Prophylactic efficacy of single dose pulmonary administration of amphotericin B inhalation powder in a guinea pig model of invasive pulmonary aspergillosis

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Objectives: Amphotericin B inhalation powder (ABIP) is a novel dry-powder amphotericin B formulation that is directly delivered to the lung, resulting in elevated lung tissue drug concentrations of this polyene. We evaluated the prophylactic efficacy of single dose administration of ABIP in a guinea pig model of invasive pulmonary aspergillosis.

Methods: Guinea pigs were immunosuppressed with cyclophosphamide and cortisone acetate and challenged with Aspergillus fumigatus conidia in an aerosol chamber. Guinea pigs received prophylaxis with a single inhaled dose of ABIP at 0.05, 0.5, 4 or 10 mg/kg administered 24 h prior to infection. Treatment with oral voriconazole at doses of 5 or 10 mg/kg twice daily beginning 24 h post-challenge served as the positive control.

Results: Improvements in survival were observed with ABIP prophylaxis. A single inhaled dose of 4 mg/kg ABIP and treatment with 5 mg/kg voriconazole both improved median and percentage survival compared with untreated controls. In addition, pulmonary fungal burden, as assessed by cfu, quantitative PCR and galactomannan, was also reduced in a dose-dependent fashion with ABIP prophylaxis as well as with both doses of voriconazole treatment.

Conclusions: Single-dose prophylaxis with inhaled ABIP as prophylaxis demonstrated a significant survival advantage and reductions in pulmonary fungal burden in this model of invasive pulmonary aspergillosis. Optimization of the dose and dosing frequency of ABIP dose may help to further enhance the anti-Aspergillus activity of this novel amphotericin B formulation.

Keywords: ABIP, animal model, Aspergillus, prophylaxis

Introduction

Despite the clinical availability of new antifungal agents with potent activity against Aspergillus species (i.e. voriconazole, posaconazole and the echinocandins), invasive aspergillosis remains a significant cause of morbidity and mortality in heavily immunocompromised patients.1–3 Although prompt diagnosis and rapid initiation of antifungal therapy may improve outcomes in invasive aspergillosis,4 disease severity and suboptimal response to available agents have led to the continuing search for other options to counter this invasive mycosis. As part of the expanded spectrum of treatment options, amphotericin B inhalation powder (ABIP; Novartis Pharmaceuticals Corporation, San Carlos, CA, USA; formerly NKTR-024, Nektar Therapeutics, San Francisco, CA, USA) is an investigational dry-powder formulation of amphotericin B that is inhaled directly into the lungs. One favourable characteristic of this novel formulation of amphotericin B is its particle size (~2–5 μm), which is similar to that of Aspergillus fumigatus conidia. Thus, the inhaled powder is deposited within the airways and alveoli of the lungs, similarly to the manner in which fungal conidia deposit within the lungs.5 Targeted delivery of ABIP to the lungs may be advantageous in that it may lead to lung-tissue amphotericin B concentrations that are greater than the MIC for Aspergillus species and other moulds, while avoiding systemic exposure and thus limiting the toxicity normally associated with intravenous administration of this drug.5,7
In this study, we used an established guinea pig model of invasive pulmonary aspergillosis to evaluate the prophylactic efficacy of ABIP, a unique formulation of amphotericin B designed for pulmonary delivery. The effectiveness of this approach was compared with that of untreated control animals and with those treated with voriconazole, the recommended first-line therapy against invasive aspergillosis.

Materials and methods

Organism

We used the A. fumigatus clinical isolate AF293 in all studies. The 48 h amphotericin B and voriconazole MICs were both 0.25 mg/L as determined by the CLSI M38A2 methodology. In preparation for aerosol challenge, AF293 was grown on potato dextrose plates at 37°C for 10 days. Conidia were harvested from the plate surface on the day of infection by washing with 8 mL of 0.9% saline (pH 7.5) with 0.2% Tween 80. Conidia were concentrated by centrifugation, hyphal elements were removed by passing through a sterile glass wool filter and the conidia were then resuspended in 30 mL of the wash solution. Conidial counts were performed using a haemocytometer and verified by plating serial dilutions on potato dextrose agar with overnight incubation at 37°C. The concentration of the final inoculum was 1 × 10⁸ conidia/mL.

Animal model of invasive pulmonary aspergillosis

Two days prior to infection, male Hartley guinea pigs (0.5 kg; Charles River Laboratories, Wilmington, MA, USA) were rendered immunosuppressed with cyclophosphamide (250 mg/kg intraperitoneally; Mead Johnson, Princeton, NJ, USA) and cortisone acetate (250 mg/kg subcutaneously; Sigma, St Louis, MO, USA). Additional doses of cyclophosphamide (200 mg/kg) and cortisone acetate (250 mg/kg) were administered 3 days post-inoculation. Ceftazidime (100 mg/kg/day subcutaneously beginning 2 days before inoculation) and tobramycin (7 mg/kg/day intramuscularly beginning on the third day post-inoculation) were administered to prevent bacterial infections. Guinea pigs were exposed to AF293 conidia at 1 × 10⁸ conidia/mL for 1 h in an aerosol chamber. Seven groups (8–24 guinea pigs per group) were included in this study: (i) untreated controls, and animals treated with (ii) 0.05 mg/kg ABIP, (iii) 0.5 mg/kg ABIP, (iv) 4 mg/kg ABIP, (v) 10 mg/kg ABIP, (vi) 5 mg/kg voriconazole and (vii) 10 mg/kg voriconazole. ABIP groups received a single dose of inhaled drug as prophylaxis 24 h prior to pulmonary inoculation. Animals treated with voriconazole (Pfizer, Inc., New York, NY, USA) received twice-daily administration with doses of 5 or 10 mg/kg beginning 24 h post-inoculation and continuing for 8 days. Animals were then followed without further treatment until 11 days post-inoculation. Guinea pigs were monitored multiple times per day, and any animal that appeared moribund was humanely euthanized. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio, and animals were maintained in accordance with the American Association for Accreditation of Laboratory Animal Care.

Administration of ABIP

ABIP doses in this paper are expressed as mg of active amphotericin B. To generate an aerosol of the ABIP dry-powder formulation (50% amphotericin B in an 18 carbon lipid excipient, distearoylphosphatidylcholine) a rotating brush aerosol generator (RBG 1000, Palas, Inc., Karlsruhe, Germany) was utilized, with samples taken at a flow rate of 2 L/min. Filters were analyzed both gravimetrically and analytically by HPLC to determine particle size and amphotericin B concentration.

Five days prior to dosing with ABIP, all guinea pigs were acclimated to the restraint tubes (CH Technologies, Westwood, NJ, USA) for increasing periods of time. On the day of the single ABIP dose, guinea pigs were placed in an exposure chamber once the air amphotericin B concentration reached the desired level. Guinea pigs were restrained in tubes and exposed for 10 to 50 min to aerosolized ABIP using a nose-only rodent exposure chamber (CH Technologies; Figure 1). Groups of animals, as determined by the desired target dose of ABIP, were dosed and then removed from the exposure chamber after a pre-determined time, based on the predicted delivered dose as determined during a pre-experimental calibration. The actual dose of amphotericin B deposited in the lungs was determined in a separate group of animals 15 min after dosing.

Figure 1. Schematic of the inhalation device and nose-only rodent exposure chamber used to administer ABIP to the guinea pigs.
after the end of the inhalation exposure as follows: 
\[ \text{inhale amphotericin B (mg/kg)} = \frac{(AC \times MV \times T \times DF)}{BW} \]

where AC = air amphotericin B concentration (mg/L), measured on the exposure day, MV = minute volume (L/min) (406 mL/min as measured by plethysmography), T = exposure duration (min), BW = body weight (kg) and DF = deposition fraction (0.15).

**Tissue drug-concentration and treatment efficacy**

Lung tissue amphotericin B concentrations were measured in infected animals that received single doses of ABIP, either 1 h after dose administration, or as the animals succumbed to infection (or at the pre-determined study endpoint, 11 days post-inoculation). Tissue amphotericin B concentrations were measured by liquid chromatography-mass spectrometry (LC-MS/MS) by an independent laboratory (MEDTOX Laboratories, Inc., St Paul, MN, USA). Therapeutic efficacy was assessed by survival and fungal burden. Fungal burden in the lungs was determined post-mortem using semi-quantitative culture (as cfu), quantitative real-time PCR (q-PCR) and galactomannan enzyme immunoassay (EIA). For cfu assessment, 1 g of lung tissue was collected and homogenized (RW 16 basic Overhead Stirrer; IKA® Works Inc., Wilmington, NC, USA) in 9 mL of sterile saline supplemented with gentamicin and chloramphenicol. Serial dilutions of the homogenate were prepared and plated on potato dextrose agar plates, and incubated at 37°C for 24–36 h, after which the number of *A. fumigatus* cfu was determined.

For q-PCR, primary lung homogenates were subjected to a secondary homogenate step using glass beads, to aid release and extraction of cell nuclei from all conidial and hyphal forms present. Aliquots (0.5 mL) of the primary homogenate were bead beaten (3200 rpm) utilizing a Biospec Bead Beater homogenizer (Biospec, Bartlesville, OK, USA) at 3200 rpm for 90 s, and immediately placed on ice. DNA was then extracted from the secondary lung homogenate (0.1 mL) using a QIAamp® DNA Mini Kit (Qiagen®, Valencia, CA, USA) according to manufacturer’s directions. The lung tissue burden of *Aspergillus* DNA per gram of lung tissue (expressed as conidial equivalents) was measured by q-PCR utilizing previously reported primers for AFK51 and AFK52, and the FKS probe that targets a 101 bp fragment of the single copy *A. fumigatus FKS* gene. The standards were derived from genomic DNA obtained from *A. fumigatus* AF293 as previously described. All assays were performed on an Applied Biosystems 7300 Real Time PCR system (Life Technologies Corp, Carlsbad, CA, USA) as previously described.

To measure galactomannan in lung tissue, a 0.4 mL aliquot from each primary lung homogenate was placed into a 1.5 mL microcentrifuge tube and centrifuged at 2300 g for 5 min, after which the supernatant (0.3 mL) was placed into a fresh tube. The galactomannan index, adjusted for sample weight within the homogenate was determined using the Plateia galactomannan EIA kits (Bio-Rad Laboratories, Redman, WA, USA) as previously described.

**Results**

**Lung tissue amphotericin B concentrations**

High lung tissue amphotericin B concentrations were achieved following single dose administration of ABIP. Concentrations measured 1 h post-administration increased in a dose-dependent fashion and ranged from 1.24 to 60.8 μg/g of lung tissue (Table 1). In addition, lung tissue amphotericin B concentrations remained elevated throughout the course of the study. Single doses of 0.5, 4 and 10 mg/kg ABIP resulted in lung tissue amphotericin B concentrations >17-fold higher than the MIC for the isolate used in this study (0.25 mg/L) on the day of terminal assessment, which was 7–12 days after the drug was administered.

**Survival**

A modest survival advantage was observed with ABIP. Single dose administration of 4 mg/kg ABIP showed significantly improved median survival (10 days) and percentage survival (41.7%) compared with untreated controls (8 days and 12.5%; *P* < 0.05) (Figure 2). The improvements in survival observed with the single inhaled dose of 4 mg/kg ABIP were similar to those with twice-daily 5 mg/kg voriconazole treatment (10.5 days and 50% survival; *P* < 0.05). Interestingly, neither the highest dose of ABIP nor that of voriconazole resulted in a significant survival advantage: the median and percentage survival values (6.5 days and 25% for 10 mg/kg ABIP, and 8.5 days and 33.3% for 10 mg/kg voriconazole) were similar to untreated controls.

**Pulmonary fungal burden**

Modest improvements in fungal burden were also observed upon pulmonary administration of ABIP (Figure 3). A single dose of 4 mg/kg ABIP resulted in significant reductions in cfu (3.5 log10 cfu/g), conidial equivalents (6.0 log10 conidial equivalents/g) and galactomannan (galactomannan index 1.3) within the lungs of guinea pigs compared with untreated controls (3.9 log10 cfu/g, 7.6 log10 conidial equivalents/g and galactomannan index 2.4, respectively; *P* < 0.05). Although 10 mg/kg ABIP did lead to significant reductions in the galactomannan index within the lungs, no significant differences in fungal burden were observed as measured by cfu or conidial equivalents. Improvements were also observed with voriconazole treatment, as each dose of this azole resulted in a significant reduction in each...
marker of fungal burden compared with controls. In addition, both 5 and 10 mg/kg voriconazole resulted in lower numbers of conidial equivalents (4.1 and 3.6 log_{10} conidial equivalents/g, respectively) and galactomannan index values (0.6 and 0.7, respectively) than each dose of ABIP.

**Discussion**

Invasive fungal infections are a significant cause of morbidity and mortality in immunocompromised patients, including patients with haematologic malignancies, those undergoing haematopoietic stem cell transplants and solid organ transplant recipients.21–23 These patient populations are at increased risk of pulmonary infections caused by invasive moulds, of which A. fumigatus is a primary cause.2,3 Although new surrogate markers [galactomannan and (1→3)-β-D-glucan] have improved the diagnosis of these infections, a number of limitations exist, and the results of these assays may not be available in a timely fashion at all institutions.19,20 Thus, antifungal prophylaxis in high-risk populations is still commonly used, and this strategy has been shown to be effective in clinical trials. Prophylactic administration of both itraconazole and posaconazole has been shown to reduce the occurrence of invasive fungal infections, including those caused by Aspergillus species.21–23 However, systemic administration of these agents predisposes patients to adverse effects and numerous drug–drug interactions.22,24,25

Recent attention has focused on the direct delivery of antifungal agents to the lungs via inhalation to prevent invasive fungal infections. This targeted delivery has the potential to achieve high antifungal concentrations that are localized in the lungs, thus avoiding toxicities that occur with systemic exposure. Aerosolized administration of amphotericin B deoxycholate, as well as lipid amphotericin formulations, has been shown to result in high lung-tissue amphotericin B concentrations and improvements in survival in animal models of invasive disease.16–18 Additionally, Rijnders et al.29 noted a significant reduction in the incidence of invasive pulmonary aspergillosis in a randomized placebo-controlled clinical trial in haematologic patients with prolonged neutropenia (<500 cells/mm³ for ≥10 days) who received prophylactic, multiple inhaled doses of aerosolized liposomal amphotericin B. Clinically, pulmonary delivery of amphotericin B has gained favour in some transplant centres and has been shown to be relatively safe and effective.30,31 However, this practice currently utilizes intravenous formulations not designed or optimized for pulmonary administration.

ABIP is a dry-powder formulation of amphotericin B specifically designed for pulmonary administration. We used an established guinea pig model of invasive pulmonary aspergillosis to evaluate the prophylactic effectiveness of ABIP across a range of doses, and compared these results with those for untreated controls and animals that received treatment with voriconazole. In this model, which uses a heavy inoculum of A. fumigatus conidia delivered to the lungs by aerosolization, a single prophylactic dose of 4 mg/kg ABIP resulted in a modest survival benefit and reduction in pulmonary fungal burden, as assessed by semi-quantitative culture, q-PCR or galactomannan. In addition, dose-dependent increases in lung tissue amphotericin B concentrations were observed at 1 h and at termination; at 11 days post-inoculation, these were well in excess of the amphotericin B MIC of 0.25 mg/L for all but the lowest dose of ABIP (0.05 mg/kg). These data demonstrate that single-dose prophylaxis with inhaled ABIP was effective as an antifungal in this model of invasive pulmonary aspergillosis; this is consistent with other studies that have assessed single-dose prophylaxis with this amphotericin B formulation. Using invasive aspergillosis in a persistently neutropenic rabbit model, Kugler et al.5 demonstrated that single doses of ABIP (0.5 and 1.5 mg/kg) administered 1 day prior to inoculation improved median survival compared with untreated controls. Similar results have also been observed when ABIP had been administered at longer intervals (up to 12 days) prior to inoculation.7 The feasibility of effective single-dose administration of ABIP could potentially improve the effectiveness and safety of multiple dosing of intravenous formulations of amphotericin B and reduce the deleterious effects of the surfactant-inhibiting property of the deoxycholate moiety in inhaled amphotericin B. Interestingly, in the current
Figure 3. Pulmonary fungal burden as assessed by cfu (a), conidial equivalents by q-PCR (b) and galactomannan (c) in untreated controls (n=24), or in guinea pigs that received a single prophylactic dose of ABIP at 0.05 (n=16), 0.5 (n=16), 4 (n=24) and 10 mg/kg (n=8) by pulmonary delivery 1 day before aerosol inoculation, or of voriconazole (VRC) treatment [5 (n=8) or 10 mg/kg (n=24) twice daily by oral gavage] for 8 days beginning 1 day after inoculation. All values are mean±SEM. *P<0.05 versus untreated controls. CE, conidial equivalents; GMI, galactomannan index.
study, 10 mg/kg ABIP did not significantly improve survival or reduce pulmonary tissue burden as measured by either cfu or conidial equivalent. During the course of the study, a saturation point may have occurred with the 10 mg/kg ABIP dose, as a build-up of dry powder was observed on the hair surrounding the animals’ noses. This may have led to higher concentrations within the upper airways. Although the total lung concentrations were higher with this dose, less drug may have reached the lower airways where the conidia were deposited following pulmonary inoculation. Another potential explanation could be toxicity with this dose. Although not directly assessed in this study, toxicity due to increased systemic exposure is unlikely. A previous study in healthy volunteers who received single ABIP doses of up to 25 mg reported minimal systemic amphotericin B exposure (plasma amphotericin B concentrations <0.025 mg/L). In addition, no changes in markers of pulmonary function (i.e. 1 s forced expiratory volume (FEV1), forced vital capacity (FVC) or forced expiratory flow (FEF25-75)) were observed in healthy volunteers administered with ABIP at up to 50 mg. In this is in contrast to what has been reported with other aerosolized formulations of amphotericin B. Clinical studies have reported decreases in markers of pulmonary function (20% decrease in FEV1 or FVC) and respiratory symptoms (cough, shortness of breath and wheezing) in patients administered aerosolized amphotericin B deoxycholate and amphotericin B/lipid complex. However, we cannot rule out the possibility of pulmonary toxicity with this dose of ABIP.

Although statistically significant compared with untreated controls, the observed improvements in survival and reductions in tissue fungal burden were modest. A previous study that used a different aerosolized amphotericin B formulation also reported improvements in survival with limited reductions in fungal burden. The reasons for this remain unclear but may be related to the extensive protein- and tissue-binding of amphotericin B, which results in reduced free-drug concentrations. In addition, viable fungi have been found within tissue samples collected from patients with invasive disease, despite total amphotericin B concentrations that were markedly above the MIC for the organism recovered.

Overall, prophylaxis with ABIP was effective at improving survival and reducing fungal burden in this guinea pig model of invasive pulmonary aspergillosis. In addition, persistently elevated lung tissue amphotericin B concentrations were achieved following single inhaled doses of this formulation. Additional studies may help to optimize the dose and dosing frequency of ABIP, as well as to evaluate the utility of this formulation as treatment against invasive pulmonary aspergillosis once infection has been established.

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References


