Efficacy of posaconazole and amphotericin B in experimental invasive pulmonary aspergillosis in dexamethasone immunosuppressed rats

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Received 8 May 2007; returned 27 June 2007; revised 6 July 2007; accepted 25 July 2007

Objectives: Invasive pulmonary aspergillosis is associated with high mortality. To assess new antifungal therapy options, animal models have to be developed to assess, in an appropriate setting, the activity of new drugs.

Methods: Male albino CD rats (125–150 g) were fed with a protein-free diet and received dexamethasone thrice weekly subcutaneously during the whole experiment. After 2 weeks, an inoculum of 10^6 conidia of *Aspergillus fumigatus* (H11-20) was injected intratracheally. Antifungal treatment was initiated and continued for a total of 7 days. Animals were grouped in numbers of 10. One group of animals served as untreated control, whereas the others were treated with amphotericin B intraperitoneally (2 and 4 mg/kg) and posaconazole via gavage (2, 4, 10 and 20 mg/kg). Survival and log_{10} cfu/g of the lungs were the endpoints. The strain H11-20 was tested for susceptibility *in vitro* to amphotericin B and posaconazole, respectively. Fungal burden of the lungs was expressed as log_{10} cfu/g. Survival analysis was performed by the Kaplan–Meier method. Differences in fungal burden were assessed by the Mann–Whitney test.

Results: All untreated animals died within a week. Amphotericin B and posaconazole at 2 mg/kg demonstrated survival benefits over control (*P* < 0.01 and *P* < 0.04). Dosages of 4 mg/kg were superior to 2 mg/kg for amphotericin B (*P* = 0.02) and posaconazole (*P* < 0.05), respectively. No further survival benefits were demonstrated beyond dosages of 10 mg/kg. Rats treated with 20 mg/kg posaconazole, however, had a lower fungal burden than all the other treatment groups (*P* = 0.0002).

Conclusions: Posaconazole and amphotericin B are effective in a dosage-dependent manner in this pulmonary aspergillosis model in immunocompromised rats.

Keywords: animal models, *Aspergillus fumigatus*, azoles, polyenes

Introduction

Invasive pulmonary aspergillosis is associated with a high mortality rate in humans.1,2 No reliable *in vitro* testing is available that could make predictions on the efficacy of various therapy options in humans and breakpoints are not yet identified especially for newer antifungal agents. Due to these limitations, animal models must be developed to assess the activity of these drugs in appropriate disease and host settings. Frequently antifungal agents are evaluated in disseminated fungal diseases of animal models.

Therefore, animal models are crucial for the preclinical evaluation of new antifungal agents. Further, the advantages of animal models are their potential for pharmacological and biological evaluation. Additionally, different disease models are accessible.

Posaconazole is an extended-spectrum triazole that demonstrates a broad *in vitro* antifungal spectrum. Posaconazole has shown *in vitro*, *in vivo* and clinical activity against various fungal pathogens, including *Aspergillus* spp., *Candida* spp., *Cryptococcus neoformans*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, zygomycetes and *Fusarium*.3–5

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Amphotericin B has broad activity against numerous fungi, however, its clinical use is frequently limited by an unfavourable safety profile, most notably dose-limiting nephrotoxicity in humans.6,7

An animal model with continuously immunocompromised rats was established to study antifungal agents (posaconazole and amphotericin B) in invasive pulmonary aspergillosis.

**Methods**

The original model consisted of Sprague–Dawley rats which were treated with cortisone acetate, infected intratracheally with $10^6$ conidia of *Aspergillus fumigatus* and followed daily for survival.8 A modification of this model was used, since in our hands, not all rats became ill within the preset time of 7 days when the original dosage of cortisone acetate was administered.

**Preparation of inocula**

*A. fumigatus*, strain H11-20, was used for the experiments. H11-20 was isolated from a rat dying of spontaneously acquired pulmonary aspergillosis while on steroid treatment.9 The isolate was incubated on Sabouraud dextrose agar plates at 35°C for 4–5 days to form conidia. The conidia were harvested in 0.05% (v/v) Tween 80 in saline and filtered through several layers of sterile gauze. Conidia were counted with a haemocytometer. The final suspension was diluted in normal saline to a concentration of $10^7$ conidia/mL. The conidial suspension was used within 24 h and stored until application at 4°C.

**Rats**

Male albino CD rats (Charles River Laboratories, Sulzfeld, Germany; 125–150 g) received doxycycline (200 mg dissolved in 750 mL of drinking water) *ad libitum* for the entire duration of the experiment. All animals were housed in groups of three rats per cage. Cages were inspected daily during the entire experiment. This animal experiment had an approval by the local authorities and was in accordance with laws for animal housing and care.

**Immunosuppression**

Rats were fed a protein-free diet and additionally given 12 mg/kg dexamethasone subcutaneously three times weekly during the whole experiment. After 2 weeks of this pretreatment, the rats were infected with *A. fumigatus* (Figure 1).

**Infection**

Rats were anaesthetized by intraperitoneal injection of ketamine hydrochloride and xylazine. The trachea was revealed by surgery. The appropriate number of conidia in a volume of 0.1 mL of normal saline was slowly administered into the trachea by a 27 gauge needle with a syringe. Rats had fully recovered within 1 h of the procedure, and no deaths resulted from the inoculation procedure. Antifungal treatment was initiated within 1 h after infection and continued for 7 days. Ten rats were in each group; one untreated group served as a control. Rats appearing moribund or surviving 2 weeks after the end of treatment were sacrificed.

**Antifungal treatment**

Posaconazole (Noxafil®; Schering-Plough, Kenilworth, NJ, USA) was made available by Schering-Plough. The suspension had a concentration of 40 mg/mL and is stable at room temperature. The suspension was diluted in sterile water to a concentration of 1 mg/mL and was stored at room temperature in the dark until use. Posaconazole was administered at doses of 2, 4, 10 and 20 mg/kg via gavage. Amphotericin B was purchased as the sodium deoxycholate formulation (Bristol–Myers Squibb, Germany) and was dissolved in 5% dextrose to produce a stock solution of 5 mg/mL. The stock solution was diluted in sterile water to produce the required concentration of 1 mg/mL. The stock solution was stored at 4°C in the dark. Amphotericin B was administered at doses of 2 and 4 mg/kg intraperitoneally. All dilutions were newly prepared for each experimental therapy cycle (1 week).

**Fungal cultures**

The main organ of interest for this experiment was the lung. Organs used for fungal culture were removed using a sterile technique and mechanically homogenized in sterile saline. Homogenates were further diluted in normal saline and quantitatively cultured for 48 h on Sabouraud dextrose agar plates at 35°C. Colonies visible on plates were counted to calculate the remaining fungal burden of the lung. Plates without any visible colonies were kept for an additional 5 days before they were discarded. Fungal burden was expressed as log$_{10}$ cfu/g of lung tissue.

**Susceptibility testing**

Portions of 0.1 mL of each spore suspension were added to microdilution tray wells that contained no drug (control wells) or amphotericin B. Plates were incubated at 35°C for 48 h. The lowest concentration of test drug that prevented visible growth was considered the MIC. Strain H11-20 was tested at least twice against amphotericin B on at least 2 days. Distinct endpoints for amphotericin B were visible after 48 h of incubation.10 An Etest® (AB
Biodisk, Solana, Sweden) was used for testing susceptibility to posaconazole. This test was utilized due to its easier procedure and was performed twice. The Etest is considered consistent with the standard testing by CLSI.11

**Results**

MICs of amphotericin B and posaconazole for _A. fumigatus_ strain H11-20 were 0.4 and 0.25 mg/L, respectively. All untreated and infected animals died within 1 week. The median survival time (days) after infection for the treated groups was longer compared with the untreated control group (Figure 2). Amphotericin B and posaconazole both administered at 2 mg/kg
demonstrated survival benefits over controls (\(P = 0.01\) and \(P = 0.04\), respectively); 4 mg/kg amphotericin B or posaconazole was superior to 2 mg/kg of the same drug (\(P = 0.02\) and \(P < 0.05\), respectively; Log-Rank). No further survival benefit was demonstrated beyond 10 mg/kg posaconazole.

Rats receiving 20 mg/kg posaconazole had a lower fungal burden than all other treatment groups (control versus 20 mg/kg posaconazole: \(P = 0.0002\); Mann–Whitney test) (Figure 3). All other groups showed no significant difference compared with the control group with regard to fungal burden of the lung. No disseminated disease of aspergillosis was seen in this model of invasive pulmonary aspergillosis (Figure 4).

Discussion

This study developed a rat animal model of non-disseminated invasive pulmonary aspergillosis in a non-neutropenic immunosuppressed state. The purpose of this model was to mimic invasive pulmonary aspergillosis, which is the most frequent manifestation of invasive aspergillosis in humans.

The majority of murine and rodent models for invasive aspergillosis use neutropenia and disseminated disease, which seldom occurs in humans as compared with invasive pulmonary disease. These animal models were usually developed to study the activity of antifungal drugs.\(^{12,13}\) So far there is no consensus on which model can be used ideally. Nevertheless, risk factors for invasive aspergillosis have been identified in various host groups including allogeneic stem cell recipients. Such risk factors for this specific risk group are prolonged neutropenia, graft failure, graft versus host disease and steroid exposure.\(^{14,15}\) This animal model was intended to mimic the immune state caused by steroid therapy in such patients. Dexamethasone was chosen since the corticosteroid potency is the highest. Important to that matter was the continuous immunosuppression with steroids since their withdrawal would allow the immune system to rapidly recover.\(^{16}\)

The intratracheal model used in this study provided a fixed spore count to each animal, in contrast to the application of conidia by intranasal aspiration which may be unreliable, or by use of inhalation chambers which have the disadvantage of possible differences in the quantity of conidia inhaled, due to variations in the respiratory rates of the animals. The invasiveness of the intratracheal procedure was well tolerated and no procedural deaths occurred.

Formal pharmacokinetic analyses were not performed in this study, but those results have been reported previously and were used for the treatment range in our model.\(^{17}\) Both posaconazole and amphotericin B do not immediately achieve high and effective tissue levels allowing immediate application of the agents within the hour of conidia application. The results from this animal model confirmed previously published results with posaconazole but in a different immunocompromised setting and

Figure 4. Histopathology of different organs of interest indicating the non-dissemination of fungal disease. Periodic acid-Schiff staining of lung tissue (a), spleen (b), kidney (c) and liver (d). Only in the lung are hyphae detectable; indicated by the arrow.
In our study, posaconazole at the highest dosage tested was able to nearly sterilize the lungs of most rats from *Aspergillus fumigatus*. The non-significant difference in survival rate might be attributable to the low numbers of animals in the group (underpowered study). The comparison of the fungal load in the lower dosage range might be misleading since no fixed time point was set for organ removal.

This model of experimental invasive pulmonary aspergillosis in immunocompromised outbred rats mimicked an immune situation similar to that of patients treated with corticosteroids. Infection without therapy resulted in death within 7 days. Pulmonary infection did not spread to other organs, although this was not studied thoroughly (data not shown). This model could be useful to study disease mechanism and various different therapeutic options. Posaconazole and amphotericin B in this model were effective in a dose-dependent manner.

**Acknowledgements**

This paper was presented in part at the Forty-fifth Annual Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, USA, 2005. This project was supported by an unrestricted grant from Schering-Plough, Kenilworth, NJ, USA, and a donation from the Biermann Family, Wörrstadt, Germany, for which we are deeply indebted. The histopathology pictures were made by Andreas Kreft, Pathology Institute of the University Mainz.

**Funding**

This project was supported by an unrestricted grant from Schering-Plough Research Institute, Kenilworth, NJ, USA and a donation from the Biermann Family, Wörrstadt, Germany.

**Transparency declarations**

A. J. U. was supported by an unrestricted grant from Schering-Plough Research Institute, Kenilworth, NJ, USA; he was also an advisor to Astellas Pharma Inc., Basilea Pharmaceutica, Gilead, MSD, Pfizer and Schering-Plough Corporation (including Essex Pharma, Germany), and has served on speakers’ bureaux for Astellas Pharma Inc., Gilead, MSD, Pfizer and Schering-Plough Corporation (including Essex Pharma, Germany). A. C. is a full-time employee of Schering-Plough Research Institute. The other authors have nothing to disclose.

**References**