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Research Paper

Combination Antifungal Therapy Involving Amphotericin B, Rapamycin and 5-Fluorocytosine Using PEG-Phospholipid Micelles

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Purpose. Rapamycin and 5-fluorocytosine (5-FC) are antifungal agents with unique mechanisms of activity, with potential for cooperative interaction with AmB. Combination antifungal therapy involving conventional AmB has been restricted by poor physical stability and compatibility with antifungal drugs and vehicles.
Methods. AmB and rapamycin were encapsulated in 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-methoxy poly(ethylene glycol) (PEG-DSPE) micelles using a solvent evaporation method. The physical stability of micelle encapsulated AmB and rapamycin with 5-FC and saline was evaluated using dynamic light scattering (DLS). *In vitro* susceptibility of *Candida albicans* isolates to 5-FC and PEG-DSPE micelle solubilized AmB and rapamycin has been evaluated. Interactive effects have been quantified using a checkerboard layout.
Results. In contrast with conventional AmB, PEG-DSPE micelles encapsulating AmB and rapamycin are compatible with saline and 5-FC over 12 h. The solubilized drugs retain high level of potency *in vitro*. The combination of solubilized AmB and rapamycin was indifferent, as fractional inhibitory concentration (FIC) index and combination index (CI) values were approximately 1. Combinations of solubilized AmB or rapamycin with 5-FC, and the three-drug combination were moderately synergistic since the FIC index and CI values were consistent less than 1.
Conclusions. These results indicate that AmB solubilized in PEG-DSPE micelles is compatible with solubilized rapamycin and 5-FC. The indifferent or moderately synergistic activity of combinations is encouraging and warrants further investigation in appropriate rodent models.

KEY WORDS: 5-fluorocytosine; amphotericin B; antifungal therapy; checkerboard analysis; combination therapy; disseminated candidiasis; PEG-DSPE; polymeric micelles; rapamycin.

INTRODUCTION

The increasing incidence of hospital acquired opportunistic fungal infections is of concern, particularly in the context of a growing population of patients with compromised or suppressed immune systems (1). Amphotericin B (AmB) solubilized as a colloidal dispersion by sodium deoxycholate (D-AmB) is a first-line antifungal agent administered to many patients with invasive candidiasis, despite severe kidney toxicity (2). Liposomal amphotericin B (L-AmB) is less toxic compared to D-AmB allowing for dose-escalation and improved tolerability; however, L-AmB has lower antifungal activity at equal doses necessitating significantly higher doses for comparable efficacy, raising doubts about a meaningful increase in the therapeutic index (3). Notably, L-AmB has not had a significant impact on the crude or attributable mortality compared to D-AmB (4).

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There is renewed interest in studying combinations of antifungal drugs to explore potential advantages such as broad-spectrum efficacy in the context of resistant organisms and/or an improved safety and tolerability profile resulting from synergistic increase in potency (5,6). There is growing evidence that the combination of D-AmB and 5-fluorocytosine (5-FC) has better activity against candidiasis compared to D-AmB alone (7–10). The *in vitro* antifungal effects of the combination of D-AmB and 5-FC are synergistic or indifferent, depending on the isolate of *Candida* (6). It is hypothesized that AmB potentiates 5-FC activity by increasing the penetration of 5-FC across fungal membranes, highlighting the value of exposing the fungal pathogen to 5-FC at the same time or after exposure to AmB (11). Recently, D-AmB in 5% dextrose and 5-FC in 0.9% NaCl, injected intraperitoneally as separate and rapid sequential injections showed additive activity in a murine model of candidiasis according to the response-surface model (12).

Rapamycin exerts potent antifungal activity by inhibiting target-of-rapamycin (TOR) kinases [minimum inhibitory concentration (MIC) against *C. albicans* <0.02 mg/l] (13,14). Early animal experiments showed effectiveness against *Candida* infection, however, enthusiasm for use as an antifungal agent was lowered on emergence of potent immunosuppression in hosts at moderate doses (15). Rapamycin-analogues

that selectively bind yeast TOR kinases present a thousand-fold reduction in immunosuppressive activity, while retaining some measure of antifungal activity (MIC<3 mg/l for *C. albicans* and *C. glabrata*) (16). The TOR signaling cascade represents a conserved pathway across yeast, involved in eliciting cell response to a wide variety of stimuli including nutrients and external stress (17). Blocking this survival pathway using rapamycin, while inducing stress using AmB, has unexplored potential for treatment of systemic fungal disease and may result in a cooperative increase in antifungal potency. Notably, the aqueous solubility of rapamycin has been reported as 2.6 μg/ml and has proven to be highly challenging for drug solubilization (18).

Several strategies have emerged which are beneficial in lowering AmB related toxicities. There is recent clinical evidence which suggests that D-AmB administered as a continuous infusion over 24 h results in a lower incidence of nephrotoxicity and infusion-related side-effects compared to the standard 2–4 h infusion at an equal dose (19–21). Although the mechanism for lowered toxicities is poorly understood, it is proposed that slow infusion results in lower levels of protein-bound drug capable of evoking host toxicity, compared to the standard infusion regimen (22). Saline loading during AmB therapy has been shown to reduce the severity of AmB related toxicities. In a double-blind, placebo-controlled study with human subjects, sodium supplementation has been shown to diminish infusion-related side effects and nephrotoxicity caused by D-AmB (23).

Combination antifungal therapy involving AmB has been restricted by factors such as poor physical stability and compatibility with antifungal drugs and vehicles, especially in the form of D-AmB. D-AmB is not compatible with saline and precipitates instantly on dilution (24). Although saline loading has gained some degree of acceptance, this procedure necessitates sequential administration of saline and D-AmB and special care must be taken to adequately flush infusion lines with 5% dextrose prior to D-AmB administration to avoid potentially hazardous drug precipitation. Multiple-agent therapy with continuous administration of D-AmB would require additional intravenous (IV) access-lines owing to incompatibility, raising concern of increased risk of infection in these critically ill patients.

We have previously reported that micelles formed from the amphiphilic polymer 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-methoxy poly(ethylene glycol) (PEG-DSPE) readily solubilize AmB (AmB|PEG-DSPE) (25). The encapsulated drug in these micelles is in a deaggregated state. Drug release studies indicated that AmB is released slowly from PEG-DSPE micelles under sink conditions at 37°C (26). The micelle encapsulated AmB resulted in low (<10%) levels of hemolysis in murine erythrocytes, while retaining potent *in-vitro* activity (27). Hence, we hypothesize that the released AmB is predominantly in a monomeric form. In this work, we report that PEG-DSPE micelles also solubilize high levels of rapamycin when prepared using an identical solvent evaporation method (rapamycin|PEG-DSPE). AmB is stably incorporated in PEG-DSPE micelles in a form which is compatible with rapamycin and 5-FC in 0.9% NaCl. We have evaluated the *in vitro* activity of two- and three-drug combinations using a broth microdilution method. Interactive effects have been quantified by two methods used in the literature: Fractional Inhibitory Concentration index (FICI), combination index (CI) calculated using median dose–effect equation.

MATERIALS

AmB was obtained as a gift from Alpharma (Copenhagen, Denmark). Rapamycin and 5-FC were purchased from LC Labs (Woburn, MA) and Sigma (St. Louis, MO), respectively. AmB formulated in sodium deoxycholate (D-AmB) was purchased from Sigma (St. Louis, MO). The drugs were stored at −20°C until use. PEG-DSPE (M_n=5,800 g/mol) was obtained from Avanti Polar Lipids (Alabaster, AL). All other reagents used were of analytical grade and were used without further purification.

MICELLE PREPARATION AND INCORPORATION OF AMB AND RAPAMYCIN

PEG-DSPE (6.0 mg/ml in chloroform) was mixed with AmB (0.25 mg/ml in methanol) or rapamycin (1 mg/ml in chloroform) in a round bottom flask. The organic solvent was evaporated under high vacuum to produce a thin film of co-precipitated drug and polymer. This film was dissolved in 10 mM HEPES, pH 7.0 and incubated at room temperature for 10 min to allow for complete equilibration. The micellar solution was filtered through a 0.45-μm polyethersulfone (PES) filter. Empty micelles were prepared using an identical procedure without drug. The concentration of AmB was quantified by diluting a 50 μl aliquot of AmB in 1.95 ml DMF and observing absorbance at 413.5 nm. This assay was tested for linearity in the 0.02–0.8 mg/ml range. For determination of rapamycin content, 5 μl samples were injected into 4.6 mm×50 mm Ace 3 C18 reverse-phase column and absorbance detected at 277 nm. The assay was tested for linearity in the 0.1–100 μg/ml range.

MICELLE CHARACTERIZATION

Dynamic Light Scattering

Particle sizes were determined using dynamic light scattering using the NICOMP ZLS380 particle sizer (Particle Sizing Systems, Santa Barbara, CA) equipped with a 639 nm

Table I. Solubilization of AmB and Rapamycin in PEG-DSPE Micelles

	Fraction of initial drug encapsulated	Drug loading (% *w*/*w*)	Level of drug solubilized (mg/ml)	Diameter (nm)
PEG-DSPE	–	–	–	16.1±2.2
AmB|PEG-DSPE	0.78	7.21	0.38±0.12	19.3±4.4
rapamycin|PEG-DSPE	0.97	7.76	0.37±0.06	26.4±2.0

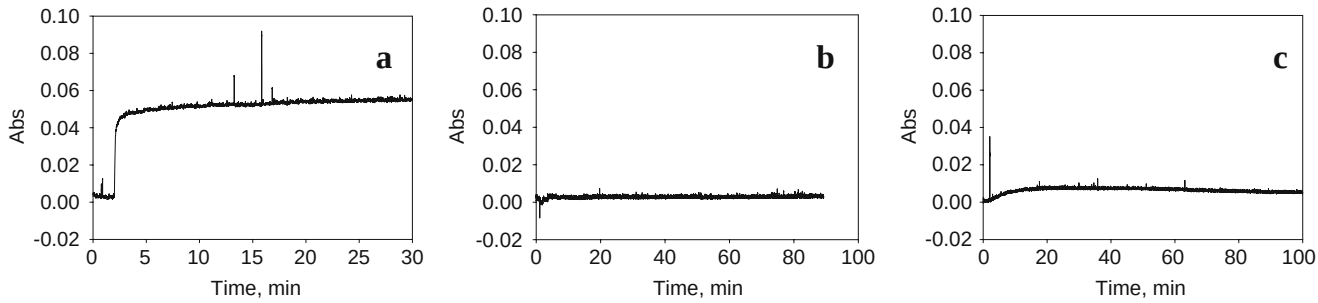

Fig. 1 Light scattering at 650 nm on admixing **a** D-AmB with 0.9% NaCl **b** D-Amb with 5% dextrose **c** AmB|PEG-DSPE with rapamycin/PEG-DSPE and 5-FC in 0.9% NaCl. The final AmB concentration was 0.1 mg/ml.

laser at a fixed angle of 90°. Data was acquired to have greater than 100 k counts in channel 1. Particle sizes were expressed as volume-weighted diameters.

SOLUTION TURBIDITY MEASUREMENTS

Precipitation of D-AmB was studied by observing solution turbidity ($OD_{650\ nm}$) using a Cary 50 UV spectrophotometer equipped with a dip probe. Aqueous solutions containing 0.9% NaCl or 5% dextrose were maintained at 25° C in a water-jacketed beaker. $OD_{650\ nm}$ was observed for the initial 2.0 min to estimate baseline turbidity. Thereafter, D-AmB was added to aqueous medium and changes in $OD_{650\ nm}$ were observed over the duration of the experiment. The final concentration of AmB was 0.1 mg/ml. Changes in solution turbidity on the addition of AmB|PEG-DSPE to 0.9% NaCl and 0.9% dextrose were similarly measured. Further, the potential for precipitation on mixing AmB|PEG-DSPE, rapamycin|PEG-DSPE and 5-FC was examined using an identical procedure.

IN VITRO SUSCEPTIBILITY OF FUNGAL ISOLATES

Estimation of Minimum Inhibitory Concentration (MIC)

C. albicans 98-17 and 98-234 were maintained on Sabouraud Dextrose Agar (SDA) plates. Susceptibility of yeast isolates was performed using broth microdilution in RPMI-1640 (supplemented with 0.165 M morpholinepropanesulfonic acid, buffered to pH 7.0) according to procedures recommended in CLSI (Clinical Laboratory Standards Institute) M27-A2 (28). Test drug solutions were incubated with yeast inoculum at 35°C in 96-well plates for a 24 h period. Growth inhibition relative to drug-free control, f_a was quantified by measuring optical density (OD) at 650 nm, using the Microplate EL 312e plate reader.

$$f_a = 1 - \frac{OD_{sample} - OD_{blank}}{OD_{control} - OD_{blank}}$$

where OD_{sample}, OD_{blank}, $OD_{control}$ are the measured OD for sample, blank buffer and drug-free control respectively.

In the CLSI method for amphotericin B, the MIC is read as the lowest drug concentration that prevents any discernable growth. We have defined the MIC values to represent drug concentrations which resulted in f_a values greater than 0.95. The MIC values were expressed as a mean of three determinations.

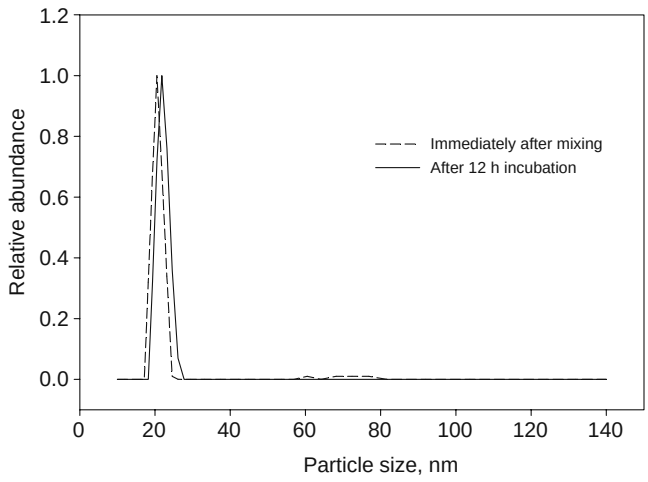

Fig. 2 Sizing of AmB|PEG-DSPE, rapamycin|PEG-DSPE and 5-FC mixture in 0.9% NaCl using DLS. **a** Immediately after mixing **b** after incubation for 12 h at room temperature.

Interactive Effects of Drug-combinations

The interactive effects of drug combinations were assessed using a checkerboard layout. For the three-drug combination experiments, the level of 5-FC was varied for a constant ratio of AmB|PEG-DSPE/rapamycin|PEG-DSPE (1:2 or 2:1). Determination of fungal growth in response to drug combinations was performed in triplicate. Interactive effects were interpreted using two methods extensively used in the literature: FIC index (FICI) analysis (6) and calculation of a combination index (CI) using a median-effect equation (29,30).

Table II. Minimum Inhibitory Concentrations (MIC) in mg/l Against *C. albicans* Isolates

	Strain of *C. albicans*	
	98-17	98-234
D-AmB	0.25	0.25
AmB| PEG-DSPE	0.05	0.05
rapamycin| PEG-DSPE	0.05	0.05
5-FC	0.1	0.1

Table III. Best Fit Parameters to Median-Effect Equation, $f_a = \frac{1}{1+(D_m/D)^m}$

	Strain of *Candida albicans*			
	98-17		98-234	
	D_m (mg/l)	m	D_m mg/l	m
AmB| PEG-DSPE	0.017	6.0±0.2	0.011	6.5±0.5
rapamycin|PEG-DSPE	0.032	6.5±2.2	0.024	3.5±0.5
5-FC	0.071	2.0±0.8	0.039	1.9±0.4

FIC Analysis

The FICI for a three drug combination is defined by the equation:

$$\mathrm{FICI} = \frac{\mathrm{MIC_{A-combination}}}{\mathrm{MIC_A}} + \frac{\mathrm{MIC_{B-combination}}}{\mathrm{MIC_B}} + \frac{\mathrm{MIC_{C-combination}}}{\mathrm{MIC_C}}$$

where $\mathrm{MIC_A}$ and $\mathrm{MIC_{A\text{-}combination}}$ represent the lowest concentration for drug A which resulted in f_a values greater than 0.95 as a single agent or in combination with drug B and drug C. A similar notation has been used for drug B and drug C respectively. The FICI values have been interpreted to indicate synergism (<0.5), additivity (0.75–1.5) or antagonism (>4) according to the recommendations in the literature (5,31–33).

Combination Indices Using Median-effect Response

Calculation of a combination index was done in two steps. First, inhibition of fungal growth, f_a in response to varying concentrations of single-drug, D was fit to median-effect function:

$$f_a = \frac{1}{1 + (D_m/D)^m}$$

Parameters D_m and m for each drug were estimated by non-linear regression using SigmaPlot (v. 9.0). D_m is the drug concentration corresponding to a 50% growth inhibition, and m is the slope-factor describing the dose–response curve.

The combination index (CI) for was estimated on the assumption of mutually exclusive effects using the CombiTool software (v. 2.001, IMB-Jena, Germany) using the following equation:

$$\mathrm{CI} = \frac{D_{\mathrm{A-combination}}}{D_{\mathrm{A},fa}} + \frac{D_{\mathrm{B-combination}}}{D_{\mathrm{B},fa}} + \frac{D_{\mathrm{C-combination}}}{D_{\mathrm{C},fa}}$$

where $D_{\mathrm{A},fa}$ is the calculated concentration of drug A corresponding to inhibition f_a, based on known values of D_m and m for drug A. $D_{\mathrm{A\text{-}combination}}$, is the actual concentration of drug A in the three-drug combination which caused growth inhibition, f_a. A similar notation has been used for drug B and drug C respectively. Combination indices (CI) were represented as color maps using Origin software (version 7.0). The graphs have been color-coded to represent synergism (dark blue) for CI less than 0.5, moderate synergism (light blue) for CI in the range 0.5–0.75, indifference (green) for CI in the range 0.75–1.5 and antagonism (red) for CI values greater than 1.5. The underside of the surface is displayed in dark yellow.

RESULTS AND DISCUSSION

Micelle Characterization

AmB was efficiently solubilized by PEG-DSPE micelles (AmB|PEG-DSPE) when prepared using the solvent evaporation method (Table I). AmB in PEG-DSPE micelles was deaggregated owing to polymer–drug interactions, as reported earlier (25). Drug release studies indicated that the encapsulated AmB was released slowly from the micelles, presumably in a monomeric form (25,26). High levels of rapamycin could be loaded into PEG-DSPE micelles (rapamycin|PEG-DSPE) using a similar procedure. The level of rapamycin solubilized was 0.38 mg/ml, with a high yield (97% of initial drug). Using dynamic light scattering, the size of AmB|PEG-DSPE micelles was determined to be 19.3± 4.4 nm, slightly larger than empty PEG-DSPE micelles (16.1± 2.2 nm). The size of rapamycin|PEG-DSPE micelles was similarly small, 26.4±2.0 nm.

We have studied changes in solution turbidity on addition of D-AmB to 0.9% NaCl or 5% dextrose solutions using a spectrophotometer equipped with a dip-probe assembly. There was an instantaneous increase in solution turbidity on addition of D-AmB to 0.9% NaCl, indicative of rapid precipitation on mixing (Fig. 1a). Addition of D-AmB to 5% dextrose did not result in increased solution turbidity, consistent with the conventional use of 5% dextrose as

Table IV. Fractional Inhibitory Concentration (FIC) Analysis for Test Isolates Against AmB–rapamycin–5-FC Combinations After 24 h

	C. albicans 98-17	*C. albicans* 98-234
AmB|PEG-DSPE–rapamycin|PEG-DSPE	1.3 (1.1–1.6)	1
AmB|PEG-DSPE–5-FC	0.6	0.8
rapamycin|PEG-DSPE–5-FC	1	1
AmB|PEG-DSPE–rapamycin|PEG-DSPE–5-FC	0.7 (0.4–0.8)	0.45 (0.4–0.5)

Results are expressed as mean (range) for test-replicates

acceptable vehicle for dilution of D-AmB (Fig. 1b). In contrast, addition of AmB|PEG-DSPE and rapamycin|PEG-DSPE to 5-FC in 0.9% NaCl led to a minor increase in $OD_{650\ nm}$, attributed to light scattering by intact PEG-DSPE micelles (Fig. 1c). Also, DLS could not detect aggregates 12 h after mixing AmB|PEG-DSPE, rapamycin|PEG-DSPE and 5-FC in 0.9% NaCl, further indicating that this mixture was stable against drug precipitation (Fig. 2).

These results are particularly encouraging since AmB|PEG-DSPE may be suitable for slow infusion over several hours without concern for drug precipitation on dilution. This novel form of AmB has a distinct advantage over D-AmB, since it may allow for co-administration of saline via a single IV access-line. Further, since the three drug combination of AmB|PEG-DSPE, rapamycin|PEG-DSPE and 5-FC remains solubilized without precipitate formation, it may become possible to explore the potential of infusing two- or three-drug combinations over AmB monotherapy.

In Vitro Susceptibility Studies

In vitro susceptibility of *C. albicans* 98-17 and 98-234 was evaluated using the broth microdilution method. MIC values for AmB|PEG-DSPE were 0.05 mg/l. The MIC for D-AmB, in which the drug is highly self-aggregated, was 0.25 mg/l (Table II). PEG-DSPE did not exhibit intrinsic antifungal activity (MIC>10 mg/l). The reason for enhanced activity of PEG-DSPE encapsulated AmB is unclear—however a similar potentiation of AmB activity has been demonstrated on encapsulation in mixed micelles formed from poly(ε-caprolactone) and poloxamer 188 (34).We hypothesize that in contrast with D-AmB that produces a mixture of monomers and water soluble aggregates, AmB dissociated from intact PEG-DSPE micelles is predominantly in a monomeric form, which results in a higher number of membrane-active units compared to the D-AmB. The MIC of rapamycin in PEG-DSPE micelles was 0.05 mg/l, comparable to that for

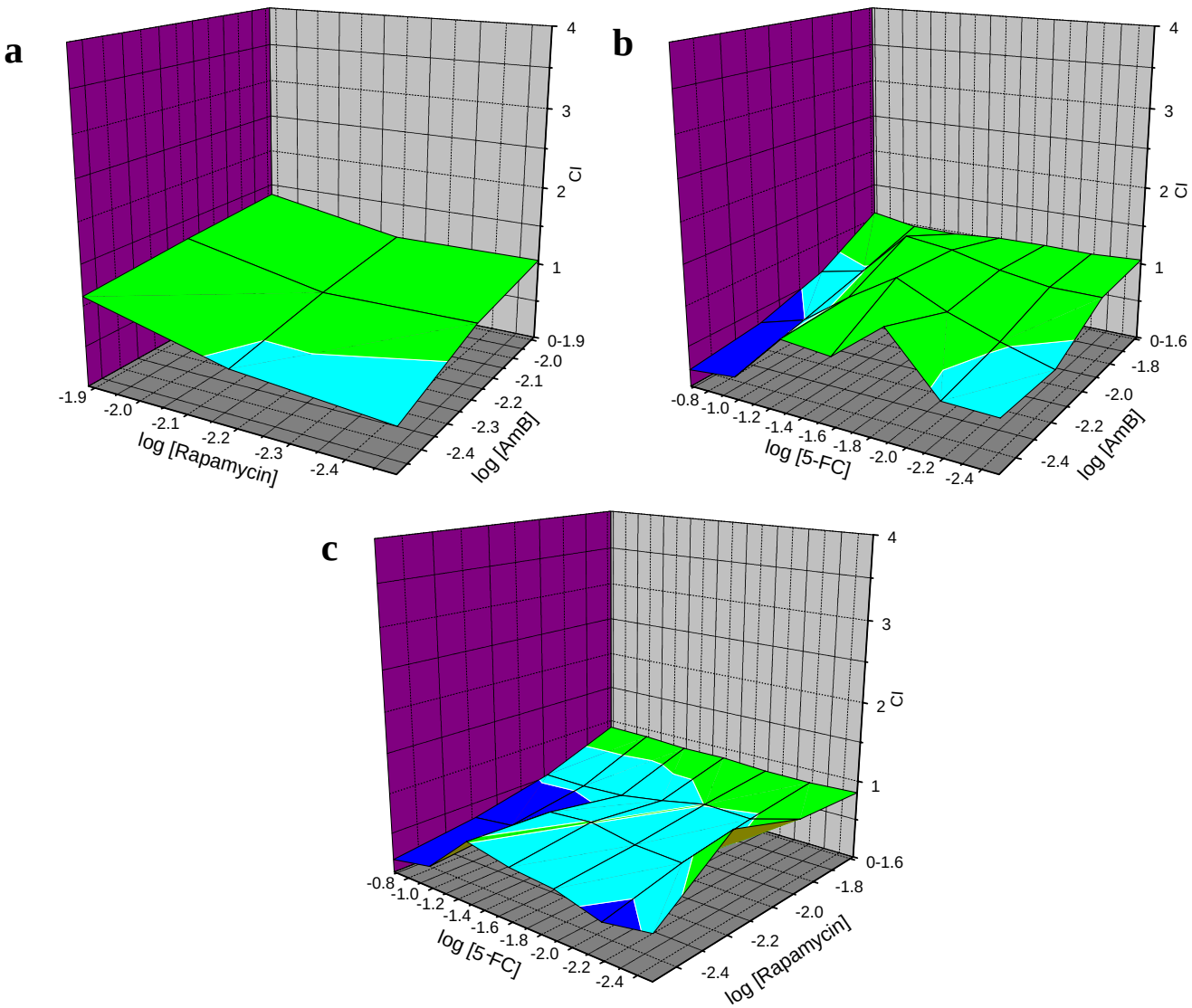

Fig. 3 Contour plots for **a** rapamycin|PEG-DSPE–AmB|PEG-DSPE **b** 5-FC–AmB|PEG-DSPE **c** 5-FC–rapamycin|PEG-DSPE combinations against *C. albicans* 98-17.

unformulated rapamycin reported in the literature (14). The MIC for 5-FC, corresponding to complete inhibition of fungal-growth was 0.1 mg/l—consistent with literature reports (10,35). Table III shows parameters obtained by fitting the dose–response curve for AmB|PEG-DSPE, rapamycin|PEG-DSPE and 5-FC to the median-effect equation. Reasonable fits to the data were obtained using non-linear regression, judged by R^2 values greater than 0.9. A high value for the shape factor, m for both AmB|PEG-DSPE and rapamycin|PEG-DSPE implied transition from no inhibition to complete growth inhibition over a narrow range of concentration. In comparison the shape factor for 5-FC was 2.0, lower in comparison with AmB|PEG-DSPE and rapamycin|PEG-DSPE.

We have studied interactive effects of AmB|PEG-DSPE, rapamycin|PEG-DSPE and 5-FC as two- and three-drug combinations *in vitro* using a checkerboard layout. The complexity of assessing drug interactions has been stressed in the literature (5,10,36). Calculation of FIC indices has been extensively used for evaluating interactive effects between antimicrobial combinations. A limitation of the FIC analysis is that this analysis presumes that drug interactions are unvariant and apply across all concentrations. Several methods have been proposed to study drug interactions which have been reviewed in detail by Greco (37). The response surface method proposed by Greco *et al.* (38) and the method of Chou and Talalay using the median-effect equation (29,33) have been frequently used in studying antiviral and antineoplastic drug interactions, each with a set of important underlying assumptions and limitations. An advantage of the method of Chou and Talalay is that it allows visualization of the combination index for different drug ratios—an important goal in studying drug combinations, *in vitro*. This approach has particular utility when the drug combination under study shows cooperativity for some ratios and antagonism at other ratios. We have calculated combination indices for two- and three-drug combinations, based on the median-effect equation using parameters in Table III.

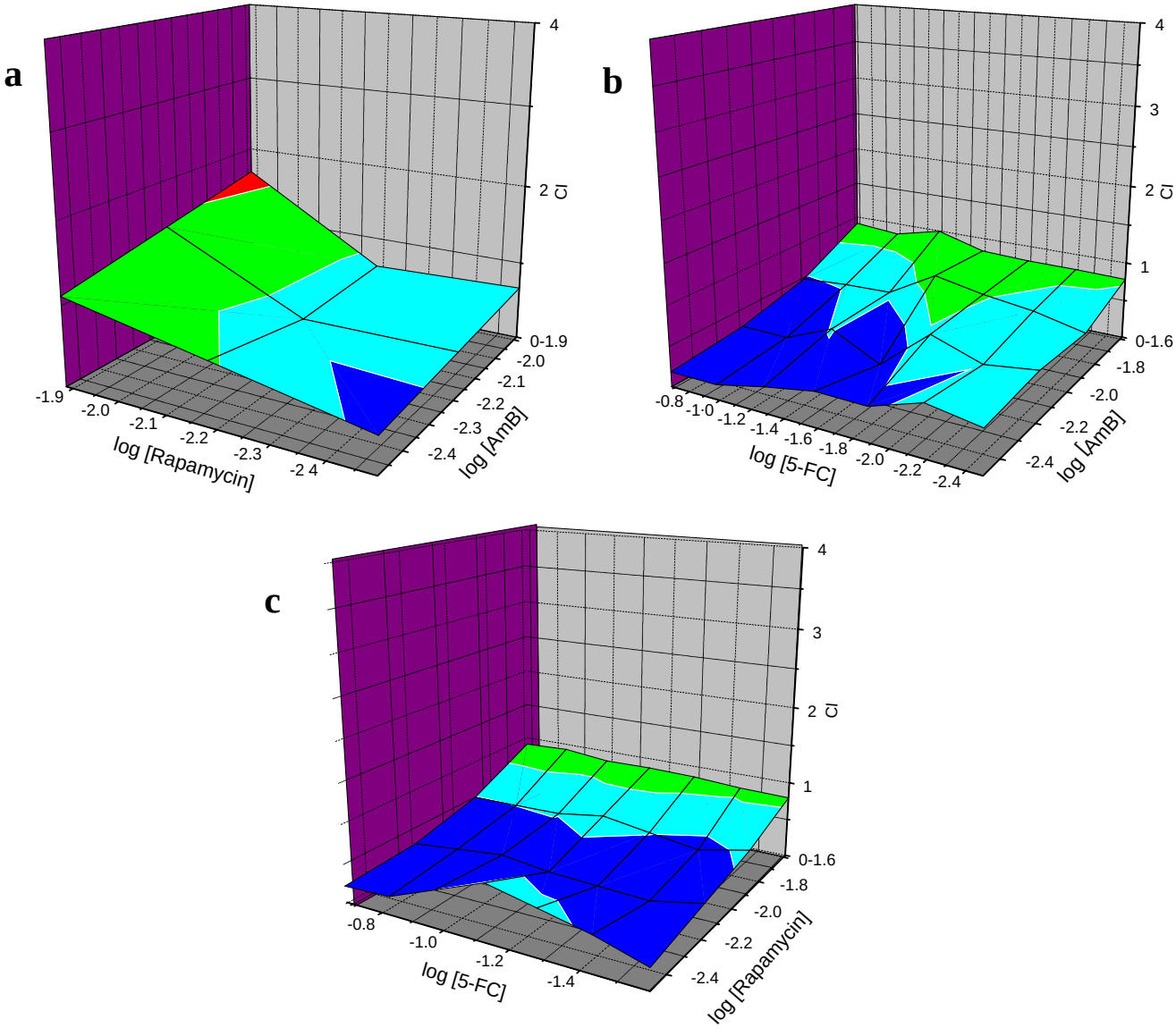

Fig. 4 Contour plots for **a** rapamycin|PEG-DSPE–AmB|PEG-DSPE **b** 5-FC–AmB|PEG-DSPE **c** 5-FC–rapamycin|PEG-DSPE combinations against *C. albicans* 98-234.

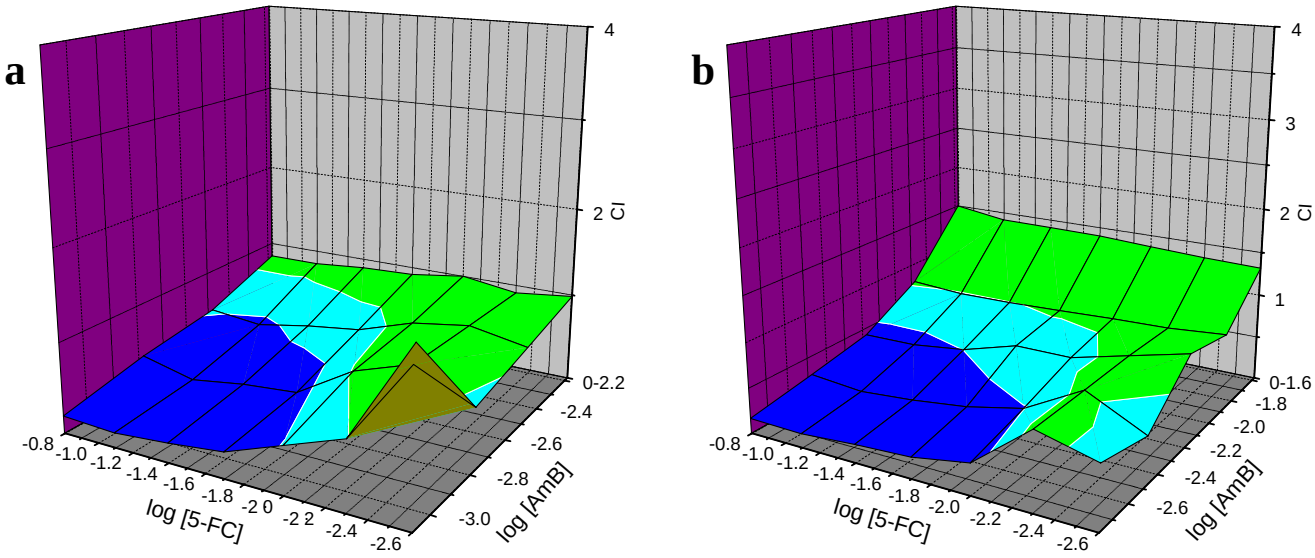

Fig. 5 Contour plots for 5-FC–AmB|PEG-DSPE–rapamycin|PEG-DSPE combinations for AmB|PEG-DSPE/rapamycin|PEG-DSPE **a** 1:2 **b** 2:1 for *C. albicans* 98-17.

Consistent variations of FICI or combination index from 1 are taken as measures of drug interaction. Although a subject of debate, there is general consensus that a synergistic interaction between drugs be claimed for FICI less than 0.5. Similarly, drug antagonism is claimed for FICI values greater than 4 (5,31,32). Additionally, we have used the terms "moderate synergy" or "moderate antagonism" in the following discussion according to recommendations of Chou (33). The graphs representing combination indices have been color coded as described in the methods section.

The FICI for the AmB|PEG-DSPE–rapamycin|PEG-DSPE combination ranged from 1.0 to 1.6 (Table IV), suggesting an indifferent interaction between the antifungal agents. This indifferent action was also noted from the interaction profile using combination indices (Figs. 3a, 4a). It is noted that a similar indifference has been reported for D-AmB and unformulated rapamycin against Aspergillus fumigatus isolates (39). The lack of antagonism between drugs is an important result since drug combinations would allow for an increase in antifungal activity in situations when dose-escalation is not an option. Mechanistic studies of drug-interactions were outside the scope of this work—however, it appears that these antifungal drugs act by relatively independent mechanisms. This combination may present a strategy which combines the rapid fungicidal action of AmB|PEG-DSPE with low doses of rapamycin|PEG-DSPE, which may work well as consolidation or clearance therapy.

The 5-FC–AmB combination has been extensively studied *in vitro* and in animal models (5,12). The FICI values for the 5-FC–AmB|PEG-DSPE ranged from 0.6 to 0.8, for *C. albicans* 98-17 and 98-234, indicating that this combination exerted moderately synergistic behavior. The D-AmB–5-FC interaction is reported to be variable and dependent on experimental conditions and on the *Candida* isolate tested

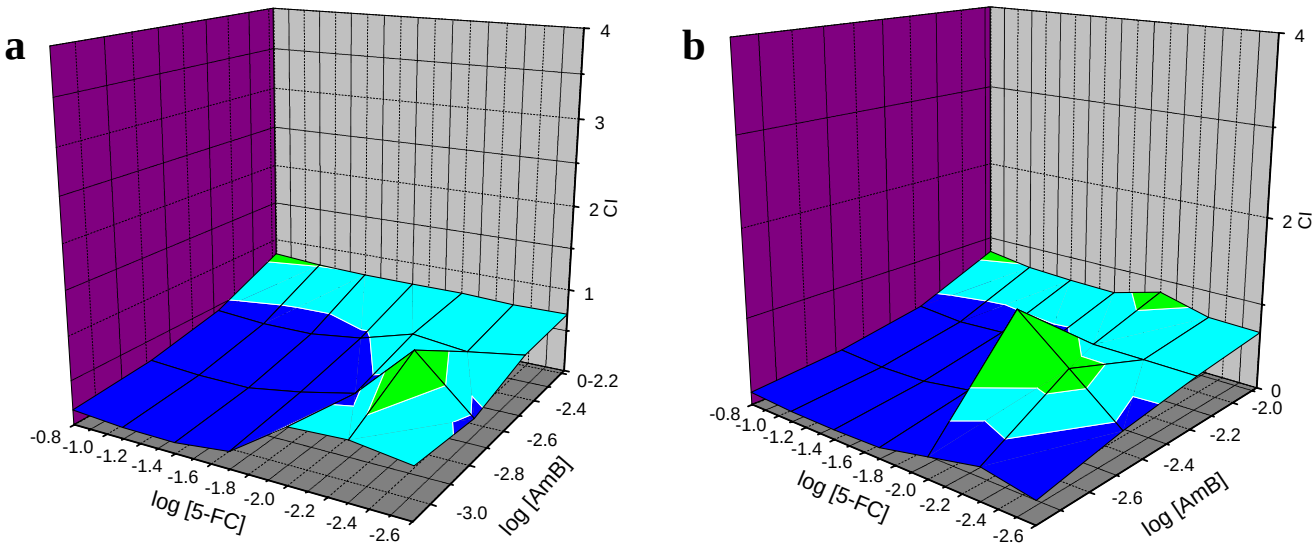

Fig. 6 Contour plots for 5-FC–AmB|PEG-DSPE–rapamycin|PEG-DSPE combinations for AmB|PEG-DSPE/rapamycin|PEG-DSPE **a** 1:2 **b** 2:1 for *C. albicans* 98-234.

(10,36). A majority of the isolates studied in the literature reported indifferent activities, whereas the interaction was synergistic for some isolates. Moderate antagonism has been reported in a small fraction of *C. albicans* isolates tested (40).

The interaction of 5-FC with AmB|PEG-DSPE was synergistic for *C. albicans* 98-17 and 98-234 (Figs. 3b, 4b). The mechanism of cooperative interaction between 5-FC and AmB|PEG-DSPE is not well understood, however, higher penetration of 5-FC through the yeast cell-membrane in the presence of small quantities of D-AmB has been proposed. It is noted that nystatin, structurally related to AmB exerted an antagonistic interaction with 5-FC (41). Alternatively, D-AmB is thought to influence processes that transport 5-FC out of the yeast cells (5).

The combination of 5-FC with rapamycin|PEG-DSPE was indifferent with FICI equal to 1 for the isolates tested. The combination index analysis indicated a trend similar to the 5-FC–AmB|PEG-DSPE combination, with a moderately synergistic interaction for *C. albicans* 98-17 and 98-234 (Figs. 3c, 4c). This aspect of the drug interaction was missed by the FIC analysis, which concludes additivity based on concentrations required to completely inhibit fungal growth.

The three-drug combination of 5-FC, AmB|PEG-DSPE and rapamycin|PEG-DSPE resulted in a synergistic interaction for *C. albicans* 98-17 and 98-234, with the FICI ranging from 0.4 to 0.8, consistently lower than 1. It is interesting to note that cooperativity is observed over a greater range of concentrations compared to pair-wise combinations (Figs. 5, 6). It appears that while AmB|PEG-DSPE and rapamycin|PEG-DSPE are largely indifferent, the three-drug combination derives synergistic potency from interactions between 5-FC–AmB|PEG-DSPE and 5-FC–rapamycin|PEG-DSPE. In summary, there was good agreement between results obtained from FIC analysis and those obtained from the method Chou and Talalay, using the median-effect equation. Each drug pair represented indifferent or moderately synergistic interactions. This analysis suggests increased potency of 5-FC in the presence of small quantity of AmB|PEG-DSPE and rapamycin|PEG-DSPE. Drug combinations exhibiting indifferent interaction are also important, particularly in the context of resistant isolates. It must be stressed that although *in vitro* studies can provide an excellent frame-work for studying drug interactions, factors such as drug pharmacokinetics, fungal burden and state of immunosuppression may play an important role in outcome *in vivo* (5).

CONCLUSIONS

AmB|PEG-DSPE represents an alternative to D-AmB, with significant advantages in the context of combination therapy. In this form, AmB is stable against precipitation in a saline vehicle over prolonged periods of time, which may enable slow or continuous administration of AmB with optional sodium supplementation in a single IV access-line. Contrary to L-AmB, which exerts lower activity compared to D-AmB, AmB|PEG-DSPE retains potent antifungal activity. Additionally, in this form AmB may be mixed with 5-FC and rapamycin|PEG-DSPE. In this investigation, we observed indifferent or moderately synergistic activity for two- or three-drug combinations against *C. albicans* isolates. The encouraging *in vitro* results present an opportunity to investigate the toxicity and efficacy profile of these combinations in appropriate rodent models.

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