

Triazole-Polyene Antagonism in Experimental Invasive Pulmonary Aspergillosis: In Vitro and In Vivo Correlation

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Combination antifungal therapy is increasingly used in the treatment of invasive aspergillosis. Whether the interaction between amphotericin B and triazoles is antagonistic against invasive aspergillosis is a controversial issue that is not likely to be resolved through a randomized clinical trial. Here, we found both in vitro and in vivo antagonism between liposomal amphotericin B and ravuconazole in simultaneous treatment of experimental invasive pulmonary aspergillosis in persistently neutropenic rabbits. Bliss independence-based drug-interaction modeling showed significant antagonism in vitro and in vivo, with the observed drug effects being 20%–69% lower than would be expected if the drugs were acting independently. These in vitro and in vivo finding of antagonism were consistent with the finding from Loewe additivity-based drug-interaction modeling. No pharmacokinetic interaction was found. The combination of a triazole and polyene may be antagonistic in the treatment of invasive pulmonary aspergillosis.

Invasive pulmonary aspergillosis is an important cause of morbidity and mortality in patients with hematological malignancies and in patients who have undergone hematopoietic stem-cell transplantation [1, 2]. Moreover, mortality in persons with this infection is high despite the availability of antifungal therapy, particularly in immunocompromised patients (>50%) [1]. Given the urgent need for more-effective chemotherapeutic approaches, much attention has been focused on antifungal drug combinations [3, 4]. However, in cases in which one drug antagonizes the action of the other, combination therapy may have deleterious effects.

Amphotericin B (AMB) remains an important compound for the treatment of invasive pulmonary aspergillosis [1]. Its dose-limiting nephrotoxicity has been ameliorated by lipid formulations [5]. The family of antifungal triazoles has been expanded through the development of new azoles with better antifungal, pharmacokinetic, and toxicity profiles. Ravuconazole (RAV) is a new triazole that has a prolonged half-life and in vivo antifungal activity against *Aspergillus fumigatus* [6]. Both liposomal AMB (LAMB) and RAV have demonstrated activity against experimental invasive pulmonary aspergillosis [7–9].

The pharmacodynamic and pharmacokinetic interactions between polyenes and triazoles in the treatment of invasive aspergillosis are not well understood. Whether this combination of antifungal compounds is antagonistic against aspergillosis is a controversial issue that is not likely to be resolved through a randomized clinical trial. Therefore, in the present study, we investigated the pharmacological interaction between RAV and LAMB in vitro and in vivo, to better characterize polyene-triazole combination therapy in the treatment

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of invasive pulmonary aspergillosis in persistently neutropenic hosts.

MATERIALS AND METHODS

In Vitro Combination Experiments

The combination of RAV and AMB was tested against *A. fumigatus* isolate NIH4215 (ATCC no. MYA-1163) in triplicate by an in vitro broth microdilution checkerboard assay based on Clinical and Laboratory Standards Institute guidelines [10], as described elsewhere [11]. Stock solutions of RAV (Eisai) and AMB (Ben Venue Laboratories) were prepared in diemthyl sulfoxide and water, respectively. Two-fold serial dilutions were prepared in RPMI 1640 buffered at a pH of 7.0 with 0.165 mol/L 4-morpholinepropanesulfonate at concentrations that were 4 times the final concentrations and that ranged from 2 to 0.06 mg/L and from 4 to 0.004 mg/L for AMB and RAV, respectively. The interaction was assessed using Bliss independence-based response-surface analysis and Loewe additivity isobolographic analysis.

Infection Model

The *A. fumigatus* isolate NIH4215 was used in the experimental model of invasive pulmonary aspergillosis in 33 persistently neutropenic rabbits (5–6 per study group). The methods for establishing this model have been described elsewhere [7, 12].

Antifungal Compounds and Treatment Regimens

RAV was provided as a phosphodiester lysine salt (Bristol-Myers Squibb Pharmaceutical Research Institute) and was dissolved in 5% dextrose injection solution (Abbott Laboratories), to produce a stock solution (37.5 mg/mL) that was maintained at -4°C . Before use, RAV was freshly diluted with 5% dextrose injection solution to a concentration of 18.75 mg/mL for the 5 mg/kg/day (RAV5) dosage. Reconstituted RAV was administered at an ambient temperature as a slow intravenous (iv) bolus over 1 min. LAMB (AmBisome; Astellas Pharma) was diluted with sterile water to a concentration of 1 mg/mL and administered iv at 1.5 mg/kg/day (LAMB1.5) and 3 mg/kg/day (LAMB3) slowly (0.1 mL every 10 s). The RAV5 dosage was chosen because it achieved the maximum effect in this rabbit model [7], has been studied in patients and healthy volunteers [13], and will likely be used for therapeutic trials [14]. RAV is a novel investigational triazole currently being studied in phase 1 trials. RAV provides sustained plasma drug exposure over the dosing interval, permitting the study of triazole activity. The LAMB3 dosage was used because this is the dose approved for the treatment of invasive pulmonary aspergillosis in both Europe and the United States [15, 16]. The LAMB3 and LAMB1.5 dosages were used in vivo to assess a range of pharmacological interactions between polyene and triazole.

Antifungal compounds were administered iv once daily, were

initiated 24 h after endotracheal inoculation, and were continued throughout the course of the experiments for up to 12 days. Thirty-six rabbits were randomly assigned to 1 of 6 groups: an untreated control group that received normal saline; a group that received the RAV5 dosage; a group that received the LAMB1.5 dosage; a group that received the LAMB3 dosage; a group that received a combination of the RAV5 dosage and the LAMB1.5 dosage; and a group that received a combination of the RAV5 dosage and the LAMB3 dosage. In the combination therapy groups, RAV was administered first followed by LAMB within 30 min. Surviving rabbits were killed by pentobarbital sodium anesthesia (65 mg/kg; pentobarbital sodium was in the form of 0.5 mL of Beuthanasia-D Special [euthanasia solution]; Schering-Plough Animal Health) on day 13 after inoculation, 24 h after the last dose of study drug was administered.

Outcome Variables

The following panel of outcome variables was used to assess antifungal efficacy: lung weight, residual fungal burden, number of pulmonary infarct lesions, and galactomannan (GM) index (as described elsewhere in detail [7, 12]). Number of pulmonary infarct lesions and lung weight were used as measures of organism-mediated pulmonary injury. For histopathological analysis, pulmonary lesions were excised and fixed in 10% neutral buffered formalin. Paraffin-embedded tissue sections were sectioned and then stained with Grocott-Gomori methenamine silver stain. Tissues were microscopically examined for pulmonary injury and organism burden.

Pharmacodynamic Drug-Interaction Analysis

Drug interactions may be misclassified depending on the analytical tools used [11, 17]. Therefore, in the present study, we analyzed the combination of RAV and LAMB by use of 2 different drug-interaction models, one based on Bliss independence theory and one based on Loewe additivity zero-interaction theory. According to Bliss independence theory, when 2 drugs do not interact, the effect of their combination can be derived from the law of probabilities for joint independent events and is equal to the sum of their individual effects minus their product [18, 19]. According to Loewe additivity zero-interaction theory, when 2 drugs do not interact, they behave as simple dilutions of each other, and the effect of their combination can be derived from the dose response curves of the individual drugs [20–22]. Furthermore, because different interactions may occur pharmacologically at various levels [22], the interaction between RAV and LAMB was assessed on the basis of the various outcome variables for efficacy.

For the purpose of this analysis, the measured variables for efficacy were transformed to a percentage reduction relative to the measured variables for the untreated control group in such a way that the percentage would indicate drug effect (E). Thus,

for the 4 outcome variables, the percentage reduction in these variables was calculated as an indicator of drug effect by the equation $100\% \times (MV_{\text{control}} - MV_{\text{treated}}) / MV_{\text{control}}$, where MV is the measured variable (i.e., lung weight in grams, residual fungal burden in colony-forming units per gram, number of pulmonary infarct lesions, and GM index, for which the MV was measured as the area under curve [AUC]).

Bliss independence-based drug-interaction modeling. In Bliss independence theory, if x mg/L RAV and y mg/L LAMB act independently, the probability of their actions occurring alone or together is described by the equation $E_{\text{EXP}} = E_{\text{RAV}} + E_{\text{LAMB}} - E_{\text{RAV}} \times E_{\text{LAMB}}$, where E_{RAV} is the effect of RAV acting alone at the dosage of 5 mg/kg/day, E_{LAMB} is the effect of LAMB acting alone at the dosage of either 1.5 or 3 mg/kg/day, and E_{EXP} is the expected effect of a noninteractive (independent) theoretical combination of the RAV dosage with either dosage of LAMB. The difference (ΔE) between E_{EXP} and the experimentally observed effect (E_{OBS}) of the combinations RAV5 plus LAMB1.5 and RAV5 plus LAMB3 (determined as described above) was calculated for each outcome variable used to assess antifungal efficacy, and its statistical significance was assessed by Student's t test. When E_{OBS} was statistically significantly higher or lower than E_{EXP} (positive or negative ΔE , respectively), statistically significant synergy or antagonism was concluded. In any other case, Bliss independence was assumed.

For the in vitro experiments, ΔE was calculated for the combination of each concentration of the 2 drugs. The sum and the mean of all statistically significant synergistic ($\Delta E \pm 95\%$

confidence interval [CI] > 0) and antagonistic ($\Delta E \pm 95\%$ CI < 0) combinations are reported as summary measures of the response surface.

Loewe additivity-based drug-interaction modeling. Loewe additivity is described by the equation $1 = c_A/C_A + c_B/C_B$, where c_A and c_B are the concentrations of drugs A and B in a combination that elicits a certain effect and C_A and C_B are the isoeffective concentrations of drugs A and B when acting alone. An isobologram is a plot in which the coordinates are the doses of the 2 drugs in arithmetic scale. An isobole is a curve that starts from a dose of drug A on the X-axis and ends at a dose of drug B on the Y-axis, connecting the doses of all combinations showing the same effect. A concave-up isobole indicates synergy, and a concave-down isobole indicates antagonism. Isobolograms were constructed for each outcome variable, and in vivo drug interaction was assessed by visual inspection of isoboles.

The in vitro interaction between AMB and RAV was assessed by isobolographic analysis of Loewe additivity for 80% growth inhibition (i.e., 20% growth) [23]. By use of a non-weighted, nonlinear regression analysis, the E_{MAX} model was fitted (by use of GraphPad Prism [version 4.0; GraphPad Software]) to the concentration-effect curves of each drug alone and in combination at the fixed AMB:RAV ratios of 1:8, 1:4, 1:2, 1:1, 2:1, 4:1, 8:1, and 16:1, on the basis of weight. The E_{MAX} model is described by the equation $E = E_{\text{MAX}} \times (EC/EC_{50})^m / [1 + (EC/EC_{50})^m]$, where E is the percentage of growth (dependent variable) at the effective concentration (EC) of drug

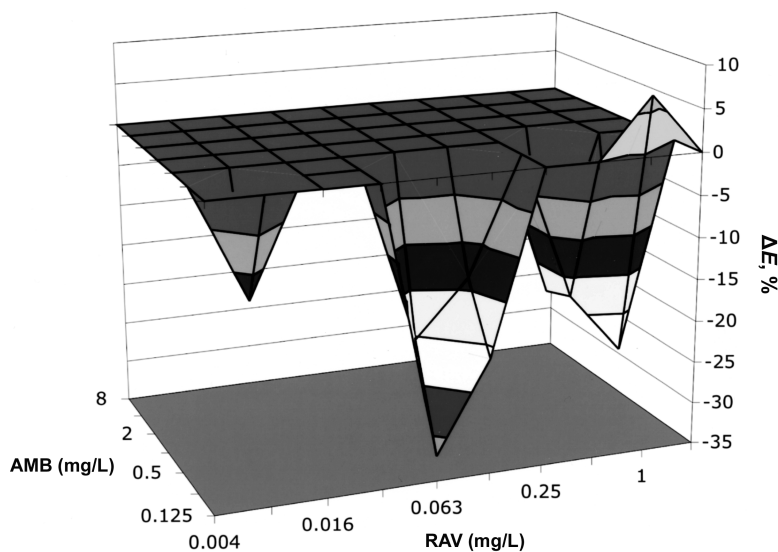


Figure 1. Interaction surface obtained from response-surface analysis of a Bliss independence-based drug-interaction model for the in vitro combination of amphotericin B (AMB) and ravuconazole (RAV) against *Aspergillus fumigatus* after 24 h of incubation. The X- and Y-axes show the concentrations of AMB and RAV, respectively, whereas the Z-axis shows the ΔE (observed effect – expected effect), given as a percentage. The zero plane indicates Bliss independent interactions, whereas values below the zero plane indicate statistically significant antagonistic interactions (negative ΔE). The magnitude of the antagonistic interaction is directly related to a negative ΔE value. The different tones in the 3-dimensional plot represent different percentile bands of antagonism.

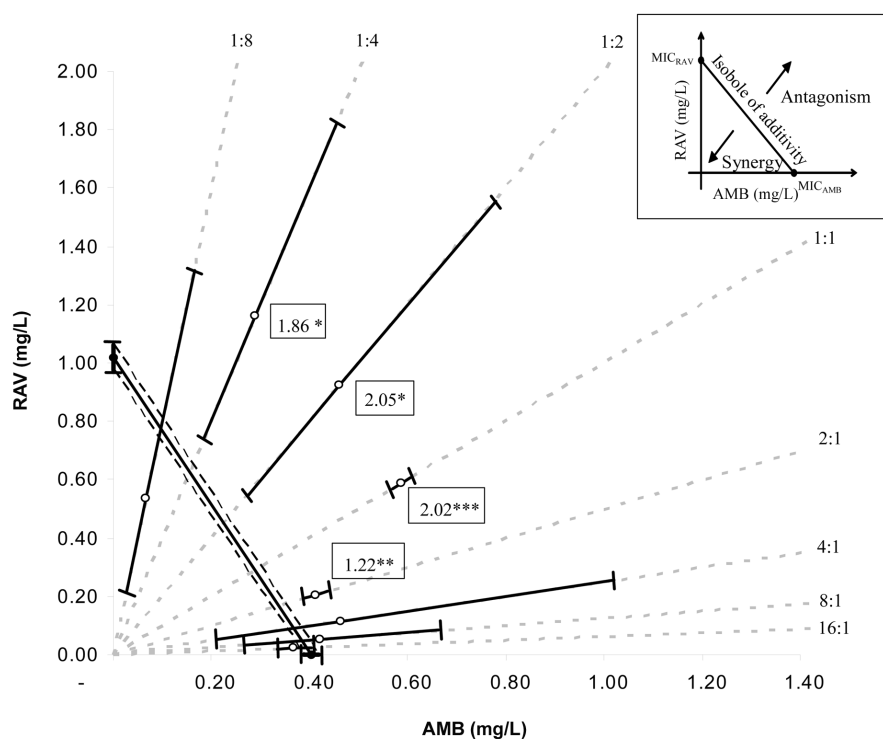


Figure 2. Isobologram showing the interaction between amphotericin B (AMB) and ravuconazole (RAV) against *Aspergillus fumigatus* at a 20% growth level. An additive isobole is a line that connects the equieffective concentration of each drug on each axis (black circles). The 2 concentrations of AMB and RAV along that line are additive in effect. The isobole of additivity is shown as a solid line drawn between the $EC_{AMB, 20}$ value (the AMB concentration that results in 20% growth) on the X-axis and the $EC_{RAV, 20}$ (the RAV concentration that results in 20% growth) on the Y-axis (black circles), and the dark dashed lines represent the theoretically additive 95% confidence interval (CI). The gray dotted rays starting from the origin of the axes represent the different AMB:RAV fixed ratios. The white circles represent the experimentally derived EC_{MIX} values (the total concentration of both drugs in combination that resulted in 20% growth), and the error bars represent their 95% CIs. The experimental EC_{MIX} values (e.g., 1.22, 2.02, 2.05, and 1.86) for the mixture AMB:RAV at the fixed-ratio combinations of 1:4, 1:2, 1:1, and 2:1 were found to be statistically significantly above the theoretical isobole of additivity (* $P < .05$, ** $P < .01$, and *** $P < .001$, compared with the theoretical additive concentrations [solid line]), indicating antagonism. Thus, more drug is required in combination to produce the same effect than the drugs have alone. The ECs and 95% CIs presented in this isobologram were obtained from regression analysis of 1 of 3 replicate experiments.

(independent variable), E_{MAX} is the maximum percentage of growth observed in the drug-free control, EC_{50} is the drug concentration producing 50% of the E_{MAX} , and m is the slope of the concentration-effect curves (Hill coefficient). The maximum and the minimum of the E_{MAX} model were kept constant at 100% and 0%, respectively. The goodness of fit of the model was interpreted using the runs test, residuals, and r^2 values; poor fit (e.g., $r^2 < 0.9$, 95% CI $> 1 \log_2$, statistically significant deviation of residuals from a normal distribution with a mean of 0, and statistically significant deviation on the basis of the runs test) were excluded from the analysis.

On the basis of the isobolographic analysis [23], for each fixed-ratio combination of AMB and RAV, the total concentration of both drugs (EC_{MIX}) was compared with the isoeffective theoretical additive total concentration (EC_{ADD}). For each fixed-ratio combination, the E_{MAX} model provides an estimate of the total concentration ($EC_{MIX} = c_{AMB} + c_{RAV}$, where c_{AMB} and

c_{RAV} are the concentrations of AMB and RAV in the combination) together with its SE for a 20% growth level. The EC_{ADD} for the same growth level is calculated by the equation

$$EC_{ADD} = EC_{AMB} / (P_{AMB} + P_{RAV} \times EC_{AMB} / EC_{RAV}), \quad (1)$$

where EC_{AMB} and EC_{RAV} are the isoeffective concentrations of, respectively, AMB and RAV alone (obtained from the E_{MAX} model of the concentration-effect curves of the drugs alone) and P_{AMB} and P_{RAV} are the proportions of AMB and RAV in the total concentration (c_{AMB} / EC_{MIX} and c_{RAV} / EC_{MIX} , respectively) [23]. The SE of EC_{ADD} is given by the equation

$$SE\{EC_{ADD}\} = [f^2 \times (SE\{EC_{AMB}\})^2 + (1 - f)^2 \times (SE\{EC_{RAV}\})^2]^{1/2}, \quad (2)$$

where $f = P_{AMB} / (P_{RAV} + P_{AMB} \times EC_{AMB} / EC_{RAV})$ and $SE\{EC_{AMB}\}$

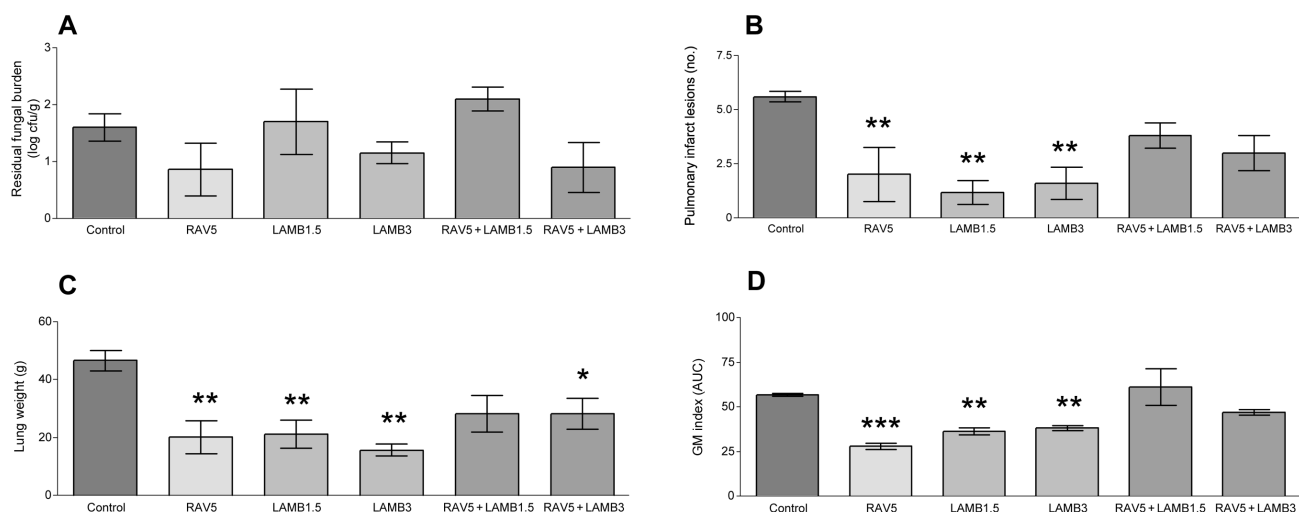


Figure 3. Effect that ravuconazole at 5 mg/kg/day (RAV5) and liposomal amphotericin B at 1.5 and 3 mg/kg/day (LAMB1.5 and LAMB3, respectively) have, alone and in combination, on residual fungal burden in lungs (A), no. of pulmonary infarct lesions (B), lung weight (C), and galactomannan (GM) index (D). Error bars represent SEs of means. * $P < .05$, ** $P < .01$, and *** $P < .001$, compared with values for the untreated control rabbits (Dunnett's posttest).

and $SE\{EC_{RAV}\}$ can be obtained from the E_{MAX} model of the concentration-effect curve of the drugs alone [23].

Because the drug concentrations were logarithmically distributed, the statistical significance of the differences between EC_{MIX} and EC_{ADD} was calculated on the basis of their logarithms by Student's t test. The $\log EC_{MIX}$ and the $SE\{\log EC_{MIX}\}$ were obtained directly from the E_{MAX} model, because the regression analysis was performed using the logarithms of the drug concentrations. The $\log EC_{ADD}$ is the logarithm of equation (1), and its SE is given by the equation $SE\{\log EC_{ADD}\} = SE\{EC_{ADD}\} / [\ln(10) \times EC_{ADD}]$, where the $SE\{EC_{ADD}\}$ is calculated using equation (2). An interaction index (I) was then calculated for each

fixe ratio at a 20% growth level as the ratio EC_{MIX}/EC_{ADD} for each replicate [24]. If the I values for all 3 replicates were statistically significant <1 or >1 , then synergy or antagonism, respectively, was concluded.

Pharmacokinetic Analysis

The plasma pharmacokinetics of LAMB and RAV were investigated in 4–6 infected rabbits per dosage group by use of optimal plasma sampling on day 6 of antifungal therapy. Blood samples were collected in heparinized syringes before iv infusion and then at 0.08, 0.25, 1, 2, 4, 8, and 24 h after drug

Table 1. Efficacy of monotherapy and combination therapy, by Bliss independence-based drug-interaction modeling.

Outcome variable	Efficacy of monotherapy			Efficacy of combination therapy						
	E_{RAV5}	$E_{LAMB1.5}$	E_{LAMB3}	RAV5 + LAMB1.5			RAV5 + LAMB3			
				$E_{OBS1.5}$	$E_{EXP1.5}^a$	ΔE , % (interaction ^b)	$E_{OBS1.5}$	E_{EXP3}^a	ΔE , % (interaction ^b)	ΔE , % (interaction ^b)
Residual fungal burden	78 ± 11	56 ± 15	70 ± 5	55 ± 10	90 ± 3	−35 ^c (A)	77 ± 11	94 ± 1	−17 (I)	
Pulmonary infarct lesions	82 ± 14	79 ± 10	72 ± 12	35 ± 9	97 ± 2	−62 ^d (A)	47 ± 14	97 ± 2	−50 ^e (A)	
Lung weight	63 ± 11	55 ± 10	66 ± 4	41 ± 11	87 ± 3	−46 ^e (A)	39 ± 12	92 ± 2	−53 ^e (A)	
Galactomannan index	51 ± 2	36 ± 2	33 ± 2	0	69 ± 3	−69 ^c (A)	17 ± 2	67 ± 3	−50 ^d (A)	

NOTE. Data are mean ± SE percentage reduction relative to measured outcome variables in untreated control rabbits, unless otherwise indicated. See Materials and Methods for a complete discussion of Bliss independence-based drug-interaction modeling; in brief, E indicates drug effect, E_{OBS} indicates the observed drug effect, E_{EXP} indicates the expected effect of a noninteractive (independent) theoretical combination of the RAV dosage with either dosage of LAMB, and ΔE indicates the difference between E_{EXP} and E_{OBS} . LAMB1.5, liposomal amphotericin B at 1.5 mg/kg/day; LAMB3, liposomal amphotericin B at 3 mg/kg/day; RAV5, ravuconazole at 5 mg/kg/day.

^a The mean ± SE $E_{EXP1.5}$ and E_{EXP3} independent effects were derived by using all possible combinations of the monotherapy data from each of the 6 RAV5-, LAMB1.5-, or LAMB3-treated rabbits, respectively.

^b A, Bliss antagonism; I, Bliss independence.

^c $P < .001$.

^d $P < .01$.

^e $P < .05$.

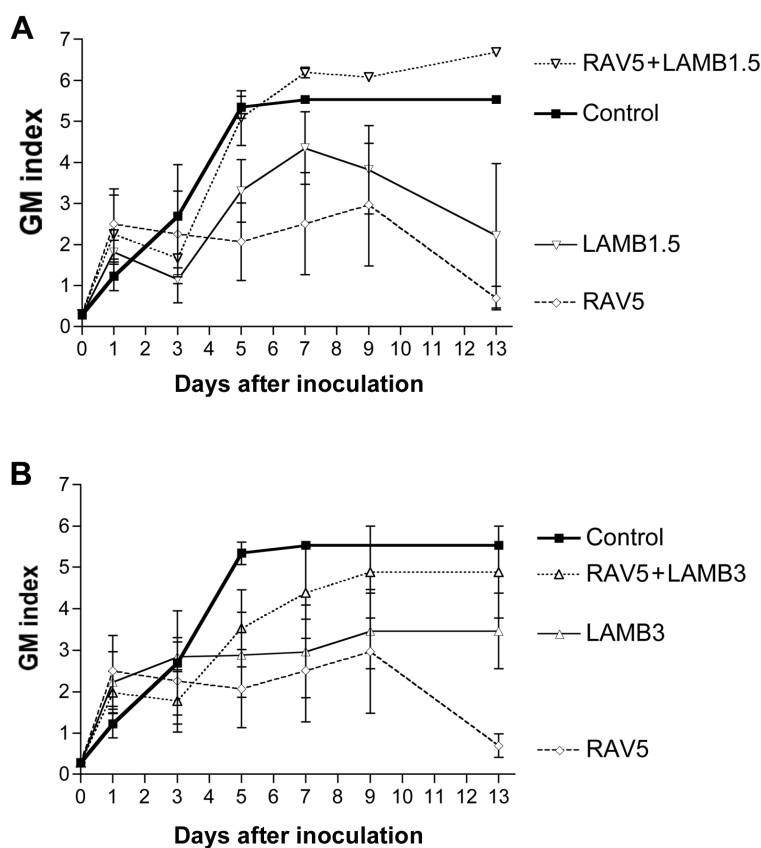


Figure 4. Kinetics curves for the galactomannan (GM) index. Groups of 5–6 rabbits received either normal saline (control), monotherapy of ravuconazole at 5 mg/kg/day (RAV5) or of liposomal amphotericin B at 1.5 or 3 mg/kg/day (LAMB1.5 and LAMB3, respectively), or combination therapy of RAV5 plus LAMB1.5 (A) or RAV5 plus LAMB3 (B). Error bars represent SEs of means.

administration, were immediately separated by centrifugation, and were stored at -70°C until being assayed.

Concentrations of LAMB in plasma were determined after liquid/liquid protein precipitation with methanol (1:2 [vol: vol]) by reverse-phase high-performance liquid chromatography (HPLC), by means of a Waters 2996 Photodiode Array Detector at wavelength of 405 nm, as reported elsewhere [25]. Concentrations of RAV in plasma were also determined after liquid/liquid protein precipitation with methanol (1:2 [vol: vol]) by reversed-phase HPLC, as reported elsewhere [7].

The pharmacokinetic parameters of minimum and maximum concentrations (C_{MIN} and C_{MAX}), AUC between 0 and 24 h (AUC_{0-24}) and between 0 h and infinity, volume of distribution, clearance, and half-life for LAMB and RAV were determined for each rabbit by a model-independent analysis conducted by means of WinNonlin (version 4.0.1; Pharsight). Plasma-concentration time profile of rabbits from all dosage groups were fitted into a noncompartmental pharmacokinetic model with iv bolus input.

Statistical Analysis

Differences in the number of pulmonary infarct lesions, residual fungal burden, and lung weight between treated and untreated

rabbits were analyzed by analysis of variance (ANOVA), followed by Dunnett's multiple-comparison test. Differences among the treated rabbits were analyzed by ANOVA, followed by Tukey's multiple-comparison test. Pharmacokinetic parameters estimated by noncompartmental analysis for each rabbit were compared between the monotherapy and combination therapy groups by the Kruskal-Wallis test. $P < .05$ was considered to be statistically significant. All statistical analyses were performed using GraphPad Prism.

RESULTS

In Vitro Combination Experiments

The MICs of RAV and AMB (visually clear well) were 1–2 mg/L and 0.5 mg/L, respectively. Bliss independence-based response-surface analysis showed 8 of 70 statistically significant antagonistic combinations, with a sum ΔE of -162% and a mean \pm SE ΔE of $-20\% \pm 2\%$ after 24 h (figure 1). Most of the antagonistic interactions were observed at 0.125–0.5 mg/L AMB and 0.063–1 mg/L RAV. As shown in figure 2, the isobolographic analysis revealed significant antagonism ($P < .05$) between AMB and RAV across a wide range of fixed ratios. The I values of statistically significant antagonistic interactions

ranged from 1.22 to 2.5 for all 3 replicates. These interactions were found at combinations including 0.41 mg/L (95% CI, 0.26–0.59 mg/L) AMB and 0.50 mg/L (95% CI, 0.08–1.24 mg/L) RAV.

In Vivo Combination Experiments

The levels for the outcome variables used to assess antifungal efficacy are shown in figure 3 for the control, monotherapy, and combination therapy groups. Drug effects (reduction in outcome variables, compared with those in the untreated control rabbits) are summarized in table 1. The residual fungal burden in the combination therapy groups (RAV5 plus LAMB1.5 and RAV5 plus LAMB3) was higher than that in the monotherapy groups (RAV5, LAMB1.5, and LAMB3). The differences in the number of pulmonary infarct lesions and in the GM index in the monotherapy groups, but not in the combination therapy groups, were significantly lower ($P < .05$, with Dunnett's

and Tukey's posttest), compared with those in the untreated control group. Figure 4 shows the GM index kinetics. The GM index AUC was significantly lower ($P < .05$) in the monotherapy groups, but not in the combination groups, than in the untreated control group (figure 3). Histopathological analysis showed more-extensive fungal growth in the rabbits receiving combination therapy, compared with that in the rabbits receiving monotherapy (figure 5).

Pharmacodynamic Drug-Interaction Analysis

The results of Bliss independence–based drug-interaction analysis for the in vivo pharmacodynamic interaction between RAV and LAMB are summarized in table 1. Statistically significant Bliss antagonism was found for the combination RAV5 plus LAMB1.5, on the basis of all outcome variables for which the observed drug effects were 35%–62% lower than the expected effects under the Bliss independence zero-interaction hypoth-

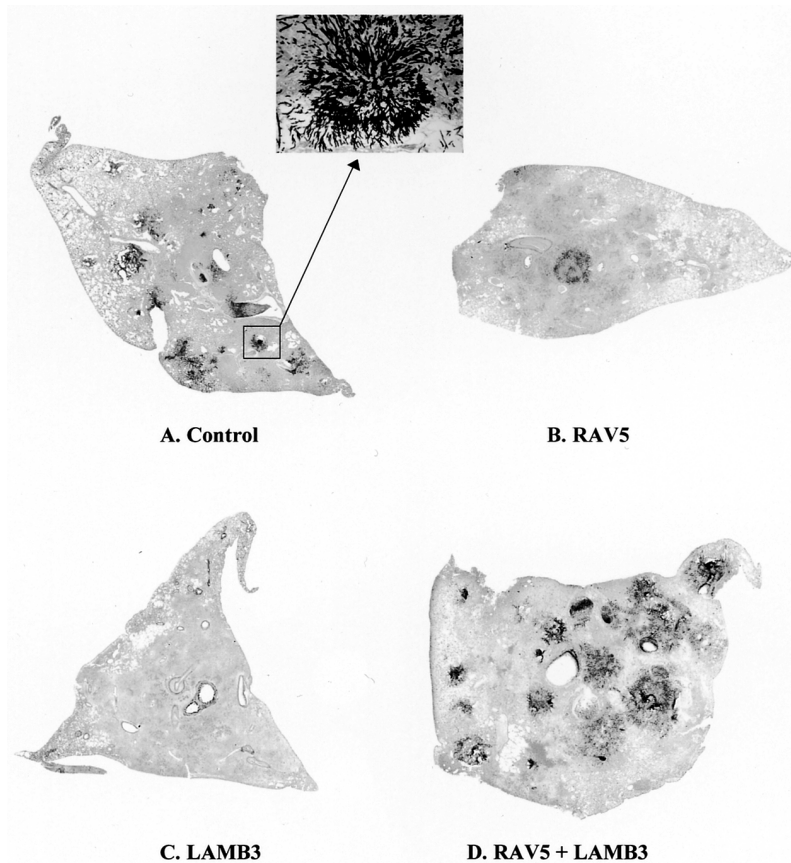


Figure 5. Photomicrographs of histopathological slides, stained with Grocott-Gomori methenamine silver (GMS) stain, of representative lung-tissue sections from untreated control rabbits (A), rabbits treated with ravuconazole at 5 mg/kg/day (RAV5) (B), rabbits treated with liposomal amphotericin B at 3 mg/kg/day (LAMB3) (C), and rabbits treated with the combination RAV5 plus LAMB3 (D). The black lesions represent *Aspergillus* hyphae stained with reduced silver, by GMS stain (see magnified insert [$\times 200$]). Note that the residual fungal burden in the lungs of the rabbits receiving combination therapy is comparable with that in the lungs of the untreated control rabbits and is more extensive than that in the lungs of the rabbits receiving monotherapy. The tissue sections depicted here are typical of the sections from the same groups when lesions were present. These findings are compatible with those shown in figures 3 and 4, which demonstrate residual fungal burden and galactomannan antigen level in the lungs of the rabbits receiving combination therapy that were comparable with those in the lungs of the untreated control rabbits.

esis. For the combination RAV5 plus LAMB3, statistically significant Bliss antagonism also was found for the number of pulmonary infarct lesions (ΔE , -50%), lung weight (ΔE , -53%), and GM index (ΔE , -50%). The interaction between RAV and LAMB also was Loewe antagonistic, as is depicted by the concave-down isoboles in figure 6.

Pharmacokinetic Drug-Interaction Analysis

The plasma concentration profile of LAMB and RAV are depicted in figure 7. There was no significant noncompartmental pharmacokinetic interaction between LAMB and RAV to account for the in vivo antagonism observed in these experiments. The mean \pm SE AUC_{0-24} values were 28 ± 4 for RAV5, 205 ± 29 for LAMB1.5, and 455 ± 54 for LAMB3. No significant differences in most pharmacokinetic parameters were found between the monotherapy groups and the combination therapy

groups. However, the C_{MAX} of RAV5 plus AMB1.5 was found to be significantly lower in the combination therapy group than in the corresponding monotherapy groups. Plasma levels of both drugs were higher than their MICs for most of the dosing interval.

DISCUSSION

The combination of RAV and LAMB demonstrated antagonism in the treatment of experimental invasive pulmonary aspergillosis. This antagonism was not due to pharmacokinetic interaction and was compatible with the results of the in vitro combination experiments, which showed statistically significant Bliss and Loewe antagonism between RAV and AMB against *A. fumigatus*.

Azole-AMB interactions may be understood conceptually as

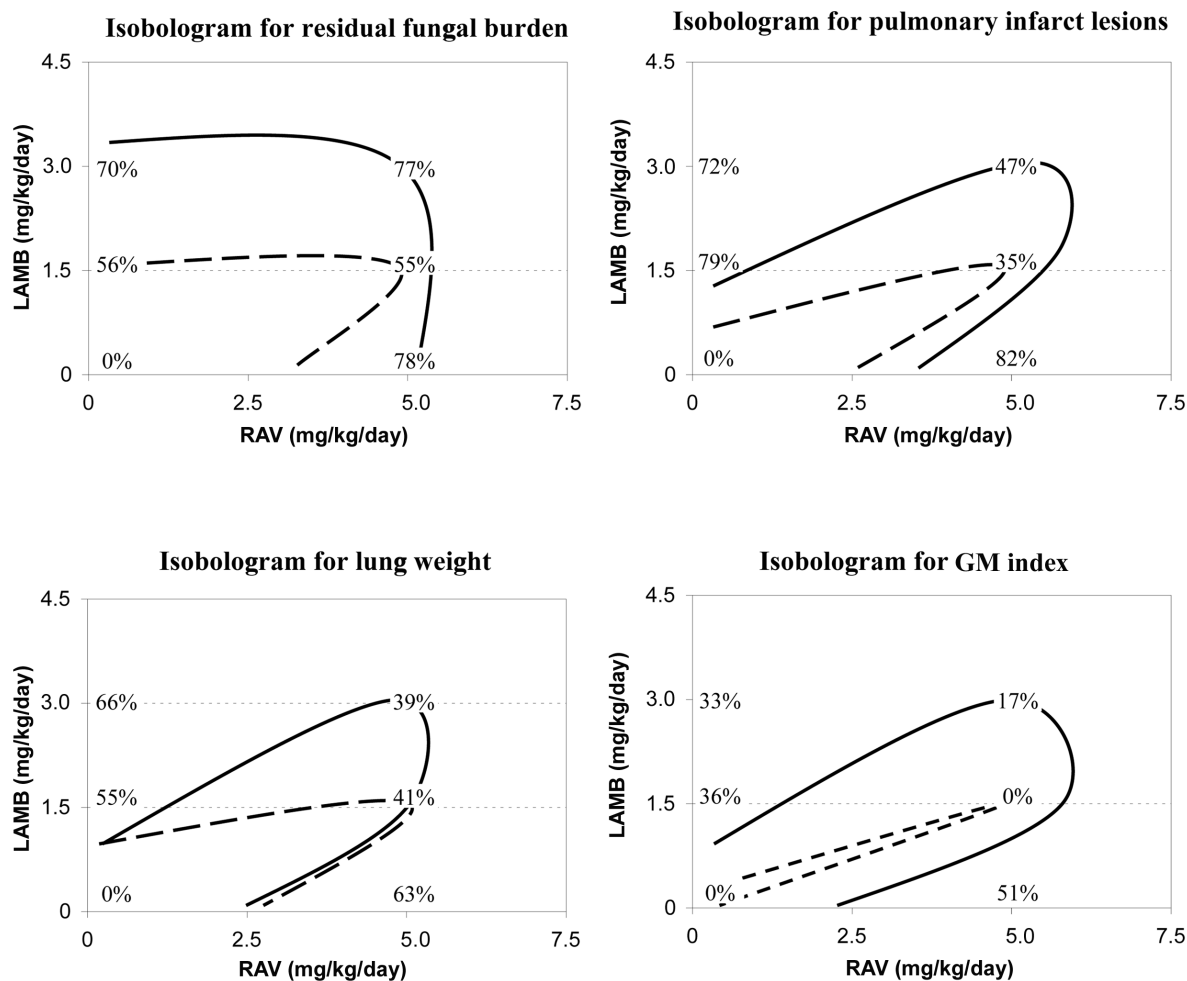


Figure 6. Isobolograms for various outcomes of efficacy of the combination of ravuconazole at 5 mg/kg/day (RAV5) and liposomal amphotericin B at 1.5 or 3 mg/kg/day (LAMB1.5 and LAMB3, respectively) for treatment of experimental invasive pulmonary aspergillosis. Values in the graph indicate the percentage of drug effects (percentage reduction relative to drug effects in untreated control rabbits) at the corresponding doses. The bold and dashed lines represent approximate (the exact isoeffective doses of the single drugs are unknown) isoboles for the combination of RAV5 and either LAMB1.5 or LAMB3, respectively, with concave-down isoboles indicating antagonism.

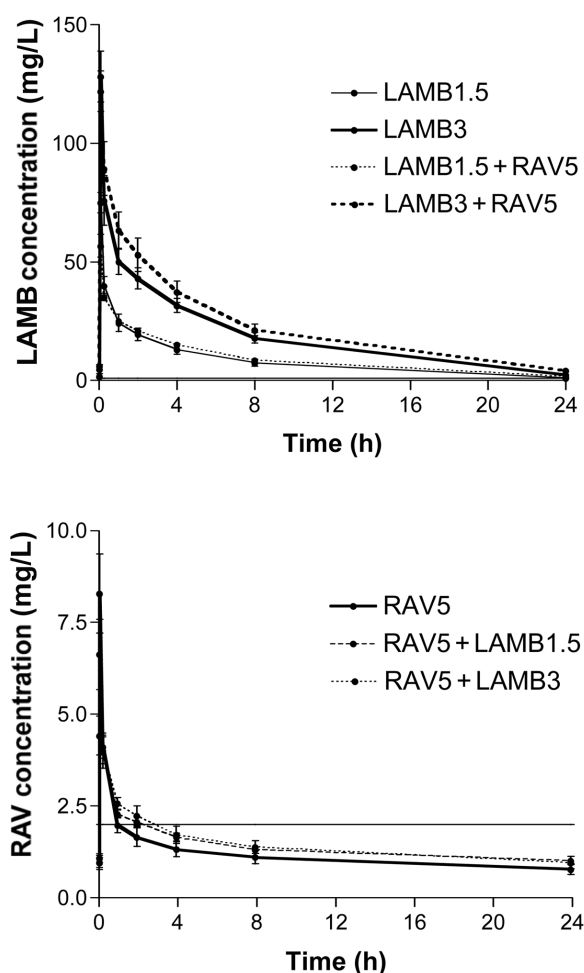


Figure 7. Plasma levels of liposomal amphotericin B (LAMB) and ravuconazole (RAV) administered alone and in combination in rabbits with invasive pulmonary aspergillosis. Error bars represent SEs of means among 4–6 rabbits. Horizontal lines parallel to the X-axis represent the mode MIC of LAMB (0.5 mg/L) and RAV (2 mg/L).

either the azole antagonizing the effect of AMB or AMB antagonizing the effect of the azole. Among the proposed mechanisms of azoles antagonizing AMB is the reduction of AMB binding to depleted fungal membrane ergosterol, resulting from inhibition of the ergosterol biosynthetic pathway by the azole [4, 26, 27]. An alternative mechanism for azole-AMB antagonism is the accumulation of azole in the cell membrane, which competitively inhibits the binding of AMB to ergosterol [28, 29]. For AMB antagonism of azole activity, other hypotheses include the interference by AMB with a cell membrane-associated permease that is likely involved in azole entry into the cell [30] and reduced azole influx due to AMB membrane damage [31].

Although most in vitro combination studies have shown antagonism or indifference against *A. fumigatus* when an azole is concomitantly combined with AMB [11, 27, 32, 33], in vitro

synergistic interactions have also been observed for some isolates [34, 35]. Moreover, AMB–itraconazole interaction was recently found to be concentration dependent in vitro, with synergistic interactions at low concentrations of AMB and antagonistic interactions at AMB concentrations near or greater than the MIC [11, 36]. In the present study, in vitro antagonism was observed at AMB concentrations near or greater than the MIC, and, for most of the dosing interval, rabbit plasma levels of AMB were also higher than the MIC of AMB for the *A. fumigatus* strain. Thus, as the isobologram and response-surface analysis demonstrated, the antagonistic interactions occurred over a wide range of concentrations near or greater than the MIC of AMB. Interaction between antifungal agents is dependent on the class of compound and the organism. Interactions observed for *A. fumigatus* are not necessarily applicable to other genera or species. Although members of the class of triazole differ with respect to their structures, the overall pattern of indifferent to antagonistic interaction appears to be a common property against *A. fumigatus*.

Previous in vivo combination studies of AMB and an azole for the treatment of aspergillosis have shown antagonism or indifference [3, 4]. Pretreatment of experimental aspergillosis with an azole may also lead to AMB resistance [27, 37, 38]. Concomitant therapy of AMB (at a dosage of >0.5 mg/kg/day) with other azoles in experimental models of aspergillosis have resulted in worse survival and/or similar fungal burdens, compared with those observed for monotherapy [39–42].

Interactions may be organ dependent. Because the lungs are the primary organ of aspergillosis, pharmacodynamic interactions in the lungs are important for controlling this infection. However, Clemons et al. have demonstrated that voriconazole (40 mg/kg/day per os) and LAMB (1 mg/kg/day iv) exerted activity in experimental murine aspergillosis of the central nervous system (CNS) that was superior to the activity of either drug alone [43]. This interaction in the CNS may be explained by the isobologram shown in figure 2, which indicates that subinhibitory concentrations of AMB may be synergistic with RAV. Such concentrations may be present in the CNS.

No prospective controlled clinical trials of the combination of AMB and triazole for the treatment of invasive aspergillosis have been conducted. A large retrospective analysis of 179 patients with invasive aspergillosis who received primary antifungal therapy of either LAMB alone or LAMB in combination with itraconazole showed an equally poor response in both study arms (10% vs. 0%, respectively) [44]. In a smaller case series of 21 patients with invasive aspergillosis, 9 (82%) of 11 patients receiving AMB and itraconazole were cured or improved, compared with 5 (50%) of 10 patients receiving AMB alone [45]. However, this difference was not significant.

In conclusion, the in vitro and in vivo findings of the present study collectively indicate that a polyene-triazole combination

may be antagonistic in the treatment of invasive pulmonary aspergillosis.

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