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Inhibition of RNA Synthesis as a Therapeutic Strategy against *Aspergillus* and *Fusarium*: Demonstration of In Vitro Synergy between Rifabutin and Amphotericin B

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We investigated the in vitro antifungal activity of amphotericin B, alone and in combination with rifabutin, an inhibitor of bacterial RNA polymerase, against 26 clinical isolates of *Aspergillus* and 25 clinical isolates of *Fusarium*. Synergy or additivism between these drugs was demonstrated against all isolates tested. Amphotericin B MICs were reduced upon combination with rifabutin from a mean of 0.65 μg/ml to a mean of 0.16 μg/ml against *Aspergillus*, and from a mean of 0.97 μg/ml to a mean of 0.39 μg/ml against *Fusarium* ($P < 0.000001$ for both). Similarly, the MICs of rifabutin were reduced upon combination with amphotericin B from a mean of >32 μg/ml to a mean of 1.1 μg/ml against both fungi ($P < 0.000001$ for both). These positive interactions were corroborated by a colony count study with two *Fusarium* isolates, for which treatment with the combination of subinhibitory concentrations of amphotericin B (at concentrations 2- and 4-fold less than the MIC) and rifabutin (at concentrations ranging from 4- to 64-fold less than the MIC) resulted in 3.2-log reductions in colony counts compared to those after treatment with either drug alone. Inhibition of RNA synthesis was shown to be the mechanism of antifungal activity. These results suggest that inhibition of fungal RNA synthesis might be a potential target for antifungal therapy.

Invasive infections caused by fungi are major causes of morbidity and mortality in the immunocompromised host. *Aspergillus* and *Fusarium* are two important emerging pathogenic fungi. Mortality rates for patients with disseminated aspergillosis and fusariosis approach 80%, despite therapy with antifungal agents. If advances in the treatment of *Aspergillus* and *Fusarium* infections are to be made, new therapeutic approaches will be necessary.

One possible approach is to combine amphotericin B, the current agent of choice against both organisms, with other antimicrobial agents. We have recently demonstrated in vitro fungicidal synergy between amphotericin B and the protein synthesis inhibitor azithromycin against *Aspergillus* and *Fusarium*. This fungicidal synergy is mediated through inhibition of fungal protein synthesis (3, 22). The inhibition of fungal RNA synthesis might also exert antifungal effects through a reduction in subsequent protein synthesis. Indeed, the agent flucytosine exerts its antifungal effects at least partly through inhibition of RNA synthesis, suggesting that this target might be exploited for therapy (4).

The combination of amphotericin B and rifampin, an antibacterial agent that inhibits DNA-dependent RNA polymerase, has been shown to interact synergistically in vitro against a variety of fungi (5, 12, 15, 17–19, 21). Results of studies of the interactions between these agents with animal models of fungal infections have been conflicting, however (1, 7, 14, 16, 21). The concentrations of rifampin required to achieve synergy in vitro and in vivo frequently exceeded those safely achievable in humans.

Rifabutin is an antibacterial agent closely related to rifampin; however, it has a broader spectrum of activity than rifampin and is accumulated at higher concentrations within tissues (2, 19). We studied the in vitro interaction between amphotericin B and rifabutin against 26 clinical isolates of *Aspergillus* and 25 clinical isolates of *Fusarium*. We further investigated the mechanism of antifungal activity of this combination.

MATERIALS AND METHODS

Isolates. Twenty-six clinical isolates of *Aspergillus* (*A. fumigatus*, $n = 16$; *A. flavus*, $n = 10$) and 25 clinical isolates of *Fusarium* (*F. solani*, $n = 11$; *F. moniliforme*, $n = 5$; *F. semitectum*, $n = 5$; *F. proliferatum*, $n = 4$) were obtained from the Clinical Microbiology Laboratory at the Shands Teaching Hospital at the University of Florida, Gainesville, or the Fungus Testing Laboratory, University of Texas Health Sciences Center, San Antonio. *Paecilomyces variotii* ATCC 22319 was incorporated into each set of experiments as a quality control isolate.

Antimicrobial agents. A stock solution of amphotericin B (Bristol-Myers Squibb, Princeton, N.J.) at a concentration of 1,600 μg/ml was prepared in sterile water and was stored at −70°C in 0.5-ml aliquots. Amphotericin B stock solutions were used within 2 months of initial preparation. A stock solution of rifabutin (Pharmacia-Upjohn, Kalamazoo, Mich.) at a concentration of 1,600 μg/ml in methanol was prepared fresh from powder for each experiment.

Susceptibility testing. A two-dimensional macrodilution checkerboard technique was used for susceptibility testing. Details of the procedure have been described elsewhere (23). Briefly, testing was performed in RPMI 1640 medium (American Biorganics, Inc., Niagara Falls, N.Y.) with L-glutamine but without bicarbonate, and the RPMI 1640 medium was buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS). Aliquots of 50 μl of each drug at concentrations 20 times the final concentrations were dispensed into polystyrene tubes (12 by 75 mm). In the case of single-drug controls, 50 μl of sterile water was dispensed along with 50 μl of drug. Final test concentrations ranged from 0.03 to 16 μg/ml for amphotericin B and 0.06 to 32 μg/ml for rifabutin. In addition, tubes containing methanol at concentrations similar to those present in the rifabutin control tubes were prepared to ascertain that methanol at these concentrations did not affect fungal growth. In total, 132 tubes were used to test each isolate.

Isolates were subcultured onto potato dextrose agar at 35°C for 7 to 10 days. Plates containing sporulated *Aspergillus* or *Fusarium* were overlaid with 5 ml of sterile water, the surface was scraped with a sterile inoculating loop, and the resulting suspension was transferred to a glass tube with a sterile transfer pipette. The suspension was vortexed and diluted 1:100 with sterile water, and the number of conidia was counted with a hemacytometer. The suspension was adjusted to a concentration of 1×10^5 to 5×10^5 CFU/ml and was diluted 1:10

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with RPMI 1640 medium to attain a final inoculum concentration of 1×10^4 to 5×10^4 CFU/ml.

Nine hundred microliters of inoculum was added to tubes containing drugs, as well as to a drug-free control tube and tubes containing diluted methanol. Tubes were incubated at 35°C, and the results were read at 24 and 48 h. The MIC was defined as the lowest concentration of each drug associated with no growth.

The minimum lethal concentration (MLC) was determined by plating 100 μl from each tube with no growth onto Sabouraud agar plates, which were incubated at 35°C. The MLC was defined as the lowest concentration of each drug yielding fewer than five colonies after 48 h of incubation.

Definitions. The interaction between two drugs was defined by the fractional inhibitory concentration (FIC) index (FICI) (6). FICI is the sum of the FIC of each of the drugs. FICI is expressed mathematically as follows:

$$\mathrm{FICI} = \mathrm{FIC_A} + \mathrm{FIC_B} = \frac{\text{MIC of drug A, in combination}}{\text{MIC of drug A, tested alone}} + \frac{\text{MIC of drug B, in combination}}{\text{MIC of drug B, tested alone}}$$

Synergy was defined as an FICI of ≤0.5. Additivity was defined as an FICI of >0.5 but ≤1. Antagonism was defined as an FICI of >1 (6).

Colony counts. Colony counts were determined with two isolates of *Fusarium*. After 48 h MICs were determined, serial 10-fold dilutions were made for tubes containing the drug-free control, amphotericin B at a concentration of 0.25 or 0.5 μg/ml (a concentration 4- or 2-fold lower than the MIC, respectively), rifabutin at concentrations of 0.5 to 16 μg/ml, and amphotericin B at a concentration 0.25 or 0.5 μg/ml combined with rifabutin at all concentrations. Inocula of 100 μl from the original tubes and all tubes containing 10-fold-diluted suspensions were streaked onto Sabouraud dextrose agar plates. The plates were incubated at 35°C for 48 h, and the colonies were counted. The numbers of CFU per milliliter were calculated and were expressed as the average of counts at all dilutions at which growth was evident.

RNA synthesis. RNA synthesis was assessed indirectly by measuring the level of incorporation of [^{3}H]uridine into RNA. In order to maximize incorporation, the tested isolates were grown in a minimally nutritive medium (yeast nitrogen base (YNB) without amino acids [Difco, Detroit, Mich.] supplemented with 1% glucose). An isolate of *A. fumigatus* (isolate 96-1011) was suspended in 5 ml of YNB at a concentration of 10^6 spores/ml. The inoculum was incubated overnight at 35°C in an incubator with shaking at 250 rpm.

Inhibitors were added to each tube containing inoculum following the overnight incubation period. The inhibitors tested were amphotericin B alone at 0.25 μg/ml (4-fold less than the MIC), rifabutin alone at 8 μg/ml, and amphotericin B (0.25 μg/ml) combined with rifabutin at concentrations of 1, 2, 4, and 8 μg/ml. Sterile water was added to the control tubes. All tubes were incubated for 15 min at 35°C in a shaking incubator prior to labelling with [^{3}H]uridine.

One microcurie of [^{3}H]uridine was added to each tube, and the tubes were incubated at 35°C in an incubator with shaking at 250 rpm for 4 h. The 4-h incubation period was determined by preliminary experiments that established that the maximum level of incorporation of [^{3}H]uridine was attained within 4 h. Following incubation, 5 ml of cold 10% trichloroacetic acid (TCA) was added to each tube, and the tubes were then cooled on ice for 30 min. The products were filtered through 2- to 4-cm Whatman glass microfiber filters (Fisher Scientific) and washed with 5 ml of cold TCA and then acetone. The filter papers were dried under a heat lamp and placed in scintillation vials, and the amount of radioactivity was measured with a liquid scintillation counter. The amount of [^{3}H]uridine incorporated was defined as the difference between the amount of radioactivity incorporated at 4 h and the amount of radioactivity incorporated at the start of the experiment. The level of incorporation for the tubes containing inhibitors was compared to that for the control tube.

Protein synthesis. Protein synthesis was measured indirectly by measuring the level of incorporation of [^{35}S]methionine. This experiment was performed simultaneously with the experiment assessing RNA synthesis described above. The inoculum of *A. fumigatus* was prepared and incubated as described above. Inhibitors were added following overnight incubation, and incubation continued for 15 min. Two microcuries of [^{35}S]methionine was added to each tube, and 50-μl aliquots were taken from each tube to establish a baseline reading of radioactivity. Aliquots were pelleted by centrifugation at 13,000 rpm for 2 min in an Eppendorf centrifuge. Five microliters of supernatant was spotted onto filter paper, and the radioactivity was counted in a scintillation counter. Similar procedures were followed every hour to establish the level of uptake of [^{35}S]methionine. The amount of [^{35}S]methionine taken up was the difference between the amount of radioactivity present in the supernatant at time zero and the amount present at the time of subsequent sampling.

After 4 h of incubation, inocula from each tube were centrifuged at 2,500 rpm for 15 min in a Sorvall GLC-2B centrifuge. Protein was precipitated by the addition of cold 10% TCA. An equal volume of cold, acid-washed glass beads (diameter, 0.4 mm) was added to the mixture, which was then vortexed for 1.5 min. Precipitates were collected by centrifugation at 13,000 rpm for 2 min in an Eppendorf centrifuge, washed with acetone, and dried. The samples were then reconstituted in 50 μl of solution containing sodium dodecyl sulfate (SDS) gel sample buffer and mercaptoethanol. Redissolved samples were separated electrophoretically on 10% polyacrylamide gels.

Statistical analysis. MIC data were logarithmically transformed prior to statistical analysis to approximate a normal distribution. Continuous variables were compared by Wilcoxon's test. *P* values of <0.05 were considered significant.

TABLE 1. Distribution of amphotericin B MICs for *Aspergillus* and *Fusarium*[a]

AmB[b] MIC (μg/ml)	No. of isolates			
	Aspergillus		*Fusarium*	
	AmB alone	AmB as part of combination	AmB alone	AmB as part of combination
0.06	0	1	0	0
0.125	0	16	0	0
0.25	1	7	0	12
0.5	15	2	5	10
1.0	9	0	16	3
2.0	1	0	4	0

[a] For all isolates of *Aspergillus* and *Fusarium*, the amphotericin B MICs were ≤2 μg/ml.
[b] AmB, amphotericin B.

RESULTS

Antifungal susceptibility testing. All isolates of *Aspergillus* and *Fusarium* grew well after 48 h of incubation at 35°C. The turbidity of control tubes containing diluted methanol did not differ from that of control tubes containing water; therefore, methanol at the dilutions used exerted no influence upon fungal viability.

(i) *Aspergillus*. The MICs of amphotericin B for *Aspergillus* ranged from 0.25 to 2 μg/ml (Table 1). For 38% (10 of 26) of the isolates, MICs were ≥1 μg/ml (1 μg/ml for 9 isolates; 2 μg/ml for 1 isolate). The MIC at which 50% of isolates are inhibited (MIC_{50}) was 0.5 μg/ml, the MIC_{90} was 2 μg/ml, and the geometric mean MIC was 0.65 μg/ml. For all isolates, rifabutin MICs exceeded 32 μg/ml (Table 2). The MLCs of both drugs were identical to the MICs for all isolates.

The interaction between amphotericin B and rifabutin was synergistic for 77% (20 of 26) of the isolates and additive for 23% (6 of 26) of the isolates; antagonism was not observed.

Amphotericin B MICs were reduced two- to eightfold upon combination with rifabutin (mean, fourfold), to a range of 0.06

TABLE 2. Distribution of rifabutin MICs for *Aspergillus* and *Fusarium*

Rifabutin MIC (μg/ml)	No. of isolates			
	Aspergillus		*Fusarium*	
	Rifabutin alone[a]	Rifabutin as part of combination with AmB[b]	Rifabutin alone[a]	Rifabutin as part of combination with AmB
0.06	0	0	0	1
0.125	0	0	0	0
0.25	0	3	0	0
0.5	0	6	0	4
1.0	0	6	0	7
2.0	0	6	0	8
4.0	0	4	0	1
8.0	0	1	0	2
16.0	0	0	0	1
32.0	0	0	0	1
>32.0	26	0	25	0

[a] For all isolates of *Aspergillus* and *Fusarium*, the rifabutin MICs were >32 μg/ml when they were tested with rifabutin alone.
[b] AmB, amphotericin B.

to 0.5 μg/ml (Table 1). The MIC_{50} was reduced to 0.125 μg/ml, the MIC_{90} was reduced to 0.5 μg/ml, and the geometric mean MIC was reduced to 0.16 μg/ml ($P < 0.000001$). Among the nine isolates for which amphotericin B MICs were 1 μg/ml, MICs were reduced to 0.125 μg/ml for three isolates, 0.25 μg/ml for four isolates, and 0.5 μg/ml for two isolates. For the isolate for which the MIC was 2 μg/ml, the MIC was reduced to 0.25 μg/ml. The MLCs of the drug combination for all isolates were identical to the respective MICs.

Similarly, the addition of amphotericin B to rifabutin resulted in reductions in the rifabutin MICs for all isolates (Table 2). Rifabutin MICs were reduced 8- to 256-fold (mean reduction, 58-fold), to a range of 0.25 to 8 μg/ml; upon combination the MIC_{50} was 1 μg/ml, the MIC_{90} was 4 μg/ml, and the geometric mean MIC was 1.1 μg/ml ($P < 0.000001$). As seen with amphotericin B, rifabutin MLCs were identical to the respective MICs upon combination with amphotericin B.

No differences in the susceptibility profiles of isolates of *A. fumigatus* and *A. flavus* were evident when they were tested with either individual drugs or the combination of drugs.

(ii) *Fusarium*. The range of MICs of amphotericin B for *Fusarium* was 0.5 to 2 μg/ml (Table 1). For 80% (20 of 25) of the isolates MICs were ≥1 μg/ml (1 μg/ml for 16 isolates; 2 μg/ml for 4 isolates). The MIC_{50} was 1 μg/ml, the MIC_{90} was 2 μg/ml, and the geometric mean MIC was 0.97 μg/ml. Rifabutin MICs for all isolates exceeded 32 μg/ml (Table 2). The MLCs of both amphotericin B and rifabutin were identical to the MICs for all isolates.

The interaction between amphotericin B and rifabutin was synergistic for 28% (7 of 25) of the *Fusarium* isolates and additive for 72% (18 of 25) of the isolates; antagonism was not observed.

Upon combination, the MICs of both amphotericin B and rifabutin were significantly reduced in comparison to the MICs when the drugs were tested alone ($P < 0.000001$ for both drugs). Amphotericin B MICs were reduced 2- to 4-fold (mean reduction, 2.5-fold), to a range of 0.25 to 1 μg/ml (Table 1). The MIC_{50} was reduced to 0.5 μg/ml, the MIC_{90} was reduced to 1 μg/ml, and the geometric mean MIC was reduced to 0.39 μg/ml. Among the 16 isolates for which the amphotericin B MIC was 1 μg/ml, the MIC was reduced to 0.5 μg/ml for 9 isolates and the MIC was reduced to 0.25 μg/ml for 7 isolates. Among the four isolates for which the MICs were 2 μg/ml, the MIC was reduced to 0.5 μg/ml for one isolate and the MIC was reduced to 1 μg/ml for three isolates. The MLCs of amphotericin B upon combination with rifabutin for all isolates were within one twofold dilution of the respective MICs.

Similarly, rifabutin MICs were reduced by 2- to 1,067-fold (mean reduction, 58-fold), from >32 μg/ml to a range of 0.06 to 32 μg/ml (Table 2). Upon combination, the MIC_{50} was 2 μg/ml, the MIC_{90} was 16 μg/ml, and the geometric mean MIC was 1.1 μg/ml. For 96% (24 of 25) of the isolates, the rifabutin MLC upon combination with amphotericin B was within one twofold dilution of the respective MIC. Synergy was maintained for the isolate for which the discrepancy between the MIC and the MLC exceeded one twofold dilution.

No differences in the susceptibility profiles of isolates of different *Fusarium* species were evident when they were tested with either individual drugs or the combination of drugs.

Colony count experiments. The positive interaction between the drugs was also demonstrated by colony count experiments with two isolates of *Fusarium*. For isolate 1083, the MICs of amphotericin B and rifabutin were 2 and >32 μg/ml, respectively. The colony counts of this isolate incubated with amphotericin B alone at 0.5 μg/ml (4-fold less than the MIC) and with rifabutin alone at concentrations ranging from 0.5 to 16 μg/ml (4- to 64-fold less than the MIC) were essentially identical to those of the isolate incubated without drugs. When the isolate was incubated with amphotericin B (0.5 μg/ml) in combination with rifabutin (4 μg/ml), the colony count was reduced 1.5 logs compared to the colony count after incubation with either drug alone. Further reductions of 2.7 and 3.1 logs were demonstrated when this subinhibitory concentration of amphotericin B was combined with 8 and 16 μg of rifabutin per ml. For isolate 1274, for which the amphotericin B MIC was of 1 μg/ml and the rifabutin MIC was >32 μg/ml, reductions of 1.2, 2.1, 3.2, 3.3, and 5.1 logs were achieved when amphotericin B at 0.5 μg/ml (2-fold less than MIC) was combined with rifabutin at 1, 2, 4, 8, and 16 μg/ml (64-, 32-, 16-, 8-, and 4-fold less than the MIC, respectively), respectively (Fig. 1).

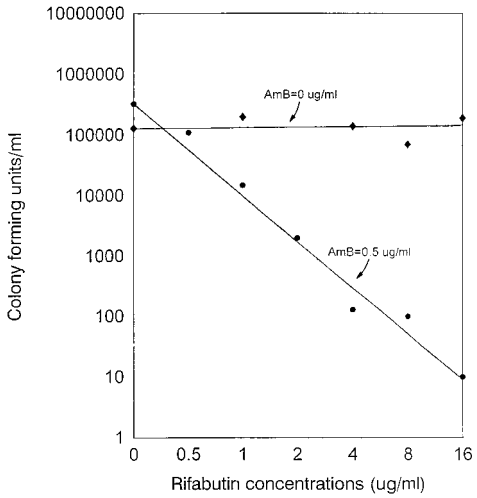

FIG. 1. Colony counts of *Fusarium* isolate 1274 in the presence of rifabutin either alone (concentration range, 0.5 to 16 μg/ml) or in combination with amphotericin B at 0.5 μg/ml. The colony count of isolate 1274 grown without drugs (first diamond) was essentially identical to the colony counts of the isolate grown in the presence of amphotericin B at 0.5 μg/ml (first circle) or in the presence of increasing concentrations of rifabutin (line labelled AmB [amphotericin B] = 0 μg/ml). With the combination of amphotericin B (0.5 μg/ml) and rifabutin (1, 2, 4, 8, and 16 μg/ml), colony counts were reduced by 1.2, 2.1, 3.2, 3.3, and 5.1 logs, respectively, in comparison with the colony counts of the controls or colonies treated with amphotericin B alone (line labelled AmB = 0.5 μg/ml).

Effects of amphotericin B and rifabutin on RNA and protein synthesis. To investigate the mechanism of the positive antifungal interaction between amphotericin B and rifabutin, we tested the hypothesis that the combination of these agents inhibited fungal RNA synthesis. *A. fumigatus* 96-1011, for which the amphotericin B MIC was 1 μg/ml and the rifabutin MIC was >32 μg/ml, was used.

RNA synthesis was assessed indirectly by measuring the level of incorporation of [^{3}H]uridine into RNA. The level of incorporation of [^{3}H]uridine was not affected by either amphotericin B at 0.25 μg/ml (fourfold less than the MIC) or rifabutin at 8 μg/ml (eightfold or more less than the MIC) compared to the level of incorporation by the drug-free control. When 0.25 μg of amphotericin B per ml was combined with 1, 2, and 4 μg of rifabutin per ml, however, the level of [^{3}H]uridine incorporation was reduced by 21, 54, and 68%, respectively.

To determine the effect of the combination of amphotericin B and rifabutin on protein synthesis, the level of [^{35}S]methionine uptake into protein was assessed. The levels of uptake were reduced by 22 and 25% after treatment with amphotericin B at 0.25 μg/ml in combination with rifabutin at 2 and 4

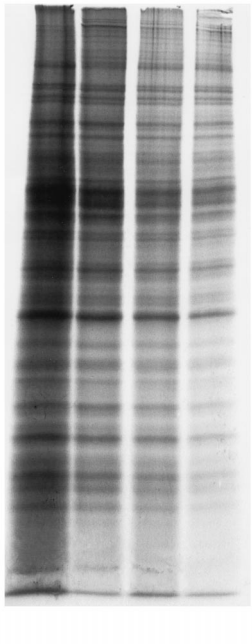

FIG. 2. Autoradiograph of 10% polyacrylamide gels of [^{35}S]methionine-labelled protein synthesized by *A. fumigatus* 96-1011 in the presence of water (control) (lane 1), amphotericin B (0.25 μg/ml) (lane 2), or rifabutin (16 μg/ml) (lane 3). The results for the combination of amphotericin B (0.25 μg/ml) and rifabutin (4 μg/ml) are presented in lane 4; the results for the combination of amphotericin B (0.25 μg/ml) and rifabutin (8 μg/ml) are presented in lane 5 (unnumbered lanes 1 to 5 in the figure are the lanes from left to right, respectively). Note the almost nonexistent banding pattern in lane 5 (arrow), reflecting almost complete inhibition of protein synthesis.

μg/ml, respectively compared to that seen after treatment with amphotericin B alone. The inhibition of protein synthesis was rapid: 75% of the total reduction of uptake occurred within 1 h of incubation with the combination of drugs.

Findings from the [^{35}S]methionine uptake experiment were corroborated by the level of [^{35}S]methionine incorporation into protein. Protein products were precipitated by trichloroacetic acid and separated by 10% polyacrylamide gels. The band patterns revealed a reduction in the amount of [^{35}S]methionine incorporation after treatment with the combination of amphotericin B (0.25 μg/ml) and rifabutin (4 μg/ml) compared to those for drug-free control or after treatment with amphotericin B alone (0.25 μg/ml) or rifabutin alone (16 μg/ml) (Fig. 2). The isolate treated with the combination of amphotericin B (0.25 μg/ml) and rifabutin (8 μg/ml) revealed almost no protein banding pattern (Fig. 2).

DISCUSSION

Intensive investigation into methods of vitro testing of filamentous fungi for susceptibility to antifungal agents has been undertaken by many laboratories within the past few years (8–10, 13, 20). The National Committee for Clinical Laboratory Standards' Subcommittee on Antifungal Susceptibility Testing has begun developing standardized testing methodologies with inoculum preparations containing conidial forms (8–10). A correlation between the results from the in vitro testing of inocula of *A. fumigatus* conidia for susceptibility to itraconazole and the in vivo response to therapy has been demonstrated (24).

Results from our in vitro susceptibility testing of *Aspergillus* and *Fusarium* isolates were consistent with clinical experience with patients infected with these organisms. For 38% (10 of 26) of the *Aspergillus* isolates and 80% (20 of 25) of the *Fusarium* isolates amphotericin B MICs were ≥1 μg/ml. Since the mean peak concentration of amphotericin B in serum after conventional dosing (dose ranging from 0.6 to 1.0 μg/ml) is 1.2 μg/ml (11), this finding might partially explain the suboptimal efficacy of amphotericin B against these organisms.

Given the high morbidity and mortality associated with aspergillosis and fusariosis and the suboptimal efficacy of amphotericin B, new therapeutic strategies will be needed to improve clinical outcomes. One strategy is to combine amphotericin B with another antimicrobial agent.

In this study, we have demonstrated that the interaction between amphotericin B and rifabutin was synergistic or additive against all 26 *Aspergillus* isolates and all 25 *Fusarium* isolates tested. Most importantly, for all 10 *Aspergillus* isolates for which the amphotericin B MICs were ≥1 μg/ml the MICs were reduced to levels that can more reliably be achieved. Among the nine isolates for which the amphotericin B MIC was 1 μg/ml, for three the MIC was reduced to 0.125 μg/ml, for four the MIC was reduced to 0.25 μg/ml, and for two the MIC was reduced to 0.5 μg/ml upon combination of amphotericin B with rifabutin; for the isolate for which the MIC was 2 μg/ml, the MIC was reduced to 0.25 μg/ml. Similarly, among the 20 *Fusarium* isolates for which the amphotericin B MIC was ≥1 μg/ml, for 17 the MIC was reduced to levels that can more reliably be achieved. For all 16 isolates for which the MIC was 1 μg/ml, the MIC was reduced to achievable levels. For seven isolates, the MIC was reduced to 0.25 μg/ml, and for nine isolates, the MIC was reduced to 0.5 μg/ml. For one of the four isolates for which the MIC was 2 μg/ml, the MIC was reduced to 0.5 μg/ml; for the other three isolates the MIC was reduced to 1 μg/ml. These findings imply that isolates exhibiting resistance to amphotericin B in vitro might be rendered susceptible to the agent at concentrations achievable in serum by the addition of rifabutin.

In addition, the concentrations of rifabutin required to exert in vitro synergy with amphotericin B were within the range achievable in human tissue. The peak concentration of rifabutin serum after the administration of a 600-mg oral dose is 0.4 to 0.6 μg/ml (2). Since rifabutin is rapidly and extensively picked up by tissues, however, the concentrations of the drug in serum can be misleading since they do not reflect concentrations within tissue sites of infection (19). Indeed, levels in tissue that exceed those in serum by greater than sixfold have been demonstrated, particularly in the lung and liver (2, 19). The median rifabutin MIC of 1.1 μg/ml for *Aspergillus* and *Fusarium* demonstrated upon combination of rifabutin with amphotericin B is significantly less than the levels of 2.8 to 3.4 μg/ml achievable in the lungs (2). Our demonstration that synergy and additivity were evident with concentrations of both drugs that are achievable in human tissue suggests that this interaction has potential clinical relevance.

The colony count experiments conducted with two *Fusarium* isolates resistant to amphotericin B corroborated the positive interactions observed in the in vitro susceptibility tests. Amphotericin B alone (0.5 μg/ml) and rifabutin alone (at concentrations ranging from 0.5 to 16 μg/ml) had no effect on the colony counts of either isolate after 48 h of incubation compared to the colony counts of the drug-free control isolates. The combination of amphotericin B and rifabutin, however, significantly reduced the colony counts (Fig. 1). For one isolate for which the amphotericin B MIC was 2 μg/ml and the rifabutin MIC was >32 μg/ml, the combination of amphotericin B at 0.5 μg/ml (fourfold less than the MIC) with rifabutin at con-

centrations ranging from 4 to 16 μg/ml resulted in 1.5- to 3.1-log reductions in colony counts compared to the counts for controls treated with each drug alone. Similarly, for the second isolate, for which the amphotericin B MIC was 1 μg/ml and the rifabutin MIC was >32 μg/ml, the combination of amphotericin B at 0.5 μg/ml (twofold less than the MIC) with rifabutin concentrations ranging from 1 to 16 μg/ml resulted in 1.2- to 5.1-log reductions in colony counts compared to the colony counts for the controls.

To our knowledge, this is the first study that has assessed the effects of rifabutin against filamentous fungi. Rifabutin is a semisynthetic derivative of rifamycin S, which has been shown to be more potent than rifampin against species of *Mycobacterium*, including isolates with documented resistance to rifampin (19). In addition to heightened potency, this agent can be concentrated at high levels at infected tissue sites (2).

The mechanism of the positive interaction between amphotericin B and rifabutin is unknown. Rifabutin alone demonstrated no antifungal activity. The combination of agents resulted in the inhibition of RNA synthesis, which is the mechanism of rifabutin's activity against bacteria and mycobacteria. We hypothesize that rifabutin's inherent lack of antifungal activity is due to its inability to penetrate the fungal cell membrane and that amphotericin B, by damaging the cell membrane, permits rifabutin to enter the cell. Once given intracellular access, rifabutin can exert its antifungal effect through the inhibition of RNA synthesis.

The precise mechanism by which rifabutin is able to inhibit RNA synthesis in eukaryotic cells such as fungi is also unknown. Against prokaryotes, the rifamycin class of antibiotics inhibits DNA-dependent RNA polymerase. Unlike prokaryotes, which use a single RNA polymerase to synthesize all types of RNA, eukaryotes possess several RNA polymerases, each with specialized transcription functions. It has been suggested that rifampin preferentially inhibits fungal rRNA synthesis (21). Since rifampin and rifabutin both belong to the rifamycin class of antibiotics, it is likely that the RNA synthesis inhibition of rifabutin was due to its activity against eukaryotic RNA polymerase I, which is responsible for rRNA transcription. Further investigation to define the precise mechanism is indicated.

Regardless of the specific mechanism of activity against RNA synthesis, we demonstrated that inhibition of fungal RNA synthesis by rifabutin leads to the inhibition of protein synthesis; inhibition of protein synthesis occurs within an hour of coincubation with amphotericin B and rifabutin. We have previously demonstrated that the combination of azithromycin, which inhibits protein synthesis, and amphotericin B results in fungicidal synergy against *Aspergillus* and *Fusarium* (3, 22). Since protein synthesis is vital to fungal growth, therapeutic strategies directed against this process might be successful against fungal infections. Both RNA synthesis and protein synthesis represent potential targets for new antifungal agents.

In summary, the activity of the combination of amphotericin B and rifabutin was superior to that of either agent alone against *Aspergillus* and *Fusarium* in vitro. Heightened fungicidal activity with this combination might assist in eradicating infection in vivo and improve patient outcomes. Synergy might also permit lower doses of amphotericin B to be used effectively, thereby reducing systemic toxicity. Most importantly, our study suggests that inhibition of fungal RNA or protein synthesis might represent a new target for future antifungal agents. Given these promising findings, in vivo evaluation is warranted to elucidate the role of rifabutin in combination with amphotericin B as a therapeutic strategy against *Aspergillus* and *Fusarium.*

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