Intrapulmonary Posaconazole Penetration at the Infection Site in an Immunosuppressed Murine Model of Invasive Pulmonary Aspergillosis Receiving Oral Prophylactic Regimens

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Adequate penetration to the infection/colonization site is crucial to attain optimal efficacy of posaconazole against Aspergillus fumigatus diseases. We evaluated posaconazole exposure in pulmonary epithelial lining fluid (ELF) in a murine model of invasive pulmonary aspergillosis. The posaconazole exposure (area under the plasma concentration-time curve from time zero to 24 h postinfusion [AUC0–24]) in ELF was 20% to 31% of that in plasma for total drug after the third dose, and the relationship between plasma and ELF exposure was linear ($r^2 = 0.97, P = 0.016$).

Inhalation of Aspergillus fumigatus conidia is the most common route of infection in invasive aspergillosis (IA), with the lung being the primary site of infection. Adequate drug penetration to the infection site is therefore crucial for optimal efficacy. Posaconazole is the recommended drug for prophylaxis of Aspergillus diseases in neutropenic patients and stem cell transplantation (SCT) recipients (1–3). Notably, despite relatively low levels of free (unbound) posaconazole exposure in plasma (4–6), there appears to be an adequate protection (adequate prophylaxis) against infection due to Aspergillus fumigatus with an MIC higher than susceptible breakpoints to posaconazole. This is somewhat surprising, compared to some other triazole antifungals in similar animal models and clinical trials of invasive candidiasis (7). This phenomenon could possibly be related to a higher-than-predicted posaconazole penetration at the infection/colonization site, particularly in case of lung infections. Therefore, we set out to explore the posaconazole concentrations and corresponding exposure in pulmonary epithelial lining fluid (ELF) compared to those in blood levels.

A clinical A. fumigatus isolate (posaconazole MIC of 0.5 mg/liter) obtained from a patient with proven IA was used in the experiments. Strain identification was confirmed by sequence-based analysis, as described previously (8). The isolate had been stored in 10% glycerol broth at −80°C. Before performing the experiment, the isolate was cultured once on Sabouraud dextrose agar (SDA) for 5 to 7 days at 35 to 37°C, and subcultured twice on 15-cm Takashio slants for 5 to 7 days at 35 to 37°C. The conidia were harvested in 20 ml of sterile phosphate-buffered saline (PBS) and 0.1% Tween 80 (Boom B.V., Meppel, the Netherlands). The conidial suspension was filtered through sterile gauze folded four times to remove any hyphae, and the number of conidia was counted in a hemocytometer. After the inoculum was adjusted to the required concentration, the conidial suspension was stored overnight at 4°C. The in vitro antifungal susceptibility test was performed based on the EUCAST guidelines, using a broth microdilution format (9).

A total of 96 outbred CD-1 (Charles River, the Netherlands) female mice, 4 to 5 weeks old, weighing 20 to 22 g, were used. To render the mice neutropenic, cyclophosphamide (150 mg/kg of body weight on day −4 and 100 mg/kg on day −1) was administered. Animals received 4, 8, 16, or 32 mg/kg posaconazole oral solution (Schering-Plough B.V., Boxmeer, the Netherlands) once daily at days $-2$, $-1$, and 0 by oral gavage. On day 0, mice were infected with the A. fumigatus isolate through instillation of conidial suspension in the nares. The infection model was confirmed by 0% survival in survival experiments with untreated control animals ($n = 44$ mice) (10, 11). Blood and bronchoalveolar lavage (BAL) samples were drawn at 8 predefined time points postinfection (0, 0.5, 1, 2, 4, 8, 12, and 24 h, 3 mice per each time point), as described previously (12). Briefly, the blood samples were drawn through the orbital vein into lithium-heparin-containing tubes and were cooled and centrifuged for approximately 10 min at 1,000 × g within 30 min of collection. Plasma was aspirated, transferred in two 2-ml plastic tubes, and stored at −80°C. BAL fluid was obtained using a technique described previously (10, 11). After being sacrificed under isoflurane anesthesia followed by cervical dislocation, the mice were secured on a plastic platform. The trachea was exposed by a 1-cm incision on the ventral neck skin for insertion of the cannula and sutured in place. Lungs were instilled 4 times with 0.5 ml of sterile 0.9% saline, with the fluid being immediately aspirated. The aspirates recovered from the instillations were pooled per mouse, placed on ice after each aliquot, and subsequently stored at −80°C.

Posaconazole concentrations in plasma and BAL fluid were measured by a validated (for human and mouse matrices) ultraperformance liquid chromatography (UPLC) method with fluorescence detection. Details of the analytical assay are described elsewhere (13).

Geometric mean concentrations of total posaconazole in plasma were calculated for each time point ($n = 3$ mice). Peak concentrations in plasma ($C_{\text{max}}$) were directly observed from the data. Pharmacokinetic parameters were derived using noncom-
partmental analysis with Phoenix, version 6.2 (Pharsight, Inc.). The area under the plasma concentration-time curve from time zero to 24 h postinfusion (AUC$_{0-24}$) was determined by use of the log-linear trapezoidal rule. The elimination rate constant was determined by linear regression of the terminal points of the log-linear plasma concentration-time curve. The terminal half-life was defined as ln2 divided by the elimination rate constant. Clearance (CL) was calculated as dose/AUC$_{0-24}$.

Concentrations of total posaconazole in BAL fluid from three mice per time point were determined as described previously (13). Urea in BAL aspirate and plasma was measured utilizing a modified enzymatic assay (QuantiChrom urea assay kit, DIUR-500; BioAssay Systems) (14, 15). The concentration of posaconazole in ELF was then determined by using the ratio of urea concentration in BAL fluid to that in plasma as described previously (10, 11, 14–19): drug concentration$_{ELF}$ = drug concentration$_{BAL}$ × urea$_{plasma}$/urea$_{BAL}$.

All data analyses were performed using Graph-Pad Prism software, version 5.0, for Windows (GraphPad Software, San Diego, CA). A regression analysis was conducted to determine the linearity between posaconazole concentration in blood and ELF. The goodness of fit was checked by use of the $r^2$ value and visual inspection. Statistical significance was defined as a P value of <0.05 (two-tailed). The $C_{max}$ and AUC$_{0-24}$ data were log$_{10}$ transformed to approximate a normal distribution prior to statistical analysis.

All animals were alive at the time of sample collection from 0 to 24 h postchallenge, and 192 blood and BAL samples were obtained.

The drug concentrations in plasma, including the $C_{max}$ of posaconazole, were higher than those in ELF for all dosages (Fig. 1). A significant correlation between mean posaconazole concentrations in plasma and ELF was noted by linear regression analysis ($r^2 = 0.61$, $P < 0.0001$) (Fig. 2a). However, a better correlation was found for AUC$_{0-24}$ in plasma and ELF in a linear fashion for the dosages of 4 to 32 mg/kg ($r^2 = 0.97$, $P = 0.016$) (Fig. 2b).

The total AUC$_{0-24}$ in plasma and ELF is shown in Table 1. The penetration of posaconazole in ELF based on total drug was between 20.21 and 31.39%. At the highest dose (32 mg/kg), the total AUC$_{0-24}$/MIC ratio was 157.56 in ELF for the isolate with an MIC of ≤0.5 mg/liter.

In the present study, we investigated the intrapulmonary posaconazole penetration in an immunosuppressed murine model of invasive pulmonary aspergillosis receiving oral prophylactic regimens. Our results indicate that posaconazole exposure (AUC$_{0-24}$) in ELF was 20% to 31% of that in plasma for total drug, and the relationship between posaconazole and AUC$_{0-24}$ in plasma and ELF was significantly linear ($r^2 = 0.97$).

Our findings are comparable to the results of two clinical observations of Conte et al. (20, 21). In the first study, the plasma and intrapulmonary concentrations of posaconazole were evaluated in 25 healthy adult subjects receiving multiple doses (13 or 14) of oral posaconazole suspension 400 mg twice daily with a high-fat meal (20). The AUC$_{0-12}$ values in ELF and plasma were 18.3 and 21.9 mg · h/liter, respectively, corresponding to an ELF-to-plasma penetration ratio of 86% of total drug. However, the corresponding exposure in alveolar macrophages (AMs) was appreciably higher (range, 46.2 to 87.7 mg/liter; AUC$_{0-12}$ of 715 mg · h/liter). Similarly, in the second study of the above-mentioned group (21),
moved nuclear leukocytes (PMNs) compared to those in plasma (22). Peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNs) demonstrated that administration of posaconazole oral solution resulted in significantly increased intracellular concentrations in the cellular membranes. On the other hand, Farowski et al. demonstrated that lipophilic characteristics of posaconazole increase the penetration of posaconazole into mammalian host cell membranes, which can mediate its efficacy in prophylactic regimens and likely explains the observed discrepancy between posaconazole low serum level and high potency (23). They exposed intracellular cells to posaconazole and removed the extracellular drug prior to infection. Epithelial cells loaded with posaconazole were able to inhibit fungal growth for at least 48 h and were protected from damage caused by infection. Cell-associated posaconazole levels were 40- to 50-fold higher than extracellular levels, and the drug was predominantly detected in cellular membranes. On the other hand, Farowski et al. demonstrated that administration of posaconazole oral solution resulted in significantly increased intracellular concentrations in the peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNs) compared to those in plasma (22). They collected 23 blood samples from 14 patients receiving posaconazole for prophylaxis of fungal infections in order to determine the intracellular concentrations of posaconazole in different compartments of the peripheral blood. These samples were separated by double-discontinuous Ficoll-Hypaque density gradient centrifugation. The intracellular posaconazole concentrations of PBMCs, PMNs, and red blood cells (RBCs) were determined by liquid chromatography-tandem mass spectrometry. The intracellular concentrations of the PBMCs and PMNs were significantly higher than those of surrounding media ($P < 0.001$). The ratios between the intracellular and extracellular concentrations (C/E) were $22.5 \pm 21.2$, $7.66 \pm 6.50$, and $0.09 \pm 0.05$ for the PBMCs, PMNs, and RBCs, respectively.

The exposure-response relationships of posaconazole have been defined previously in experimental models of Aspergillus infections (4, 6, 24). Similar to the other triazoles, posaconazole displays exposure-dependent pharmacodynamic characteristics, for which a free AUC$_{0-24}$/MIC ratio ranging from 1.67 to 1.78 was the value predictive of success associated with half-maximal efficacy. In general, it is accepted that only the unbound fraction of a drug in serum/plasma is pharmacologically active. Considering the high degree of posaconazole protein binding in plasma (98 to 99%) and negligible bounds in ELF, the results of the current study indicated that effective local concentrations might be achieved even at the lowest dose (4 mg/kg) with a free AUC$_{0-24}$/MIC ratio of 1.76 in plasma and 14.69 in ELF, which is probably high enough to prevent infection with *A. fumigatus* isolates with an MIC of $\leq 0.5$. However, for the isolates with resistant phenotypes and higher MIC to posaconazole ($\geq 16$ mg/kg), the obtained free AUC$_{0-24}$/MIC in plasma was $\leq 0.18$ in plasma and $\leq 4.92$ in ELF at the highest dose (32 mg/kg), which indicates the possibility of breakthrough IA.

We conclude that penetration of posaconazole in ELF is relatively high, which is consistent with lipophilic characteristics and its increased intracellular permeability. The high intrapulmonary penetration ratio of posaconazole indicates that this agent is an optimal therapeutic choice in prevention of IA due to azole-susceptible and/or azole-resistant *A. fumigatus*.

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**TABLE 1** Pharmacokinetic parameters of posaconazole in plasma and ELF following 3 days of once daily oral administration of 4, 8, 16, and 32 mg/kg in immunosuppressed mice

<table>
<thead>
<tr>
<th>Posaconazole dose (mg/kg)</th>
<th>$C_{\text{max}}$ (mg/liter) Plasma</th>
<th>AUC$_{0-24}$ mg·h/liter Plasma</th>
<th>AUC$_{0-24}$ ELF</th>
<th>ELF-plasma $C_{\text{max}}$ ratio (%)</th>
<th>ELF-plasma AUC$_{0-24}$ ratio (%)</th>
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<tr>
<td>4</td>
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Posaconazole Penetration in ELF Compared to the Blood

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REFERENCES