Comparative study on the efficacy of AmBisome and Fungizone in a mouse model of pulmonary aspergillosis

Koji Takemoto1*, Yutaka Yamamoto1, Yutaka Ueda1, Yoshihiro Sumita1, Koichiro Yoshida2 and Yoshihito Niki2

1Discovery Research Laboratories II, Sumitomo Pharmaceuticals Research Division, 1-98, Kasugadenaka 3-chome, Konohana-ku, Osaka 554-0022, Japan; 2Division of Respiratory Disease, Department of Medicine, Kawasaki Medical School, 577, Matsushima, Kurashiki 701-0192, Japan

Received 28 July 2005; returned 25 September 2005; revised 8 November 2005; accepted 23 December 2005

Objectives: The aim of this study was to evaluate the efficacy and tissue concentration of AmBisome and Fungizone in murine pulmonary aspergillosis, and to investigate the localization of AmBisome at the infection site.

Methods: Mice were infected intratracheally with Aspergillus fumigatus. A single dose of each of the antifungals was administered intravenously 4 h after infection. The efficacy of the antifungal treatment was assessed by the pulmonary fungal burden at 20 h post-treatment and the survival time over 1 month. The pulmonary amphotericin B (AMB) concentration was measured until 48 h after administration. The distribution of AmBisome in the lung was evaluated using rhodamine-labelled AmBisome and an anti-AMB antibody.

Results: AmBisome at a dose of ≥1 mg/kg significantly prolonged the survival time of infected mice compared with the control group. At the maximum tolerated dose, 10 mg/kg AmBisome exhibited greater efficacy than 1 mg/kg Fungizone in terms of increasing survival and reducing the fungal burden. The pulmonary AMB concentration of 10 mg/kg AmBisome was higher than that of 1 mg/kg Fungizone. Tissue distribution analysis showed that AmBisome was localized at the infection site in the lung, and this might explain the potent in vivo efficacy in this infection model.

Conclusions: AmBisome is localized at the infection site in the lung and consequently may fully exhibit its in vivo activity. The efficacy of AmBisome is superior to that of Fungizone against pulmonary aspergillosis.

Keywords: liposomal amphotericin B, amphotericin B deoxycholate, Aspergillus fumigatus, localization

Introduction

Pulmonary aspergillosis is an important cause of morbidity and mortality in immunosuppressed patients1–4 and its incidence has gradually increased in recent years.1–3,5 Amphotericin B (AMB) has been considered the ‘gold standard’ therapy for fungal infection, but its use is limited due to its dose-dependent nephrotoxicity.6 Hence, the therapeutic dose is often determined by renal tolerance rather than infection severity.7 The liposomal formulation of AMB (AmBisome), a homogeneous suspension of unilamellar vesicles containing AMB within their hydrophobic membranes, was developed to induce fewer side effects, particularly with regard to reducing renal toxicity, while maintaining AMB efficacy. In addition, as a consequence of AMB integration into the liposomal membrane, AmBisome is known to show different pharmacokinetics from AMB and produces a high plasma concentration and low distribution volume.8

We previously compared the efficacy of AmBisome and AMB deoxycholate (Fungizone) in a mouse model of disseminated aspergillosis.9 Although the MIC of AmBisome against Aspergillus was higher than that of Fungizone, there was no difference in the survival times at a dose of 1 mg/kg, which is the maximum tolerable dose of Fungizone.9 Moreover, at the maximum tolerated dose for AmBisome (10 mg/kg), its protective effect was superior to that of Fungizone at 1 mg/kg.9 It is notable that the in vivo efficacy of the same doses of AmBisome and Fungizone was identical.
Localization of AmBisome at the infection site

whereas the in vitro activity differs between the two. A possible explanation for this discrepancy may be the difference in their pharmacokinetic profiles. However, little is known about the correlation between the pharmacokinetic profile and the in vivo efficacy of AmBisome in a model of aspergillosis.

In this study, we compared the efficacy and tissue AMB concentration of AmBisome and Fungizone in a model of pulmonary aspergillosis, which is a major tissue infection caused by Aspergillus. Moreover, we investigated the distribution of AmBisome in pulmonary fungal infection lesions and its relationship with the in vivo activity of AmBisome.

Materials and methods

Antifungal agents

Liposomal amphotericin B (AmBisome; Gilead Sciences, Inc., San Diams, CA, USA) and AMB deoxycholate (Fungizone; Bristol-Myers K.K., Tokyo, Japan) were stored at 4°C in the dark. Immediately before use, AmBisome and Fungizone were reconstituted with sterile water according to the manufacturer’s instructions to obtain solutions of 4 and 5 g/L, respectively.

N-lissamine rhodamine B sulphonyl phosphoethanolamine-labelled AmBisome (Rho-PE AmBisome) was prepared as described previously. A 2.4 mL sample of 4 g/L AmBisome was lyophilized and then dissolved in 7 mL of chloroform. The suspension was mixed with 0.1 mL of 0.5% 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulphonyl) (Avanti Polar Lipids, Inc., Alabaster, AL, USA) in chloroform. Chloroform was evaporated under nitrogen at room temperature. Liposomes were prepared by hydrating the lipid films with 5 mL of 9% sucrose in 10 mM succinate buffer (pH 5.5) and by sonication for 15 min at 65°C. The liposomes were filtered through a 0.22μm filter. The unincorporated rhodamine-lipid was then removed using a PD-10 column. Rho-PE AmBisome were identical to AmBisome.

These solutions of AmBisome, Rho-PE AmBisome and Fungizone were diluted in 5% glucose immediately before use. In this manuscript, the concentration of antifungals is expressed as that of AMB.

Animals

Male albino ddY mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and were used at 5 weeks of age. The mice were randomized by a stratified sampling method and all animal experiments were performed under institutional animal guidelines.

Organism

Aspergillus fumigatus H11-20 was isolated from a rat dying of spontaneously acquired pulmonary aspergillosis while on steroid treatment. The MICs of AmBisome and Fungizone against A. fumigatus H11-20 were 4 and 1 mg/L, respectively. The susceptibility of this isolate against these agents is typical. Aspergillus conidia were stored at –80°C with silica gel. For each experiment, the fungus was grown at 32°C on Sabouraud dextrose agar (Becton, Dickinson and Company, Sparks, MD, USA), and 3- to 5-day-old conidia were harvested. The conidia were overlaid with 0.1% Tween 80 in water and gently removed with a bent plastic rod, and the suspension was filtered to remove hyphae. The conidia suspension was then centrifuged at 2000 g for 5 min at room temperature. The conidia obtained were re-suspended in sterile saline. Next, the number of conidia was counted on a haemocytometer and adjusted to 2 × 10⁸ or 4 × 10⁸ conidia/mL.

Animal model of pulmonary aspergillosis

Cyclophosphamide (Shionogi & Co., Ltd., Osaka, Japan) was administered subcutaneously at 200 and 100 mg/kg 2 days before and 1 day after infection, respectively, resulting in temporary leucopenia at least until 4 days after infection. On the day of infection, mice were anaesthetized with 90 mg/kg subcutaneous pentobarbital sodium (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). As previously described, a 50 μL aliquot of the conidial suspension was injected into the trachea via a catheter (1 × 10⁷ or 2 × 10⁷ conidia/mouse).

Efficacy of AmBisome and Fungizone against pulmonary aspergillosis

We previously evaluated the tolerated dose of a single administration in ddY mice. The maximum tolerated doses of AmBisome and Fungizone were 10 and 1 mg/kg, respectively, determined from mortality, body weight change, and blood biochemical parameters of renal and hepatic functions.

Mice were treated intravenously with a single dose of antifungals or 5% glucose 4 h after infection. The number of mice that survived for 30 days was recorded.

The pulmonary fungal burden was determined 1 day after infection. Mice were sacrificed by bleeding from the abdominal veins under ether anaesthesia. Lungs were aseptically removed and rinsed with sterile saline. The lungs were weighed and homogenized in four volumes of sterile saline (4 mL/g of tissue) with a Polytron homogenizer (NS-310E; NITI-ON. Co., Ltd, Chiba, Japan) at 30,000 rpm. Ten-fold serial dilutions of lung homogenates were prepared with sterile saline, and 0.05 mL of each dilution was plated onto Sabouraud dextrose agar. The plates were incubated at 32°C for 2 days and the colonies were then enumerated. The lower limit of detection was 1.00 log₁₀ cfu/lung.

Determination of AMB concentration in the lungs and serum

Blood samples were collected 4, 12, 24 and 48 h post-administration by cardiac puncture under ether anaesthesia and lungs were excised as described above. Serum was separated by centrifugation. Tissue samples were frozen at –80°C until the measurement of AMB concentrations by HPLC was carried out.

AMB was extracted and quantified by modifying the procedure of Gondal et al. The internal standard solution was obtained by dissolving 1-amino-4-nitronaphthalene (Sigma-Aldrich, Tokyo, Japan) in methanol at 0.1 g/L. The tissue samples were mixed with four volumes of methanol and 10 μL of the internal standard solution, and were homogenized as described above. The serum samples of 10 mg/kg AmBisome were diluted with blank serum so that their concentrations were within the range of the standard curve. Homogenates were then centrifuged at 10,000 g for 10 min at room temperature. Next, the supernatants were collected and evaporated. The residue was re-suspended in acetoni-trile-2.5 mM EDTA (pH 5.0) (2:3) and centrifuged as described above. The supernatants (50 μL) were separated on a CAPCELL PAK C₁₈ SG120 reverse-phase column (4.6 by 250 mm; Shiseido Co., Tokyo, Japan) with an acetoni-trile-2.5 mM EDTA (pH 5.0) (2:3) mobile phase delivered at 1.0 mL/min. The HPLC system (LC-2010C, Shimadzu Co., Kyoto, Japan) was controlled by CLASS-VP workstation (Shimadzu) and the wavelength for the detection of AMB and the internal standard was 405 nm. AMB concentration was determined from the ratio between the area of AMB and that of the internal standard.

Standard curve samples were prepared with AMB powder (Sigma Chemical Co., St Louis, MO, USA), which was dissolved in methanol/ dimethyl sulphoxide (1:1) and serially diluted with methanol. Blank
serum or lung was mixed with four volumes of methanol, 10 or 20 μL of AMB solution, and 10 μL of internal standard solution. Subsequently, standard curve samples for HPLC analysis were prepared as described above.

Localization of AmBisome at the infection site

A single dose of Rho-PE AmBisome, Fungizone or 5% glucose was administered into the tail veins 4 h after infection. Pulmonary tissues were excised 12 hours later, fixed in 10% neutral-buffered formalin and cryoprotected by sucrose replacement. These tissues were then frozen and sectioned. The fluorescence signal from Rho-PE AmBisome in the section was observed by fluorescence microscopy (BX61, Olympus Optical Co., Tokyo, Japan). For immunohistochemical detection of AMB, frozen sections of formalin-fixed tissue were blocked with Tris-buffered saline (TBS) containing 1% BSA for 15 min at 37°C. Affinity-purified rabbit anti-AMB polyclonal antibody, which cross-reacts with AmBisome (data not shown), was provided by Gilead Science. The sections were incubated with anti-AMB polyclonal antibody (1:5000 dilution) for 1 h at 37°C, and were washed with TBS, treated with biotinylated goat anti-rabbit immunoglobulin G antibody (1:500 dilution) (DakoCytomation, Kyoto, Japan) for 30 min at 37°C, washed with TBS and then allowed to react with fluorescein isothiocyanate-conjugated streptavidin (DakoCytomation) (1:500 dilution) for 30 min at 37°C. After washing with TBS, fluorescence signals from AMB in the sections were observed by fluorescence microscopy. Adjacent serial sections were stained with Gomori methanamine silver (GMS) and fungal infection sites in the pulmonary lesions were observed microscopically.

Statistical analysis

The 50% effective dose (ED₅₀) was calculated by probit analysis of the number of survivors in each group on the final day. The survival time between the control group, AmBisome treatment groups and Fungizone treatment group was compared using the Shirley–Williams test for multiple comparisons, the Wilcoxon rank sum test and Steel’s test between the control group, AmBisome treatment groups and Fungizone (1 mg/kg) for 30 min at 37°C. The ED₅₀ of Fungizone was 4.16 mg/kg (95% confidence intervals, 1.85–14.5 mg/kg). No significant difference in survival time between 1 mg/kg Fungizone and 3 mg/kg AmBisome was observed. However, treatment with AmBisome at 10 mg/kg was superior to Fungizone at 1 mg/kg in terms of survival time (P < 0.05). These observations with the in vivo efficacy of AmBisome and Fungizone were consistent with those seen in our previous study of murine disseminated aspergillosis.

Results

In vivo experiments in murine pulmonary aspergillosis

Survival time. The survival rate and median survival time for this infection model are shown in Table 1 and survival curves are illustrated in Figure 1. All control mice died within 6 days after the infection. The survival times of all drug-treated groups were longer than that of the control group (P < 0.05). A dose-dependent effect of AmBisome was observed: treatment with 1, 3 and 10 mg/kg AmBisome resulted in a survival rate of 10, 50 and 70% at the end of the experiment, respectively. The ED₅₀ of AmBisome was 4.16 mg/kg (95% confidence intervals, 1.85–14.5 mg/kg). No significant difference in survival time between 1 mg/kg Fungizone and 1 mg/kg AmBisome was observed. However, treatment with AmBisome at 10 mg/kg was superior to Fungizone at 1 mg/kg in terms of survival time (P < 0.05). These observations with the in vivo efficacy of AmBisome and Fungizone were consistent with those seen in our previous study of murine disseminated aspergillosis.

Table 1. Efficacy of antifungal treatment in a mouse model of pulmonary aspergillosis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Median (days)</th>
<th>I²</th>
<th>II²</th>
<th>III²</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AmBisome</td>
<td>1 3.5</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 &gt;30</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 &gt;30</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fungizone</td>
<td>1 3</td>
<td>&lt;0.05</td>
<td>R</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All mice were infected with 2 × 10⁷ conidia of A. fumigatus H11-20 via intratracheal inoculation.
Ten mice from each group received antifungals intravenously 4 h after infection.
For 30 days.
R, reference value; NS, not significant (P > 0.05).
Compared with the control by the Shirley–Williams test for multiple comparisons.
Compared with the control by the Wilcoxon rank sum test.
Compared with 1 mg/kg Fungizone by Steel’s test for multiple comparisons.

Figure 1. Survival curves of ddY mice infected intratracheally with A. fumigatus H11-20 at 2 × 10⁷ conidia/mouse. Antifungals were administered intravenously 4 h after infection (n = 10). Filled squares, AmBisome 1 mg/kg; filled triangles, AmBisome 3 mg/kg; filled circles, AmBisome 10 mg/kg; open diamonds, Fungizone 1 mg/kg; open circles, control. *Significantly different from Fungizone 1 mg/kg (P < 0.05 by Steel’s test for multiple comparison).

Serum AMB concentration

The validation data measurement of AMB in serum was as follows. Six-point standard curves (0.05–10 mg/L) were linear with R² > 0.99. The lower limit of quantification was 0.05 mg/L. The precision was 2.2–5.8% and the accuracy was –14.0 to 11.0%.

The serum AMB concentrations in infected mice until 48 h after administration are depicted in Figure 2. The serum AMB concentrations of 1 mg/kg AmBisome (from 0.069 to 5.91 mg/L) and 10 mg/kg AmBisome (from 1.41 to 123 mg/L) were higher than that of 1 mg/kg Fungizone (from 0.134 to 0.479 mg/L), except for 48 h after the administration of 1 mg/kg AmBisome. This tendency was also observed in the previous investigation using normal mice. The volume of distribution at steady state (Vₐ) of AmBisome was smaller than that of Fungizone (1 mg/kg AmBisome,
antifungals at the infection site. Therefore, we investigated the efficacy and distribution of these antifungals. The administration of 10 mg/kg AmBisome resulted in a reduction in the fungal burden than 1 mg/kg Fungizone (log10 6.05 < 0.01). On the other hand, the cfu in the lungs for the 1 mg/kg Fungizone-treated group was slightly lower than the control. In addition, the cfu in the lungs with 2.5 mg/kg AmBisome, 1 mg/kg AmBisome, 1 mg/kg Fungizone and the control was 4.96 ± 0.24, 5.05 ± 0.17, 5.11 ± 0.16 and 5.14 ± 0.19 log10 cfu/lung, respectively (n = 6). Although there was no significant difference among these four groups, the mean fungal burdens in the lungs for both agents were slightly lower than the control. In addition, the cfu in the lungs with 2.5 mg/kg AmBisome-treated group was reduced compared with that with 1 mg/kg Fungizone-treated group. Thus, these results demonstrated a correlation between the survival times and fungicidal activity in the pulmonary tissue.

**Fungal burden in the lungs**

To ascertain drug efficacy at an infection site, the number of viable organisms is generally determined. However, in the case of filamentous fungi, it is difficult to accurately count the number of viable cells using cfu count. One possible reason is that indistinguishable filamentous hyphae composed of many cells appear as a large single colony. Therefore, we investigated the colony morphology in each infection period. No large colonies were detected in the lungs of infected mice on day 1, whereas large colonies were frequently observed on and after day 2. Furthermore, infected mice started to die about 2 days after infection. If the data of dead mice are omitted, the pulmonary fungal burden is underestimated. Taken together, it is thought that the in vivo efficacy of antifungals was appropriately evaluated by analysing the microbiological clearance of *A. fumigatus* 1 day after infection as reported previously.

The numbers of organisms in pulmonary tissue are presented in Figure 3. The administration of 10 mg/kg AmBisome resulted in a reduction in *A. fumigatus* growth compared with the control group (log10 5.72 ± 0.20 versus log10 6.12 ± 0.23 cfu/lung, respectively; P < 0.01). On the other hand, the cfu in the lungs for the 1 mg/kg Fungizone-treated group (log10 6.05 ± 0.34 cfu/lung) was slightly reduced compared with that for control group (not significant). Treatment with 10 mg/kg AmBisome was more effective in reducing the fungal burden than 1 mg/kg Fungizone (P < 0.05).

In another experiment, the number of viable organisms in the lungs of mice given 1 mg/kg of antifungals was measured in the inoculum size of 1 × 107 conidia/mouse 1 day after infection. The cfu in the lungs with 2.5 mg/kg AmBisome, 1 mg/kg AmBisome, 1 mg/kg Fungizone and the control was 4.96 ± 0.24, 5.05 ± 0.17, 5.11 ± 0.16 and 5.14 ± 0.19 log10 cfu/lung, respectively (n = 6). Although there was no significant difference among these four groups, the mean fungal burdens in the lungs for both agents were slightly lower than the control. In addition, the cfu in the lungs with 2.5 mg/kg AmBisome-treated group was reduced compared with that with 1 mg/kg Fungizone-treated group. Thus, these results demonstrated a correlation between the survival times and fungicidal activity in the pulmonary tissue.

**AMB concentration in the lungs**

The validation data measurement of AMB in lung was as follows. Six-point standard curves (0.10–20 mg/kg) were linear with R² > 0.98. The lower limit of quantification was 0.10 mg/kg. The precision was 7.0–9.5% and the accuracy was –12.9 to 14.2%.

The AMB concentrations in the lungs at 4, 12, 24 and 48 h after the administration of AmBisome and Fungizone are shown in Figure 4. The pulmonary AMB concentrations of 1 and 10 mg/kg AmBisome ranged from 0.234 to 0.959 mg/kg of lung and 12.7 to 17.6 mg/kg of lung, respectively. Contrary to our expectations, the pulmonary AMB concentration of 1 mg/kg Fungizone ranged from 1.78 to 2.47 mg/kg of lung and was between that of 1 and 10 mg/kg AmBisome. Moreover, from the viewpoint of the pharmacokinetic/pharmacodynamic profile, the AMB concentration in the lungs of mice treated with 1 mg/kg AmBisome was always lower than its MIC, while that with 1 mg/kg Fungizone was higher than its MIC. In addition, pulmonary AMB concentrations of 10 mg/kg AmBisome and 1 mg/kg Fungizone were over each MIC until 48 h after administration, but the in vivo outcome differed between the two. These results indicated that the survival time and fungal burden of mice treated with antifungals did not differ. The AMB concentrations in the lungs of mice infected intratracheally with *A. fumigatus* H11-20 at 2 × 107 conidia/mouse. Antifungals were administered intravenously 4 h after infection (n = 10). The number of *A. fumigatus* in the lungs was determined 20 h later. The values represent the mean and standard deviation. *Significantly different from control (P < 0.01 by Tukey–Kramer multiple comparisons test). ¶Significantly different from Fungizone 1 mg/kg (P < 0.05 by Tukey–Kramer multiple comparisons test).
Localization of AmBisome at the infection site

To ascertain whether AmBisome was distributed throughout the pulmonary tissue or at the fungal infection site, liposome and AmBisome-derived AMB in a pulmonary section from infected mice were detected using Rho-PE AmBisome and anti-AMB antibody, respectively.

GMS staining and fluorescent images of the lungs at 12 h after administration are presented in Figure 5. With GMS staining, the infection sites were coloured dark blue (Figure 5b and e), and black-stained conidia were observed (Figure 5c). The lungs of mice given 10 mg/kg Rho-PE AmBisome showed bright red fluorescence localized at fungal infection sites [Figure 5; compare (a) with (b)]. In contrast, the pulmonary tissue of mice treated with 5% glucose showed only faint, diffuse autofluorescence [Figure 5; compare (d) with (e)]. In immunohistochemical study, 10 mg/kg Rho-PE AmBisome-derived AMB was detected near areas of fungal infection in the lung using an anti-AMB antibody [Figure 6; compare (a) with (d)]. In addition, the increased dose of 20 mg/kg resulted in a stronger fluorescent signal than 10 mg/kg (data not shown). Meanwhile, no signal was observed in the pulmonary tissue of mice treated with 1 mg/kg Fungizone and 5% glucose, except for autofluorescence (Figure 6b, c, e and f). These results suggested that AmBisome itself was localized at the infected sites, and this distribution might contribute to the in vivo activity of AmBisome against pulmonary aspergillosis.

Discussion

AmBisome is less toxic than Fungizone, particularly with regard to renal impairment. The reduced toxicity of AmBisome may be explained as follows: First, since AmBisome remains as intact liposomes in plasma, the release of AMB molecule is not easily caused by the administration of AmBisome. Second, as a result of the $V_{ss}$, AmBisome is hard to distribute to uninfected tissue, except for liver and spleen, compared with Fungizone. Third, AmBisome is less cytotoxic to mammalian cells than Fungizone. Taken together, it is thought that AmBisome can be administered safely in much higher doses than Fungizone.

This study revealed that AmBisome had a significant protective effect against a model of pulmonary aspergillosis in prolonging survival and reducing the number of organisms in the lungs. Treatment with AmBisome resulted in a dose-dependent increase in the survival rate from 1 to 10 mg/kg. Comparisons of AmBisome and Fungizone showed no difference in the in vivo outcome between both agents at 1 mg/kg. As AmBisome could be safely administered at more than 1 mg/kg, the survival rate of AmBisome at a dose of 3 mg/kg, about one-third of the maximum tolerated dose, was higher than that of Fungizone at the maximum tolerated dose (1 mg/kg). Finally, 10 mg/kg AmBisome demonstrated significantly greater in vivo activity than 1 mg/kg Fungizone. Similar results have been reported by other investigators using a rat or rabbit model of pulmonary aspergillosis. On the other hand, Otsubo et al. reported that AmBisome is not superior to Fungizone for reducing mortality in a rat model. However, since the maximum tolerable dose of AmBisome was not used in the latter study, the in vivo activity of AmBisome might be underestimated.

The distribution analysis of AmBisome in pulmonary tissue using Rho-PE AmBisome and an anti-AMB antibody suggested that AmBisome itself was localized in the pulmonary infection sites. Moreover, using AmBisome encapsulating the aqueous fluorescent dye sulphorhodamine, Adler-Moore et al. also reported that AmBisome is distributed to the infected sites in the kidney with systemic candidiasis, and this supports the above interpretation. Although the AMB concentration in lungs with 1 mg/kg AmBisome was below its MIC, the in vivo efficacy of AmBisome was equivalent to that of Fungizone. Concerning the determination method of tissue AMB concentration, the infected and uninfected sites were mixed together when the tissue homogenate was prepared. Therefore, the AMB concentration at the infected site in the lungs of mice treated with AmBisome might be higher than that of the whole tissue, and probably more than the MIC in the case of 1 mg/kg AmBisome. Unlike AmBisome, no fluorescence signal was observed in a pulmonary section from infected mice treated with Fungizone in our immunohistochemical study. This could be ascribed to the low tissue concentration of Fungizone; the 12 h post-dose lung tissue level of AMB of 1 mg/kg AmBisome was approximately 5-fold lower than that of 10 mg/kg AmBisome. In addition, since the $V_{ss}$ of 1 mg/kg Fungizone (1.37 L/kg) was larger than the sum of plasma and extravascular volume of a mouse (0.230 L/kg), it tends to penetrate throughout the pulmonary tissue, and the degree of localization of Fungizone at fungal infection sites might be smaller than AmBisome. Taken together, although the distribution of Fungizone in the lung was not fully...
elucidated, differences in the pharmacokinetic profile between AmBisome and Fungizone as described above might affect the in vivo outcome of both agents in the model of pulmonary aspergillosis.

In the relationship between in vivo activity and the pulmonary tissue level of antifungals at the maximum tolerated dose, the pulmonary AMB concentrations of 10 mg/kg AmBisome and 1 mg/kg Fungizone were higher than each MIC until 2 days after infection, whereas 10 mg/kg AmBisome exhibited greater efficacy than 1 mg/kg Fungizone in terms of increasing survival and reducing the fungal burden. This may be because the ratio of the mean pulmonary concentration to the MIC for 10 mg/kg AmBisome (values ranging from 3.18 to 4.40) was higher than that for 1 mg/kg Fungizone (values ranging from 1.78 to 2.47). Moreover, the results of AmBisome localization as described above suggest that a difference in drug concentration between 10 mg/kg AmBisome and 1 mg/kg Fungizone at the fungal surround might be much greater than that observed from the whole tissue concentration.

Moreover, pulmonary host defences against Aspergillus spp., including resident pulmonary alveolar macrophages, might involve the in vivo antifungal effect of AmBisome. The elimination of inhaled conidia by macrophages begins 6–12 h after phagocytosis, but 100% killing does not occur within 24 h. AmBisome can kill fungi phagocytosed by macrophages, suggesting that macrophages that take up AmBisome are able to kill more...

Figure 5. Distribution of Rho-PE AmBisome in the lungs of mice infected intratracheally with A. fumigatus H11-20 at 2 × 10⁷ conidia/mouse. Four hours after infection, 10 mg/kg Rho-PE AmBisome (a–c) or 5% glucose (d and e) was administered intravenously, and the lungs were removed 12 h later. Panels (a and d) were examined by fluorescence microscopy; Panels (b and c) and (e), which are adjacent sections of panels (a) and (d), respectively, were stained with GMS and examined by light microscopy. Original magnification: ×1000, panel (c); ×40, all other panels.
Aspergillus than macrophages alone, thus contributing to the in vivo activity of AmBisome against pulmonary aspergillosis.

According to the report by Bekersky et al., three forms of AMB, unbound, protein-bound and liposomal, exist in the body after administration of AmBisome. The percentage of total AMB in plasma that was liposome-associated was >90% until 24 h after administration. The authors discussed that the total AMB pharmacokinetic parameters approximate the pharmacokinetics of the liposomal AMB, because the concentrations of non-liposomal AMB were low compared with total AMB concentrations. Adler-Moore et al. and Boswell et al. also reported that the AmBisome sphere remains intact for 24 h in plasma. These observations indicate that pharmacokinetics of the liposome-associated AMB can be considered as that of the total AMB pharmacokinetics. With this mechanism of action, AmBisome remains intact in the blood and is localized at the infection site in tissues. Disruption of AmBisome is observed after its attachment to the outside of fungal cells, resulting in fungal cell death. In other words, liposome-associated AMB contributes significantly to the exertion of an antifungal effect of AmBisome. On the other...
hand, Fungizone is highly protein bound (>95%). Considering the protein binding, free AMB concentration in lungs of 1 mg/kg Fungizone (<0.124 mg/kg of lung) was below its MIC (1 mg/L). However, since Fungizone had an in vivo activity, its efficacy cannot be simply explained by free AMB concentration. Furthermore, by total AMB concentration, Andes et al. characterized the pharmacodynamic parameter predictive of efficacy of Fungizone in a mouse model of disseminated candidiasis. For these reasons, it is thought that the correlation between the pharmacokinetic profile and the in vivo efficacy of both agents can be discussed by total AMB concentration.

In conclusion, at the maximum tolerated dose, AmBisome was more effective than Fungizone in significantly prolonging the survival time and reducing the fungal burden in a mouse model of pulmonary aspergillosis. The localization of AmBisome at the infection site in the lung may have a positive effect on its in vivo activity. These results suggest that the clinical efficacy of AmBisome is superior to that of Fungizone in the treatment of pulmonary aspergillosis.

Acknowledgements
We appreciate the excellent technical assistance of K. Urasaki, E. Takemoto and Y. Hirai.

Transparency declarations
No declarations were made by the authors of this paper.

References