on 170 test points. The smooth curve represents the best fit to these points. The curve marked 0.0 shows growth inhibition by AmphB with no 5FC present, while the remaining curves show inhibition at progressively higher 5FC concentrations. For example, the curve labeled 0.061 represents inhibition by AmphB in the presence of 0.061 μg of 5FC per ml. At low concentrations of AmphB on this curve, addition of 5FC enhanced inhibition. However, at higher concentrations of AmphB (0.5 to 1.5 μg/ml), *C. albicans* grew better in the presence of the combination than with AmphB alone. Thus, an antagonistic interaction exists between these two drugs at higher concentrations and different ratios of AmphB to 5FC. This same observation, that the enhancement or antagonism of AmphB-mediated inhibition of *C. albicans* growth is dependent on the absolute 5FC concentration, was noted for each 5FC concentration tested.

Our drug interaction experiments (Fig. 1 and 2) examined only limited ranges of possible ratios for each agent. Numerous curves of this sort are required to present an overall picture of interactions among drugs. Figure 3 provides a more comprehensive representation of interactive effects between drugs over the entire range of concentrations tested. Data are presented in the form of contour plots showing inhibition of growth of *C. neoformans* by combinations of AmphB and FLU (Fig. 3A), AmphB and 5FC (Fig. 3B), and FLU and 5FC (Fig. 3C). The points overlaid on the Fig. 3A contours indicate the concentrations of AmphB and FLU used for growth inhibition measurements and contour generation. These points are well distributed over the entire concentration range. All of the plots in Fig. 3 and 4 were based on similar distributions of datum points. Contour plots successfully illustrate how growth, indicated on each contour as a percentage of control growth, varies with the concentrations of the drug pairs. Minor deviations in these curves are not significant. Only the overall shapes of the contour patterns should be considered. The level of growth is the same at all points on a given contour line. For example, in Fig. 3C, all of the FLU and 5FC concentrations defined by coordinates at any point on the curve labeled 50 give 50% growth inhibition. With each of these three drug combinations, the overall shape of the contours is concave downward for *C. neoformans.*

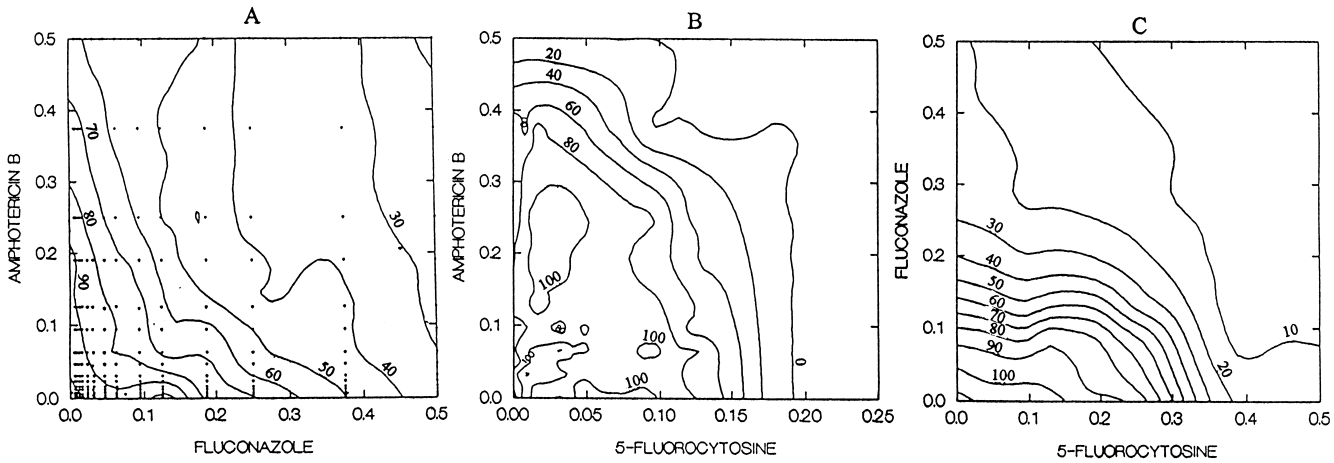

FIG. 4. Contour plots showing percentages of maximum *C. albicans* growth in the presence of (micrograms per milliliter) AmphB plus FLU (A), AmphB plus 5FC (B), and FLU plus 5FC (C). Equations describing the best fit to the growth data of *C. albicans* ATCC 36082: AmphB-FLU (A), Growth = 100 − 174(FLU) − 83(AmphB) + 85(FLU)(AmphB) + 99(FLU)^2; AmphB-5FC (B), Growth = 100 − 149(5FC) − 284(AmphB) + 148(5FC)(AmphB) + 201(5FC)^2; FLU-5FC (C), Growth = 95 − 138(FLU) − 136(5FC) + 160(FLU)(5FC) + 33(FLU)^2.

A similar set of contour plots is presented in Fig. 4 to show inhibition of growth of *C. albicans* by these same drug pairs. With *C. albicans*, the contours for the combination of AmphB and FLU (Fig. 4A) have a generally concave upward form, while contours are concave downward for the combinations AmphB-5FC and FLU-5FC (Fig. 4B and C). In this respect, two-drug combinations resulting in contours that have a generally concave upward form suggest that the two drugs interact more favorably to inhibit organism growth than do drug combinations resulting in concave downward contours.

Growth inhibition by three-drug combinations. When a third antifungal agent is included in tests of growth-inhibitory activity, an increased number of experimental measurements are required to define the combined drug responses. Graphical representation of the results follows the same form used for two-drug combinations but becomes correspondingly more complex. One way of illustrating the effects of three-drug combinations on growth is presented in Fig. 5 for *C. neoformans* ATCC 36556. This plot summarizes the inhibition by AmphB at different concentrations of FLU and a single fixed concentration of 5FC (0.181 μg/ml). Percent growth refers to the percentage of the maximal growth with no drugs present. The highest points in each column are growth measurements at low FLU concentrations. Thus, the column of points at each concentration of AmphB shows growth from 0 FLU (at the top) to a high FLU concentration (at the bottom). A line connects the highest point in each column showing the growth rate in the presence of AmphB without FLU. As the concentrations of FLU increased, growth inhibition by the two combinations increased also.

A series of contour plots can be prepared showing responses to any two of the drugs, with each graph in the series representing responses at a separate concentration of the third drug. Figure 6 was plotted with 5FC maintained at 0.061 μg/ml in the lower plane and increased to 0.092 and 0.122 μg/ml in the center and top planes, respectively. As the concentration of 5FC increased from 0.061 to 0.092 and 0.122 μg/ml, growth was further inhibited, as is evident from the decreased values on the contours at the higher concentration of 5FC. In the presence of 5FC, the combined effects of AmphB and FLU on *C. neoformans* ATCC 36556 growth remained indifferent. Figure 6 illustrates that when the AmphB concentration was greater than about 1.0 to 1.2 μg/ml, addition of FLU had no further effect on growth.

A similar series of contour plots was developed for the response of *C. albicans* growth to AmphB, FLU, and 5FC (data not shown).

DISCUSSION

The limited number of in vitro studies of combined drug effects on fungal inhibition are often conflicting (17). These conflicting results have been attributed to differences in experimental design, pathogens studied, drug concentrations, and

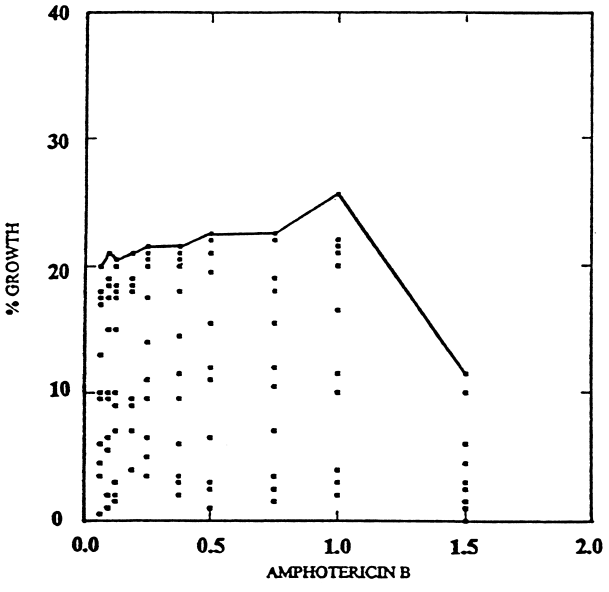

FIG. 5. Inhibition of *C. neoformans* ATCC 36556 by AmphB at different levels of FLU and a fixed level of 5FC (0.181 μg/ml). Percentage of maximum control growth is plotted against AmphB concentrations. The points of each column at a fixed AmphB concentration represent different levels of FLU. The points giving the highest percentages of growth are at the lowest concentrations of FLU.

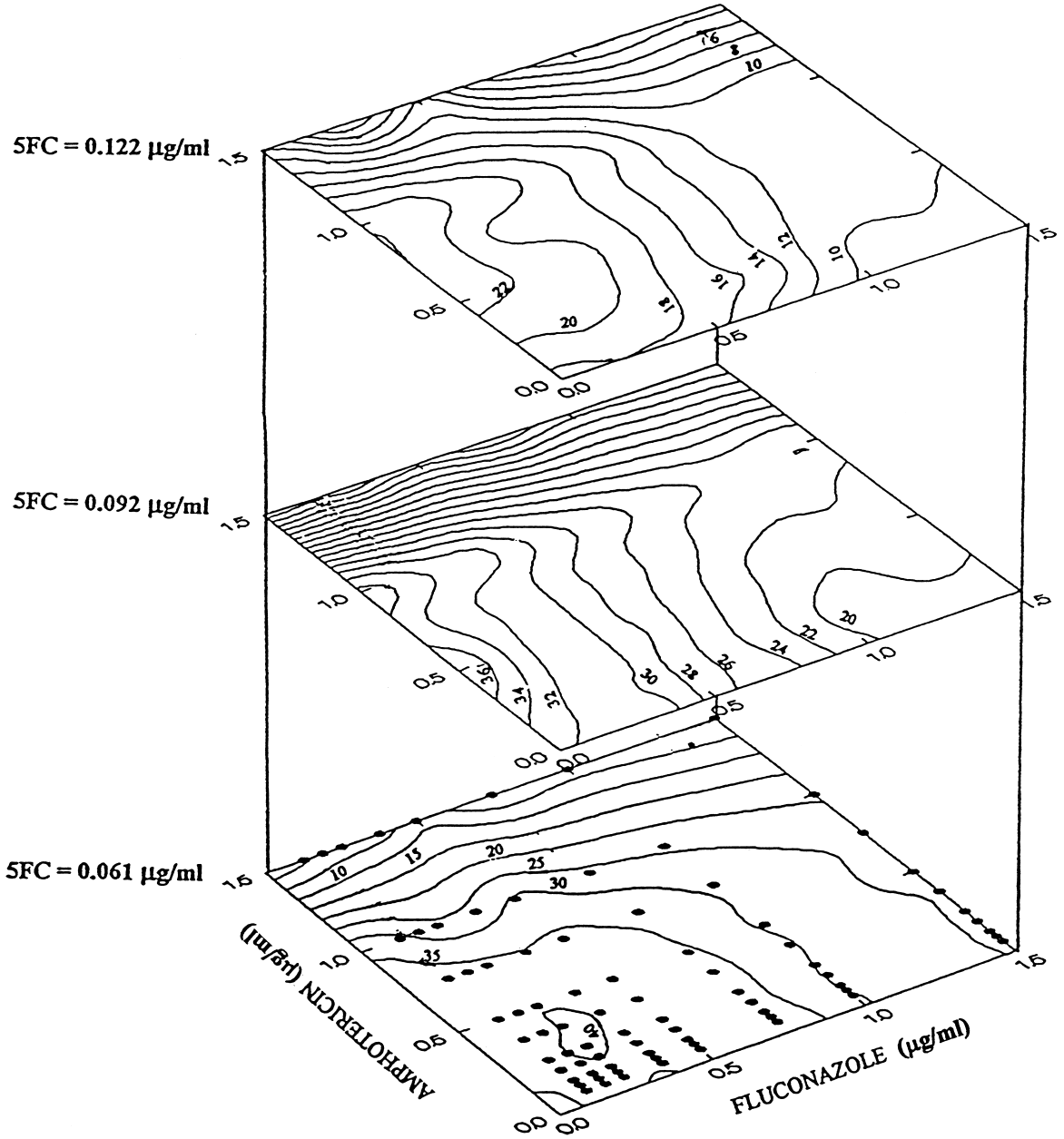

FIG. 6. Stacked contour plots showing the combined effects of three drugs on the growth of *C. neoformans* ATCC 36556. The numerical values on the contours indicate the percentages of maximal fungal growth in the presence of the various combined antifungal concentrations. The points on the lower contour plane indicate the concentrations of AmphB and FLU used at each of the three levels of 5FC shown to develop the contour plots. Growth data at these 80 points (in duplicate) were used to generate the contours. Equation describing the best fit to the *C. neoformans* ATCC 36556 growth data for a three-drug combination: Growth = 101 − 27(FLU) − 33(AmphB) − 173(5FC) + 7(FLU)(AmphB) + 19(FLU)(5FC) + 36(AmphB)(5FC) − 7(FLU)(AmphB)(5FC) + 2(FLU)^2 + 78(5FC)^2. Equation describing the best fit to the *C. albicans* ATCC 36082 growth data for a three-drug combination: Growth = 98 − 83(FLU) − 161(AmphB) − 208(5FC) + 56(FLU)(AmphB) + 201(FLU)(5FC) + 97(AmphB)(5FC) − 94(FLU)(AmphB)(5FC) + 27(FLU)^2 + 65(5FC)^2.

regimens used (1, 6). The studies reported here support an additional explanation for the conflicting results. The effects of drug combinations on fungal growth in vitro heavily depend upon the ratios and concentrations of the drugs employed, as well as the fungal strains tested. Drug combinations can have a synergistic, additive, antagonistic, or indifferent effect, depending upon the nature and relative concentrations of the drugs used.

Different experimental designs, such as isobolograms in checkerboard titration assays and indices such as the fractional inhibitory concentration, have been proposed to address the complexities of combined-drug interactions (4). These approaches have been useful but do not effectively describe the relative and absolute concentrations of drugs required for optimum inhibition of fungal cells over a broad range of concentrations. We previously (8) developed methods that described interactive effects among two- and three-drug antifungal-antineoplastic agent combinations. Other models for three-dimensional analysis of antiviral drug combinations, such as those used by McKinley and Rossman (14), Kong et al. (12)

and Prichard et al. (19), could equally be employed in analyzing the results of our studies of antifungal combinations. With the large number of datum points available in our studies, it is not difficult to satisfactorily fit the experimental data by a variety of methods to obtain graphical representations or equations that accurately describe the results. The polynomial model that we developed earlier (8) and employed here compares favorably with any of the other methods in describing drug interactions among two- or three-drug combinations. Furthermore, our experimental methods and analyses allow automation and efficient evaluation of multiple drug combinations with multiple organisms.

Our data showed that AmphB-FLU combinations had additive effects against *C. albicans* over a wide range of concentrations for each drug and had an indifferent inhibitory effect on *C. neoformans*. The finding that no antagonism was observed in combinations employing AmphB and FLU in this in vitro model is of critical importance, since it argues against the current theoretical concept of antagonism between these two drugs.

The effect of combined AmphB and 5FC on both *C. neoformans* and *C. albicans* (Fig. 3B and 4B) is complex. There appears to be a downward concavity in curves representing combinations of less than 0.5 μg of AmphB per ml and less than 0.15 μg of 5FC per ml, which implies indifferent interaction between these two antifungal agents in combination. In contrast to our findings, the combination of AmphB and 5FC has been suggested to be synergistic against *C. albicans* in vitro (15). Although our data showed that more growth inhibition is obtained with the combination of AmphB and 5FC than with either antifungal agent alone, no significant synergy between these two antifungal agents was observed.

The use of triple drug combinations may provide further improvement in antifungal treatment compared with two-drug combinations. However, it is necessary to obtain a full description of individual and combined drug effects over a wide range of the three drug concentrations to fully realize the potential benefits of such combinations. Our methods show that it is as conceptually feasible to describe growth-inhibitory responses to three drugs as it is for two agents in combination. In this study, we have shown that AmphB, FLU, and 5FC at all concentrations in combination are more inhibitory than any one or two of the drugs together. Equations developed from the data allow calculation of growth inhibition in the presence of any selected antifungal agent combinations. Thus, we can define minimal concentrations of antifungal agents needed for effective inhibition in vitro.

There were no drug combinations detected in this study with highly synergistic inhibitory effects on fungal growth comparable to those reported earlier for specific combinations of antineoplastic and antifungal drugs (8). The large number of drug combinations used in the current study (between 616 and 1,080 datum points) make it likely that most combinations which could prove significantly effective in vitro are included. Therefore, the search for significant synergy must shift to other drug combinations. With the development of microdilution assay methodology and the multifactorial approach outlined here, it will be possible to do equally detailed screening of large numbers of drugs and organisms to define optimum conditions for growth inhibition and to find highly synergistic (or antagonistic) interactions when they exist. With these methods, we provide an accurate description of the efficacy of single and combined antifungal agents against strains of *C. albicans* and *C. neoformans* in vitro. These methods made the evaluation of complex antifungal drug-drug interactions possible and helped to define two problematic areas in antifungal agent combination studies: (i) the role of relative concentrations of component drugs in altering the effectiveness of the combination and (ii) a method to determine rational combinations for future testing to correlate in vitro activity with in vivo response.

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