We investigated the efficacy of posaconazole prophylaxis in preventing invasive aspergillosis due to azole-resistant *Aspergillus fumigatus* isolates. Using a neutropenic murine model of pulmonary infection, posaconazole prophylaxis was evaluated using three isogenic clinical isolates, with posaconazole MICs of 0.063 mg/liter (wild type), 0.5 mg/liter (F219I mutation), and 16 mg/liter. A fourth isolate harboring TR34/L98H (MIC of 0.5 mg/liter) was also tested. Posaconazole prophylaxis was effective in *A. fumigatus* with posaconazole MICs of ≤0.5 mg/liter, where 100% survival was reached. However, breakthrough infection was observed in mice infected with the isolate for which the posaconazole MIC was >16 mg/liter.

### MATERIALS AND METHODS

#### Fungal isolates.

Four clinical *A. fumigatus* isolates were studied in a neutropenic murine model of pulmonary infection. Three isolates were obtained sequentially from respiratory samples of a patient with aspergillosis. The first isolate exhibited a wild-type phenotype (POS MIC of 0.063 mg/liter) and harbored an A9T mutation in the CYP51A gene, which was not associated with azole resistance. The patient was treated with itraconazole, during which an F219I mutation was gained that corresponded with itraconazole resistance (MIC of >16 mg/liter) and low POS resistance (MIC of 0.5 mg/liter). The patient was then treated with POS, and a phenotype emerged which was POS highly resistant (MIC of >16 mg/liter) although no additional changes in the Cyp51A gene were found. Microsatellite typing indicated that these isolates were isogenic. A fourth genetically unrelated isolate harboring the highly prevalent TR34/L98H resistance mechanism with low POS resistance (MIC of 0.5 mg/liter) was also investigated. This isolate was obtained from a patient with proven IA and was previously evaluated in a nonneutropenic murine model of IA (Table 1). Strain identification was confirmed by sequence-based analysis, as described previously (12). The isolates were stored in 10% glycerol broth at −80°C, and the inoculum was prepared as described before (34). An in vitro antifungal susceptibility test was performed based on the EUCAST guidelines using a broth microdilution format (35).

#### In vitro kinetic growth assay.

In order to rule out important fitness costs associated with the acquisition of resistance mechanisms, the growth characteristics of the four isolates were determined using a previously described microbroth kinetic system (36). Briefly, the inocula were prepared by diluting an overnight culture grown on agar plates with 0.9% NaCl to 1 × 10^6 to 5 × 10^6 CFU/ml. The fungal suspensions were then further diluted in RPMI 1640 medium (with l-glutamine, without sodium bicarbonate) (Sigma, USA) supplemented with 0.165 M morpholinepropanesulfonic acid (MOPS) to give a final inoculum between 0.5 × 10^6 to 5 × 10^6 CFU/ml. The fungal suspensions were then further diluted in RPMI 1640 medium (with l-glutamine, without sodium bicarbonate) (Sigma, USA) supplemented with 0.165 M morpholinepropanesulfonic acid (MOPS) to give a final inoculum between 0.5 × 10^6 to 5 × 10^6 CFU/ml.
TABLE 1 Characteristics of four A. fumigatus isolates used in the prophylaxis model

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Strain</th>
<th>Prior azole exposure</th>
<th>Cyp51A substitution(1)</th>
<th>MIC (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AMB</td>
</tr>
<tr>
<td>1</td>
<td>V74-61</td>
<td>ITC</td>
<td>A97</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>V76-03</td>
<td>ITC</td>
<td>A97, F219I</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>V79-63</td>
<td>POS</td>
<td>A97, F219I</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>V52-35</td>
<td>No</td>
<td>TR6a/L98H</td>
<td>1</td>
</tr>
</tbody>
</table>

* Isolates 1, 2, and 3 were isogenic and recovered from an aspergilloma patient (29). Isolate number 4 was unrelated to isolates 1 to 3 and was obtained from a patient with proven invasive aspergillosis.

* Isolates 1 to 3 harbored an A9T mutation Cyp51A, but this was found not to be associated with a change of the susceptibility to azoles. The F219I mutation was found to be associated with itraconazole resistance (29).

During POS therapy the phenotype changed for voriconazole (VRC) and associated with a change of the susceptibility to azoles. The F219I mutation was found.

During POS therapy the phenotype changed for voriconazole (VRC) and associated with a change of the susceptibility to azoles. The F219I mutation was found.

During POS therapy the phenotype changed for voriconazole (VRC) and associated with a change of the susceptibility to azoles. The F219I mutation was found.

During POS therapy the phenotype changed for voriconazole (VRC) and associated with a change of the susceptibility to azoles. The F219I mutation was found.

During POS therapy the phenotype changed for voriconazole (VRC) and associated with a change of the susceptibility to azoles. The F219I mutation was found.

During POS therapy the phenotype changed for voriconazole (VRC) and associated with a change of the susceptibility to azoles. The F219I mutation was found.

During POS therapy the phenotype changed for voriconazole (VRC) and associated with a change of the susceptibility to azoles. The F219I mutation was found.

During POS therapy the phenotype changed for voriconazole (VRC) and associated with a change of the susceptibility to azoles. The F219I mutation was found.
**RESULTS**

*In vitro* susceptibility and fitness. The characteristics and *in vitro* susceptibilities of the four *A. fumigatus* isolates are shown in Table 1. All isolates grew well after 48 h of incubation at 35 to 37°C. The three isolates showed increasing POS MICs although the increase from 0.5 mg/liter to 16 mg/liter was not associated with additional mutations in the *cyp51A* gene. Growth curves did not differ in shape and growth rates between the wild-type and two POS-resistant isogenic *A. fumigatus* isolates. Similar growth characteristics were also observed for the nonisogenic isolate harboring the TR34/L98H resistance mechanism (data not shown).

**PK of POS.** The PK parameters of POS prophylaxis are shown in Table 2. The penetration of POS in ELF based on total drug was between 20.21 and 31.39%. At the range of 4 to 32 mg/kg dosing regimens, the total AUC0–24/MIC was 145.4 to 581 in plasma and 29.38 to 157.56 in ELF for the isolates with MICs of ≤0.5 mg/liter. As a comparison, the recommended dose of the oral suspension of POS is 200 mg three times a day for the prophylaxis of invasive fungal infections, which corresponds to an AUC of 115.06 mg · h/liter and a Cmax of 0.58 mg/liter in plasma (6).

**Efficacy of POS prophylaxis.** (i) Survival curves. Figure 1 shows the survival curves of POS-treated mice by dose. The survival curves for all control groups receiving 0.9% saline orally showed a mortality of 100%. The survival at day 10 postinfection was significantly better for POS-treated mice than for controls (Fig. 1). A dose-response relationship was observed for each isolate. The maximum effect (100% survival) was reached at a dose of 16 mg/kg for the isolates with MICs of ≤0.5 mg/liter, independent of the corresponding genotype. Yet for the isolate with the POS MIC of >16 mg/liter, maximum effect was lower (less than 70%) than for other isolates, even with the highest dose (32 mg/kg).

(ii) Dose-response analysis. The dose-response curves for dosing regimens and control groups of POS prophylaxis are shown in Fig. 2. POS prophylaxis improved the survival of the mice in a dose-dependent manner. A dose-response relationship was observed that depended on the POS dose level but was inde-
dependent of the genotypes and azole resistance mechanisms. The
Hill equation with a variable slope fitted the relationship between
the dose and 10-day survival data well, with $R^2$ values of 0.96
(V74-61, wild type; MIC of 0.63 mg/liter POS), 0.98 (V76-03,
F219I mutation; MIC of 0.5 mg/liter POS), 0.95 (V79-63, F219I
mutation; MIC of $>16$ mg/liter POS), and 0.97 (V52-35, TR34/
L98H mutation; MIC of 0.5 mg/liter POS), respectively. The 50%
effective dose (ED$_{50}$) was 3.35 mg/kg (95% confidence interval
[CI], 2.19 to 5.12 mg/kg), 2.83 mg/kg (95% CI, 1.81 to 4.43 mg/
kg), and 14.08 mg/kg (95% CI, 9.06 to 21.90 mg/kg) for three
genetically identical isolates with wild type phenotype and low and
high POS resistance, respectively, and 3.03 mg/kg (95% CI, 1.91 to
4.80 mg/kg) for the isolate with different genotype and low resis-
tance to POS.

(iii) Exposure-response analysis. The AUC for each dose, de-
determined from PK experiments (Table 2), was used to calculate
the AUC$_{0-24}$/MIC ratio for each isolate, as shown in Fig. 3. The
exposure-response relationship had a sigmoidal shape. Increased
POS exposure was required to obtain maximum efficacy in mice
infected with the isolate with a MIC of $>16$ mg/liter compared to
those infected with the isolate with a MIC of $\leq0.5$ mg/liter.
The Hill-type model with a variable slope fitted the relation-
ship between the 24-h AUC/MIC ratio and 14-day survival well,
with an $R^2$ value of 0.77 ($P < 0.05$). The 50% effective AUC for
POS prophylaxis was 37.38 (95% confidence interval [CI], 7.130
to 196). We also determined the relationship between the in vivo
efficacy and the peak level $C_{\text{max}}$/MIC (50% effective concentra-
tion [EC$_{50}$] of 1.74; CI, 0.073 to 41.28; $R^2$ value of 0.76). However, the
AUC$_{0-24}$/MIC appeared to be the most important pharmacodynamic index correlating with prophylaxis, which was significantly different between *A. fumigatus* isolates with POS MICs of $\leq 0.5$ mg/liter and the isolate with a POS MIC of $16$ mg/liter ($P < 0.05$).

Notably, in the current prophylaxis study, the 50% effective total AUC$_{0-24}$/MIC was 93.58 (95% CI, 13.90 to 629.9) in order to prevent invasive pulmonary infection caused by the *A. fumigatus* isolate (V 52-35) with a POS MIC of 0.5 mg/liter. However, in preclinical treatment studies, we have previously shown that a two-times-higher exposure was required to treat infection caused by this isolate ($E_{50}$ of 184.2 [95% CI, 33.21 to 1022]) (Fig. 4).

(iv) Comparative efficacy of POS prophylaxis against the four isolates. In order to compare the efficacy of POS prophylaxis in preventing infection caused by the different isolates, the best-fit values for the curves were defined based on the $E_{50}$, $E_{80}$, and $E_{90}$ of the AUC of POS and compared to each other (Table 3). The efficacy of POS prophylaxis was significantly different between *A. fumigatus* isolates with POS MICs of $\leq 0.5$ mg/liter and the isolate with a POS MIC of $>16$ mg/liter ($P < 0.05$). The null hypothesis was rejected in an $F$ test ($P = 0.0014$, $F = 10.72$, degrees of freedom, numerator $[df_n] = 3$; degrees of freedom, denominator $[df_d] = 11$), indicating that $E_{50}$ was significantly different (Table 3).

**DISCUSSION**

Our model indicated that the efficacy of POS prophylaxis in low-resistant isolates (MIC of 0.5 mg/liter) was similar to that of mice infected with the isolate with the wild-type phenotype. In a previous study that investigated the efficacy of POS treatment of azole-resistant isolates, we observed that the efficacy of POS was reduced in mice infected with the isolate harboring the TR34/L98H resistance mechanism (MIC of 0.5 mg/liter) compared to that of mice infected with a wild-type isolate, indicating that higher doses of POS were required to achieve efficacy similar to that for the wild-type-infected mice (24). Our current model indicates that when

**TABLE 3** The comparison of efficacy of POS prophylaxis between four *A. fumigatus* isolates based on the $E_{50}$, $E_{80}$, and $E_{90}$ values of the 24-h AUC$^a$

<table>
<thead>
<tr>
<th>MIC group and <em>A. fumigatus</em> strain (mutation [POS MIC])$^b$</th>
<th>$E_{50}$ (95% CI)</th>
<th>$E_{80}$ (95% CI)</th>
<th>$E_{90}$ (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC $\leq 0.5$ mg/liter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V 74-61 (A9T [0.63])</td>
<td>16.36 (1.28 to 21.97)</td>
<td>31.85 (21.16 to 47.94)</td>
<td>47.03 (26.14 to 84.61)</td>
</tr>
<tr>
<td>V 76-03 (A9T F219I [0.5])</td>
<td>13.60 (9.29 to 19.91)</td>
<td>29.18 (18.87 to 45.12)</td>
<td>45.60 (23.40 to 88.88)</td>
</tr>
<tr>
<td>V 52-35 (TR34/L98H [0.5])</td>
<td>13.81 (8.92 to 21.38)</td>
<td>33.80 (21.68 to 52.70)</td>
<td>57.06 (28.95 to 112.5)</td>
</tr>
<tr>
<td>MIC $&gt; 16$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V 79-63 (A9T F219I [&gt;16])</td>
<td>51.22 (38.44 to 68.24)</td>
<td>135.8 (74.38 to 247.9)</td>
<td>240.2 (96.39 to 598.5)</td>
</tr>
</tbody>
</table>

$^c$ For all isolates, differences between prophylactic efficacy of isolates with MICs of $\leq 0.5$ mg/liter and the isolate with a MIC of $>16$ mg/liter were significant.
POS is given as prophylaxis, the efficacy against isolates with a POS MIC of 0.5 mg/liter is similar to that of the wild-type isolate.

It has been reported that the POS levels in the lung, at the site of infection/colonization, are relatively high (49, 50), which is consistent with the drug’s lipophilic characteristics and its increased intracellular permeability (51, 52). In our model we previously reported high POS levels in epithelial lining fluid, which might explain the high efficacy against low-POS-resistant isolates (38, 39). However, reduced efficacy was observed in isolates with high POS resistance (MIC of >16 mg/liter).

It is unlikely that differences in POS efficacies were due to differences in A. fumigatus fitness levels. Although the acquisition of resistance mechanisms duringazole therapy has been associated with a fitness cost (33), isolates with resistance mechanisms in the cytochrome P450 51A gene were shown to be as virulent as wild-type controls (54). In our current study we selected three isogenic isolates with increasing POS MICs and demonstrated similar in vitro growth characteristics, which indicates that the acquisition of a resistance mechanism was not associated with a fitness cost.

The exposure-response relationships of POS have been defined previously in experimental models of aspergillosis infections (24, 25, 55), for which a total AUCτ₀–24/MIC ratio between 167 and 178 was predictive of half-maximal efficacy, given that only the unbound fraction of a drug in serum/plasma is pharmacologically active. Considering the high degree of POS protein binding in plasma (98 to 99%) and negligible protein binding in ELF, the results of the current study indicated that effective local concentrations (less than 50% survival) might be achieved even at the lowest dose (4 mg/kg), with a free AUCτ₀–24/MIC ratio of 1.45 in plasma and 14.69 in ELF. Our model indicates that this level is high enough to prevent infection with A. fumigatus isolates with MICs of ≤0.5 mg/liter (Fig. 4). However, for the isolates with higher POS MICs (≥16 mg/liter), the obtained free AUC τ₀–24/MIC was ≤0.18 in plasma and ≤4.92 in ELF at highest dose (32 mg/liter), which indicates the possibility of breakthrough IA. Given that a significant proportion of isolates harboring an azole resistance mechanism exhibit a POS MIC of <0.5 mg/liter (36), the effective exposure is highly probable to be achieved with the recommended dose of POS.


REFERENCES


