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Activity of Posaconazole Combined with Amphotericin B against *Aspergillus flavus* Infection in Mice: Comparative Studies in Two Laboratories

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Posaconazole and/or amphotericin B was given to mice pretreated with a steroid and then infected by inhalation of *Aspergillus flavus* conidia. Two laboratories conducted studies using almost identical protocols to evaluate both survival and lung tissue burdens 8 days after infection. The results of the in vivo studies performed at both laboratories were consistent. We found that (i) up to 5 mg of amphotericin B per kg of body weight was poorly effective in treating invasive aspergillosis; (ii) posaconazole at 2 or 10 mg/kg/dose prolonged survival and reduced lung tissue CFU; and (iii) there was generally no antagonistic interaction of the drugs in combination, even when the experiments were designed to maximize the likelihood of antagonism. These studies do not confirm the antagonistic interaction of triazoles and polyenes reported by others.

Acute invasive aspergillosis (AIA) is one of the most feared infections of immunosuppressed patients. Both corticosteroids and neutropenia predispose to this infection, which almost invariably occurs after inhalation of infectious conidia (1). The speed of dissemination or pulmonary spread of the infection, and ultimately survival, depend in large part on the nature and severity of the predisposing host immune defect(s). In patients with the most fulminating forms of AIA, it has been difficult to demonstrate efficacy of antifungal therapy (7, 11). To date, there have been three approaches in the management of aspergillosis. The first is reversal of the predisposing conditions if possible. The second is antifungal therapy, and the third is resection when possible (18). Animal studies have been increasingly used to help determine whether a particular antifungal drug is effective against invasive aspergillosis. Some advantages of animal studies include (i) information in ascertaining the relative efficacy and dose-dependent toxicity of antifungal agents; (ii) assessing the contributions of immune deficiency to the outcome of aspergillosis; (iii) evaluating combinations of antifungals; and (iv) controlling for a variety of conditions that are thought to contribute to human invasive aspergillosis and its outcome but that cannot be controlled in clinical studies.

The experiment quoted most often, an experiment using amphotericin B and ketoconazole, was conducted by Schaffner and Frick (15) some years ago. When mice with AIA were treated with ketoconazole before amphotericin B, Schaffner and Frick noted a marked decrease in the efficacy of amphotericin B (15). They theorized that the azole blocked the synthesis of the ergosterol target necessary for the binding of amphotericin B. Polak et al. also found somewhat similar results (13, 14). These studies led to the concern that azole antifungal agents would antagonize the effects of amphotericin B in humans and that therefore they should not be used in combination with amphotericin B clinically.

In a large review of 595 cases of aspergillosis, Patterson et al. found that patients treated with amphotericin B and then with itraconazole fared no worse than those given amphotericin B alone (11). However, most of these patients had begun amphotericin B therapy before itraconazole was administered. The sequence used by Schaffner and Frick has seen limited clinical use. This is likely to change. The use of itraconazole antifungal prophylaxis and the recent phase III study of voriconazole, indicating the superiority of voriconazole over amphotericin B in AIA, all suggest that we shall be seeing increasing numbers of patients who receive a triazole before amphotericin B (R. Herbrecht, D. W. Denning, T. F. Patterson, W. V. Kern, K. A. Marr, D. Caillot, E. Theil, and P. Ribaud, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother. **41:**378, 2001).

The present studies were undertaken (i) to check for interactions of amphotericin B and posaconazole and (ii) to explore the potential reproducibility of triazole-polyene combinations in the same animal model of AIA studied in two different laboratories. In order to control the variables and to limit the number of animals used, we chose to use infection by inhalation of conidia, because this most closely approximates clinical infection. We chose corticosteroids for immune suppression because they predispose to a lethal infection in mice and are a major predisposing factor for AIA in humans. We anticipated that the results of these studies would give us some guidance on whether new triazoles with activity against *Aspergillus* can be used safely together with amphotericin B or should not be used in specific circumstances. Such information may ultimately help guide the use of these agents in clinical therapy.

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MATERIALS AND METHODS

Sites. Studies were conducted at two different laboratories, Schering Plough Research Institute (SPRI) and The University of Texas Health Science Center at

San Antonio (UTHSCSA). Both institutions utilized as close to identical methods as was possible.

Description of model. The pathogen used was *Aspergillus flavus* strain ND83, obtained from SPRI. This is available from the American Type Culture Collection as ATCC MYA-1004. Prior to infection, 11- to 13-g CF-1 (SPRI) or outbred ICR (UTHSCSA) male mice were treated with cortisone acetate (100 mg/kg of body weight) given subcutaneously on day −1, day 0 (day the mice were infected), and day +1. One-liter Erlenmeyer flasks with eight circumventing tubes were used for the inhalation infection. Each tube was approximately 4 in. long and had a 1-in. internal diameter, and the tubes were placed equidistant from each other. A small, 3- to 4-mm-long, orifice was made in the wall of the flask at the center of each tube to allow communication between the tube and flask. The outer ends of the tubes around each flask were plugged with cotton, and the flasks were sterilized. This inhalation chamber is a SPRI modification of the chamber first described by Piggot and Emmons (12) and routinely used at SPRI (8).

Two weeks before infection, 150 ml of liquid malt extract (SPRI) or potato flake (UTHSCSA) agar was added to the sterile flask. The agar was allowed to cool 3 h or longer and then heavily inoculated with *A. flavus* conidia. A rubber stopper with a glass tube, curved 45 degrees at the lower end (2 to 4 cm above the agar) and a second central glass tube was placed on top of the agar, and the flask was incubated for 13 days at room temperature to allow a dense mycelial mat to form.

At the time of infection, eight unanesthetized mice were inserted into the tubes. One mouse was inserted into each tube. Each mouse was inserted far enough that their nostrils protruded into the central chamber. The tubes were plugged with cotton to prevent the mice and inoculum from leaving the tube. A 60-ml syringe was attached to the central glass tube, and one vigorous blast of air was delivered into the chamber by plunging the syringe. This disrupted the mycelia, forming a cloud of conidia. Mice were allowed to breathe the conidial suspension for 1 min and were then rapidly removed from the chamber. The tubes were then refilled with a second cohort of mice. The glass tube was rotated so that the end was pointed at undisrupted mycelia, and the infection process was repeated. A third cohort of mice was similarly exposed. No more than three cohorts of mice were used for any one flask.

From each chamber, one mouse was sacrificed at 1 h after pulmonary infection for quantitative counts of *Aspergillus* conidia, done by serial colony count dilutions of homogenates of the total lungs. These were considered the baseline counts. In order to examine the natural course of this infection, in two studies, two mice from each flask were also sacrificed at days 3 and 5 after infection. Lungs were removed and homogenized in 2 ml of saline, and serial dilution colony counts were made.

To minimize variability from different flasks, mice from each flask were randomly assigned to different cages, and the cages were randomly assigned to control or treatment groups. There were 8 to 12 mice/group. Treatment began 24 h after infection. A clinical oral suspension of posaconazole was obtained from SPRI. Further dilutions were made in sterile water. Both laboratories used amphotericin B (Fungizone [Bristol-Myers Squibb Co., Princeton, N.J.]) prepared according to the manufacturer's directions. UTHSCSA further diluted amphotericin B with 5% dextrose, while SPRI further diluted amphotericin B in sterile water for injection. In some studies, amphotericin B, posaconazole, or both were given on day 1 after infection (concurrent treatment). In other studies, posaconazole therapy was begun on day 1, and amphotericin B therapy was not begun until day 2 (sequential treatment). Drug doses used included 2 or 10 mg of posaconazole per kg administered once a day orally or 1 or 5 mg of amphotericin B per kg administered intraperitoneally once a daily. Treatment continued through day 7. For tissue burden studies, quantitative cultures of the lungs of mice were performed for all mice when they died or were sacrificed on day 8. For survival studies, treatment continued through day 7, and mice were terminated on day 8.

In vitro MICs were determined by using the National Committee for Clinical Laboratory Standards (NCCLS) method modified for mycelial fungi, with incubation at 24 and 48 h for end points (10). At 48 h, the posaconazole MIC was 0.06 μg/ml, and the amphotericin B MIC was 2 μg/ml.

Statistics. The Mann-Whitney U test was used to compare tissue counts. The log rank test was used to compare survival of the groups. Because each group was compared both with the untreated control and with groups given the individual drugs for efficacy, a P of <0.03 was used to determine significant differences.

RESULTS

It was our initial intention to study AIA with *Aspergillus fumigatus*, as this is the most significant pathogen of the genus *Aspergillus*. In preliminary studies, eight isolates of *A. fumigatus* were evaluated using the inhalation model. *A. fumigatus* caused a fulminating infection which was lethal too quickly to allow studies of combined antifungal therapy. In other models, posaconazole is effective against *A. fumigatus* (3).

A less virulent species, *A. flavus*, was then chosen. Using isolate ND83 (ATCC MYA-1004), we found that untreated mice usually succumbed 6 to 10 days after infection. To determine the actual inoculum size, the quantity of viable conidia inhaled was measured. Therefore, in several studies, one group of mice was sacrificed 1 h after infection, and the lungs were quantitatively cultured. As shown in Fig. 1A (data from UTHSCSA), the fungal burden fell about 1 log unit between 1 h and 1 day after infection and was then stable for 5 days after infection. However, as the conidia germinate into mycelia within 24 h, measurements after 1 day were in essence fragmented mycelia from lung abscesses. Figure 1B shows the results of a typical experiment at SPRI and UTHSCSA, using mice treated with water (controls). Note that the median tissue count for the SPRI mice was 1 log higher than for the UTHSCSA mice. We could not determine whether this was due to more vigorous dispersion of the conidia, more fragile mycelial cultures, or other aspects of culture. This systematic difference was maintained from 1 h to 8 days after infection.

Survival. Survival studies were conducted with posaconazole given at 2 or 10 mg/kg/day and with amphotericin B given at 1 or 5 mg/kg/day. Figure 2 presents the results of posaconazole (10 mg/kg/day) and amphotericin B (5 mg/kg/day) therapy, with both drugs begun concurrently 1 day after infection. Control mice at SPRI, inoculated more heavily, began to succumb about a day earlier than those at UTHSCSA. Mice at SPRI and UTHSCSA were not protected by amphotericin B. This result is similar to the results of previously published studies where we found inconsistent protection by amphotericin B in steroid immunosuppressed animals challenged intranasally with *A. fumigatus* (3). Posaconazole gave almost 100% survival both at SPRI and UTHSCSA when it was given alone at a dose of 10 mg/kg or combined with amphotericin B (5 mg/kg). Because of the high efficacy of posaconazole, this study would have been able to detect only antagonism, not any additive effects of the combination therapy. There was no antagonism seen with the combination.

Lower drug doses were also studied (results not shown). An amphotericin B dose of 1 mg/kg conferred no protection to mice when the survival of treated and control mice was compared ($P > 0.3$ for both laboratories). Mice receiving posaconazole (2 mg/kg) were significantly protected compared to the controls, with survival percentages of 60 and 67 at the study termination ($P < 0.008$ for both laboratories). At SPRI and UTHSCSA, mice concurrently treated with both posaconazole and amphotericin B had survival rates similar to those given posaconazole alone ($P > 0.4$). Therefore, at these lower drug doses, there were neither additive nor antagonistic effects.

Tissue burden. Figure 3 shows the lung tissue burden results of mice treated with the same doses used in Fig. 2. Two studies done at UTHSCSA with similar results were combined, while only one study was done at SPRI. The mean control counts were 5.95 $\log_{10}$ CFU/lung at SPRI and 4.66 $\log_{10}$ CFU/lung at UTHSCSA. Posaconazole treatment at 10 mg/kg significantly reduced tissue burden levels below those of the control mice in

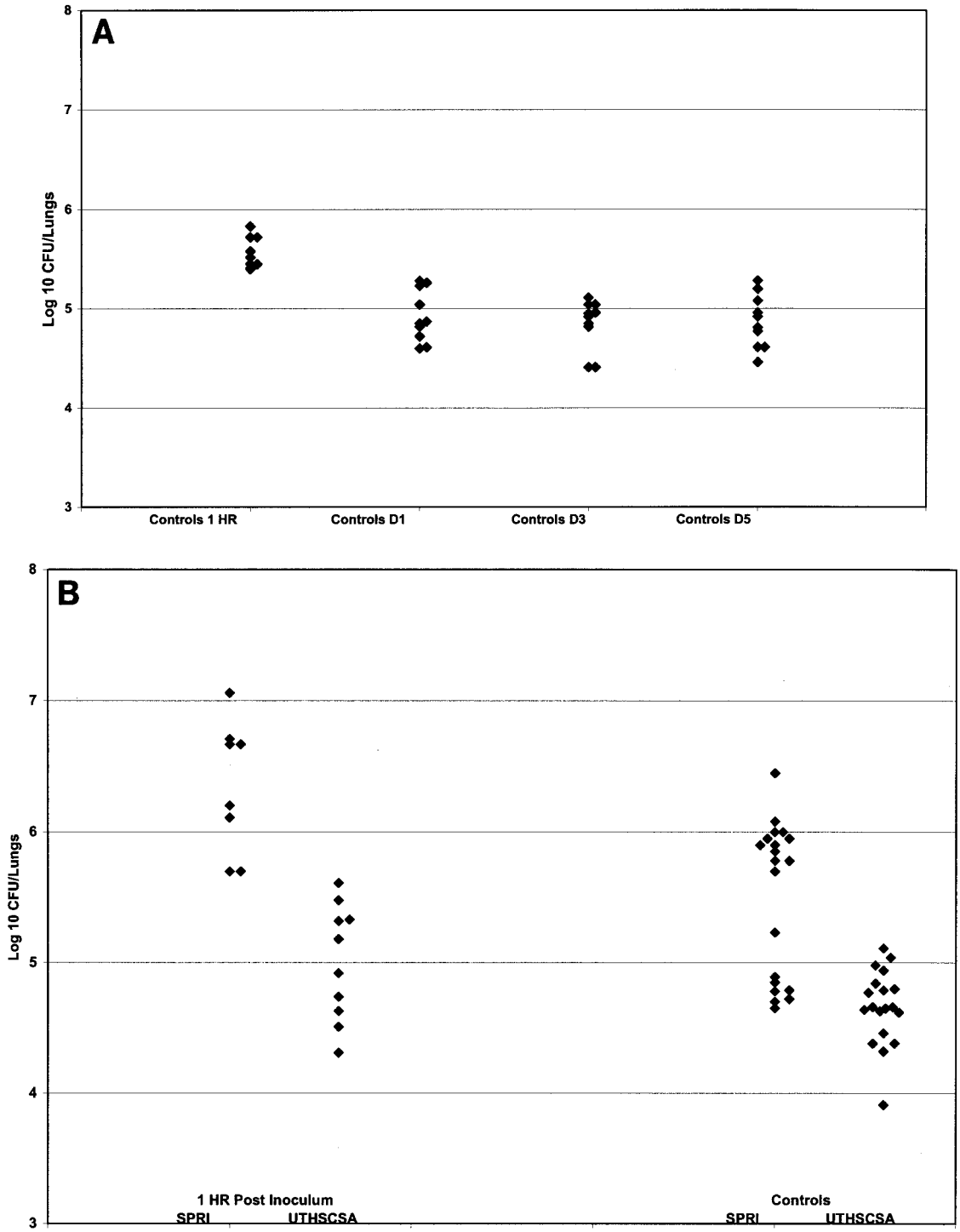


FIG. 1. Total lung tissue burdens of mice infected with *A. flavus* and sacrificed after infection. (A) Mice were infected with 5.57 $\log_{10}$ CFU/lung (geometric mean) and sacrificed at 1 h, 1 day (D1), 3 days, and 5 days after infection. (B) Mice were infected with 6.34 $\log_{10}$ CFU/lung at SPRI and 4.99 $\log_{10}$ CFU/lung at UTHSCSA (both values are geometric means). Lung tissue burdens were measured 1 h after infection. The lung tissue burdens in the controls were measured at the time of death or 8 days after infection. Data from two studies each were combined. Control mice were treated orally with water (0.2 ml/day) from days 1 to 7.

both laboratories. At SPRI, amphotericin B treatment at doses of 5 mg/kg (5.88 $\log_{10}$ CFU/lung) did not significantly reduce the tissue counts relative to those of controls, but at UTHSCSA, the same dose of amphotericin B significantly reduced the mean CFU to 4.31 $\log_{10}$ CFU/lung. Mice treated with the combination of posaconazole at 10 mg/kg and amphotericin B at 5 mg/kg had counts significantly below those of control mice and mice given amphotericin B alone. Both at

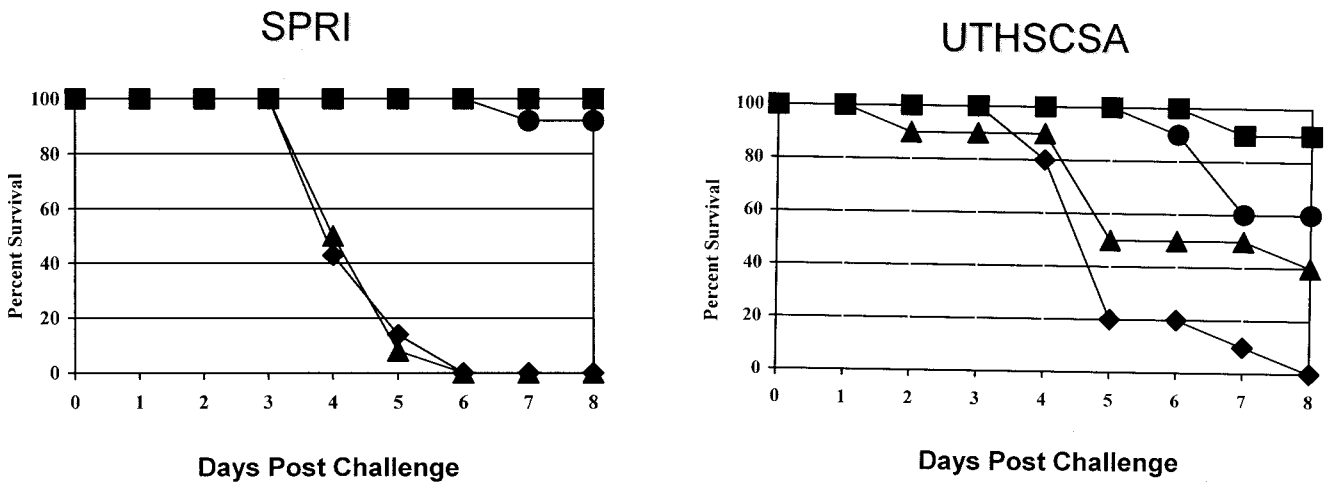
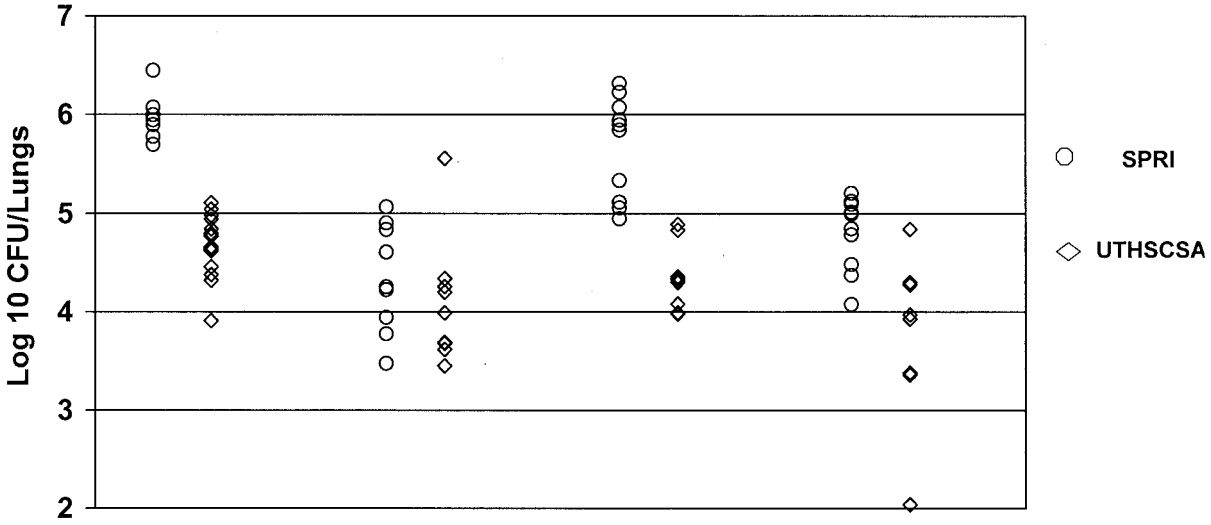


FIG. 2. Concurrent therapy survival study. The results of survival studies with posaconazole (10 mg/kg) (■), amphotericin B (5 mg/kg) (▲), and the combination of posaconazole and amphotericin B (●) are shown. Both drugs were begun concurrently 1 day after infection and continued daily through day 7 postinfection. Posaconazole (10 mg/kg) and combined posaconazole-amphotericin B treatment prolonged survival over that of controls (◆) at both laboratories ($P \leq 0.03$). Amphotericin B was ineffective in prolonging survival. The combination of posaconazole and amphotericin B gave results similar to those of POS given alone.

SPRI and UTHSCSA, the combination did not give results not significantly different from the results when posaconazole alone was given. These studies were also done with posaconazole at 2 mg/kg and amphotericin B at 1 mg/kg. The results (not shown) were qualitatively similar to those at the higher doses.

Figure 4 shows the results of survival studies in mice treated sequentially. In these studies, posaconazole treatment (10 mg/



	Control	POS 10	AMB 5	COMBO
N/group	10/19	10/9	10/10	9/10
Comparison with controls (P value, Mann Whitney test)				
SPRI	--	<0.0001*	0.3150	<0.0001*
UTHSCSA	--	0.0027*	0.0116	0.0005*

FIG. 3. Concurrent therapy tissue burden survival study. The lung tissue burden results of mice treated with posaconazole at 10 mg/kg (POS 10), amphotericin B at 5 mg/kg (AMB 5), or a combination of POS and AMB (COMBO). Both drugs were begun concurrently 1 day after infection and continued daily through day 7 postinfection. POS and COMBO groups reduced lung fungal burden from those of the control groups at both laboratories. AMB reduced counts only at UTHSCSA and not at SPRI. The COMBO results were similar to those of POS alone. Values that are significantly different ($P \leq 0.03$) from the control values are indicated by an asterisk.

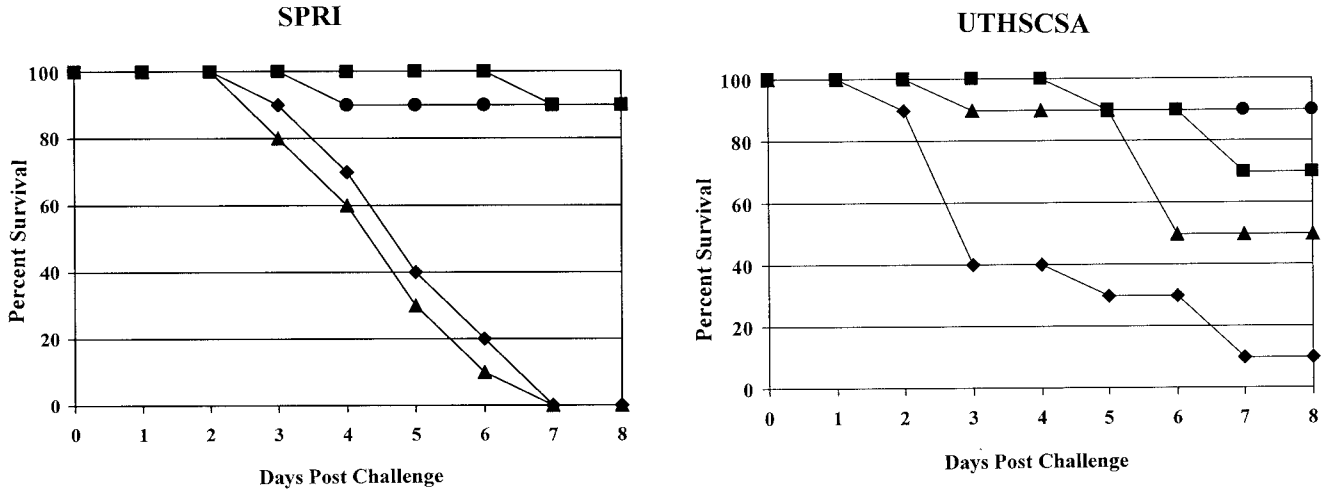


FIG. 4. Sequential therapy survival study. The results of survival studies in mice treated sequentially with posaconazole at 10 mg/kg initiated on day 1 after infection (■), amphotericin B at 5 mg/kg begun on day 2 after infection (▲), or the combination of posaconazole and amphotericin B (●). Therapy continued daily for 7 days. The groups of mice treated with posaconazole and the posaconazole-amphotericin B combination showed prolonged survival compared to control groups (◆) ($P \leq 0.03$) at both laboratories. However, amphotericin B at 5 mg/kg prolonged survival ($P \leq 0.03$) only at UTHSCSA. The survival of mice treated with the posaconazole-amphotericin B combination was similar to that of mice treated with posaconazole alone.

kg/day) was initiated on the first day after infection, and amphotericin B treatment (5 mg/kg/day) was begun on the second day after infection. This was similar to the initiation of ketoconazole treatment before amphotericin B treatment in the studies of Schaffner and Frick (15). Sequential therapy survival results at SPRI were similar to those given concurrent therapy (Fig. 2). Control mice survived a median of 5 days in both laboratories. Posaconazole treatment at both laboratories prolonged survival to 8 days ($P < 0.0001$). Amphotericin B treatment was ineffective at SPRI (median survival time, 5 days; $P = 0.5010$) but prolonged survival significantly over that of controls at UTHSCSA (median survival time, 8 days; $P = 0.0095$). Combination therapy recipients had significantly prolonged survival compared to the survival of controls at SPRI and UTHSCSA, but not compared to mice given posaconazole alone.

Both laboratories found prolonged survival for mice given the lower dose of posaconazole (2 mg/kg) compared to that of the controls (median survival time, 8 days at SPRI and 7 days at UTHSCSA; $P < 0.0001$) (data not shown). Amphotericin B at 1 mg/kg was not protective. In both laboratories, the survival times of mice given the combination of posaconazole (2 mg/kg) and amphotericin B (1 mg/kg) were similar to that of mice given posaconazole alone.

Figure 5 shows lung tissue counts in mice treated sequentially. Treating mice with 5 mg of amphotericin B per kg did not affect the lung tissue counts of mice compared with those of control mice at both laboratories. At SPRI and UTHSCSA, treating mice with 10 mg of posaconazole per kg significantly lowered lung tissue burden from that of the controls. The lung tissue counts of mice given combination therapy were significantly lower than the counts of the controls but similar to the counts for mice given posaconazole alone.

In summary, we found generally similar results for studies performed in the two laboratories. Posaconazole treatment (2 or 10 mg/kg) significantly prolonged survival and reduced lung tissue burden of *A. flavus* compared with those of controls. Amphotericin B treatment (1 or 5 mg/kg) was irregularly effective and less protective than posaconazole treatment alone. Combined therapy, either concurrent or sequential, gave similar results to posaconazole therapy alone. Therefore, in these experiments there were no antagonistic or additive effects of amphotericin B (in doses up to 5 mg/kg) on posaconazole.

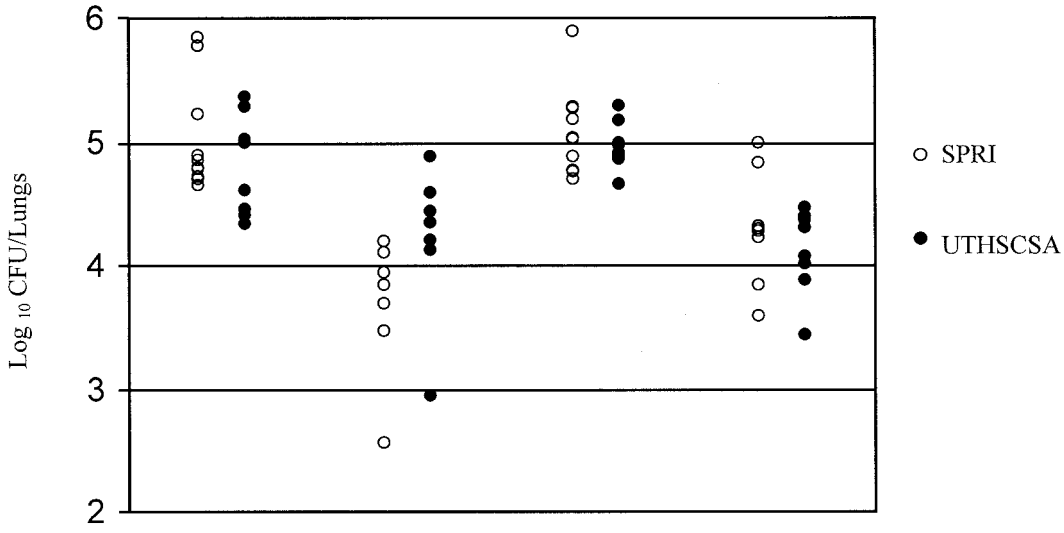
DISCUSSION

The present studies illustrate both the benefits and limitations of animal studies in reproducing clinical disease. One of the benefits of the present studies was that the natural inhalation route of infection was used in mice pretreated with corticosteroids (a predisposing factor). This is similar to the clinical environment. The target organ is the lungs, just as in clinical disease. However, *A. fumigatus* is a more significant cause of clinical disease than *A. flavus*. In the present inhalation model, we were unable to use *A. fumigatus* due to its consistently high virulence and had to rely on *A. flavus*, a less common pathogen.

Another benefit of animal studies is the standardization of inoculum size and use of reference isolates of fungal pathogens. Standardizing these variables is necessary for the reproducibility of experimental aspergillosis, but the clinical outcome of aspergillosis depends in part on the inoculum size and virulence of the infecting fungal isolate. Thus, at the time treatment is initiated, patients may have much heavier fungal burdens and later stages of fungal disease than animal models can reproduce.

One advantage of animal models is that the influence of specific predisposing immune defects can be studied. However, patients often have combined immune deficiencies (i.e., neutropenia and steroid administration). Most animal models are limited by use of a single immune deficiency (neutropenia or steroid administration).

Additional considerations include variable drug absorption,



	Control	POS 10	AMB 5	COMBO
N/group	10/8	10/8	10/8	10/8
Comparison with controls				
SPRI	--	<0.0001*	0.4359	0.0029*
UTHSCSA	--	0.0207*	0.6454	0.0030*

FIG. 5. Sequential therapy tissue burden study. Lung tissue counts in mice treated sequentially with posaconazole at 10 mg/kg (POS 10) initiated on day 1 after infection, amphotericin B at 5 mg/kg (AMB 5) begun on day 2 after infection, or the POS-AMB combination (COMBO). Therapy continued daily for 7 days. Both POS- and COMBO-treated groups showed prolonged survival ($P \leq 0.03$) over that of controls at both laboratories. AMB was not effective. Results with COMBO treatment were similar to those of POS alone. Values that were significantly different from the control values ($P \leq 0.03$) are indicated by an asterisk.

distribution, and metabolism among animal species. As one example, voriconazole (and to a lesser degree itraconazole) is cleared so rapidly in mice that it is very difficult to show efficacy, whereas in guinea pigs, clearance is slow and voriconazole is efficacious in aspergillosis (4, 16, 17). In the present studies, we used posaconazole, which is cleared slowly in mice and is more effective than either voriconazole or itraconazole.

Finally, in animal models, both the doses and sequence of drugs in combinations can be tightly controlled. However, patients often present with a treatment history including multiple prior or current antifungals.

All of these factors, while allowing us to reproduce a model of a serious fungal infection in vivo and manipulate it in multiple ways, significantly limit the degree to which one can extrapolate results of animal studies to the clinical situation.

Finally, there is the as yet unanswered question of reproducibility of in vivo studies. The NCCLS has spent considerable effort in standardizing in vitro antifungal testing, but little has been done in vivo (9, 10). We considered that a similar effort at standardization might be undertaken using an in vivo murine model of aspergillosis, with single and combination drug regimens. Even with multiple controlled parameters are used in animal studies, the same drug may yield highly disparate results in different laboratories. This may be due to different *Aspergillus* isolates, inoculation routes, treatment regimens, methods of assessing outcome, or strains or species of animals used (2). These problems are further confounded when one attempts to evaluate combinations of antifungals. To search for additive effects in combination studies, one must use a suboptimal dose of each drug and then show that the combination is superior to each component. This is often very difficult to achieve, especially when drug efficacy depends highly on inoculum size and timing of initiation of therapy in a very short window. Conversely, to search for antagonism, one should use highly effective regimens and then seek to determine whether efficacy is compromised by the combination. Therefore, the sequence of drugs further influences results (5, 6).

In recent years, there has also arisen an ethical emphasis on limiting the numbers of animals in each treatment group and in limiting the repetition of studies for confirmation and statistical significance. Therefore, it is not surprising that different laboratories may report additive, neutral, or antagonistic effects with the same drugs used against the same fungal species.

In the present studies, we found that similar protocols generally gave similar results in the two different laboratories. Three systematic variables, different mouse strains, different media used for culture of the *A. flavus* isolate, and a higher inoculum at SPRI (approximately 10 times higher), did not affect the overall similarity of responses to antifungal therapy. In both laboratories, treatment results were strikingly similar. Posaconazole treatment at doses of 2 and 10 mg/kg was effective, prolonging survival and reducing lung tissue count. Treatment with amphotericin B alone at both 1 and 5 mg/kg was mostly ineffective. In the inhalation model of steroid-immunosuppressed mice, amphotericin B is often poorly effective (3). Both drugs thus performed similarly at both laboratories.

These studies are also the first to evaluate the combination of posaconazole and amphotericin B, using both concurrently initiated and sequential treatment regimens. The sequential studies were performed in regimens set to maximize the po-

tential for antagonism, per the studies of Schaffner and Frick (15), Kontoyiannis et al. (5), and Lewis et al. (6). In the present studies, there was no evidence that amphotericin B treatment, at doses as high at 5 mg/kg, had an antagonistic effect to posaconazole.

It is not clear why the results of our studies conflict with those of Kontoyiannis et al. (5). We are assured by our results, conducted both with concurrently initiated and with sequential therapy, using both survival and reduction of lung tissue burden, that there is no antagonism of the triazole antifungal posaconazole and the polyene amphotericin B. Therefore, we conclude that in vivo models can be standardized to give predictable results in murine aspergillosis. Just as in vitro studies, a number of parameters need to be standardized as far as possible.

How do the results of these studies translate to the clinician? First, they should encourage the clinical investigator that posaconazole has potential as an effective antifungal against AIA. AIA carries such a high mortality that many clinicians are now utilizing combination therapy as treatment. This is in part the result of a number of in vitro studies supporting additive or synergistic effects and in spite of conflicting animal studies. Whether combinations are superior to individual antifungal drugs is a matter that may require clinical studies for resolution. Our studies revealed no combination effect of posaconazole and amphotericin B in this model. There was little evidence of antagonism of posaconazole and amphotericin B. Therefore, these studies provide no reason to avoid clinical studies of posaconazole and amphotericin B combination therapy.

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