Efficacy of Abelcet and caspofungin, alone or in combination, against CNS aspergillosis in a murine model

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Objectives: Currently, few options exist to treat central nervous system (CNS) aspergillosis, which is usually fatal. We tested the efficacy of Abelcet and caspofungin, alone and in combination for treatment of this disease.

Methods: Male CD-1 mice were immunosuppressed with 200 mg/kg cyclophosphamide 2 days prior to infection and every 5 days thereafter. In the first study, mice were infected intracerebrally with 2.1 × 10⁶ conidia/mouse of Aspergillus fumigatus; 10 days of once daily therapy began one day later. Groups of 10 received 0.8, 4, or 8 mg/kg of Abelcet, intravenously (iv), or caspofungin, intraperitoneally, 0.8 mg/kg of conventional amphotericin B (AmB) iv, or no treatment. In a second study, mice were challenged with 6.4 × 10⁶ conidia and given no treatment, 8 mg/kg of Abelcet or caspofungin, alone or in combination. On day 14, cfu were determined in survivors by plating of organ homogenates.

Results: In the first study, mice given any regimen of Abelcet or caspofungin had a survival rate ≥80% whereas untreated had 90% mortality. All drug regimens prolonged survival (P ≤ 0.0008) and reduced cfu (P ≤ 0.0001–0.003) recovered from the brains and kidneys compared with untreated. Abelcet showed an apparent dose-related reduction of cfu in the brains. Abelcet at 4 or 8 mg/kg were equivalent to AmB in reducing cfu from both organs (P > 0.05); AmB was superior to 0.8 mg/kg of Abelcet in the brain only (P < 0.02). Abelcet at 8 mg/kg or AmB at 0.8 mg/kg were superior to all regimens of caspofungin in reducing cfu (P ≤ 0.05–0.001). In the second study, Abelcet alone significantly prolonged survival and reduced cfu in the organs versus the controls. Caspofungin did not significantly prolong survival or reduce cfu in comparison with the controls. In combination, Abelcet and caspofungin were equivalent to Abelcet alone.

Conclusions: Abelcet proved to be efficacious, but not curative, in the treatment of CNS aspergillosis and was equivalent overall to conventional AmB. Caspofungin was not as effective against the larger inoculum, but did not enhance or interfere with the efficacy of Abelcet. Since Abelcet displayed dose-responsive efficacy, it is possible higher doses could produce superior results, yet not show toxicity.

Keywords: Aspergillus fumigatus, antifungal combination therapy, echinocandins

Introduction

Aspergillus fumigatus is a ubiquitous filamentous fungus. It can become a serious pathogen for immunodeficient or immunocompromised patients, such as those undergoing bone marrow or solid organ transplants.¹,² Once established, dissemination of this pathogen to the central nervous system (CNS) causes one of the most lethal forms of aspergillosis, where mortality is greater than 95% despite available therapies.¹–⁴ Until recently, conventional deoxycholate-formulated amphotericin B (AmB) has been the first line of therapy for most forms of aspergillosis, such as invasive pulmonary aspergillosis (IPA).⁴ However, AmB demonstrates dose-related nephrotoxicity, which limits the amount and frequency at which it can be administered.⁴–⁷ This poses a severe drawback to the aggressive therapy required to successfully treat CNS aspergillosis.⁴–⁸

With the advent of a murine model for CNS aspergillosis, it is now possible to re-examine antifungal agents already used for...
treatment of other mycoses, to specifically determine their comparative efficacies against A. fumigatus infection of the CNS.3,9,10 For this reason, we examined Abelcet (Amphotericin B Lipid Complex) and caspofungin, independently and in combination, to evaluate their potential in treating this disease. Abelcet is a formulation of standard AmB complexed with two lipids to improve target delivery while reducing toxicity.6,11–14 Caspofungin, an echinocandin, works by disrupting β-glucan synthesis of the fungal cell wall.15,16 Although studies in vitro show that caspofungin is not lethal to all cells in an A. fumigatus mycelial mat,17 it has been shown to be as effective as AmB with little toxicity in treating fungal infections, including aspergillosis.18–21 Caspofungin has been approved as salvage therapy for cases of invasive aspergillosis not resolved by conventional treatment or in patients intolerant to such treatment.20,22 Abelcet has been approved as alternative therapy for aspergillosis in patients who cannot use AmB due to pre-existing renal distress or intolerance.7

Materials and methods

Animals

Five-week-old male CD-1 mice, with an average weight of 22–23 g, were purchased from Charles River Laboratories. Mice were housed five per cage, provided sterilized food, and acidified water ad libitum. Animals were maintained under standard environmental conditions and experiments were performed as approved by the animal care and use committee of the California Institute for Medical Research, which follows the guidelines set forth by the Office of Laboratory Animal Welfare at the National Institutes of Health.

Preparation of the inocula

Aspergillus fumigatus (10AF) was used for both experiments. The isolate was stored at –80°C. Before the start of each experiment, the isolate was grown on potato dextrose agar (PDA) plates at 35°C for 72 h. The conidia were harvested in 0.05% (v/v) Tween 80 saline and stored at 4°C until used.23–25 Colony forming units (cfu) in the conidial suspensions were determined by culture on Sabouraud dextrose agar (SDA) + chloramphenicol (50 mg/L) plates and by haemocytometer count. For experiment 1, the stock was adjusted to a challenge of 2.08 × 10^6 conidia/mouse. For the second experiment, the challenge was 6.35 × 10^6 conidia/mouse. Inocula numbers were confirmed by serial dilutions on SDA plates.

Immunosuppression

All mice were immunosuppressed with 200 mg of cyclophosphamide (Cytoxan, Mead Johnson, Princeton, NJ, USA) per kg body weight by intraperitoneal injection 2 days before infection (day –2).3,9,10 This was re-administered once every 5 days thereafter to maintain neutropenia.

Infection

On day 0, mice were anaesthetized by methoxyflurane vapour inhalation (Metofane, Schering Plough, Union, NJ, USA). The inoculum, in a volume of 50 μL, was administered with a 27-gauge needle through a direct intracerebral puncture, 2–3 mm deep, at a midline point 4–5 mm posterior to the eyes.3,9,10

Drugs

Stock Abelcet (originally provided for these studies by Elan Pharmaceuticals, Inc., Princeton, NJ, now available from Enzon Pharmaceuticals, Inc., Fairfield, NJ, USA) was filtered per manufacturer’s instructions, and dilutions were made in sterile 5% dextrose water (D5W). Caspofungin acetate (Cancidas; Merck, Whitehouse Station, NJ, USA), was diluted in sterile saline. Conventional amphotericin B (AmB) (Fungizone; Bristol-Myers Squibb, Princeton, NJ, USA), was diluted in sterile 5% dextrose water. Abelcet, from the filtered stock, and caspofungin were prepared fresh daily.

Therapy regimens

All therapies began on day 1 and continued once daily for 10 days post-infection. Mice were randomized into groups of 10–11 mice. For the monotherapy study, one group of mice served as untreated controls; a second group was treated with 0.8 mg of AmB per kg of body weight. Previous studies in this laboratory have shown that higher doses of AmB given intravenously (iv) to infected animals produce some toxicity. Additional groups (all n = 10) were treated with regimens of 0.8, 4, or 8 mg of Abelcet per kg of body weight, or 0.8, 4, or 8 mg of caspofungin per kg of body weight. Abelcet and AmB were given iv, while caspofungin was given intraperitoneally, the route used in prior published studies.18,19 For the combination therapy study, one group (n = 11) served as untreated controls. Groups 2, 3 and 4 (all n = 10) were treated with Abelcet at 8 mg/kg, iv + saline, intraperitoneally; caspofungin at 8 mg/kg, intraperitoneally + D5W, iv; or Abelcet at 8 mg/kg, iv + caspofungin at 8 mg/kg, intraperitoneally, respectively. Abelcet or D5W (diluent for Abelcet) were administered iv (0.25 mL/mouse), while caspofungin or saline was administered intraperitoneally (0.2 mL/mouse). Hereafter, these will simply be referred to as Abelcet at 8 mg/kg or caspofungin at 8 mg/kg only, without the additional reference to the diluent control treatment. These diluents were given to ensure that all mice received equal numbers and forms of injections and to eliminate any confounding effects from the diluents.

Mice were observed daily and cumulative mortality was recorded through day 14 post-infection, with the last 4 days being post-therapy observation. All surviving mice were euthanized using CO₂ asphyxia. Brains and kidneys were removed aseptically, placed in 5 mL of sterile saline + penicillin and streptomycin, and mechanically homogenized with a Tissumizer (Tekmar; Cincinnati, OH, USA). Fungal burdens were determined by quantitative plating of these organ homogenates (SDA + chloramphenicol).3,9,24,25 Plates were incubated at 35°C and colonies were counted after 24 and 48 h.

Statistics

Kaplan–Meier survival plots were analysed using a log rank test with GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA, USA). Fungal burdens were converted to log_{10} colony forming units (cfu) per entire organ. Mice not surviving to the conclusion of the studies were assigned an arbitrary number of log_{10} 5 cfu, chosen to exceed cfu in sacrificed mice to assure that death was assumed a worse outcome than survival with any amount of residual fungal burden.26,27 It should be noted that with non-parametric statistics, the actual value assigned does not affect the P value, since its rank is higher than that of any survivor. The resulting data were analysed using the Mann–Whitney U-test (GraphPad Prism). P values were considered significant at the 0.05 level.

Results

Monotherapy therapy study

Prolongation of survival and residual fungal burdens recovered from surviving mice are the standard evaluation end points for determining the efficacy of a drug. The Kaplan–Meier plots in Figure 1 illustrate the survival curves for both untreated mice and those who received various treatment regimens. Ninety percent
mortality was observed in untreated mice by day 10, with only one untreated mouse surviving throughout the study (Figure 1). In contrast, all mice treated with AmB survived, and those treated with any regimen of Abelcet or caspofungin had ≥80% survival (Figure 1). In addition, with the exception of one death that occurred on day 6 for the group of mice treated with Abelcet at 8 mg/kg, initial deaths for all other groups treated with Abelcet did not occur until 3 days after therapy ceased, on day 13 post-infection (Figure 1a). For mice treated with caspofungin, initial deaths occurred only a few days apart, on days 4 and 6; no additional deaths occurred even after treatment ended (Figure 1b). These results indicate that any treatment regimen of Abelcet or caspofungin significantly prolonged survival of mice over that of no treatment ($P \leq 0.0008$). Moreover, survival of mice treated with Abelcet or caspofungin was equivalent to survival of mice treated with AmB ($P > 0.05$).

All surviving mice had detectable cfu in their brains, with the exception of one mouse from the group treated with caspofungin at 4 mg/kg. Although mice treated with any regimen of Abelcet had comparable prolongation of survival among their groups, a distinguishable dose-related reduction of cfu was noted (Figure 2a). Fungal burdens of mice treated with Abelcet at 0.8, 4, or 8 mg/kg had median log$_{10}$ 2.77, 2.19 and 1.73 cfu/brain, respectively. These cfu medians suggested that as the dosages of Abelcet increased, fungal burdens decreased; Abelcet at 8 mg/kg was superior to Abelcet at 0.8 mg/kg ($P = 0.005$), but equivalent to Abelcet at 4 mg/kg. Mice treated with AmB at 0.8 mg/kg had a median fungal burden of log$_{10}$ 1.97 cfu/brain, which was superior to Abelcet at 0.8 mg/kg ($P = 0.02$); higher doses of Abelcet (4 or 8 mg/kg) were equivalent to AmB at 0.8 mg/kg. Mice treated with various regimens of caspofungin showed no significant differences in reducing fungal burdens among their groups, with median log$_{10}$ ranging between 2.46 and 2.74 cfu/brain (Figure 2a).

In the kidneys, the median cfu recovered was generally lower than those in the brain, as would be expected since the brain is the primary site of infection, and the kidneys are the secondary sites. Kidneys from seven treated animals had no detectable infection. Mice treated with Abelcet at 0.8, 4, or 8 mg/kg (Figure 2b) again showed dose-related reduction of $A. fumigatus$, with median log$_{10}$ cfu at 2.03, 1.84 and 0.88, respectively. The median log$_{10}$ cfu for mice treated with AmB was 0.89 cfu/kidneys. Caspofungin showed no dose-related reduction of fungal burdens in the kidneys.
All doses of all drugs significantly reduced the log_{10} cfu recovered from the brains and kidneys of treated mice, compared with untreated controls (P < 0.0001–0.003). Abelcet at 4 or 8 mg/kg was equivalent to AmB in reducing or eliminating fungal burdens. In addition, Abelcet at 8 mg/kg and AmB at 0.8 mg/kg were superior to any dose of caspofungin, in reducing cfu in both organs (P < 0.04–0.001, dependent on comparison).

Combination therapy study

The survival curve (Figure 3) illustrates a mortality of 73% by day 11 for untreated controls, with only two mice surviving to the end of the study. Mice treated with caspofungin at 8 mg/kg had lower survival than the first study, with only 40% surviving to the end of the experiment. In contrast, mice treated with Abelcet at 8 mg/kg or Abelcet at 8 mg/kg + caspofungin at 8 mg/kg both had 90% survival, which was comparable to mice treated with any dose of Abelcet in the first study. Abelcet at 8 mg/kg alone or in combination with caspofungin at 8 mg/kg was efficacious in prolonging survival over no treatment (all P ≤ 0.03).

The scattergrams (Figure 4) of cfu for the brain and kidneys show mice treated with Abelcet at 8 mg/kg or Abelcet at 8 mg/kg + caspofungin at 8 mg/kg had significantly lower median log_{10} cfu than untreated controls or those treated with caspofungin at 8 mg/kg (all P = 0.0005–0.03). Abelcet at 8 mg/kg and Abelcet at 8 mg/kg + caspofungin at 8 mg/kg were not significantly different in their capacity to reduce cfu from either organ (P > 0.05). Thus, the combination of Abelcet and caspofungin showed no significantly increased efficacy over that of Abelcet given alone, nor was antagonism noted.

Discussion

Various animal models of experimental aspergillosis have been used to examine the potential therapeutic efficacy of antifungal agents. Among the newer agents that have shown promise in these models are Abelcet and caspofungin. Abelcet has demonstrated efficacy against systemic and pulmonary aspergillosis experimental models of infection.28–31 In addition, open-label trials with Abelcet have shown equivalent, if not better, results than AmB in treating various other fungal diseases, including IPA, and with less toxicity than AmB.12,14 Similarly, caspofungin had shown efficacy against experimental aspergillosis in preclinical trials,18,19,32 as well as in clinical trials against aspergillosis.20,22 However, little is known about the efficacies of these antifungals for the treatment of CNS aspergillosis. Therefore, our objective was to determine the therapeutic efficacies of these two drugs against CNS aspergillosis established in a murine model.

Our monotherapy study demonstrated that Abelcet and caspofungin were efficacious in treating CNS aspergillosis in a murine model. Furthermore, they had efficacy comparable to each other as well as to AmB in prolonging survival. In humans, fivefold or
greater doses of Abelcet, compared with AmB, are less toxic.\textsuperscript{6,12,14} This would suggest amphotericin B efficacy in CNS aspergillosis could be retained, with less toxicity with Abelcet. A dose-responsive reduction of \textit{A. fumigatus} from key organs of mice treated with the various regimens of Abelcet was observed. However, the highest doses of either drug used in our studies were not curative. Although the cfu data showed dose-responsiveness for Abelcet, and the cfu data for caspofungin were consistent with the survival curves, it has been suggested that cfu determinations may not be an accurate measure of the effect of echinocandins on mycelial organisms, since only the growing parts of the mycelia are affected by the drug,\textsuperscript{17} and the cfu may not reflect this partial inhibition.\textsuperscript{19} However, recent studies indicate the validity of cfu determinations as an end point in echinocandin drug therapy studies.\textsuperscript{33} Comparisons of a cfu method for fungal burden determination with quantitative PCR-based assays of fungal DNA indicate the latter method gives more accurate results correlating with fungal growth in untreated animals, but both methods give similar results in assessing the effects of therapy for aspergillosis.\textsuperscript{33}

Because neither Abelcet nor caspofungin given alone were curative, and both drugs affect different targets of the fungal cell, combination therapy studies were conducted to determine whether an improvement in efficacy, or possibly cure of CNS infection, could be attained over either drug used alone. The combination study verified the superiority of Abelcet at 8 mg/kg to prolong survival and decrease fungal burdens in the organs. Caspofungin at 8 mg/kg did not significantly prolong survival compared with untreated mice, as it had in the first study. Nevertheless, the lower and delayed mortality suggests partial efficacy of caspofungin. The discrepancy between the effectiveness of caspofungin between the two studies could be due to the three-fold higher inoculum administered to the mice in the combination therapy study, resulting in higher tissue burdens, though not necessarily significant differences in survival of animals (e.g. untreated controls). The combination of the two drugs did not show a marked improvement of efficacy over Abelcet at 8 mg/kg given alone.

In summary, these studies show promise for Abelcet or caspofungin in treating CNS aspergillosis. Although no regimen of Abelcet or caspofungin alone or in combination was able to effect cure (complete clearance of pathogen from the animal), no tested regimen displayed signs of cumulative lethal toxicity. The lack of antagonism or observable toxicity by the combination of Abelcet and caspofungin in our studies gives an initial indication these two agents may be used together without adverse effects. Since Abelcet showed dose-responsive efficacy, and no toxicity was noted at the maximal dose studied, it is possible higher doses could produce superior results, yet not show cumulative toxicity. Although data on the safety of higher doses of caspofungin used in experimental models are scant, it is also possible that higher doses of caspofungin could be more efficacious. Additional studies of combination therapy with Abelcet or caspofungin with other antifungal agents could be conducted to ascertain possible synergic or additive effects to promote cure for this CNS disease.

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References

Treatment of CNS aspergillosis


