Dose-response relationships of three amphotericin B formulations in a non-neutropenic murine model of invasive aspergillosis

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New lipid-associated formulations of amphotericin B (AmB) have been developed in order to reduce toxicity and enhance the efficacy of AmB by allowing administration of higher doses of the drug. We determined the in vivo dose-response relationships of 1 day and 7 day treatment of AmB, Ambrisome (AmBi) and Abelcet (ABLC) in a non-neutropenic murine model of invasive aspergillosis by using survival as an endpoint. Female CD-1 mice were infected intravenously 48 h prior to start therapy with Aspergillus fumigatus (1 × 10⁷ conidia/mouse). Groups of 10 mice were treated iv for 1 day or 7 days with increasing 2-fold doses of AmB, ABLC and AmBi up to a maximum of 20 mg/kg/day. Mortality was determined twice daily until day 15. Results were analyzed using product-moment survival analysis and by determining the dose response relationships on day 15. Survival at day 15 of mice with 7 day AmBi or ABLC treatment was significantly better than that of controls or AmB. The ED50s of AmBi and ABLC were 0.06 (95% CI: 0.03−0.127) mg/kg and 0.21 (0.06−0.66) mg/kg respectively. In addition, the maximum effect was higher for AmBi than ABLC, 90% survival versus 68%, respectively. Most of the effects of treatment with AmBi were reached after 1 day of treatment, indicating that the first dose given is most important in predicting survival. This study shows that AmBi and ABLC were significantly more efficacious than AmB in a non-neutropenic murine model of invasive aspergillosis, and that the effect observed was primarily dependent on the first dose administered.

Keywords Aspergillus, amphotericin B, animal model

Introduction

New lipid-associated formulations of amphotericin B (AmB) have been developed in order to reduce toxicity and enhance efficacy of AmB by allowing administration of higher doses of the drug. In addition, because of its lipid formulated properties, the concentration time profiles and the mode of distribution of these formulations differ from AmB depending on the properties of the carrier [1–3]. For instance, the pharmacokinetics of amphotericin B formulated as a unilamellar liposome (AmBisome (AmBi)), a complex ribbon form (Abelcet (ABLC)) or liposomes based on polyethylene glycol (PEG) have been shown to be clearly different, both in serum and in infected organs [4–10]. Indeed, the advantage of the lipid formulations is not only the possibility of higher doses without toxicity, but also its altered pharmacokinetic and pharmacodynamic properties, such as targeting the drug [3,8,11]. However, despite being on the market for a number of years, relatively little is known of the pharmacodynamics and dose-response relationships of the lipid formulations...
with respect to *Aspergillus* infections. There are several reasons for this situation, e.g., *Aspergillus* infections, unlike bacterial infections, develop over a longer period of time. Thus, conclusions as to the effects of the drugs require treatment over days and this in turn, has impeded until recently the development of animal models. If animals are rendered neutropenic, superinfections may arise. Another important factor is the relatively cumbersome way to determine the effect of a drug in the treatment of *Aspergillus* infections. Colony forming unit (CFU) counts, as is usually performed for bacteria, are not very reliable and difficult to standardize because of fungal hyphal growth. Alternatively, PCR based methods to measure fungal burden have been developed recently [8, 12] and seem promising.

To study the pharmacodynamics of AmB and compare those with lipid formulations of the drug, we developed a model of *Aspergillus* infection in immunocompetent mice. We specifically aimed at a chronic infection model that would allow treatment for more than a week, to more realistically compare the effects of drug over time, as opposed to more acute models of infection. Treatment was started 48 h after infection to allow outgrowth of hyphae. The aim of the present study was to determine the differential dose effect relationships of AmB, AmBi and ABLC during a dosing regimen of 1 week.

**Materials and methods**

**Microorganism**

A clinical isolate of *Aspergillus fumigatus* (AZN 8196) was used in all experiments. The isolate was obtained from the private collection of the Department of Medical Microbiology, Radboud University Nijmegen Medical Centre, The Netherlands. The isolate was stored in glycerol broth at ~80°C and was revived by subculturing twice on Sabouraud dextrose agar supplemented with 0.02% chloramphenicol (SDA) for 5–7 days at 35°C. The MIC of AmB for the strain is 1.0 mg/l [13,14].

**Antifungal agent**

Commercial formulations of AmB (Bristol-Myers BV, Woerden, NL), AmBi (Gilead Sciences Int, UK) and ABLC (Wyeth, Hoofddorp, NL) were obtained from the respective manufacturers. Drug solutions were prepared on the day of study following instructions of the manufacturer, diluted with a standard 5% glucose solution to obtain the desired concentration and administered intravenously.

**Animals**

Female CD-1 outbred mice (Charles River Laboratories, Sulzfeld, Germany), weighing 20–29 g, were used for all studies. Animal studies were conducted in accordance with recommendations of the European Community (Directive 86/609/EEC, 24 November 1986), and all animal research procedures were approved by the institutional animal care and use committee of the Radboud University, Nijmegen.

**Infection model**

Non-neutropenic mice were infected with 1×10^7 CFU/mouse, corresponding to the LD<sub>50</sub> by injection of 0.1 ml of a conidial suspension into the orbital vein. Conidia were harvested by washing the agar surface of a 48-hour-old SDA culture with sterile saline containing 0.05% Tween 80. Conidia suspensions were filtered three times through twice folded sterile gauze to remove hyphae, counted in a hemacytometer, and adjusted to the required concentration in sterile saline containing 0.05% Tween 80.

**In vivo efficacy**

Therapy was started 48 h after infection and consisted of a single treatment (except for AmB) or each day for 7 days. Groups of 10 mice were treated intravenously with twofold increasing doses of AmB ranging from 0.125 to 1 mg/kg and an additional dose of 1.5 mg/kg, as compared to dosages of ABLC and AmBi ranging from 0.063–16 mg/kg (7 days treatment) or 0.109–14 mg/kg and 20 mg/kg (1 day treatment). Control mice were infected but received only 5% glucose. Animals were checked twice daily for mortality until 7 days after end of treatment.

**Data analysis**

The relationship between the *in vivo* efficacy (survival) and dose was determined by nonlinear regression analysis using the GraphPad Prism Software 5.0 (Graphpad Inc., San Diego CA). A sigmoid dose-response model (with variable slope) was used with no weighting and model fits compared using the F-test. Survival curves were compared by the log rank test. Statistical significance was accepted at *P*<0.05 (two-tailed).

**Results**

Fig. 1a–c show the survival curves for 7 day treatments with AmB, ABLC and AmBi, respectively. The lowest daily dose that differed significantly from the survival of the controls was 0.063 mg/kg for AmBi and 1 mg/kg for ABLC, while none of the dosing regimens of AmB resulted in a significantly enhanced survival. Fig. 2 shows the relationship between daily dose (mg/kg) and mortality at day 15 for
each of the three formulations. The effect of both AmBi and ABLC was dose dependent and could be well described by the Hill equation, with $R^2$ values of 0.90 and 0.64, respectively. Alternatively, the effect of AmB was measurable at the 1.0 and 1.5 mg/kg dose only and was not significantly different from controls. The dose-effect relationships between AmBi and ABLC were significantly different ($P<0.05$). The ED50s of AmBi and ABLC were 0.06 (95% CI: 0.03–0.127) mg/kg and 0.21 (0.06–0.66) mg/kg, respectively. Although this indicates a possible better activity of AmBi on a mg/kg basis, this difference was not significant ($P=0.104$). The increased scatter at higher ABLC doses is reflected in the relatively wide confidence interval of its ED50. However, the maximum effect in terms of survival was significantly higher ($P=0.05$) for Ambi then for ABLC and was 90 (79–102)% and 68 (51–85)%, respectively.

Fig. 3 shows efficacy of ABLC and AmBi after a single dose. While there was some increased survival after single doses of ABLC, the outcome was not very predictable. A clear dose effect relationship could not be found, although the higher doses did result in increased survival. In contrast, AmBi showed a very clear dose effect relationship and a mortality reduction of 70% was reached at relatively low doses. Interestingly, the dose effect relationship is much steeper than with the 7 day dosing regimen, while the ED50 is only slightly higher, 0.15 mg/kg.

**Discussion**

In this study, we demonstrated that AmBi and ABLC exhibit a dose dependent effect on survival after seven days of treatment in a murine model of invasive aspergillosis, while AmB showed hardly any effect at all. Since we applied doses over the whole dosing range of the drugs, this allowed us to fit a sigmoid dose response model with variable slope (the Hill equation) to the obtained data. This model has been used extensively to describe the effects of antibacterials [15,16] and antifungals against Candida [17–19], but rarely against filamentous fungi [20]. One of the main reasons is that full dose response curves are seldom prepared and comparisons are made using only a few doses [4,8,21–23]. One of the reasons is probably that until recently good quantitative measures of in vivo effect were absent for antifungal agents active against moulds [8,12]. In this study, we used survival as an endpoint, but the disadvantage remains that a relatively large number of animals is needed to determine dose response relationships, stressing the need for reproducibility between experiments. In addition, other effects of the drug formulations (e.g., immunomodulatory) cannot be ruled out [24–26]. It does, however, provide a detailed dose response relationship and also allows for comparison with the differential effect of the drugs.

A comparison of these dose-effect relationships of the three formulations of AmB tested leads to the conclusion that AmB is significantly less efficacious than the two lipid based formulations. Even at the highest doses, the beneficial effect of AmB was almost unmeasurable indicating that the animal model we used was relatively stringent. In contrast, both the lipid-based formulations resulted in a significant increase in survival, be it not 100%. While AmBi was slightly more, but not significantly more, efficacious than ABLC in terms of ED50 the maximum effect reached was significantly higher for AmBi than for ABLC. The effect was also less predictable for ABLC as compared to AmBi, which may have been the reason for this difference. We do not have an explanation for the observation as it was observed during the 7 day, as well as during the 1 day treatment studies. An experimental caveat could be that the inter-experimental error is for some reason larger for ABLC than for AmBi, as the results were obtained over several experiments (for both drugs). Otherwise, the physical-chemistry properties of the two formulations are different resulting in differences in pharmacokinetic and, therefore, pharmacodynamic properties. Nevertheless, this should then also translate to clinical efficacy. Alternatively, higher doses could have caused renal toxicity. However, we did not specifically monitor toxicity in these experiments, since we were interested in the effect of the drugs after prolonged exposure. The duration of therapy in this study was 7 days. The choice of the experimental design was therefore to use a non-neutropenic instead of a neutropenic model, to prevent animals dying from superinfection due to other pathogens, or the necessity to treat them with a variety of antimicrobial agents as prophylaxis [21]. More importantly, there is an increasing number of non-neutropenic patients that develop invasive aspergillosis. Although prolonged neutropenia remains an important risk factor, invasive aspergillosis occurs in patients with severe graft-versus-host disease and, increasingly, in critically ill patients [27]. In our own hospital, more than 60% of Aspergillus infections occur in non-neutropenic patients. Whether the results described in our non-neutropenic model also apply to neutropenic animals remains unclear.

The efficacy of the two lipid formulations was also investigated following a single dose as opposed to 7 days treatment in order to determine a first dose effect. The results indicate that for AmBi in particular, the first dose is important and is therefore primarily dependent on the Cmax. The dose-effect relationship following a single dose of AmBi is much steeper compared to the 7 days treatment, indicating an important first dose effect. The Cmax dependency has also been found in another study using neutropenic animals with a more limited duration of treatment and fungal burden as an endpoint [20]. The 0.25 and 0.5 mg/kg effects of AmBi were very similar when given
Fig. 1 Survival curves of amphotericin B (a, upper panel), abelcet (b, middle panel) and ambisome (c, lower panel). Animals were treated for seven days and observed for 15 days.

either once or 7 day-dosing yielding a survival of approximately 80%. Continuing AmBi after the first day has some additional benefit but a major effect is obtained after the first dose. This may also, apart from other considerations, partly explain the results of Becker et al., who applied an extra dose of AmB at the start of treatment [28].
Fig. 2 Dose effect relationships of amphotericin B (a, upper panel), abelcet (b, middle panel) and ambisome (c, lower panel) after 15 days of observation. Animals were treated for seven days.

Conclusion

We conclude that in this non-neutropenic model of invasive aspergillosis AmBi and ABLC were more efficacious than AmB and that an adequate first dose is the most important predictor for survival.

Fig. 3 Dose effect relationships of abelcet (a, upper panel) and ambisome (b, lower panel) after 15 days of observation. Animals received one dose only.

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Disclosure of conflicts of interest

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