

Surface Response Modeling to Examine the Combination of Amphotericin B Deoxycholate and 5-Fluorocytosine for Treatment of Invasive Candidiasis

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The strategy of combining antifungal drugs in a treatment regimen may improve the outcome of invasive candidiasis. Using a well-validated pharmacodynamic murine model of invasive candidiasis, we defined the effect of the combination of amphotericin B deoxycholate (AmB) and 5-fluorocytosine (5FC) by use of the Greco model of drug interaction. The combination was additive, meaning that the experimental effect did not deviate in a statistically significant manner from the null reference model (or additive surface) of the combined effect. From a clinical perspective, the addition of 5FC to a regimen of AmB may enable the near-maximum effect to be reached in circumstances in which the administration of a given dose of AmB alone produces a submaximum effect but an increase in the dose is not possible, because of dose-related toxicity. Our methods provide a way in which some of the complex issues surrounding antifungal combination treatment can be addressed.

Invasive candidiasis (INVC) is a life-threatening illness. The attributable mortality is ~38%–49% [1, 2], and the rate of therapeutic failure is 20%–50% [3–5]; these data provide the major impetus to continually develop and refine treatment strategies. The combination of antifungal drugs is increasingly touted as a useful approach [6, 7]; the potential benefits include an attainment of effect that is greater than the effect of either drug administered alone, the use of smaller doses than is oth-

erwise possible with monotherapy, and the maximization of the spectrum of antifungal coverage [7].

Amphotericin B deoxycholate (AmB), despite its well-documented toxicity, remains the reference standard for the treatment of INVC and in clinical trials examining new antifungal drugs [3, 5]. 5-Fluorocytosine (5FC) is a fluorinated pyrimidine analogue that acts as a subversive substrate within the pyrimidine salvage pathway and disrupts both DNA and RNA synthesis [8]. 5FC is active against most *Candida* species, but, because of the rapid induction of resistance, it is generally prescribed in combination with other antifungal drugs.

The combination of AmB and 5FC is considered to be the standard of care in cryptococcal meningitis [9]. The addition of 5FC to a regimen of AmB may also be useful in cases of INVC that are refractory to AmB alone or in the treatment of infection with *Candida* species at sites where drug penetration may be compromised. In the present study, we sought to examine, using the Greco model of drug interaction in a well-validated pharmacodynamic murine model of INVC, the nature and the magnitude of the interaction between AmB and 5FC.

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MATERIALS AND METHODS

Drugs, drug measurement, and the MICs of AmB and 5FC.

AmB powder (Fungizone) and 5FC solution (10 mg/L) were obtained from Bristol-Myers Squibb Pharmaceuticals and Valeant Pharmaceuticals, respectively. Serum AmB concentrations were measured by high-performance liquid chromatography (HPLC) using a modification of the method of Groll et al. [10]. Briefly, 500 μ L of HPLC-grade methanol was added to 100 μ L of mouse serum, vortexed, and centrifuged, and the methanolic supernatant was then evaporated to dryness. The residual was reconstituted in 200 μ L of mobile phase, which consisted of methanol:acetonitrile:0.0025 mol/L NaEDTA in a ratio of 500:350:240 (vol:vol:vol), and 100 μ L of this solution was used for detection. HPLC analysis was performed using an Alltech Altima C18 column (250 \times 4.6 mm; Knauer). The flow rate of the mobile phase (composition as above) was 1.2 mL/min. AmB was eluted after \sim 6.8 min of flow, and its concentration at 382 nm was measured with a UV detector (Kratos). The coefficients of variation (CVs) were 7% and 13% for 0.5 and 0.05 mg/L, respectively. The limit of quantification was 0.05 mg/L.

Samples for the measurement of serum 5FC concentrations were prepared by adding 100 μ L of 5% trichloroacetic acid to 20 μ L of mouse serum. HPLC analysis was performed using a Hichrom SCX 10 cm \times 4.6 mm column (Thermo Electron) and 10 μ L of sample. The mobile phase consisted of 10 mmol/L ammonium acetate buffer (pH 4.1), and the flow rate was 1.0 mL/min. 5FC was eluted after 2 min of flow, and its concentration at 270 nm was measured with a UV detector (Thermo Electron). The CV was $<$ 7% between 0.2 and 13.0 mg/L. The limit of detection was 0.1 mg/L. The MICs for AmB and 5FC were determined using a microtiter modification of the National Committee for Clinical Laboratory Standards M27-A method [11] and the method recommended by the European Committee on Antimicrobial Susceptibility Testing [12], respectively.

Model of invasive candidiasis. All in vivo models were approved by the institutional ethics committees. A well-characterized clinical isolate of *Candida albicans*, F/6862, was used. The isolate was retrieved 24 h before use from beads stored at -70°C , was placed in Sabouraud liquid medium (Oxoid), and was incubated at 35°C on a shaker. The final inoculum was determined by progressive dilution in PBS and was verified by quantitative culture. Male CD1 mice (Charles River Laboratories), 24–26 g of body weight, were immunosuppressed with 200 mg/kg cyclophosphamide (Pharmacia) in 0.2 mL of 0.9% saline, which was administered intravenously (iv) via the lateral tail vein 3 days before infection (day -3). On day 0, mice were injected iv with 2×10^4 *C. albicans* organisms in 0.2 mL of PBS via the lateral tail vein. At 5 h after infection (time 0), AmB in 0.2 mL of 5% dextrose, 5FC in 0.2 mL of 0.9% saline, and the combination of AmB and 5FC in their respective diluents were administered intraperitoneally (ip) in 2 separate and im-

mediately sequential injections. An early control group (4 mice) was killed immediately after the administration of the relevant agent (time 0); early control data were subsequently compared with late control data, which were obtained at 24 h after the initiation of therapy, to ensure that logarithmic growth had been established. Treated mice were killed at 24 h after the initiation of therapy. Both kidneys were dissected, weighed, and processed together by homogenization in 2 mL of PBS. The homogenates were serially diluted to a concentration of 1:10 in PBS, and quantitative cultures were determined.

Pharmacokinetics of AmB, 5FC, and the combination of AmB and 5FC. All pharmacokinetic relationships were determined in infected mice. Three mice were used for each data point. For AmB, 0.625, 1, 2.5, and 5 mg/kg were administered ip once, and blood samples were collected at 0.5, 1, 3, 6, 10, 17, and 24 h after the initiation of therapy (i.e., 84 mice were used to study 7 time points for each of the 4 dose regimens). For 5FC, 6.25, 25, 100, and 200 mg/kg were administered ip once, and blood samples were collected at 0.5, 1, 1.5, 2, 3, 4, and 6 h after the initiation of therapy (i.e., 84 mice were used to study 7 time points for each of the 4 dose regimens). Blood was collected by terminal cardiac puncture and was allowed to clot on ice. Samples were stored at -70°C until analysis.

To investigate the possibility of a pharmacokinetic interaction between AmB and 5FC, both drugs were administered alone and in combination. Six cohorts, which were composed of groups of 3 mice, received AmB and/or 5FC in the following combinations: 0:3, 0:6, 2:0, 0.3:6, 2:3, and 2:6 mg/kg. AmB was administered once at time 0, and 5FC was coadministered at time 0 and then alone at 8 and 16 h after the initiation of therapy. The doses were chosen on the basis of the dose-response relationships defined for each drug when administered alone (see below). Blood samples were collected at 0.5, 3, 16.5, 17, 17.5, 18, and 24 h after the initiation of therapy. Blood samples were collected intensively during the third dosing interval (i.e., between 16 and 24 h after the initiation of therapy) to ensure that there was no evidence of progressive drug accumulation.

Pharmacokinetic data analysis. The concentrations of AmB and 5FC were modeled using a population pharmacokinetic analysis. The nonparametric adaptive grid (NPAG) with adaptive γ program of Leary et al. was used [13]. Data were weighted by the inverse of the observed variance of the measured drug concentrations for each group of mice. For both drugs, an open 2-compartment model was used. This model was composed of a central compartment (c), with volume V_c (in L), and a peripheral compartment (p) that were connected by the first-order transfer rate constants K_{cp} (h^{-1}) and K_{pc} (h^{-1}) and had bolus input into the peritoneal cavity, first-order absorption from the peritoneal cavity into the central compartment (K_a [h^{-1}]), and first-order clearance (CL) from the central

compartment (in L/h). Bayesian parameter estimates were obtained using the “population of one” utility within NPAG.

Determination of the dose-response relationships of 5FC and AmB administered alone. The dose-response relationships for AmB and 5FC administered alone were defined before the combination matrix was designed. Groups of 3 mice were used for each drug dose. For AmB, the effect induced by 0, 0.01, 0.0625, 0.1, 0.3, 1, and 2 mg/kg administered once at time 0 was determined. For 5FC, the effect induced by 0, 0.125, 0.1, 0.4, 0.6, 0.9, 1.56, 2, 3, and 6.25 mg/kg administered at time 0 and at 8 and 16 h after the initiation of therapy was determined. For both drugs, the observed effect, in terms of the reduction of fungal burden (in log₁₀ cfu/g of kidney), was assessed by fitting to the data the following inhibitory sigmoid E_{\max} model:

$$E = E_{\text{con}} - \frac{E_{\text{max}} \times (\text{exposure})^H}{IC_{50}^H + (\text{exposure})^H},$$

where E is the effect of drug exposure (in log₁₀ cfu/g of kidney), E_{con} is the fungal burden (in log₁₀ cfu/g of kidney) in the absence of treatment, E_{max} is the maximum reduction in fungal burden (in log₁₀ cfu/g of kidney) induced by drug exposure, “exposure” is the dose or other pharmacodynamic variable, and H is the Hill (or slope) constant. Observed data were weighted by the inverse of the observed variance. Pharmacokinetic and MIC data were used to convert the exposure term from dose (mg/kg) to the relevant pharmacodynamic variable, which was taken to be the area under the curve (AUC):MIC ratio, for AmB, and the fraction of the dosing interval (T) that serum concentrations were greater than the MIC ($T > \text{MIC}$), for 5FC. The model was implemented and fitted using the identification module of the ADAPT II software package [14].

Assessment of the combined effect of AmB and 5FC. A matrix composed of 15 treatment groups of 4 mice each, an early control group of 4 mice killed at the initiation of treatment (time 0), and a late control group of 4 mice killed at 24 h after the initiation of therapy (68 mice total) was used to examine the combined effect of AmB and 5FC. The dosing regimens were specifically chosen to span the steep portion of the dose-response relationship, as determined when AmB and 5FC were administered alone (see Results and figure 1). For the groups of mice receiving combination treatment, the drugs were dissolved in 0.2 mL of their respective diluent (total volume, 0.4 mL) and administered in 2 separate and immediately sequential ip injections. For the groups of mice receiving only 1 drug, 0.2 mL of the respective diluent of the other drug was also administered. For the 5FC treatment groups (both monotherapy and combination treatment groups), 2 additional 0.2-mL injections were given ip at 8 and 16 h, before animals were killed at 24 h after the initiation of therapy.

Drug interaction modeling. To model drug interaction, the interaction model of Greco [15] (equation [1]) was used and implemented in the identification module of the ADAPT II software package [14]. The Greco model provides parameter estimates and their associated 95% confidence intervals (CIs), thus enabling the quantification of the combined effect as well as the level of statistical significance to be determined. The Greco equation, when applied to the present study, takes the form

$$1 = \frac{D_{\text{AmB}}}{IC_{50,\text{AmB}} \times \left(\frac{E}{E_{\text{con}} - E}\right)^{1/m_{\text{AmB}}}} + \frac{D_{5\text{FC}}}{IC_{50,5\text{FC}} \times \left(\frac{E}{E_{\text{con}} - E}\right)^{1/m_{5\text{FC}}}} + \frac{\alpha \times D_{\text{AmB}} \times D_{5\text{FC}}}{IC_{50,\text{AmB}} \times IC_{50,5\text{FC}} \times \left(\frac{E}{E_{\text{con}} - E}\right)^{(1/2m_{\text{AmB}} + 1/2m_{5\text{FC}})}}, \quad (1)$$

where D_{AmB} and $D_{5\text{FC}}$ are the concentrations of AmB and 5FC, respectively, that produce effect E ; m_{AmB} and $m_{5\text{FC}}$ are the respective slope parameters for the 2 drugs; $IC_{50,\text{AmB}}$ is the AUC:MIC ratio for AmB that produces 50% of the maximum effect; $IC_{50,5\text{FC}}$ is the fraction of the dosing interval that the serum concentration of 5FC is above the MIC that produces 50% of the maximum effect; and α is the interaction parameter.

The first 2 terms on the right side of equation (1) define the additive effect (Loewe additivity); the third is the interaction term and contains the interaction parameter, α . If the 95% CI near the point estimate of α crosses 0, the combined effect is additive. If α is positive and the lower bound of its 95% CI does not cross 0, synergy is present. If α is negative and the upper bound of its 95% CI does not cross 0, antagonism is present.

RESULTS

MICs. The MICs of AmB and 5FC for *C. albicans* were 0.03 mg/L and 0.125 mg/L, respectively, in 2 experiments.

Model of INVC. IV injection of immunosuppressed mice with *C. albicans* F/6862 generated a reproducible and sublethal infection. The mean \pm SD fungal burden of *C. albicans* at 5 h after infection (time 0) and at 24 h after the initiation of therapy in control mice was 2.68 ± 0.27 and 5.87 ± 0.32 log₁₀ cfu/g of kidney, respectively.

Pharmacokinetics of 5FC and AmB alone and in combination. A total of 28 sampling points were available for use in the pharmacokinetic analysis for both AmB and 5FC. The estimates of the mean \pm SD values for each parameter derived from the NPAG analysis of the 2 compartment models are summarized in table 1. Concentrations of both AmB and 5FC when given in combination were adequately accounted for by the model for either drug alone, and this indicates that there was no significant pharmacokinetic interaction, at least at the levels proposed and subsequently used in the combination matrix (data not shown).

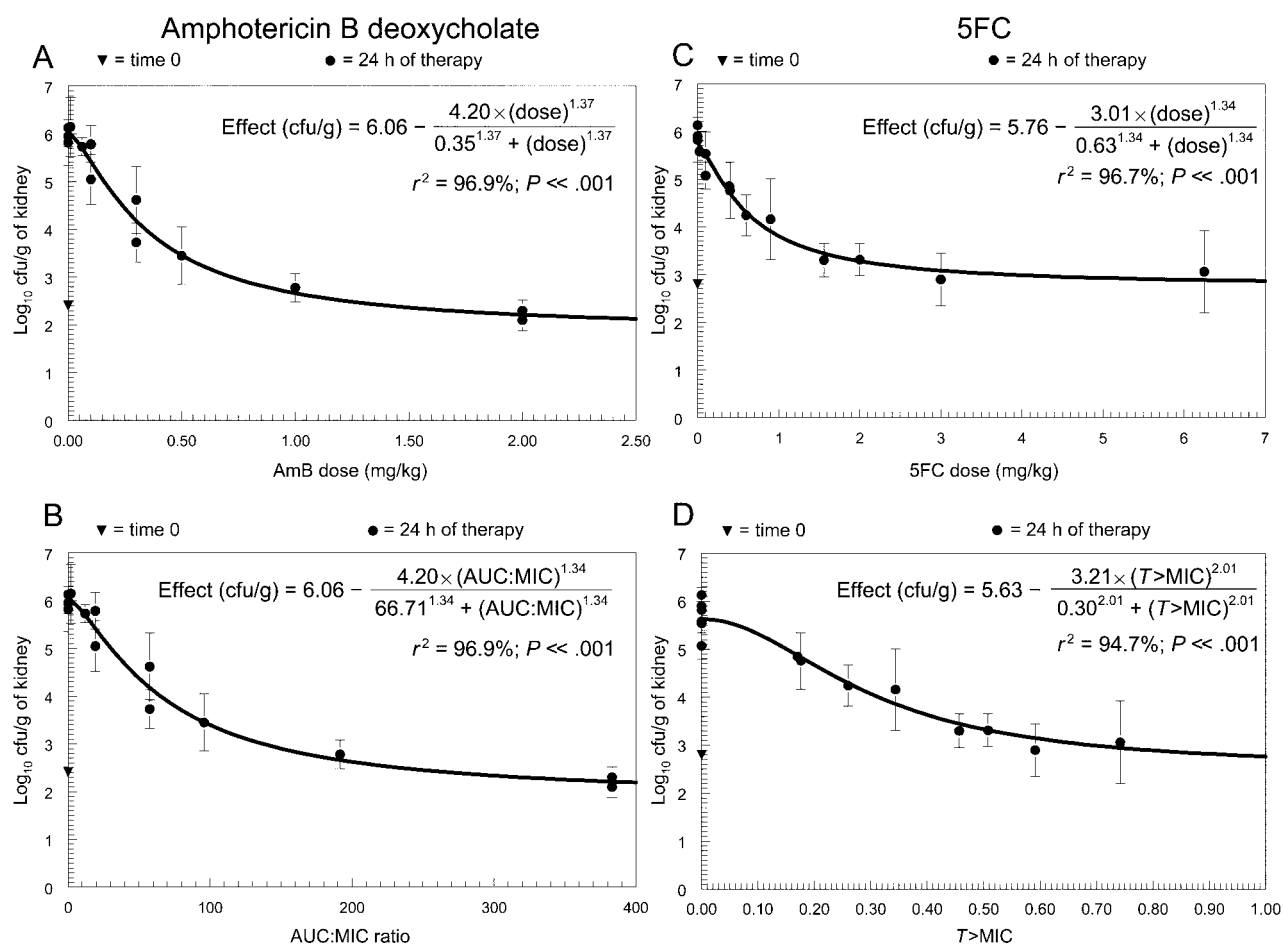


Figure 1. Exposure-response relationships and the associated r^2 values for amphotericin B deoxycholate (AmB) and 5-fluorocytosine (5FC) given as monotherapy. Drug exposure is presented both in terms of dose (A and C) and the respective pharmacodynamic parameters for AmB and 5FC (B and D). Data points represent the mean \pm SD fungal burden (in \log_{10} cfu/g of kidney) obtained from groups of 3 mice. A, Dose of AmB vs. effect. B, Area under the curve (AUC):MIC ratio for AmB vs. effect. C, Dose of 5FC given at 5 h after infection (time 0) and at 8 and 16 h after the initiation of therapy vs. effect. D, Fraction of the dosing interval (T) that the 5FC serum concentration is above the MIC ($T > \text{MIC}$) vs. effect. Shown are the fungal burden (in \log_{10} cfu/g of kidney) at 5 h after infection (time 0; inverted black triangles) and at 24 h after the initiation of therapy (black circles).

Exposure-response relationships for 5FC and AmB administered alone. The exposure-response relationships for AmB and 5FC were defined over several experiments, and the data were analyzed collectively. The dose-response relationships for AmB and 5FC are shown in figure 1A and 1C, respectively. The dose-response relationships were then transformed to refer to the AUC:MIC ratio vs. response and the $T > \text{MIC}$ vs. response for AmB and 5FC, respectively (figure 1B and 1D).

Combination model. A total of 44 regimens were evaluated to model the interaction between AmB and 5FC; all the data from the combination matrix as well as all the data for AmB and 5FC from the monotherapy experiments were analyzed collectively to generate the model of drug interaction. The doses of AmB and 5FC used in the combination matrix encompassed the steep portions of the dose-response relationships for either drug alone, as is illustrated in figure 1; thus, the doses of AmB

used in the combination matrix were 0, 0.1, 0.3, and 2 mg/kg (administered at time 0), which corresponded to AUC:MIC ratios of 0, 19.16, 57.48, and 191.6, respectively; the doses of 5FC used were 0, 0.1, 0.4, 0.6, 0.9, 2, and 3 mg/kg (administered at time 0 and 8 and 16 h after the initiation of therapy), which corresponded to 0%, 0%, 17.6%, 26.0%, 34.4%, 50.8%, and 59.2% of the dosing interval, respectively, in which the serum concentration of 5FC exceeded the MIC of 0.125 mg/L. Andes et al. [16] had reported elsewhere that the near-maximum effect induced by 5FC occurred when its serum concentration exceeded the MIC for 25%–40% of the dosing interval, which was concordant with our findings (see figure 1D).

The final estimates for the means and 95% CIs for the parameters describing the combined effect of AmB and 5FC are given in table 2, and the model of the combined effect is illustrated in figure 2A. The fit of the Greco model to the data

Table 1. Parameters for the nonparametric adaptive grid population pharmacokinetic analysis for amphotericin B deoxycholate (AmB) and 5-fluorocytosine (5FC) administered intraperitoneally to mice in a single dose.

| Drug | V_{cr} , L | K_{br} , h^{-1} | K_{cp} , h^{-1} | K_{pcr} , h^{-1} | CL, L/h |
|------|-----------------|---------------------|---------------------|----------------------|-----------------|
| AmB | 0.0549 ± 0.0180 | 5.11 ± 3.60 | 3.48 ± 2.55 | 28.49 ± 0.78 | 0.0030 ± 0.0004 |
| 5FC | 0.0218 ± 0.0167 | 19.58 ± 9.66 | 13.44 ± 7.33 | 24.07 ± 6.02 | 0.0209 ± 0.0130 |

NOTE. Data are mean ± SD. CL, clearance from the central compartment; K_{br} , first-order transfer rate constant linking the peritoneal cavity with the central compartment; K_{cp} , first-order transfer rate constant linking the central and peripheral compartments; K_{pcr} , first-order transfer rate constant linking the peripheral and central compartments; V_{cr} , volume in L of the central compartment.

was highly acceptable ($r^2 = 92.5\%$). A plot of the weighted residual values (i.e., the difference between the predicted and observed effect) (figure 2B) indicates that there was no systematic bias in the fit of the model to the data. The estimate of α was essentially 0, and the lower bound of its 95% CI crossed 0, which reflects that the observed experimental effect resulting from the combination of AmB and 5FC was not statistically greater than that predicted when the joint effect of the 2 drugs was modeled; correspondingly, the combination of AmB and 5FC has an additive effect when the killing of fungal cells is used as the end point. Finally, it is worth acknowledging that the exposure of AmB and 5FC required to produce 50% of the total effect (i.e., the $E_{50,AmB}$ and the $E_{50,5FC}$) is larger when the drugs are used in combination than when either drug is used alone; this is because the overall effect is larger with combination treatment than with monotherapy.

DISCUSSION

The combination of AmB and 5FC in the treatment of INVC is attractive, in theory, because the membrane damage induced by AmB may facilitate the intracellular accumulation of 5FC and thereby cause an effect beyond that observed with either drug alone [17, 18]. In the present study, we have demonstrated, using a rigorous definition of drug interaction and a well-validated pharmacodynamic murine model of INVC, that the combination of AmB and 5FC has an additive effect. On the basis of previous work with 5FC, we employed $T > MIC$ as the pharmacodynamic variable linked to outcome [16]. In the case of AmB, Andes et al. suggested that the peak concentration:MIC ratio is the relevant pharmacodynamic parameter [19]. In the present study, only a single dose of AmB was administered; in this circumstance, there is complete colinearity between peak concentration:MIC and the AUC:MIC ratio, and, for the purposes of computational tractability, we chose to employ the AUC:MIC ratio as the relevant linked pharmacodynamic variable.

The combination of AmB and 5FC against *Candida* species has been studied in vitro, in vivo, and in a limited number of clinical contexts. In vitro, different definitions and modeling techniques, including the fractional inhibitory concentration and surface response methods, have been used to assess the combined

effect [20–24]. The majority of studies have concluded that the combination of AmB and 5FC is potentially beneficial, although disparities in methodological methods, analysis, and interpretation prevent any definitive conclusions regarding the overall nature and magnitude of the combined effect. In vivo studies examining the combination of AmB and 5FC have used survival [25, 26] and fungal tissue burden [27] as therapeutic end points; in this context, the combination has been shown to improve survival and reduce fungal tissue burden to a greater extent than is observed with monotherapy. Finally, limited clinical data that describe the safety and efficacy of the combination of AmB and 5FC in a small number of patients in a variety of clinical contexts are available [18, 28], although, at present, no clinical data support the routine addition of 5FC to a regimen of AmB for the treatment of INVC. Thus, the potential benefits of combining AmB and 5FC for INVC remain unclear. The present study enables some of the potential advantages of the combination to be further explored.

A variety of definitions have been employed to define the interaction between 2 drugs; these have been extensively reviewed elsewhere [15]. The Greco model, which is based on the concept of Loewe additivity, was used in this study. The interaction pa-

Table 2. The estimates of the means and 95% confidence intervals (CIs) for the parameter values from the Greco model.

| Parameter | Mean (95% CI) |
|---------------|--------------------------|
| E_{con} | 5.95 (2.26 to 9.64) |
| $IC_{50,5FC}$ | 0.62 (−0.75 to 1.97) |
| m_{5FC} | 0.84 (−2.57 to 4.26) |
| $IC_{50,AmB}$ | 137.8 (−172.80 to 448.4) |
| m_{AmB} | 0.96 (−0.68 to 2.60) |
| α | 0.000005 (−4.64 to 4.64) |

NOTE. α , interaction parameter; E_{con} , fungal burden (in \log_{10} cfu/g of kidney) in the absence of treatment; $IC_{50,AmB}$, amphotericin B deoxycholate (AmB) area under the curve (AUC):MIC ratio that produces 50% of the maximum effect; $IC_{50,5FC}$, fraction of the dosing interval that 5-fluorocytosine (5FC) is above the MIC that produces 50% of the maximum effect; m_{AmB} , slope parameter for AmB; m_{5FC} , slope parameter for 5FC.

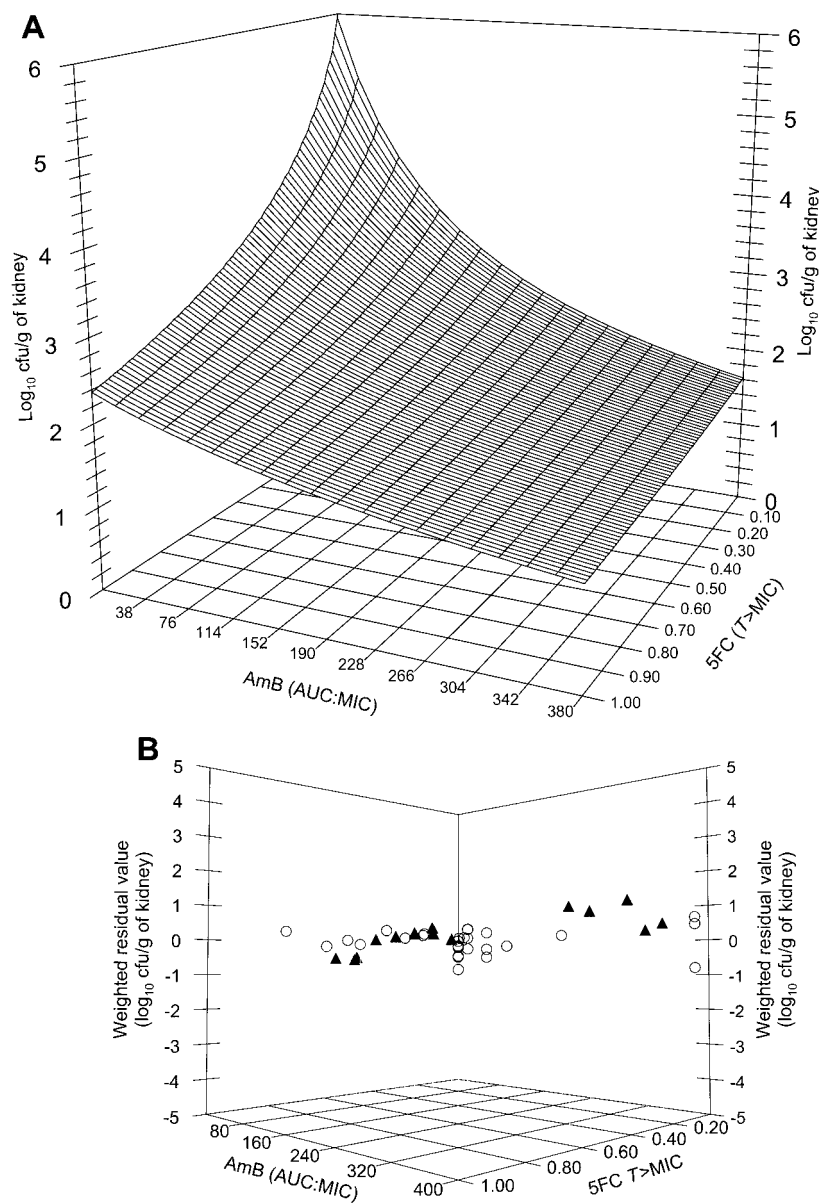


Figure 2. *A*, The effect (in \log_{10} cfu/g of kidney) induced by the combination of amphotericin B deoxycholate (AmB) and 5-fluorocytosine (5FC) expressed as the area under the curve (AUC):MIC ratio for AmB and the fraction of the dosing interval (T) that the 5FC serum concentration is above the MIC of 0.125 mg/L. The fitted response surface alone is shown; this was derived by comodeling monotherapy and combination treatment data. The fitted surface does not deviate in a statistically significant manner from the theoretically derived additive surface (not shown). A synergistic interaction would produce a more concave surface, whereas an antagonistic interaction would produce a convex surface. *B*, Three-dimensional scatter plot of the weighted residual values (predicted minus observed values). The weighted residual values cluster near 0 and conform to a Gaussian distribution (data not shown), which suggests that there is no systematic bias in the fit of the model to the data. The white circles represent the effect induced by AmB or 5FC when given alone, and the black triangles represent the weighted residual values from the various combination regimens.

rameters in equation (1) are estimated from the entire data set, at once, using a weighted nonlinear least-squares approach [29]. In the Greco model, the null reference model (or additive surface) is defined when $\alpha = 0$; consequently, the third term in equation (1) is also 0, and the Greco model defaults to an equation of Loewe additivity [29]. If α is positive, a greater-than-expected effect (synergy) is present; conversely, if α is negative, a less-than-

expected effect (antagonism) is present [29]. In the present study, the combination of AmB and 5FC has an additive effect, because the model estimate of α , the interaction term, is essentially 0, and the lower bound of the 95% CI crosses 0; thus, the observed experimental effect does not deviate in a statistically significant manner from the null reference model (or additive surface) of the combined effect.

The absence of demonstrable synergy does not necessarily invalidate the combination of AmB and 5FC as a useful therapeutic strategy in humans. Rather, a deeper understanding of the nature of the additive interaction provides an insight into ways in which this combination can be rationally employed to spare the toxicities associated with the administration of larger doses of either drug alone. In circumstances in which the administration of a given dose of AmB is associated with sub-optimal killing of fungal cells (i.e., when the maximum effect has not been obtained) and the administration of higher doses is not possible, because of dose-related toxicity, the addition of 5FC may enable the near-maximum effect to be safely reached. In our analysis of the combination of AmB and 5FC, the effect of 5FC is near the maximum when $T > MIC$ is ~70%–75% (see figure 2A). Whether this is also the case in humans with INVC could be studied in a prospective clinical trial in which combinations of AmB and 5FC in different dosing regimens, chosen on the basis of the experimental data and with suitable reference to human pharmacokinetics, could be compared. The simultaneous collection of pharmacokinetic, microbiological, and therapeutic outcome data would enable the magnitude of the drug exposure required to produce a near-maximum effect to be established and would provide validation of the experimental data.

The present study has many limitations. First, only a single isolate was studied; ideally, multiple *C. albicans* isolates would be investigated to examine whether there are significant strain-to-strain differences in terms of the combined effect of AmB and 5FC. Second, the results are applicable only to isolates with the same susceptibility profile we examined; isolates with higher AmB and 5FC MICs may need higher doses of drugs to produce a near-maximum effect. Third, the results are not directly applicable to the treatment of infections with *Candida* species at sanctuary sites, where drug penetration may be compromised. Fourth, the results cannot be extrapolated to other organisms, such as *Cryptococcus* species, although the same methods could be used to answer similar clinically relevant questions. Fifth, the potential differences in outcome induced by prolonged and sequential dosing used in human infections, in comparison with the short duration of treatment often used in pharmacodynamic models, remains poorly elucidated and is a matter for further study. Finally, unrecognized differences in protein binding between experimental animals and humans may limit the extent to which the results obtained in the murine model can be confidently extrapolated to a clinical context.

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