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Multifactorial Analysis of Effects of Interactions among Antifungal and Antineoplastic Drugs on Inhibition of *Candida albicans* Growth

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Interactions among antineoplastic and antifungal drugs affecting the inhibition of *Candida albicans* growth are complex functions of the nature of the drugs used in combination, their absolute concentrations, and also their relative concentrations. Studies of drug interactions involving the use of test drugs in fixed concentration ratios can lead to inaccurate conclusions about synergism or antagonism among the drugs. A multifactorial experimental design procedure in which the concentrations of all drugs in test combinations were simultaneously varied has been used to identify and quantify drug interactions. The methods have been applied to combinations of two, three, and four drugs, including antineoplastic drugs, antifungal drugs, and combinations of antineoplastic and antifungal drugs. Results were obtained which allow predictions of effects of combinations and provide maximum effectiveness in growth inhibition with minimum levels of the test drugs.

Cancer therapy often entails the use of combinations of antineoplastic agents and/or radiotherapy with the aim of reducing tumor cell populations with minimal side effects (1). These treatments render patients deficient in all known parameters of immune defenses and, as such, predispose them to infection (9). Thus, combinations of one or more antimicrobial agents together with one or more antineoplastic drugs are frequently used in treatment. Taking into consideration the high incidence of yeast infections, particularly those due to *Candida* spp., among cancer patients (8, 11, 12, 16), it is important to investigate the magnitude of interactions among the drugs used in combined antifungal and anticancer treatments.

Both antineoplastic and antifungal agents cause undesirable side effects as a result of their high toxicities. Accordingly, a major goal in drug therapy has been to select drug combinations with synergistic action. Although interactions among drugs when used against microorganisms are well documented (2, 7, 10, 14, 15, 17, 19, 20, 22, 25), studies of interactive effects among antineoplastic and antifungal agents have not yet been reported.

Studies investigating the effects of combinations of drugs on microorganisms have generally been limited to tests of only two drugs. Frequently, analyses are made in a one-factor-at-a-time fashion in which the concentration of a single drug is varied in the presence of a constant level(s) of the second. Such experiments have provided valuable information about interactive properties of the drugs, but cannot be used to quantitatively define interactions or to provide information about interactive effects at concentration levels other than those tested. The common approach to solving this dilemma has been to run tests at more concentrations, but the possible number of trials becomes prohibitive (4). A better approach to tests for two-factor interactions has been to use the so-called checkerboard titration, in which concentrations of both drugs are varied simultaneously. Mathematical treatment of this type of data can yield a quantitative description of all main and interactive effects of drug action. However, because of the number of tests involved, checkerboard titration becomes impractical for combinations of three or more drugs.

There is, however, an important need for testing combinations of more than two drugs. Berenbaum (4) has devised a method for determination of the level of synergy or antagonism for combinations of several drugs with much less effort than would be required in a checkerboard titration. Odds (22) has used the Berenbaum method to study interactions among four antifungal drugs in inhibiting 11 fungal strains. The results clearly show drug interactions and also show the importance of using different assay procedures and conditions which may affect interpretations of the magnitudes of the interactions. Odds (22) draws attention to the power of the Berenbaum method in defining multiple drug responses and, simultaneously, points out some of the limitations of the procedure. Quantitative estimates of synergy and inhibition are given, but these values hold strictly at only the concentrations and the particular ratios of drugs tested. Only when it is assumed that the interactive effects of the antifungal agents are constant over all concentration ranges of interest and not dependent on changing ratios of drugs can the values determined be used as a general index of synergy of the drugs. Because of the known modes of action of antimicrobial drugs, it appears unlikely that interactive effects among drugs would be constant at different concentrations and under different reaction conditions.

We have tested interactive effects by using multifactorial design techniques (5) in an attempt to (i) quantitatively determine drug interactions over broad ranges of concentrations, (ii) define conditions which maximize synergistic effects, and (iii) obtain these results with a minimum number of experimental tests. The present studies show that reports of synergy among drugs must clearly state all concentrations and conditions used. What may be measured as synergism between two drugs at one concentration ratio and set of reaction conditions may turn to antagonism under different conditions. We have shown complex interactions among

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antineoplastic and antifungal drugs and selected combinations which strongly inhibit the growth of *Candida albicans* while containing minimal levels of agents likely to cause toxicity to infected hosts treated with these drug combinations.

MATERIALS AND METHODS

Organism. *C. albicans* KCCC14172 is a clinical isolate obtained from the oral cavity of a patient undergoing head and neck radiation therapy at the Kuwait Cancer Control Center. The isolation and identification techniques were as described previously (12).

Drugs. The following antineoplastic and antifungal drugs commonly used for clinical treatments were used: the antineoplastic agents methotrexate (MT) (Cyanamid International Corp., Basel, Switzerland), cyclophosphamide (CP) (Asta-Werke AG-Chemische Fabrik, Brackwede, Federal Republic of Germany), and 5-fluorouracil (5-FU) (Hoffmann-La Roche and Co. Ltd., Basel, Switzerland) and the antifungal agents amphotericin B (AB), flucytosine (FC), and miconazole nitrate (MN). The antifungal agents were purchased from Sigma Chemical Co., St. Louis, Mo. All antifungal agents used were dissolved in a minimal amount of dimethyl sulfoxide; an equivalent amount of dimethyl sulfoxide was added to control flasks as well as to a blank flask which contained only medium. Antineoplastic drugs were dissolved in water.

MICs. An agar dilution method was used to determine the MICs of the drugs for the *C. albicans* isolate. Serial dilutions were prepared for both the antifungal and antineoplastic drugs in molten Sabouraud dextrose agar (SDA) (Difco Laboratories, Detroit, Mich.); they were then poured and left to solidify. Plates were inoculated with an overnight culture of the organism to give a final concentration of 10^7 cells per ml. The MICs were noted after 48 h. MIC is defined, for this study, as the drug concentration in the highest dilution causing zero visible growth.

Multifactorial analyses of interactive effects. The effects of combinations of antineoplastic and antifungal drugs on *C. albicans* growth were examined by multifactorial analysis techniques. Initially, combinations of three antifungal drugs or three antineoplastic drugs were used in 2^3 factorial designs. Subsequently, two antifungal and two antineoplastic drugs were combined in 2^4 factorial experimental designs. The concentration ranges to be used in analysis of drug interactions were determined by preliminary inhibition tests. High levels selected for each drug tested corresponded to the MICs of a single drug by the agar dilution technique. Low levels were selected as the concentrations at which the first sign of growth inhibition was observed (as compared with controls with no drugs). Thus, the ranges of concentrations used were as follows: MT, 400 to 1,800 μg/ml; CP, 250 to 1,250 μg/ml; 5-FU, 78 to 312.5 μg/ml; MN, 0.242 to 3.9 μg/ml; FC, 1.8 to 24 μg/ml; and AB, 0.015 to 0.25 μg/ml.

Inhibitory levels of each drug combination were determined by using a Dently multipoint inoculator to inoculate the test culture (10^7 yeast cells per ml) into SDA containing serial twofold dilutions of the drugs. Again, the inhibitory concentration was defined as the drug concentration in the highest dilution causing zero visible growth. These values were used to score the inhibitory concentrations of each drug mixture. All experimental points were replicated for measurement of random error. Bias error was minimized by randomization of the experimental order. For data analysis we used an initial logarithmic (to base 2) transformation of the inhibitory concentration data, followed by linear regression analyses. Tests of the fit of the transformed data to the linear model were made by quadruplicate experimental analysis of inhibition at a center point in the factor space (on the $\log_2$ scale) for comparison with values predicted by the regression equations. Experimental error was estimated from pooled standard deviations determined from all test replicates.

Coefficients in the regression equations were evaluated relative to the pooled standard deviations to determine minimum significant factor effects (MSFE), defined as follows: MSFE = $ts\sqrt{2/mk}$, where t is the value of Student's t test at the desired probability level (95%), $m = 2^{(p-1)}$ (where p is the number of factors tested), k is the number of replicates in each trial, and s is the pooled standard deviation for a single response. The fit of data to the linear transformed model was expressed as a curvature, i.e., the differences between experimental values and the values predicted by the regression equations. A minimum significant curvature (Min C) was calculated as Min $C = ts\sqrt{1/mk + 1/c}$, where definitions of t, s, and k are as above, $m = 2^p$, and c is the number of center point replicates (i.e., 4 in this study). Regression equations for four-factor tests were obtained with the form $y = C_0 + C_1X_1 + C_2X_2 + C_3X_3 + C_4X_4 + C_5X_1X_2 + C_6X_1X_3 + C_7X_1X_4 + C_8X_2X_3 + C_9X_2X_4 + C_{10}X_3X_4 + C_{11}X_1X_2X_3 + C_{12}X_1X_2X_4 + C_{13}X_1X_3X_4 + C_{14}X_2X_3X_4$.

The response value, y, is the dilution (concentration) of each test drug mixture at which zero visible growth of *C. albicans* is noted. The coefficients C_0 to C_{14} define the primary and interactive effects of each drug on y. X_1 through X_4 are the concentrations of the four drugs.

For convenience in experimental design and computations, drug concentrations were coded to set high-concentration values equal to +1, low values as −1, and concentrations at midpoints half way between high and low values (on a $\log_2$ scale) as 0. Regression equations are then written to fit data in either the coded forms or actual concentrations.

Further attempts to model interactive effects among drugs involved three-level Box-Behnken experimental designs (5). This method allows tests of fit of the growth inhibition curves to second-order polynomial models by determinations of second-order concentration dependence of each drug as well as estimation of primary and interactive terms. Tests of the fit of polynomials to the experimental data were again analyzed as above (5).

Screening experiment to evaluate effects of incubation variables on drug interactions. The effects of multiple variables in the assay conditions for evaluating drug inhibition were examined in a 20-run Plackett-Burman screening experiment (23). This type of screening experiment may be used to determine which of many test parameters are important in evaluation of drug effects. Twelve potentially important variables were identified, and two levels of each variable were tested as follows: (i) growth medium, SDA or yeast nitrogen base (Difco) supplemented with 2.5% glucose (YNBG); (ii) pH, 5.0 or 6.0; (iii) assay time, 24 or 48 h; (iv) incubation temperature, 30 or 37°C; (v) inoculum size, 10^5 or 10^7 cells per ml; (vi) Tween 80, zero or 0.1% by weight; (vii) bovine serum albumin, 0 or 5 mg/ml of media; (viii) antifungal drug solvent, dimethyl sulfoxide or dimethyl formamide; (ix) to (xii) MN, AB, 5-FU, and MT, respectively, at the high and low levels listed in the factorial analysis above. Analysis of the primary effects of each of these variables on the inhibition of the test drugs was performed as described by Plackett and Burman (23).

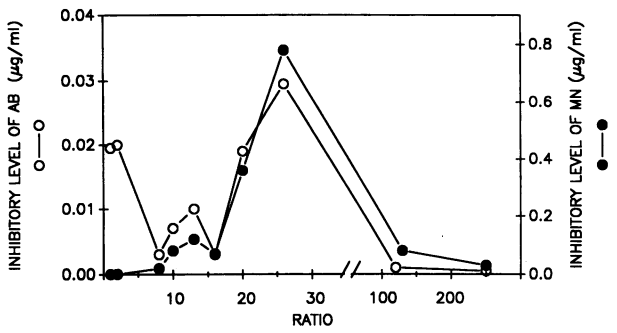

FIG. 1. Effects of changing ratios of MN to AB on levels required to inhibit the growth of *C. albicans* when the two drugs were added in different ratios in the presence of a constant concentration (60 μg/ml) of 5-FU.

RESULTS

Experiments testing inhibitory effects of drug combinations are typically conducted by varying the level of one drug while holding all others constant. Results from such studies have only limited application, since drug effects may be interactive so that relative as well as absolute concentrations of each drug in the mixture determine the inhibition of microbial growth. This is the case whenever either synergistic or antagonistic behavior of drugs is noted. The data in Fig. 1 illustrate this behavior much more graphically than typical plots of inhibition versus the concentration of one drug. Here we simultaneously varied the concentrations of both MN and AB to show that their absolute and relative concentrations have a marked influence on the levels of these drugs required to inhibit *Candida* growth in the presence of a constant level of 5-FU (60 μg/ml).

These tests were not intended to describe relative effects as a continuous function over the entire concentration range of the tests, but only to illustrate the magnitude of dependences on relative concentrations in such an interactive system at a few test points. The data are plotted as the ratios of MN to AB concentrations in each test solution versus the minimum concentrations of AB and of MN in each test combination at the maximum dilution which still caused zero visible growth.

The relative concentrations of the two drugs obviously constitute a strong determinant of the levels required for toxicity. For example, at some ratios (e.g., ratios of MN to AB near 8, 16, and, particularly, >130), low levels of both MN and AB are sufficient for inhibition of growth (Fig. 1). In contrast, when the drugs were present in ratios near 2, relatively high levels of AB but low levels of MN are required for inhibition. At ratios around 26, high levels of both AB and MN are required for inhibition. Conclusions about cooperative or antagonistic interactions between these drugs in the inhibition of *C. albicans* are clearly a function of the ratios of drug concentrations in the test solutions. It is equally clear that although experiments of this type can demonstrate interactive effects among drugs, they are of little value in the quantitative prediction of interactions at other than the test concentrations.

To obtain quantitative data on drug interactions affecting *C. albicans* growth over wider ranges of drug combinations, we performed multifactorial analyses. Test drug concentrations were varied simultaneously by using 2^n factorial designs, i.e., tests of n factors at two concentration levels each. Two sets of studies of this type were run. First, three-factor combinations of either antifungal or antineoplastic drugs were analyzed. Then, combinations of two antineoplastic and two antifungal drugs were used for four-factor analyses.

The basic experimental design for a 2^3 factorial study is shown in Fig. 2. In Fig. 2A, a cube is depicted with concentrations of each of the three antifungal drugs shown along the axes. The concentrations of drugs indicated at each corner of the cube were used as initial concentrations for each drug in the solution for an endpoint determination test to measure minimum concentrations causing zero visible growth of *C. albicans*. The corresponding coded value for each drug concentration is also presented. These show that initial drug concentrations for each dilution test were varied (as defined in Materials and Methods) from low levels (−), at which the drug alone caused minimal inhibition, to high levels (+), which were MIC levels.

Successive twofold dilutions of drug combinations were tested for inhibition. The highest dilution at which zero visible growth was noted was scored. The actual concentrations of drugs present at that dilution were calculated. Both the dilution at which inhibition was observed (shown as a circled number) and the calculated inhibitory concentrations were then plotted in Fig. 2B.

The concentrations causing inhibition depend in a complex fashion on both the absolute and relative concentrations of all drugs in the mixture. For example, the lower right rear point on the cube in Fig. 2B gives one of the lower sets of values for drug concentrations causing inhibition. These concentrations are 0.49, 0.0019, and 3 μg/ml for MN, AB, and FC, respectively. These values are 1/8, 1/256, and 1/8 of the individual MICs of these drugs, respectively. The weight ratios of the drugs at this test point are 16:1:258. Note that at points such as the upper left front corner, where the corresponding ratios are 0.5:1:3.75, inhibition of growth is achieved at 0.12, 0.24, and 0.90 μg/ml for MN, AB, and FC, respectively. Interactive effects among these drugs is evident. Either high or low levels of drugs may be necessary for inhibition, depending upon the ratios of the drugs used.

Figure 2C graphically presents the lowest concentrations of each of three drugs in various mixtures of MN, AB, and FC causing inhibition of growth. The levels vary greatly for different ratios of the three drugs in the mixtures. Points 1 and 6 show inhibition at relatively low levels of all three drugs. Point 5 is low in MN and AB but high in FC. Point 2 is high in MN but low in AB and FC. Points 4 and 8 show that with high levels of both MN and AB, inhibition is essentially invariant with FC.

Studies of three-factor interactions among antineoplastic drugs indicate similarly complex responses to changes in concentrations and ratios of drugs. Figure 3 shows the concentrations of these drugs which caused zero visible growth. The lowest overall combination of drug concentrations for inhibition was 39, 50, and 31 μg/ml for 5-FU, MT, and CP, respectively. When the drugs were mixed in these ratios, complete growth inhibition could be obtained with each of the drug concentrations well below its MIC. In contrast, some drug combinations, e.g., those shown at the top front right corner of the cube, required individual antineoplastic drug concentrations equal to or higher than levels required for inhibition when any one of the drugs alone was used.

Figure 4 shows results of tests of the combined effects of different ratios of two antineoplastic and two antifungal drugs. The minimum values of concentrations of the four drugs MN, AB, 5-FU, and CP which caused zero visible growth in a 2^4 factorial experiment are plotted. Drugs combined in certain concentration ratios proved highly

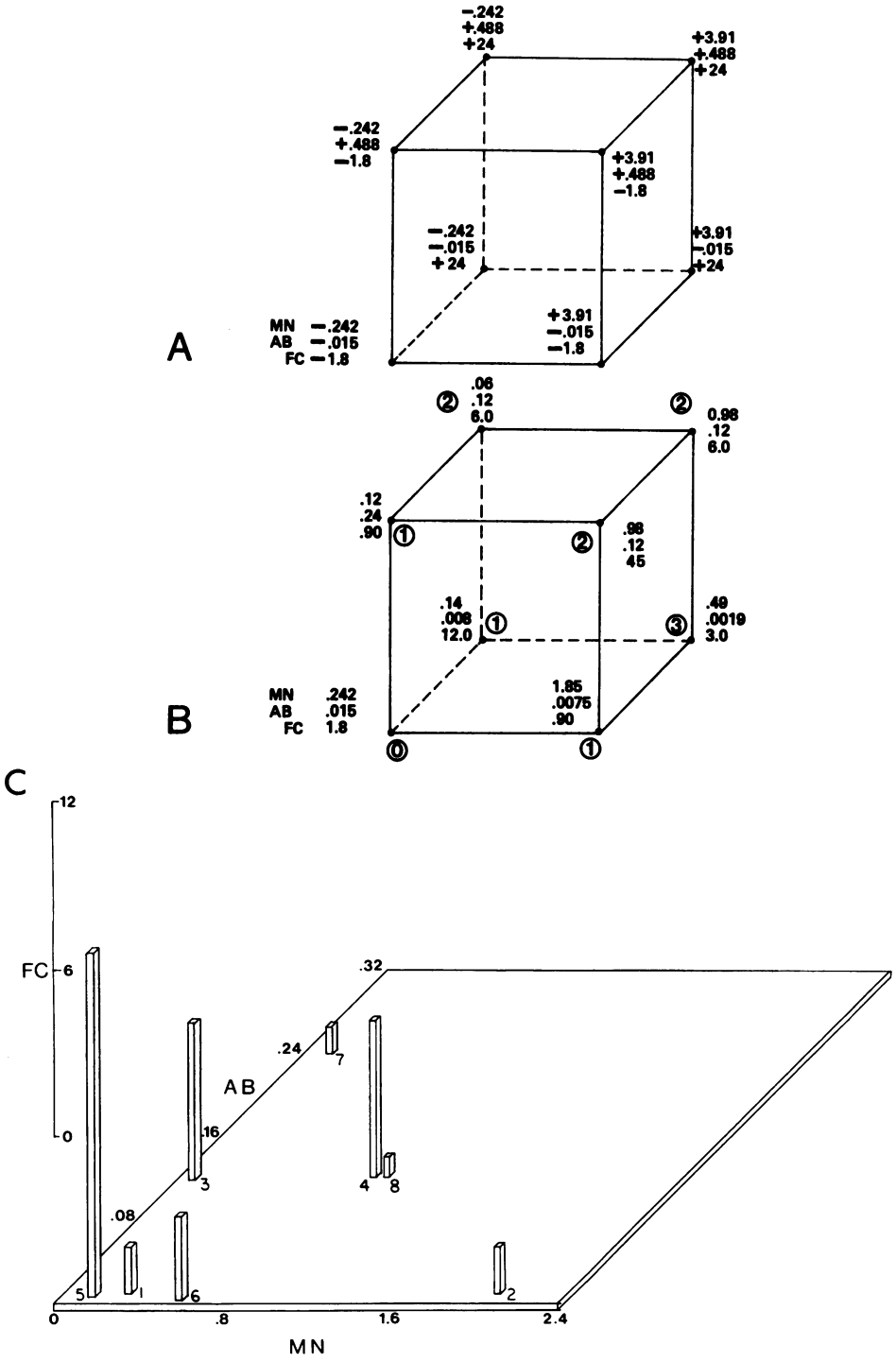

FIG. 2. Inhibition of *C. albicans* growth by combinations of antifungal drugs. (A) The experimental design for test points used in endpoint assays of drug inhibition is shown. The initial concentrations for each drug in the combinations are indicated at the corners of the cube. Also, the coded level of each drug (−, low; +, high) in the two-level analysis is shown at each test point. Endpoint determinations were run with the drugs mixed at the concentrations shown for a twofold dilution series to find inhibitory values. (B) The number of twofold dilutions of the drug combinations in panel A which caused zero visible growth is shown as circled numbers. The minimum concentrations of MN, AB, and FC are represented at each test point. The regression equation describing the responses of *C. albicans* to inhibition by the three drugs is $y = 1.5 + 0.5(MN) + 0.25(AB) - 0.25(FC) + 0.5(MN)(AAB) - 0.25(AB)(FC) - (MN)(AB)(FC)$. (C) Graphic representation of values of each of the three antifungal drugs at the minimum concentration which caused zero visible growth. The positions of the bars within the AB-MN plane and the heights of the bars along the FC axis define concentrations (in micrograms per milliliter) of each of the drugs.

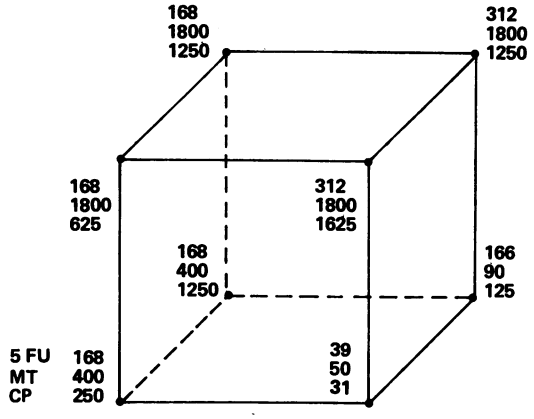

FIG. 3. Concentrations of the antineoplastic drugs 5-FU, MT, and CP in mixtures causing inhibition of *C. albicans* growth. The values are the minimum concentrations of drugs which caused zero visible growth. The regression equation describing the responses of *C. albicans* to combinations of these drugs is $y = 0.5 + 0.5(5\text{-FU}) - 0.5(\text{MT}) + 0.13(\text{CP}) - 0.25(5\text{-FU})(\text{MT}) - 0.13(5\text{-FU})(\text{CP}) + 0.25(\text{MT})(\text{CP}) + 0.25(5\text{-FU})(\text{MT})(\text{CP})$.

effective in blocking growth at low total drug concentrations. For example, consider the lower right rear corner of cube A, where growth inhibition is noted at 0.043, 0.00017, 3.45, and 2.76 μg/ml for MN, AB, 5-FU, and CP, respectively. With this mixture of drugs, inhibition of growth is noted at levels 1/360, 1/2,900, 1/360, and 1/170 of the respective MICs of these drugs when used alone.

The linear regression equations and the coefficients generated to fit the results of the 2^4 factorial analyses are shown in Table 1. These equations are expressed in terms of concentrations (not coded) so that actual magnitudes may be compared. Large significant coefficients are indicated by asterisks. The substitution of MT for CP alters the magnitudes of all the coefficients. Note that the coefficients for MN and AB are positive, whereas the C_5 coefficients are negative, indicating an antagonistic interaction between MN and AB.

The curvature parameters, which test the fit of experimental data to these linear models by comparing experimentally observed values with predicted values at the center point, standard deviation about the regression, and R^2 values, are also shown in Table 1. The values of curvature were 0.43 and 0.16 for the two different drug combinations, where a value of 1.0 is equivalent to a twofold concentration difference. These values are not greatly different from the minimum significant values for curvature in this experiment (i.e., 0.266), indicating that error in fit of the data to a linear model is of the same magnitude as experimental error (i.e., curvature may be significant). A higher-order concentration dependence may exist for at least one of the drugs. However, the linear model fits the data with an accuracy that is within a single dilution factor in a normal endpoint analysis. Analysis of residuals shows no deviation from the random, indicating that the linear model holds.

To test whether inhibition data for combinations of two antineoplastic plus two antifungal agents could be fit more accurately with a higher-order polynomial, we used a three-level Box-Behnken experimental design (5). A general second-order polynomial was developed to describe the effects of MN, AB, 5-FU, and MT on the inhibition of *C. albicans* growth over the same concentration ranges used above. A fully randomized, replicated, four-variable experiment with 27 experimental values yielded the following polynomial: $y = 4 + 0.46(X_1) + 0.25(X_2) - 0.91(X_3) + 0.21(X_4) - 0.125(X_1X_3) - 0.125(X_2X_3) - 0.375(X_2X_4) + 0.166(X_1)^2 -$

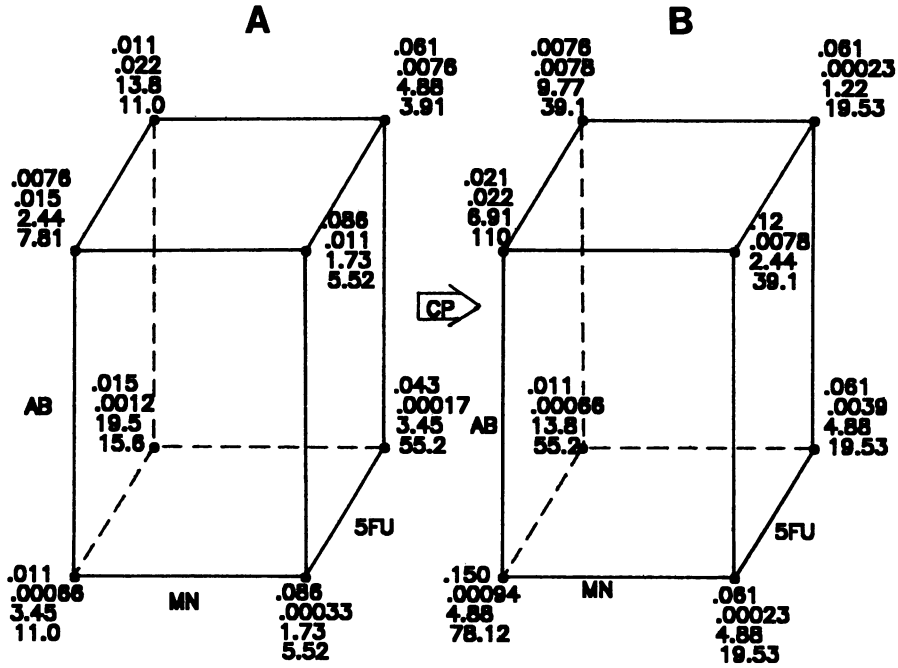

FIG. 4. Minimum concentrations of MN, AB, 5-FU, and CP, in combinations of all four drugs, which cause zero visible growth of *C. albicans*. Sixteen test points are shown. These were generated from a 2^4 factorial design in which MN, AB, and 5-FU levels were varied from low (−) to high (+) along the axes as shown on cubes A and B, whereas CP was (−) in cube A and (+) in cube B. See Materials and Methods for definitions of + and − levels for each drug.

TABLE 1. Value of the coefficients for linear regression equations describing inhibition by MN(X_1), AB(X_2), 5-FU(X_3), and MT(X_4) and by MN(X_1), AB(X_2), 5-FU(X_3), and CP(X_4)[a]

Run	Coefficient[b]													
	C_0	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_9	C_{10}	C_{11}	C_{12}	C_{13}	C_{14}
A	1.644*	1.308*	3.771*	0.01148	0.000319	−2.393*	−0.00453	−0.000166	−0.0128	0.00121	−0.000002	0.00741	0.00206	0.000001	0.000006
B	4.75*	0.0707*	2.176*	−0.00325	−0.000775	−0.349*	0.00213	0.000396	−0.000381	−0.00329	0.000004	−0.000617	0.000144	−0.000002	0.000011

[a] Pooled standard deviation, 0.44 dilutions. MSFE (95% confidence interval) = 0.17 dilutions. Standard deviation about the regression: A, 0.488; B, 0.125. R^2: A, 0.977; B, 0.998. Midpoint dilutions (predicted), A, 3.57; B, 4.65; (measured), A, 4.0; B, 4.49. Curvature: A, 0.43; B, 0.16. [Min C]: A, 0.266; B, 0.266.

[b] Asterisks denote significant effects (95% confidence interval). C_0 through C_{14} are the coefficients of the equation $y = C_0 + C_1(X_1) + C_2(X_2) + C_3(X_3) + C_4(X_4) + C_5(X_1X_2) + C_6(X_1X_3) + C_7(X_2X_3) + C_8(X_1X_4) + C_9(X_2X_4) + C_{10}(X_3X_4) + C_{11}(X_1X_2X_3) + C_{12}(X_1X_2X_4) + C_{13}(X_1X_3X_4) + C_{14}(X_2X_3X_4)$. X_1 to X_4 refer to concentrations of the drugs.

$0.021(X_2)^2 - 0.146(X_3)^2 + 0.042(X_4)^2$, where X_1 through X_4 are again the concentrations (coded) of the drugs MN, AB, 5-FU, and MT, respectively, and y is the dilution at which complete inhibition of growth is noted.

This equation accurately predicts measured experimental points near the center of the variable factor space but is less accurate in predictions of inhibitory levels of drugs near the corners of the cubes in Fig. 4. The inhibitory concentrations of MN, AB, 5-FU, and MT measured at the center of the factor space from the quadratic equation are 0.043, 0.0027, 7.4, and 37.4, respectively. Predicted values were 0.036, 0.0023, 6.2, and 31.5. Thus, measured and predicted values differ by about 20%. This is in somewhat better agreement than that achieved by the linear model and is less than one dilution factor in an endpoint determination analysis. Note that any increased accuracy obtained by such studies is purchased at the expense of increasing the number of experiments required by more than 50%.

In contrast to the centerpoint predictions, the quadratic equation gives a much poorer fit to the data at points near the corners of the factor space. For example, at the corner point of Fig. 4 with low levels of all four test drugs, (i.e., −, −, −, −), the minimum measured concentrations causing inhibition are 0.011, 0.00066, 3.45, and 11.0 μg/ml. Corresponding predicted concentrations are 0.020, 0.0126, 6.56, and 33.6 μg/ml. This is not as good an agreement as obtained with the linear equations. In fact, the predicted and measured values at this point differ by nearly one twofold dilution in an agar dilution assay. A linear model with interactions gives the best overall fit to the experimental data.

Many common experimental variables in inhibition assay conditions have been proposed to alter the measured concentrations of drugs required to cause inhibition in various assays. We tested the effects of changes in our agar dilution method to see whether this influenced the values obtained. The results are summarized in Table 2. Four drugs were used in varied combinations, together with the eight listed experimental variables, to identify factors with significant effects on the assay. Drug combinations identical to those in Fig. 4 were used in a 20-trial Plackett-Burman design (23). Three of the tested variables, in addition to the test drugs, had significant effects on assay values (>95% confidence interval). Changing the medium from SDA to YNBG enhanced drug susceptibility. Raising the incubation temperature also increased susceptibility (i.e., lower levels of a given drug mixture caused inhibition). The size of the inoculum also affected endpoint values; tests with 10^5 cells indicated inhibition at lower drug levels.

The other factors tested had no significant effects in this study. The sizes of the coefficients in Table 2 indicate the magnitude of the dependence on changes in each variable. To illustrate the relative effects clearly, each variable was once again coded with a value of +1 or −1. A coded value of −1 for continuous variables indicates the low level tested, whereas +1 is the high value; e.g., for pH, −1 corresponds to pH 5, +1 corresponds to pH 6. For discontinuous variables, e.g., the use of SDA or YNBG medium, an arbitrary assignment of −1 or +1 was given. As a result of this coding of coefficients, it was possible to evaluate the size and direction of the effects of each variable on endpoint values. The equation presented in the footnote to Table 2 can be used to describe quantitative changes in endpoints by entering the coded values −1 or +1 for each variable. For example, changing just the medium from SDA (−1) to YNBG (+1) would result in a change in the observed endpoint value from 6.05 − 1.5 to a value of 6.05 + 1.5. This

TABLE 2. Effects of varied growth conditions or endpoint dilution values

Test variable[a]	Coefficient[b]
Medium	+1.5*
pH	−0.75
Time (h)	−0.75
Temp (°C)	+1.75*
No. of cells	−2.05*
Tween (%)	+0.45
BSA (μg/ml)	−0.15
Solvent	+0.45

[a] Variations are as follows (coded values −1 and +1, respectively: medium, SDA to YNBG; pH, 5 to 6; time, 24 to 48 h; temperature, 30 to 37°C; number of cells, 10^5 to 10^7; Tween, 0 to 0.1%; BSA (bovine serum albumin), 0 to 5 μg/ml; solvent, dimethyl sulfoxide to dimethyl fluoride.

[b] Regression equation, endpoint dilution = $y = 6.05 + 1.5$ (medium) − 0.75 (pH) − 0.75 (time) + 1.75 (temperature) − 2.05 (number of cells) + 0.45 (Tween) − 0.15 (BSA) + 0.45 (solvent). Significant error (95% CI) = 0.4. Minimum significant coefficient = 0.87 (significant coefficients are denoted by asterisks). Equation for effects of significant variables on endpoint values: $\log_2{}^{-1}$ (endpoint value) = $\log_2{}^{-1}$ [6.05 + 1.5 (medium) + 1.75 (temperature) + 2.05 (number of cells)]. Note that coded values of variables (−1 to +1) are to be inserted in solving this equation.

indicates that the endpoint value would be increased by three dilutions (an eightfold concentration decrease) in a 2^n dilution series. Similar calculations can be made for the temperature and inoculum size variables. Maximum susceptibility to the test drugs was observed in YNBG medium at 37°C and with a low level of inoculum. Tests run under these conditions can give results which differ in endpoint concentrations by a factor of more than 500 from results of tests run in SDA at 30°C and a high level of inoculum.

DISCUSSION

Studies concerning the interactive effects of various drugs against bacteria as well as fungi are well documented (3, 18, 19). The complexity of such interactions has been stressed repeatedly (6, 22), and the difficulty in assessing and detecting the complex interactions among drugs has been noted. Our findings once again demonstrate inhibitory drug interactions and illustrate their complexities. In addition, we provide methods for quantitative analysis of multiple drug interactions over broad ranges of concentrations.

The complexity of interactions among the drugs tested here is clearly evident from Fig. 2 to 4. Our first tests involving multifactorial analyses examined inhibition by combinations of three antifungal agents (Fig. 2) or three antitumor agents (Fig. 3). The results showed that (i) major cooperativity occurs among antineoplastic agents in inhibition of *C. albicans*, (ii) certain combinations of drugs actually potentiate the ability of *C. albicans* to grow in the presence of drugs, (iii) certain antineoplastic drug mixtures can inhibit fungal growth at relatively low levels, (iv) the ratios of each of the three drugs in the test mixtures have a large effect on the individual and total drug concentrations that are required for inhibition, and, finally, (v) it would be impossible to predict these complex patterns from any set of one-factor-at-a-time analyses.

Each datum point shown in Fig. 2 or 3 identifies the concentrations of all three or four drugs required to inhibit *C. albicans* growth when that particular ratio of the drugs is used. Since all these mixtures inhibit growth, the question of which particular mixture of drugs is best for inhibition may be answered only in terms of what the experimental objectives are. If we consider possible future clinical application of these results in animal treatments, the drug combination of choice may be the particular mix of drugs which will inhibit *Candida* spp. but not cause major toxicity to the host animal.

Some of the results in Fig. 4 appear well suited to future clinical consideration. For example, tests with initial concentrations of MN, AB, 5-FU, and CP as (+, −, +, −) and as (+, +, −, −) both showed *C. albicans* growth inhibition at great dilutions of all four drugs. The concentration of each of the drugs necessary for growth inhibition in these two tests was far below the individual MIC. Since AB in particular causes major toxicity in animals, this lowering of the level required for blocking *C. albicans* growth may be advantageous. It will be of interest to learn whether animal cells are similarly affected by these drug combinations.

Drug combinations may be tailored to meet specific requirements for clinical applications. The regression equations of Table 1 provide the predictive means for selecting drug combinations with appropriate properties. Substitution of selected concentrations of each of the four drugs at levels anywhere within the concentration ranges tested can be used to predict corresponding responses of *C. albicans* growth. The linear regression equations fit the data with an R^2 value of better than 0.975 and give a good indication of concentrations at which growth inhibition occurs, even though the test of linearity showed that some curvature was present. The curvature, measured at the midpoint of our factor space (where it should be a maximum), was less than $2^{0.5}$. Although this level of curvature is significant on the basis of error analyses, in a practical sense ignoring curvature introduces an error of less than one dilution in the normal tests of toxicity by serial dilution methods. Other data show that differences in susceptibilities among various strains of *C. albicans* exceed this error term, so that its clinical significance is probably negligible. Moreover, a positive value for curvature is not unexpected from our scoring method, in which the endpoint value is always greater than or equal to the actual concentration required for inhibition.

When additional experiments were run to allow fitting of a quadratic equation to the data, little improvement on the fit of calculated to measured points was obtained. A better fit of data was achieved near the center of the factor space, but a poorer fit was noted near some of the edges. The data could be fit to some higher-order polynomial, but the number of experiments required to obtain this fit becomes prohibitive from a practical point of view.

For this study, the linear regression equations provide a reasonable approximation of the data and may be used as a working model for design of drug combinations. Going to a higher-order polynomial is not justified in this study, since errors resulting from the use of linear approximations are less than a single dilution in an endpoint assay. Errors in the reproducibility of drug inhibition assays from laboratory to laboratory and from fungal isolate to fungal isolate exceed this value.

Linear equations for description of interactions are also useful in that they allow a more intuitive insight into the dependence of test results on individual drug concentrations and the interactions among the drugs. In each regression equation (Table 1), C_0 is an average response to all drugs. The values C_1 to C_n are coefficients indicating dependences on drug concentrations. Large positive values of C_n indicate large positive contributions toward inhibition; e.g., a large positive value of C_1 in the term C_1 (MN) indicates a strong dependence on the concentration of MN. Negative coefficients indicate that increasing concentrations of the drug

actually decrease the effectiveness of the mixture in inhibiting growth. Coefficients (both + and −) of cross-product terms such as (MN)(5-FU) indicate the magnitude and direction of interactive effects between (or among) the drugs. Solutions to the regression equations of Table 1 at varied levels of the combined drugs can produce graphical representations, which can illustrate the magnitude of pairwise interactions among the drugs tested.

The results of the three-factor antineoplastic agent study (Fig. 3) lead to a conclusion that CP may not be a good candidate for combined drug therapy, since it interacted negatively with 5-FU and MT. Comparison of data from Fig. 3 and 4 shows, however, that the most effective drug mixture tested contained CP at low levels; higher concentrations of CP were less effective. This again emphasizes that conclusions drawn from many of the earlier studies may not lead to correct conclusions about the potential of drug combinations for synergistic interactions. It should be noted in this regard that combinations of three antineoplastic agents were not particularly effective in stopping *C. albicans* growth. Somewhat surprisingly, combinations of three antifungal agents were not much better in cooperative action. However, some combinations of antifungal and antineoplastic drugs work very well. This raises hope that appropriate combinations of these may be of use in cancer therapy.

The Plackett-Burman screening experiment (23), analyzing effects of potential experimental variables, was very informative (Table 2). This study ruled out significant effects on our results due to pH change from 5 to 6, added detergent (which could increase drug solubility and permeability), added bovine serum albumin, and increased incubation time (within the range tested) before reading the plates. These conclusions may be contrasted with those of Brajtburg et al. (6), who found the effects of antifungal agents to be dependent on both the incubation time and the presence of proteins in serum. We found that the size of cell inoculum, incubation temperature, and nature of the growth medium all had significant effects on measured inhibitory levels of the drug combinations. This is in agreement with results of others, who reported that inhibitory activity of antifungal agents often varies with the test conditions chosen (13, 21, 24). However, our procedures go beyond simply establishing the effects of variables in that they provide a quantitative estimate of the consequences of varying one or more variables. It is clear that all experimental parameters must be carefully controlled to obtain reproducible results in any experimental tests of inhibition by drug combinations and that extrapolation of results to any other test system is questionable.

In conclusion, our studies show that drug interactions are even more complex than could be anticipated from previously reported data and stress the fact that synergism in vitro is dependent on a large number of variables involved in the experimental conditions applied (e.g., drug concentration, ratio of drug concentrations, medium used, incubation temperature, inoculum size, method used, mechanism of action of various drugs involved, etc.). The complexity of the situation becomes further apparent since all of these factors must be considered simultaneously. Thus, the use of one-factor-at-a-time studies, checkerboard analysis, and even the more elaborate methods of Berenbaum (4) fall short in terms of elucidation of the quantitative interactions that take place among drugs used in combination and the prediction of possible responses of microorganisms. The need for adopting multifactorial analysis methods is obvious.

The complexity of interactions among drugs illustrated makes the relevance of our in vitro studies to the in vivo situation difficult to assess and necessitates cell culture and animal studies. These studies provide an important stepping stone toward minimizing the numbers of animal studies which may be required to achieve success (6, 22).

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