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Comparative Efficacies of Conventional Amphotericin B, Liposomal Amphotericin B (AmBisome), Caspofungin, Micafungin, and Voriconazole Alone and in Combination against Experimental Murine Central Nervous System Aspergillosis

Karl V. Clemons,[1,2,3]* Marife Espiritu,[1] Rachana Parmar,[1] and David A. Stevens[1,2,3]

California Institute for Medical Research[1] and Department of Medicine, Division of Infectious Diseases,[2] Santa Clara Valley Medical Center, San Jose, California 95128, and Department of Medicine, Division of Infectious Diseases and Geographic Medicine, Stanford University, Stanford, California 94305[3]

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Central nervous system (CNS) aspergillosis is a severe disease that responds poorly to current therapies. The current studies examined the efficacies of several antifungal agents alone or in combination with a murine model of CNS aspergillosis. Immunosuppressed mice were infected intracerebrally with *Aspergillus fumigatus* and treated with an amphotericin B preparation, an echinocandin, or voriconazole (VCZ) given alone or in combination. Monotherapy studies showed that micafungin (MICA), caspofungin (CAS), VCZ, conventional amphotericin B (AMB), Abelcet (ABLC) (a lipid-carried AMB formulation; Enzon Pharmaceuticals, Inc.), and AmBisome (AmBi) (liposomal AMB; Gilead Sciences, Inc.) were efficacious. However, doses of AmBi above 15 mg/kg of body weight showed reduced efficacy. Neither MICA nor CAS showed dose responsiveness at the doses tested (1, 5, or 10 mg/kg). Only the 40-mg/kg dose of VCZ was effective. AmBi and ABLC showed dose responsiveness, with 10-mg/kg doses causing a significant reduction in fungal burden; they had equivalent activities at the 10-mg/kg dose. Suboptimal dosages of AmBi in combination with MICA, CAS, or VCZ were effective in prolonging survival. However, significantly enhanced activity was demonstrated only with AmBi and VCZ in combination. AmBi in combination with MICA or CAS showed a trend toward enhanced activity, but the combination was not significantly superior to monotherapy. The use of AmBi with CAS or VCZ at optimal doses did not improve efficacy. Cure was not attained with any dosage combinations. These results indicate that AmBi in combination with VCZ may be superior for treatment of CNS aspergillosis; combinations of AmBi and MICA or CAS were not antagonistic and may have a slight benefit.

Among the most serious manifestations of opportunistic fungal infection is aspergillosis with dissemination to the central nervous system (CNS) (10, 28, 42, 44). Current therapeutic options are mostly ineffective, and mortality rates remain high. With newer drugs in use, little is known about their effectiveness against CNS aspergillosis. Various preparations of amphotericin B have also been used with varied success (10, 28, 42, 44). Recently, clinical studies on the use of combination therapy for invasive aspergillosis have suggested a potential benefit of salvage combination therapy for the prolongation of survival and that combination therapy may be warranted as a front-line treatment (29).

Whether combination therapy against CNS aspergillosis would be of benefit is unknown and will be difficult to study clinically. Thus, the use of an infection model of CNS aspergillosis allows for the study of potentially useful combination therapies. In our current studies, we have examined the use of liposomal amphotericin B (AmBisome; Gilead Sciences, Inc., Foster City, Calif.) (AmBi) alone and in combination against murine CNS aspergillosis. Each of these drugs has been shown to have efficacy for various models of aspergillosis (1, 5, 7, 8, 13–15, 24, 25, 33, 50, 51). Against CNS infections studied with animal models, AmBisome has demonstrated efficacy against coccidioidomycosis (12), and conventional amphotericin B, itraconazole, posaconazole, and caspofungin have demonstrated efficacy against CNS aspergillosis (9, 24). However, no data on the efficacy of combinations of these drugs in CNS disease have been published.

* Corresponding author. Mailing address: Division of Infectious Diseases, Santa Clara Valley Medical Center, 751 South Bascom Ave., San Jose, CA 95128-2699. Phone: (408) 998-4557. Fax: (408) 998-2723. E-mail: clemons@cimr.org.

MATERIALS AND METHODS

Murine model. A murine model of CNS aspergillosis was established with 5-week-old male CD-1 mice as described previously (9, 10, 24). In brief, 200 mg/kg of body weight of cyclophosphamide was given itraperitoneally (i.p.) 2 days prior to infection and once every 5 days thereafter. On the day of infection, mice were anesthetized using methoxyflurane fumes. Three separate studies were done. In the monotherapy study, mice were given 5.8×10^6 conidia, with *Aspergillus fumigatus* strain 10AF and direct intracerebral inoculation (9, 10, 24). In the suboptimal-dose combination therapy model, mice were given 6.8×10^6 conidia intracerebrally. In the third study, of optimal-dose combination therapy and high-dose AmBi, mice were given 5.5×10^6 conidia intracerebrally.

Therapy was initiated on day 1 postinfection and continued for 10 consecutive days in all three studies; each therapy group consisted of 10 mice. On day 14 of infection, all surviving mice were euthanatized and the brain and kidneys removed aseptically. The numbers of CFU remaining in these tissues were determined by homogenization of the tissues and quantitative plating of serial dilutions (7, 9–11, 22, 24).

Monotherapy regimens. For the monotherapy study, the treatment regimens were as follows: 5% dextrose water controls (D5W), intravenously (i.v.) once a

day (QD); conventional amphotericin B (AMB) (Fungizone; Bristol-Myers Squibb Co., Princeton, N.J.), 1 mg/kg i.v. QD; AmBi, 1, 5, or 10 mg/kg i.v. QD; Abelcet (ABLC) (a lipid-carried AMB formulation; Enzon Pharmaceuticals, Inc., Fairfield, N.J.), 1 or 10 mg/kg i.v. QD; caspofungin (CAS) (Cancidas; Merck & Co., Inc., West Point, Pa.), 1, 5, or 10 mg/kg/day i.p. twice a day (BID); or micafungin (MICA) (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan), 1, 5, or 10 mg/kg/day i.p. BID. Additional groups of animals received grapefruit juice (Ocean Spray) in lieu of water beginning on day 3 prior to infection to improve the serum concentrations of voriconazole by inhibiting metabolism, similar to previously described studies using mice (6, 18, 45, 46). These animals were treated with 4% polyethylene glycol (PEG) 400 (grapefruit juice controls) orally (p.o.) QD or with voriconazole (VCZ) (Pfizer Inc., Groton, Conn.) in 4% PEG 400 at 5, 10, or 40 mg/kg p.o. QD.

Suboptimal-dose combination therapy regimens. The results of the monotherapy study were used to decide which dose of each of the antifungal drugs would be chosen for use in the combination study. To allow the demonstration of enhanced activity by the combination versus each respective monotherapy, we chose to use the lowest effective dose. A highly effective monotherapy could abrogate the opportunity to demonstrate enhancement with the combination regimen. The treatment regimens for the monotherapy arms were as follows: D5W, i.v. QD; AMB, 1 mg/kg i.v. QD; AmBi, 1 mg/kg i.v. QD; CAS, 1 mg/kg/day i.p. BID; MICA, 1 mg/kg/day i.p. BID, or VCZ in 4% PEG 400, 40 mg/kg p.o. QD. Groups receiving VCZ were given grapefruit juice (50% in water) beginning on day 3 prior to infection. Using the same monotherapy doses, the groups for combination therapy were as follows: AmBi plus CAS, AmBi plus MICA, AmBi plus VCZ, AmB plus CAS, and AmBi for the initial 3 days followed by VCZ for 7 days.

Optimal-dose combination therapy and high-dose AmBisome regimens. The treatment regimens for this third study for the monotherapy arms were as follows: D5W, i.v. QD; AmBi, 10, 15, 20, or 25 mg/kg i.v. QD; CAS, 10 mg/kg/day i.p. BID; or VCZ in 4% PEG 400, 40 mg/kg p.o. QD. The groups for combination therapy were as follows: AmBi, 10 mg/kg, plus CAS, 10 mg/kg; AmBi, 10 mg/kg, plus VCZ, 40 mg/kg; and AmBi, 10 mg/kg for the initial 3 days, followed by VCZ, 40 mg/kg for 7 days.

VCZ pharmacokinetics. Uninfected mice given grapefruit juice were dosed orally once daily either a single time or for 10 days with VCZ at 5, 10, or 40 mg/kg. Blood was collected from two mice each at various times postdose, and the serum concentration of VCZ was determined by a bioassay similar to that described previously using *Candida kefyr* SA as the indicator organism (35, 47). By this bioassay method, VCZ was detectable (≥0.063 μg/ml), with a between-runs coefficient of variation of 11.7%.

Statistical analysis. The statistical analysis of survival was done using by a log rank test. For the analyses of comparative organ burdens of *A. fumigatus*, samples missing due to death were assigned a value of $\log_{10}$ 5 CFU, which assures that death is considered a worse outcome than is survival with any amount of fungal burden (27, 38). This value also approximates the fungal burden just prior to death. Assignment of any number, as long as it assigns death as a worse outcome, does not affect the statistical analysis by this method. Comparative analyses were done by a nonparametric Kruskal-Wallis analysis of variance followed by a Dunn's test for multiple comparisons. Comparative analyses were done only within experiments, not across experiments.

RESULTS

VCZ serum pharmacokinetics. Voriconazole has been only minimally utilized with murine models of fungal infection because of undesirable pharmacokinetic parameters due to metabolism. We examined the use of grapefruit juice to inhibit P_{450} metabolism, as reported previously (6, 18, 45, 46), to determine whether clinically relevant serum concentrations could be attained and allow the study of VCZ in our murine model. The serum pharmacokinetics of VCZ in mice was determined after single and multiple doses. After a single dosage of VCZ, a single peak of 0.075 μg/ml at 1 h, 0.325 μg/ml at 1 h, and 12.5 μg/ml at 4 h postdose were observed for animals given 5, 10, or 40 mg/kg of VCZ, respectively. The animals given VCZ (40 mg/kg) had the largest area under the concentration-time curve from 0 to 24 h (Fig. 1). The estimated serum half-life was between 1 and 2 h after a single dose. After 10 days of dosing, only those animals given 40 mg/kg VCZ had detectable concentrations in serum (Fig. 1). The peak value was after 1 h at 11.5 μg/ml and declined thereafter.

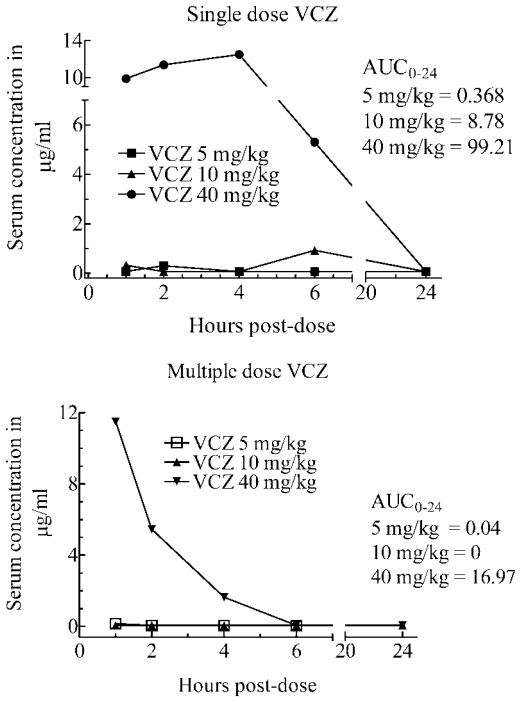

FIG. 1. Serum pharmacokinetics of VCZ in mice after one or multiple doses. Average from two mice at each time point after the last dose. AUC_{0-24}, area under the concentration-time curve from 0 to 24 h.

These data indicate that VCZ serum concentrations are attainable in mice given grapefruit juice, although dosages of less than 40 mg/kg may not result in serum concentrations sufficient to be clinically useful. In addition, VCZ did not accumulate in the mice after 10 days of dosing, and it appears VCZ is cleared more rapidly from the serum of these animals. Thus, VCZ may be inducing its own metabolism with continued dosing. In addition, it was noted during the course of these studies that the animals given grapefruit juice as the sole source of fluids progressively lost weight and in some instances did not appear to readily consume the juice. This may have contributed to the results of both the pharmacokinetics and the drug efficacy for treatment of infection. To that end, we provided 50% grapefruit juice in water to five mice to determine whether they would consume the juice and whether drug levels could be obtained after dosing. The animals readily consumed the diluted grapefruit juice and showed no loss of weight or ill effects over 10 days. Drug levels after a single 40-mg/kg dose were similar to that presented in Fig. 1 and indicated that 50% grapefruit juice in lieu of normal drinking water was suitable to result in detectable serum concentrations of VCZ.

Drug efficacy with monotherapy. An initial study of dose escalation using only monotherapy for each of the compounds was done to determine dosages to be used in subsequent combination therapy work. Figure 2 presents the Kaplan-Meier plots of cumulative mortality for each of the respective treatment arms in the monotherapy study. The model proved to be

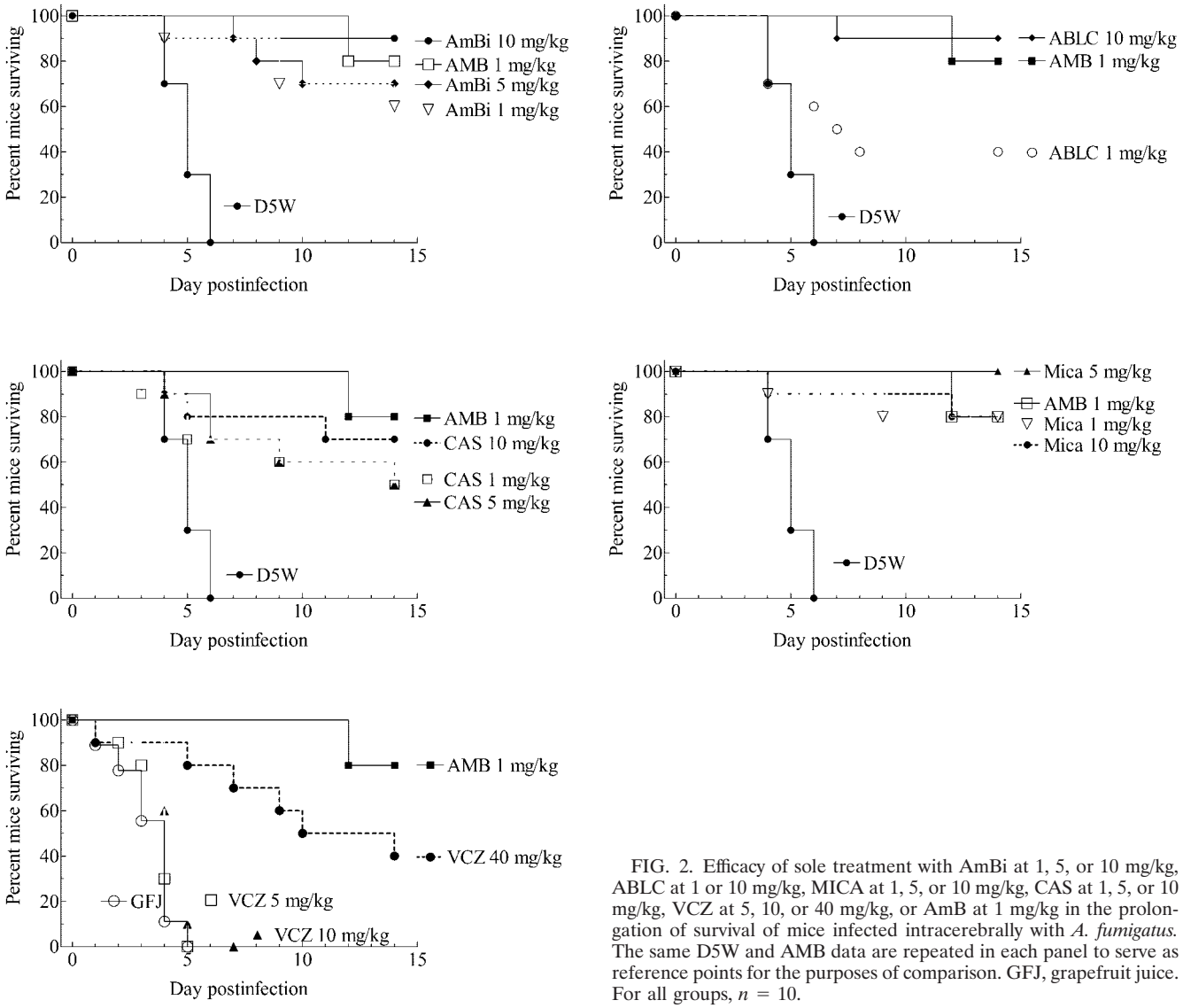

FIG. 2. Efficacy of sole treatment with AmBi at 1, 5, or 10 mg/kg, ABLC at 1 or 10 mg/kg, MICA at 1, 5, or 10 mg/kg, CAS at 1, 5, or 10 mg/kg, VCZ at 5, 10, or 40 mg/kg, or AmB at 1 mg/kg in the prolongation of survival of mice infected intracerebrally with *A. fumigatus*. The same D5W and AMB data are repeated in each panel to serve as reference points for the purposes of comparison. GFJ, grapefruit juice. For all groups, $n = 10$.

highly lethal, with all control animals succumbing to infection. All regimens of AmBi, ABLC, AMB, CAS, and MICA prolonged survival over that of the D5W controls ($P = 0.02$ to 0.0001, dependent on comparison). For VCZ-treated mice, only those given VCZ at 10 or 40 mg/kg had significantly prolonged survival over that of the grapefruit juice controls ($P = 0.008$ or 0.0004, respectively). Because the D5W and grapefruit juice controls were not equivalent, with mice given 100% grapefruit juice in lieu of drinking water dying significantly earlier than did D5W-treated mice ($P = 0.003$), comparisons of VCZ to the other treatments should be interpreted cautiously.

A limited number of comparisons between VCZ (40 mg/kg) and other treatment regimens were done. VCZ proved superior to D5W ($P = 0.001$), whereas AMB, AmBi at 10 mg/kg, ABLC at 10 mg/kg, and MICA at 5 mg/kg proved superior to VCZ ($P = 0.04$ to 0.0004, depending on comparison).

Overall, MICA at 5 mg/kg proved the most effective in the prolongation of survival and was superior to AmBi at 1 or 5 mg/kg, ABLC at 1 mg/kg, CAS at 1 or 5 mg/kg, and VCZ at 40 mg/kg ($P = 0.03$ to 0.004 depending on comparison). Least effective among the treatments were VCZ at 5 or 10 mg/kg and ABLC at 1 mg/kg. AmBi could be considered equivalent to AMB, whereas ABLC is less than 10-fold as efficacious. However, AmBi and ABLC could be considered equivalent.

Clearance of residual fungal infection. The recovery of *A. fumigatus* from the organs of surviving mice is graphed in Fig. 3. These data indicate that no treatment regimen cured more than one mouse of residual infection in the brain and more than four mice of infection in the kidneys. AmBi at 10 mg/kg cured a single mouse of infection in both organs, whereas no other regimen cured any animals. Both AmBi and ABLC appear to be acting in a dose-responsive manner in both the brain and the kidneys, whereas neither MICA nor CAS showed dose responsiveness in the clearance of infection.

Statistical analyses using the highly conservative nonparametric Kruskal-Wallis analysis of variance followed by a Dunn's test for multiple comparisons showed that AmBi at 10

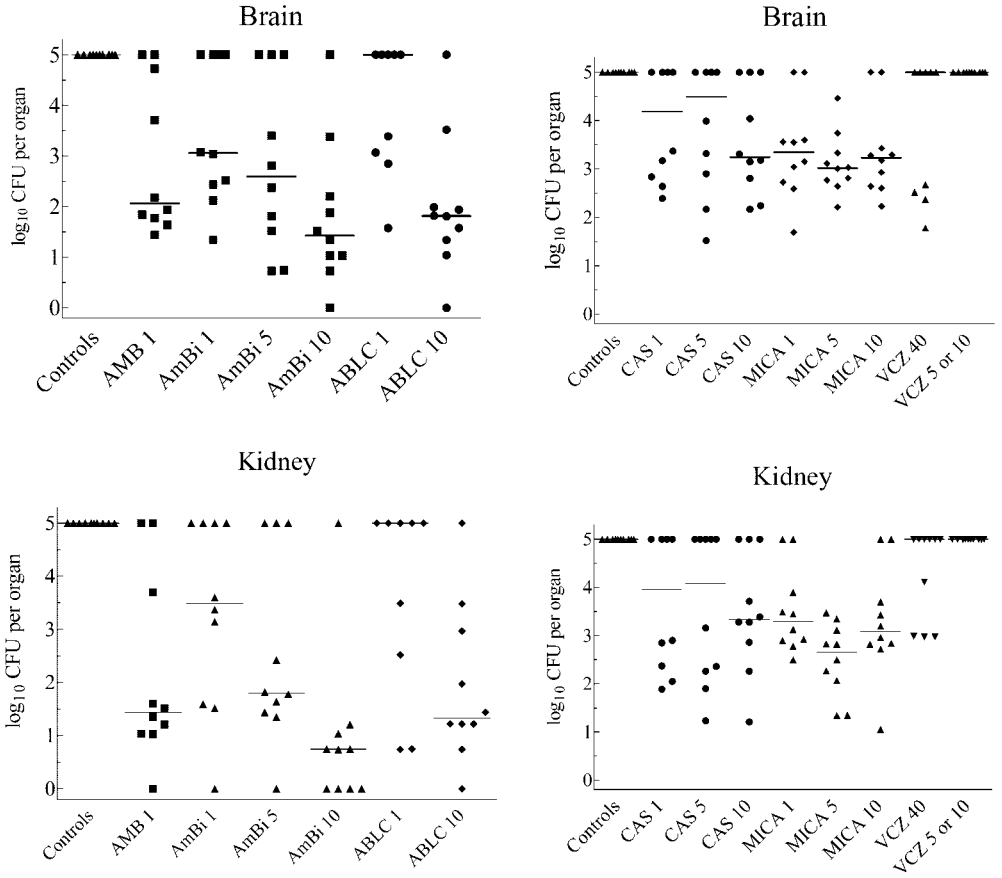

FIG. 3. Recovery of *A. fumigatus* from the organs of surviving mice (see Fig. 2 and legend) given one of the indicated monotherapies. A $\log_{10}$ value of 5 indicates death of the animal, and a value of 0 indicates that the number of CFU present, if any, was below the detectable number of approximately 10 CFU per entire organ. For all groups, $n = 10$. The bar represents the median.

mg/kg and ABLC at 10 mg/kg were the only treatments that significantly reduced the numbers of CFU in both organs ($P < 0.001$) compared with controls. These treatments were also superior to VCZ at 5 or 10 mg/kg in both organs ($P < 0.001$). In the kidneys, AMB and MICA at 5 mg/kg were superior to controls or to VCZ at 5 or 10 mg/kg ($P < 0.05$). Furthermore, AmBi at 10 mg/kg was superior to ABLC at 1 mg/kg or VCZ at 40 mg/kg in the kidneys ($P < 0.05$ and 0.01, respectively). Overall, the CFU data indicate that AmBi and ABLC at 10 mg/kg were most effective in reducing the fungal burden in the organs and were equivalent to each other. However, neither was superior to AMB at 1 mg/kg, and they could then be considered less efficacious on a mg/kg basis. Both CAS and MICA were effective in prolonging survival but not in causing a significant reduction of fungal burden, particularly from the brain.

Drug efficacy with suboptimal-dose combination therapy. The model proved to be highly lethal, with all control animals succumbing to infection by day 5. All regimens, except AmBi alone, prolonged survival over that of the D5W controls ($P = 0.015$ to 0.0001, depending on comparison). Figure 4 presents the Kaplan-Meier plots of the cumulative mortality for each of the respective treatment arms. The monotherapy regimens had higher mortality in the current study than did the same regimens in the monotherapy study. This was likely due to the higher number of conidia in the inoculum. Each of the various combination regimens was shown to be effective in the prolongation of survival. Although each of the echinocandin combinations resulted in greater survival versus controls than did the monotherapies, this was a trend only toward enhanced efficacy, since neither combination showed significantly enhanced survival over the respective monotherapy regimens. Both combinations were superior to AmBi alone ($P = 0.03$ or 0.008) but were equivalent to the respective echinocandins. Similarly, AMB in combination with CAS showed no enhanced efficacy and was equivalent to the respective monotherapies. Significant enhancement of efficacy for the prolongation of survival was attained with the AmBi-plus-VCZ regimen ($P = 0.0001$). The combination was superior to AmBi or VCZ given alone ($P = 0.005$ to 0.0001 depending on comparison). Thus, a demonstration of a potentially useful combination was found.

Clearance of residual fungal infection. The recovery of *A. fumigatus* from surviving mice from the combination therapy study is graphed in Fig. 5. These data indicate that no treatment regimen cured any mice of residual infection in the brain or the kidneys. This reflects the more-severe infection established in the current study versus that of the initial study and the use of nonoptimal dosing regimens.

No monotherapy regimen was significantly better than D5W in the brain. However, each combination regimen was superior

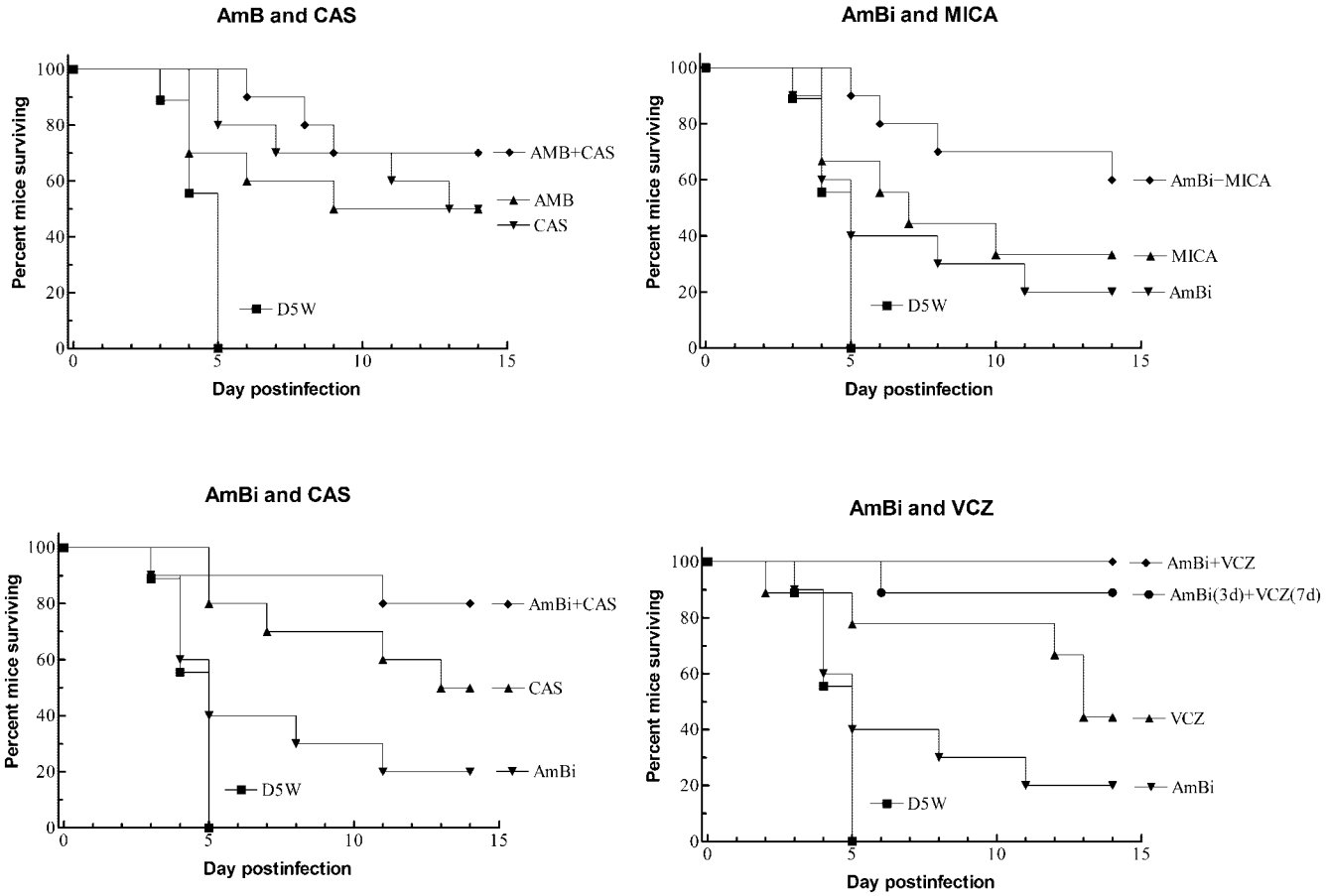

FIG. 4. Efficacies of the various combination therapies and monotherapies in the prolongation of survival of mice infected intracerebrally with *A. fumigatus.* The D5W group is repeated in each panel to serve as reference. Abbreviations: D5W, 5% dextrose water; AMB, 1 mg/kg; GFJ, grapefruit juice; AmBi, 1 mg/kg; VCZ, 40 mg/kg; MICA, 1 mg/kg; CAS, 1 mg/kg. For all groups, $n = 10$.

to D5W ($P < 0.05$ or 0.01), significantly reducing numbers of CFU in brain. Similar to the survival data, only the combination of AmBi plus VCZ achieved enhanced significant efficacy in the reduction of numbers of CFU from the brain and kidneys versus the respective monotherapies ($P < 0.05$). No other combination regimens showed significant enhancement of efficacy over the monotherapies alone.

The regimen of AmBi at 1 mg/kg on days 1 to 3 of treatment followed by VCZ on days 4 to 10 also proved to be efficacious for survival in comparison with the D5W controls ($P = 0.0001$). Comparison of survival showed no significant prolongation of survival by AmBi (3 days) plus VCZ (7 days) versus VCZ alone, but it was better than AmBi alone ($P = 0.0001$); the sequential regimen was equivalent to the AmBi-plus-VCZ combination given for the entire treatment duration. The AmBi (3 days)-plus-VCZ (7 days) regimen also significantly reduced fungal burden in both organs versus the controls ($P < 0.05$). This regimen was superior to AmBi ($P < 0.05$), but not better than VCZ, for both organs, and yet it was equivalent to the AmBi-plus-VCZ combination regimen for both organs. Thus, enhanced efficacy over VCZ monotherapy was not found. Regardless, this initial result is encouraging for the potential of this dose scheme, particularly since no apparent antagonism was noted.

Optimal-dose combination therapy and high-dose AmBisome regimens. These studies were done to determine whether improved efficacy or cure could be attained by using higher dosages of AmBi alone or in combination with CAS or VCZ, using doses that had shown optimal efficacy given as monotherapy. In addition, dosages of AmBi higher than 10 mg/kg were included to determine if improved efficacy or cure could be attained using AmBi alone.

All of the control mice succumbed. All treatment regimens provided significant protection in prolonging survival ($P = 0.01$ to <0.0001). However, no treatment group had 100% survival, with 10 to 50% of treated mice in each treatment group succumbing (Fig. 6). Comparisons among the regimens showed all were equivalent, with the exception that AmBi at 15 mg/kg was superior to CAS at 10 mg/kg ($P = 0.03$). The survival of mice given AmBi alone showed that dose escalation above 15 mg/kg did not improve survival, with more deaths occurring in higher-dosage groups; whether these deaths were due to cumulative toxicity is not known. Mice given AmBi at 10 mg/kg in combination with CAS at 10 mg/kg or VCZ at 40 mg/kg showed no improvement in survival over either monotherapy. These results are somewhat different from those of the suboptimal-dose study, where AmBi at 1 mg/kg and VCZ at 40 mg/kg in combination proved significantly more efficacious, and they may

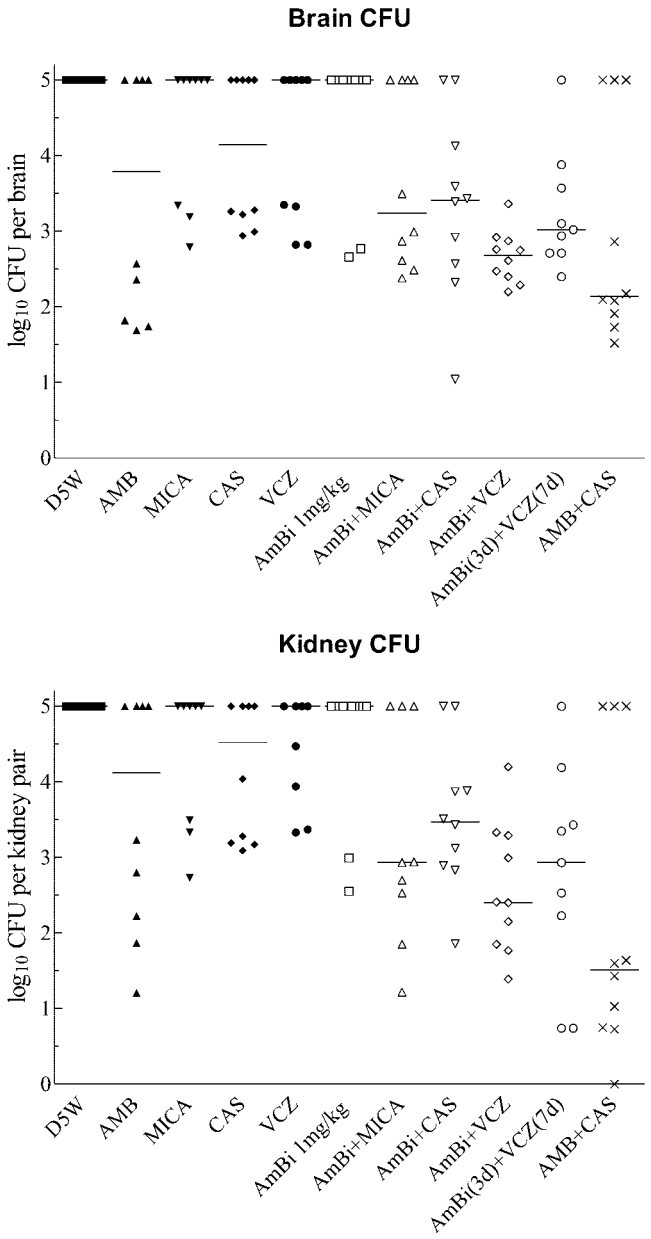

FIG. 5. Recovery of *A. fumigatus* from the organs of surviving mice given one of the indicated monotherapies or combination therapies (see Fig. 4 and legend). A $\log_{10}$ value of 5 indicates death of the animal, and a value of 0 indicates that the number of CFU present, if any, was below the detectable number of approximately 10 CFU per entire organ. For all groups, $n = 10$. The bar represents the median.

relate to the better survival protection with higher doses of monotherapy.

Because one aim of the current study was cure of infection, clearance of CFU from the brains and kidneys of surviving mice was a key parameter of evaluation. Only two mice total from all treatment groups were free of infection in both organs, and in similarity to the other studies, the kidneys were more readily cleared than the brain (data not shown). Interestingly, all surviving mice given 25 mg of AmBi per kg of body weight were free of detectable infection in the kidneys. However, only 50% survival was obtained with that dosage. Reduction of CFU from either organ did not appear solidly dose responsive for escalating doses of AmBi. The use of high doses of AmBi and CAS in combination did not improve efficacy. AmBi and VCZ in combination (together or sequentially) had proven significantly better than monotherapy in the suboptimal-dose study. In contrast to those results, there was no significant enhancement of efficacy by using AmBi at 10 mg/kg and VCZ at 40 mg/kg together or given sequentially, thus indicating no benefit in escalating the dose of AmBi in the combination.

DISCUSSION

Successful treatment of parenchymal fungal disease of the CNS is elusive and may reflect penetration of the drug into the CNS, the type of lesions (i.e., granulomatous or abscess), and the organism causing the disease. Thus, CNS disease caused by an organism like *A. fumigatus*, which grows rapidly and causes an abscess that could limit drug penetration into the lesional sites, is extremely difficult to treat. We studied drug dosages for each antifungal tested that produced blood levels considered to be clinically relevant. CAS pharmacokinetics are very similar across species, and our choice of doses ranged from 1 to 10 mg of CAS per kilogram of body weight; others have shown that a dose of 5 mg/kg results in blood levels similar to those in patients (greater than 1 μg/ml at 24 h postdose) (14, 19, 21, 37); similar serum concentrations of MICA have been reported (15, 20, 30, 31). Although the amphotericin B preparations used in our study have slightly different pharmacokinetics in humans (2–4, 23), the dosages used with the mice in our study result in serum concentrations equivalent to those in humans (32, 34, 36, 48). In addition to these considerations, the dose ranges chosen have been reported to be efficacious in various animal models of aspergillosis (1, 13, 25, 26, 30, 33, 39–41, 51).

The results of our current studies indicate that each of the drugs tested had some efficacy against CNS aspergillosis when used as monotherapy. Both lipid-formulated preparations of amphotericin B were effective at higher dosages, showing them to be potentially useful for treatment of this disease. In similarity to other studies (13), each was less effective on a milligram of amphotericin B-per-kilogram-of-body-weight basis than deoxycholate amphotericin B, and they were equivalent to each other at the highest dose tested. Likewise, both MICA and CAS were found to be highly effective as sole therapy for the prolongation of survival. In spite of this effectiveness, neither showed dose-responsive reduction of CFU numbers in either the brain or the kidneys. We have noted a similar lack of dose-responsive reduction of CFU numbers for these two echinocandins during other studies of systemic aspergillosis (24) and CNS aspergillosis (39) (J. G. Singh, J. Imai, K. V. Clemons, and D. A. Stevens, Abstr. XVth Congr. Int. Soc. Hum. Anim. Mycol., abstr. 144, 2003). Evidence has been provided that the CFU method of determination of fungal burden showed utility similar to those from a quantitative PCR method in demonstrating efficacy (39).

The inclusion of VCZ in our studies was dependent on the utility of administering grapefruit juice to the mice to inhibit rapid VCZ metabolism, which would result in serum concentrations below clinically useful values. Similar to reports by

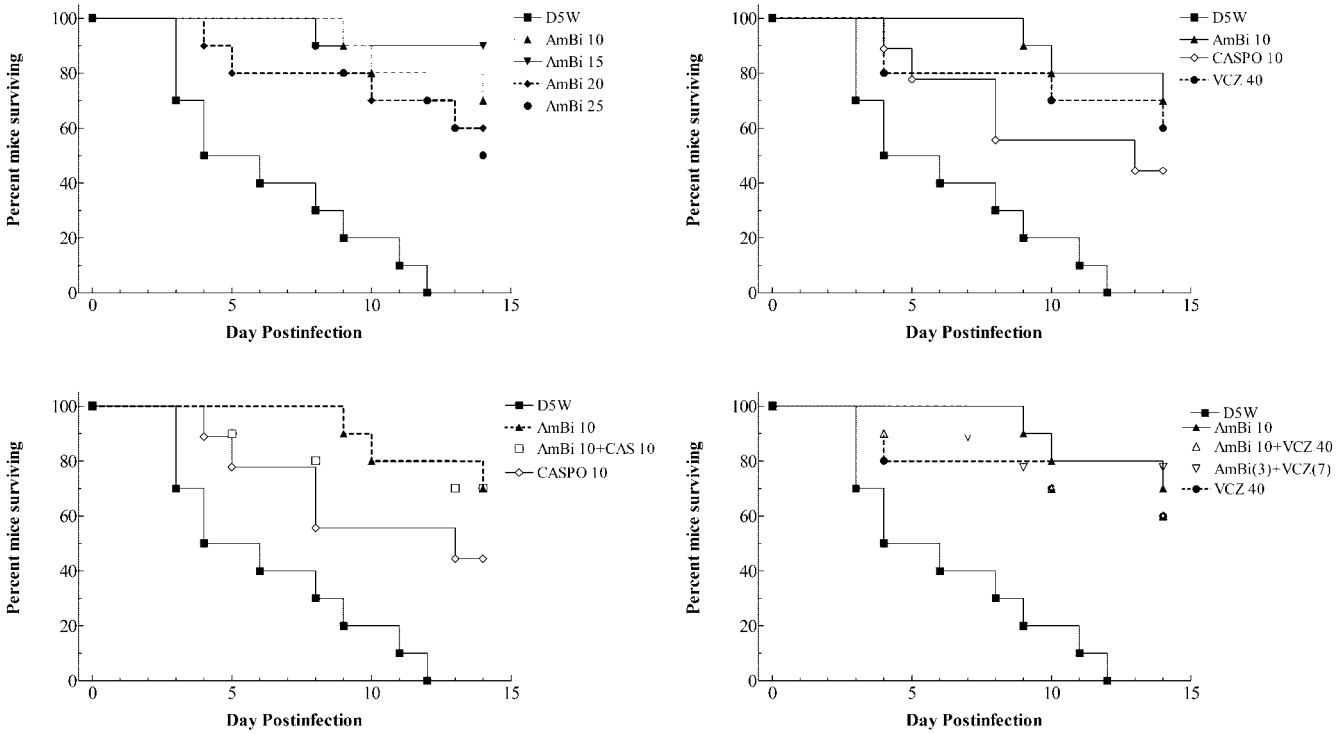

FIG. 6. Cumulative mortalities of mice infected intracerebrally with *A. fumigatus* and given one of the respective treatments. Treatment began 1 day postinfection (day 0). AmBi, 10, 15, 20, or 25 mg/kg; CAS (CAS or CASPO), 10 mg/kg; VCZ, 40 mg/kg. One mouse in each of the CAS 10 and AmBi (3 days)-plus-VCZ (7 days) groups died prior to the beginning of treatment; for each of these groups, $n = 9$. For all other groups, $n = 10$.

other investigators (6, 18, 45, 46), we were able to attain clinically relevant serum concentrations of VCZ (16) and demonstrate efficacy against an experimental infection in mice. However, it should be noted that only the higher dosage of VCZ, 40 mg/kg, showed efficacy in the CNS model and that this correlated with the presence of clinically significant serum concentrations of VCZ. The lack of even better efficacy by VCZ than was seen may be due to the possible increased metabolism of VCZ by the mice during the course of the experiment and daily dosing. Even so, our results substantiate the efficacy and utility of VCZ against aspergillosis, which has become a first-line treatment for aspergillosis (8, 25, 26, 44, 52). Our results further expand its potential as a treatment for the clinical manifestation of CNS disease.

A primary aim of our study was to examine the potential of combination therapy for treatment of CNS aspergillosis. In other studies against experimental invasive pulmonary or systemic aspergillosis, various combinations of drugs have proven effective or at least not antagonistic (40, 41). These combinations include MICA and nikkomycin Z (7), VCZ with MICA or AMB (8, 26), MICA and AmBi (17), and VCZ and CAS (26). Similarly, our results showed that combination therapy was potentially useful, with the survival data and the CFU data indicating that AmBi at 1 mg/kg and VCZ at 40 mg/kg in combination had a significantly enhanced efficacy versus either agent given alone. The addition of CAS or MICA at 1 mg/kg to AmBi at 1 mg/kg or CAS at 1 mg/kg to AMB at 1 mg/kg did not significantly enhance efficacy over the that of echinocandins alone or AMB alone, although there was a trend towards improvement that resulted in the combinations proving superior to controls (D5W). AmBi at the suboptimal dose of 1 mg/kg was not significantly efficacious, whereas VCZ at 40 mg/kg, MICA at 1 mg/kg, and CAS at 1 mg/kg were effective alone. In spite of the enhanced efficacy of the VCZ (40 mg/kg)-plus-AmBi (1 mg/kg) combination, cure was not attained.

Increasing the dosage of AmBi above 15 mg/kg did not improve survival, nor did it cure. However, one could draw the conclusion that 10 to 15 mg/kg of AmBi may be the optimal dose for monotherapy, since lower doses were less effective and higher doses also appear less effective. CAS at 10 mg/kg used in combination with AmBi at 10 mg/kg showed only a trend toward benefit, similar to the results of the suboptimal-dose study. The apparent enhanced efficacy of AmBi and VCZ together was lost when using AmBi at 10 mg/kg rather than 1 mg/kg, with no improvement of efficacy over that of AmBi given alone; likely the enhanced efficacy is most apparent using the low dose of AmBi. However, no obvious antagonism was observed with the higher-dose combination therapies either. These results indicate no benefit to high-dose (>15 mg/kg) AmBi monotherapy and also suggest that combination therapy with AmBi and CAS or VCZ may not be an improvement over sole AmBi therapy.

Another approach to therapy for aspergillosis is that of sequential therapy with different drugs. A patient with *Aspergillus* meningitis given intravenous and intraventricular AMB and intravenous VCZ sequentially showed clinical improvement (49). An i.v.-to-oral switch of agents also mimics a logistically appealing sequence, facilitating step-down of the level of care

as the seriously ill patient responds, and provides a regimen that would enable continuation therapy for an outpatient (43). In previous studies with coccidioidomycosis, we found AmBi superior to AMB for the treatment of coccidioidal meningitis (12). Thus, we chose to examine AmBi as part of a sequential treatment: AmBi initially, followed by VCZ. The order of administration was chosen in part on the basis of the monotherapy results indicating early deaths in the VCZ-treated groups, whereas AmBi-treated mice survived longer initially and deaths occurred later in the course of the experiment. The regimen of AmBi at 1 mg/kg for days 1 to 3 of treatment, followed by VCZ from days 4 to 10, also proved to be efficacious for survival in comparison with controls. Although no significant prolongation of survival by AmBi at 1 mg/kg (3 days) plus VCZ at 40 mg/kg (7 days) versus VCZ alone was found, the combination was better than the suboptimal AmBi regimen alone; the regimen was equivalent to the AmBi (1 mg/kg)-plus-VCZ (40 mg/kg) combination. The AmBi (3 days)-plus-VCZ (7 days) regimen also significantly reduced fungal burden in both organs versus that of the controls. This regimen was superior to AmBi, but not significantly better than VCZ, for both organs, and yet it was equivalent to the AmBi-plus-VCZ combination regimen (the best regimen in these studies) for both organs. Although enhanced efficacy over that of VCZ monotherapy was not found, this might have been due to the suboptimal dosage of AmBi used. Interestingly, we have found similarly enhanced efficacy using another lipid-carried amphotericin B formulation, ABLC, in combination with VCZ (K. V. Clemons, M. Espiritu, R. Parmar, and D. A. Stevens. Abstr. 42nd Annu. Mtg. Infect. Dis. Soc. Am., abstr. 632, p. 165, 2004).

The second study using AmBi, now at 10 mg/kg, for 3 days, followed by VCZ 40 mg/kg for 7 days, showed no enhanced efficacy over those of the respective monotherapies. Regardless, these results are encouraging for the potential of this dose scheme, particularly since no apparent antagonism was noted. Additional studies and various schemes are needed to further clarify the utility of sequential dosing with respect to schedule, drug sequence, and dosages.

In summary, our results further support the potential of combination therapy as a mechanism to improve the outcome in CNS aspergillosis. The combination of suboptimal doses of AmBi and VCZ given concurrently was significantly efficacious in comparison with either monotherapy. In addition, AmBi in combination with MICA or CAS showed strong trends toward improved efficacy. Taken together, these data support additional studies to better define dosages, treatment durations, and the potential of these combinations to attain cure.

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