Azole resistance is an emerging problem in *Aspergillus fumigatus* which translates into treatment failure. Alternative treatments with new azoles may improve therapeutic outcome in invasive aspergillosis (IA) even for strains with decreased susceptibility to current azoles. The in vivo efficacy of 0.25, 1, 4, 16, 64, 128, 256, and 512 mg/kg of body weight/day prodrug isavuconazonium sulfate (BAL8557) (isavuconazole [ISA]-equivalent doses of 0.12, 0.48, 1.92, 7.68, 30.7, 61.4, 122.9, and 245.8 mg/kg/day, respectively) administered by oral gavage was assessed in an immunocompetent murine model of IA against four clinical *A. fumigatus* isolates: a wild-type isolate (ISA MICEUCAST, 0.5 mg/liter) and three azole-resistant isolates harboring substitutions in the cyp51A gene: G54W (ISA MICEUCAST, 0.5 mg/liter), M220I (ISA MICEUCAST, 4 mg/liter), and TR34/L98H (ISA MICEUCAST, 8 mg/liter). The maximum effect (100% survival) was reached at a prodrug isavuconazonium sulfate dose of 64 mg/kg for the wild-type isolate, 128 mg/kg for the G54W mutant, and 256 mg/kg two times per day (q12) for the M220I mutant. A maximum response was not achieved with the TR34/L98H isolates with the highest dose of prodrug isavuconazonium sulfate (256 mg/kg q12). For a survival rate of 50%, the effective AUC0–24/MIC ratio for ISA total drug was 24.73 (95% confidence interval, 22.50 to 27.18). The efficacy of isavuconazole depended on both the drug exposure and the isavuconazole MIC of the isolates. The quantitative relationship between exposure and effect (AUC0–24/MIC) can be used to optimize the treatment of human infections by *A. fumigatus*, including strains with decreased susceptibility.
lease, 17 July 2014, Basel, Basel, Switzerland), on the basis of the 
SECURE registration study (26).

There are only limited preclinical data on the in vivo efficacy of 
isaconizole in azole-resistant IA (27). Therefore, the objective 
of the present study was to investigate the pharmacodynamics (PD) and dose-response and exposure-response relationships of 
isaconizole resistant against wild-type and clinical azole-resistant A. fumigatus isolates harboring different substitutions in the 
Cyp51A gene in an immunocompetent murine model of disseminated 
aspergillosis. Survival and reduction in kidney fungal burden de-
determined by real-time quantitative PCR were used as primary and 
secondary endpoints (respectively) to determine the dose-effect 
and the exposure-effect relationships of isaconizole for suscept-
able as well as azole-resistant isolates in comparison with the 
other azoles.

(Parts of these results were presented at the 53rd Interscience 
Conference on Antimicrobial Agents and Chemotherapy, Denver, 
CO, 10 to 13 September 2013, and at the 24th European Congress 
of Clinical Microbiology and Infectious Diseases [ECCMID], Bar-
celona, Spain, 10 to 13 May 2014.)

MATERIALS AND METHODS

Fungal isolates. Four clinical A. fumigatus isolates obtained from patients 
with proven IA (classified according to European Organization for 
Research and Treatment of Cancer/Invasive Fungal Infections Cooperative 
Group and National Institute of Allergy and Infectious Diseases Mycoses 
Study Group [EORTC/MSG] consensus definitions) (28) were used in the 
experiments: a wild-type isolate without mutations in the cyp51A 
gene (AZN8196) and three azole-resistant isolates harboring substitutions in the 
cyp51A gene: G54W (V 59-73) and M220I (V 28-77) isolates that have 
become resistant during patient azole therapy and a TR28-77-98 (V 52-35) 
isoate that has become resistant through environmental azole exposure. 
Strain identification and the cyp51A gene substitions were confirmed by 
sequence-based analysis as described previously (9). In addition, micro-
satellite genotyping of the isolates was performed to confirm that they are 
encodedically distinct (29). The isolates were stored in 10% glycerol broth at 
−80°C and were cultured on Sabouraud dextrose agar (SDA) supple-
mented with 0.02% chloramphenicol for 5 to 7 days at 35°C to 37°C. All 
isolates were cultured again on SDA for 5 to 7 days at 35°C to 37°C before 
preparation of the inoculum.

Preparation of inoculum for antifungal susceptibility testing. The 
suspensions of conidia were harvested in normal saline containing 
0.025% Tween 20. The appropriate dilutions in normal saline were made 
to obtain a final inoculum concentration of 2 × 107 to 5 × 107 CFU/ml 
(30).

In vitro antifungal susceptibility testing. In vitro antifungal suscepti-
bility testing (MBCs and minimum effective concentrations [MECs]) 
was performed by using the EUCAST (European Committee for Anti-
microbial Susceptibility Testing) (30) and CLSI (Clinical and Laboratory 
Standards Institute) (31) broth microdilution guidelines. The final con-
centrations of the antifungal agents ranged from 0.016 to 16 mg/liter for 
the antifungal agents. Survival and reduction in kidney fungal burden de-
determined by real-time quantitative PCR were used as primary and 
secondary endpoints (respectively) to determine the dose-effect 
and the exposure-effect relationships of isaconizole for suscept-
able as well as azole-resistant isolates in comparison with the 
other azoles.

Verweij et al., have been used for classifying azole-susceptible and 
azole-resistant isolates (32).

The MIC was defined as the lowest concentration that completely 
inhibited growth in comparison to the drug-free well (control) as assessed 
by visual inspection. The MEC was defined as the lowest concentration in 
which abnormal, short, and branched hyphal clusters were observed in 
contrast to the long, unbranched hyphal elements that were seen in the 
growth control well (30).

Antifungal agents. Itraconazole (BAL4815) and the prodrug isavu-
conazone sulfate (BAL8557) were provided by Astellas Pharma B.V. 
For in vitro studies, isaconizole was dissolved in dimethyl sulfoxide 
(DMSO) prior to susceptibility testing. The concentration of DMSO in 
the culture medium tubes was adjusted at 1%, and the concentration of 
antifungal agents was 2× final concentration.

For in vivo studies, the prodrug was dissolved in sterile water prior to 
oral administration in each experiment. Amounts of prodrug dissolved 
dereed for its 89% purity. The conversion factor for determining 
the equivalent isaconizole active dose from the prodrug dose was 0.48 
(provided by Astellas Pharma B.V.) on a milligram-per-kilogram-of-
body-weight basis. Thus, for every mg/kg of prodrug administered orally, 
the equivalent in vivo isaconizole dose was considered 0.48 mg/ 
kg. In the pharmacokinetic study, only isaconizole (active drug 
BAL4815) concentrations were quantified. The purity of isaconizole 
powder (BAL4815) for in vitro susceptibility testing was >99%.

Infection model. The efficacy of isaconizole monotherapy was de-
termined in an immunocompetent mouse model of disseminated asper-
gillosis following intravenous inoculation. Animals were infected via 
injection of 0.1 ml of the conidial suspension into the lateral tail of the 
mouse, corresponding to the 90% lethal dose (LD90) for each isolate (23, 
33, 34). A total of 756 outbred CD-1 (Charles River, the Netherlands) 
female mice, 4 to 5 weeks old, weighing 20 to 25 g, were randomized into 
groups of 14 mice (11 for survival analysis; 3 for quantitative real-time 
PCR [qPCR]) to control or prodrug doses.

Before performing the experiment, the isolates were cultured once on 
SDA for 5 days at 35°C to 37°C and subcultured once on 15-cm Takashio 
slants for 5 days at 35°C to 37°C. The conidia were harvested in 20 ml of 
sterile phosphate-buffered saline (PBS) plus 0.1% Tween 80 (Boon 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 ad-
justed to the required concentration, the conidial suspension was stored 
overnight at 4°C. The 90% lethal dose (LD90) was determined for each 
isoate, separately. The LD90 was 2.4 × 107 (wild-type control), 1 × 107 
(G54W strain), 5 × 106 (M220I strain), and 2.5 × 106 (TR28-77-98 strain) 
conidia. Confirmatory postinfection viability counts of the injected inoc-
ula were determined to ensure that the correct inoculum had been in-
jected.

Treatment was started 24 h after infection and continued for 14 days. 
The prodrug solution was administered in doses of 0.25, 1, 4, 16, 64, 128, 
and 256 mg/kg by oral gavage once daily in a volume of 0.12 ml or divided 
to two or three daily doses where applicable. The highest dose (256 
mg/kg) was used twice per day, corresponding to 512-mg/kg prodrug 
isaconizone sulfate (BAL8557) in days of animals where 100% efficacy was not achieved with a once-daily dose. The above-men-
tioned dosages were equivalent to 0.12, 0.48, 1.92, 7.68, 30.7, 61.4, and 
122.9 mg/kg, respectively, of the active moiety isaconizole. The control 
group received single doses of saline. In addition, dose fractionation stud-
ies were performed to determine which pharmacokinetic/pharmacody-
namic (PK/PD) index correlated with efficacy. Mice were infected with 
the A. fumigatus isolate through the lateral tail vein, and after 24 h, treat-
ment was initiated according to total daily dosing every 8 h (q8) or 12 h 
(q12) for 14 days. The animals were housed under standard conditions, 
with drink and feed supplied ad libitum. The animal studies were con-
ducted in accordance with the recommendations of the European Com-

munity (Directive 2010/63/EU revising Directive 86/609/EEC on the pro-

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tection of animals used for scientific purposes adopted on 22 September 2010), and all animal procedures were approved by the Animal Welfare Committee of Radboud University (RU-DEC 2012-050).

In all survival studies, the monitoring was performed by experienced individuals blinded to the animal treatment. The infected mice were examined at least three times daily. Clinical inspections focused on dehydration, torticollis, staggering, severe weight loss (a decrease of 15% within 48 h or 20% within 24 h), or body temperature drop to below 33°C. Mice demonstrating these clinical signs were humanely terminated according to strict protocols. On day 15 postinfection, all surviving mice were humanely euthanized under isoflurane anesthesia, and blood and internal organs were collected.

Survival and reduction in fungal burden were the primary end-point in groups of 11 and 3 mice, respectively. The survival in days postinfection was recorded for each mouse in each group and considered primary outcome-effect measure to assess the therapeutic efficacy of isavuconazole (35). On day 3 postchallenge, a quantitative real-time PCR (qPCR) was performed in groups of 3 mice. In these groups, the mice were sacrificed on day 3 postinfection (before they began to die from infection) and the fungal load in the kidney was determined. In the systemic aspergillosis model with i.v. infection, the kidneys are the main target organs, which may indicate intraluminal localization of the fungus in the renal tubules initially protects them from inflammatory cells. The reduction in kidney fungal burden was then correlated with the survival of the remaining 11 mice from each corresponding group at day 15 postinfection.

Determination of fungal burden in kidney. Left and right kidneys from each animal were homogenized using a TissueLyser (Qiagen; TissueLyser Type MM 301) and UV irradiated beads with magnetic metal cores (3 mm) and 30 Hz in 2 min. Tissue samples were transferred to MagNA Lyser Green bead tubes (Roche Applied Science). Five hundred microliters of Tris-EDTA (TE) buffer was added, and homogenization was performed for 20 s at 6,500 rpm by using the MagNA Lyser instrument. Supernatant was used for DNA isolation by using the automated MagNA Pure (MP96) system and the MagNA Pure LC total nucleic acid isolation kit according to the manufacturer’s protocol (Roche Applied Science). Phocine herpesvirus (PhHV) was added to all samples as an internal extraction and inhibition control. The concentration of total isolated DNA was measured by using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Aspergillus loads were determined by qPCR using the LC480 instrument and the probe master kit (Roche Applied Science). Phocine herpesvirus (PhHV) was added to all samples as an internal extraction and inhibition control. The concentration of total isolated DNA was measured by using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

Pharmacokinetic analysis. Geometric mean concentrations of isavuconazole were calculated for each time point (n = 3 mice). Pharmacokinetic parameters (area under the concentration-time curve from 0 to 24 h \( [\text{AUC}_{0-24}] \), maximum concentration of drug in serum \( [C_{\text{max}}] \), the trough concentration 24 h after the start of treatment \( [C_{24h}] \), half-life \( [t_{1/2}] \), volume of distribution \( [V] \), clearance \( [CL] \), and terminal elimination rate constant \( [k_{el}] \) were calculated using noncompartmental analysis (Phocinx version 6.3). The area under the concentration-time curve from 0 h to infinity \( (\text{AUC}_{0-\infty}) \) was calculated using the linear–log down trapezoidal rule. In addition, \( C_{\text{max}} \) and \( C_{24h} \) were directly observed from the data. Half-life was calculated by \( \ln 2/k_e \), in which \( k_e \) was determined by linear regression of the terminal points of the log-linear plasma concentration–time curve. \( V \) was calculated using the formula \( V = \text{dose}/(\text{AUC} \times k_{el}) \), and CL was calculated as dose/AUC_{0-\infty}.

(i) Calculation of isavuconazole concentration in ELF. Concentrations of isavuconazole in BAL fluid from three mice per time point were determined as described for plasma. Urea in plasma and BAL fluid aspirate was determined utilizing a modified enzymatic assay (Quantichrom urea assay kit, DIUR-500; BioAssay Systems) (39, 40). The concentration of isavuconazole in epithelial lining fluid (ELF) was then determined by using the ratio of urea concentration in BAL fluid to that in plasma. The drug concentration in ELF was then estimated, as described previously (37, 39–44): drug concentration_{ELF} = drug concentration_{BAL\ fluid}\times\ \text{urea}_{BAL\ fluid}/\text{urea}_{BAL\ fluid}.

(ii) Statistical analysis. All data analyses were performed using GraphPad Prism, version 5.3, for Windows (GraphPad Software, San Diego, CA). A regression analysis was conducted to determine the linearity between isavuconazole concentration in blood and that in ELF. The \( C_{\text{max}} \) data were log_{10} transformed to approximate a normal distribution prior to statistical analysis. Mortality data were analyzed by the log rank test. The survival data were plotted against dose/MIC and AUC/MIC. The Hill equation with a variable slope was fitted to the data. The fits were performed for survival data of each strain and all strains simultaneously. The goodness of fit was checked by \( R^2 \) and
visual inspection. Dose/MIC and AUC 0–24/MIC ratios were calculated by dividing the dose (milligrams per kilogram) or AUC by the MIC. Dose/MIC and AUC 0–24/MIC ratio data were log_{10} transformed to approximate a normal distribution prior to statistical analysis. The 50% effective PK/PD indexes (E_{50}, E_{80}, and E_{90}) best correlating with efficacy were determined. In addition, the 50% effective doses (ED_{50}) of isavuconazole best correlating with efficacy were determined. For comparison between strains, an F test was performed to define whether ED_{50} differed among the four groups. Statistical significance was defined as a P value of <0.05 (two-tailed).

RESULTS

**In vitro susceptibility.** 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. Variable isavuconazole activity was found in azole-resistant isolates, in which isavuconazole showed cross-resistance to voriconazole, but not with itraconazole and posaconazole. In comparison to a MIC_{EUCAST} of 0.5 mg/liter for the wild-type isolate, isavuconazole showed similar activity against the isolate harboring the G54W resistance mechanism (MIC_{EUCAST}, 0.5 mg/liter) but reduced in vitro activity against M220I and TR_{34/L98H} isolates, with MICs_{EUCAST} of 4 and 8 mg/liter, respectively. There was no difference in the amphotericin B (AmB) and anidulafungin (AFG) activity between the isolates.

**Pharmacokinetics of isavuconazole.** A total of 210 samples from 210 mice (3 mice per time point, 10 time points, 7 different dosages) were analyzed. All 210 mice were alive at the time of sample collection. The observed plasma concentrations versus-time profiles of isavuconazole are shown in Fig. 1. The corresponding pharmacokinetic parameters are tabulated in Table 2 for plasma and in Table 3 for epithelial lining fluid (ELF). The dose-normalized isavuconazole AUC in plasma ranged from 0.54 to 0.84 mg · h/liter/(mg/kg), was slightly lower for the 4-mg/kg dose, and could not be reliably determined for lower doses. The concentrations of isavuconazole in ELF correlated well with those obtained in plasma but were lower, including the maximum total drug concentrations (C_{max}) of isavuconazole (Table 3). A significant relationship between mean isavuconazole concentrations in plasma and ELF was noted by linear regression analysis (r^2 = 0.86, P < 0.0001) (Fig. 2). The penetration of isavuconazole in ELF based on total drug was between 35.8 and 72.5% with a mean of

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Aspergillus disease^a</th>
<th>Prior azole exposure</th>
<th>Cyp51A substitution</th>
<th>MIC or MEC (mg/liter) of drug by standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZN8196</td>
<td>Proven IA</td>
<td>None</td>
<td>None</td>
<td>AmB EUCAST/CLSI</td>
</tr>
<tr>
<td>V 59-73</td>
<td>Clinical, disease entity unknown</td>
<td>Unknown</td>
<td>G54W</td>
<td>0.5</td>
</tr>
<tr>
<td>V 28-77</td>
<td>Proven IA</td>
<td>Yes</td>
<td>M220I</td>
<td>0.5</td>
</tr>
<tr>
<td>V 52-35</td>
<td>Proven IA</td>
<td>No</td>
<td>TR_{34/L98H}</td>
<td>0.5</td>
</tr>
</tbody>
</table>

^a Classification according to EORTC/MSG consensus definitions.
TABLE 2 Pharmacokinetic parameters of isavuconazole (BAL4815) following oral administration of various dosages administered as prodrug isavuconazonium sulfate (BAL8557) ranging from 0.25 to 256 mg/kg (ISA-equivalent dosages ranging from 0.12 to 122.9 mg/kg)\(^a\)

<table>
<thead>
<tr>
<th>Dose group (mg/kg)</th>
<th>C(_{\text{max}}) (mg/liter)</th>
<th>C(_{\text{last}}) (mg/liter)</th>
<th>Half-life (h)</th>
<th>AUC(_{\text{INF, pred}}) (h · mg/liter)</th>
<th>AUC(_{\text{INF, D, pred}}) (h · mg/liter/kilos · kg)</th>
<th>CL(_{\text{ss,F}}) (liters/ (h · kg))</th>
<th>V(_{\text{z,F}}) (liters/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.12</td>
<td>0.12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.11</td>
<td>0.11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.54</td>
<td>0.08</td>
<td>1.12</td>
<td>0.97</td>
<td>0.24</td>
<td>4.09</td>
<td>6.62</td>
</tr>
<tr>
<td>16</td>
<td>2.31</td>
<td>0.24</td>
<td>2.49</td>
<td>9.68</td>
<td>0.60</td>
<td>1.68</td>
<td>6.04</td>
</tr>
<tr>
<td>64</td>
<td>5.69</td>
<td>0.52</td>
<td>3.00</td>
<td>34.70</td>
<td>0.54</td>
<td>1.87</td>
<td>8.11</td>
</tr>
<tr>
<td>128</td>
<td>9.84</td>
<td>2.42</td>
<td>6.06</td>
<td>107.41</td>
<td>0.84</td>
<td>1.31</td>
<td>11.46</td>
</tr>
<tr>
<td>256</td>
<td>25.62</td>
<td>0.15</td>
<td>3.06</td>
<td>146.68</td>
<td>0.57</td>
<td>1.76</td>
<td>7.75</td>
</tr>
</tbody>
</table>

\(^a\) Abbreviations: C\(_{\text{max}}\), last observed quantifiable concentration; AUC\(_{\text{INF, pred}}\), Predicted area under the plasma concentration-time curve from time zero to infinity; AUC\(_{\text{INF, D, pred}}\), dose-normalized AUC\(_{\text{INF, pred}}\); CL\(_{\text{ss,F}}\), total systemic clearance; V\(_{\text{z,F}}\), volume of distribution.

54.1\% (Table 3). For the ELF data from the 128-mg/kg dose, \(k\)_el (terminal elimination rate constant) could not be determined with the consequence that the predicted AUC\(_{\text{0–12s}}\) could not be calculated reliably. Hence, the AUC\(_{\text{0–12s}}\) in plasma and ELF were determined. The variable penetration of isavuconazole into ELF might be explained due to the difference in lysis of the cells available in interstitial spaces over the course of infection that limits passage through alveolar epithelial cells in various levels; in addition, measurements at the same time in plasma and a third compartment are liable to significant variation.

Efficacy of isavuconazole. (i) Survival and quantitative PCR as outcome parameter to monitor therapeutic efficacy of ISA.

Figure 3 shows the survival curves of isavuconazole-treated mice by produg dose. The survival curves for all control groups receiving saline by oral gavage showed a mortality of 100%. The results show that for each produg dose, survival decreased as the MIC increased. Similarly, when the produg dose was increased, an improved response was observed. The maximum effect (100% survival) was reached at a prodose of 64 mg/kg for the wild-type isolate (MIC, 0.5 mg/liter), 128 mg/kg for the G54W isolate (MIC, 0.5 mg/liter), and 256 mg/kg for the M220I isolate (MIC, 4 mg/liter). However, the response was lower in the mice infected with isolates with higher MICs to isavuconazole (≥4 mg/liter) was shifted to the right compared to those infected with isolates with the susceptible profile for isavuconazole (MIC, 0.5 mg/liter), indicating that higher doses of isavuconazole were required to achieve similar efficacy.

The Hill-type model with a variable slope fitted the relationship between the dose and 14-day survival well, with \(R^2\) values of 1 (wild type), 0.99 (G54W isolate), 0.95 (M220I isolate), and 0.91 for the TR\(_{34}/L98H\) isolate. The 50% effective dose (ED\(_{50}\)) based on survival was 24.15 mg/kg produg isavuconazonium sulfate (BAL8557) (95% confidence interval [CI], 23.96 to 24.33 mg/kg) for the wild type, 28.93 (95% CI, 24.23 to 34.54 mg/kg) for the G54W isolate, 109 (95% CI, 50.69 to 234.6 mg/kg) for the M220I isolate, and 485 (95% CI, 24.23 to 34.54 mg/kg) for the G54W isolate, 109 (95% CI, 50.69 to 234.6 mg/kg) for the M220I isolate.

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(iii) Dose-response analysis. The dose-response curves for the dosing regimens and control groups of isavuconazole administered to the immunocompetent mice are shown in Fig. 5. Isavuconazole treatment improved the survival of the mice in a dose-dependent manner. A dose-response relationship was observed that depended on the isavuconazole dose and theazole resistance mechanisms. The dose-response curve for mice infected with the isolates with higher MICs to isavuconazole (≥4 mg/liter) was shifted to the right compared to those infected with isolates with the susceptible profile for isavuconazole (MIC, 0.5 mg/liter), indicating that higher doses of isavuconazole were required to achieve similar efficacy.

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isolate, and 483.8 (95% CI, 103.2 to 2,266 mg/kg) for the TR 34/L98H isolate. Notably, the dose-response curves were significantly different ($P < 0.05$) between the wild-type $A. fumigatus$ isolate with an isavuconazole MIC EUCAST of 0.5 mg/liter and the azole-resistant isolates harboring M220I and TR 34/L98H substitutions in the $cyp51A$ gene with isavuconazole MIC EUCASTs of 4 and 8 mg/liter, respectively (Table 4).

As a comparison, the ED$_{50}$ based on survival was 11.6 mg/kg isavuconazole active moiety (95% confidence interval [CI], 11.5 to 11.7 mg/kg) for the wild type, 13.9 (95% CI, 11.6 to 16.6 mg/kg) for the G54W isolate, 52.3 (95% CI, 24.3 to 112.6 mg/kg) for the M220I isolate, and 232.3 (95% CI, 49.52 to 1,089 mg/kg) for the TR34/L98H isolate.

(iii) Exposure-response analysis. The AUC for each dose (Table 2) was used to calculate the AUC$_{0-24}$/MIC ratio for each isolate, as shown in Fig. 6. The exposure-response relationship has a sigmoidal shape. Increased isavuconazole exposure was required to obtain maximum efficacy in mice infected with the M220I (MIC, 4 mg/liter) and TR$_{34}$/L98H (MIC, 8 mg/liter) strains compared to those infected with the wild-type and G54W strains (MIC, 0.5 mg/liter).

The Hill equation with a variable slope fitted the relationship between the 24-h AUC/MIC ratio and 14-day survival well ($R^2 = 0.96$), as statistically significant pharmacodynamic indices (PDIs) for isavuconazole single-agent regimens ($P < 0.05$). The 50% effective pharmacodynamic index (total AUC$_{0-24}$/MIC$_{EUCAST}$) for isavuconazole was 24.73 (95% confidence interval, 22.50 to 27.18), to be the PD index most closely predictive of efficacy. Using MICs determined with the CLSI method, the effective AUC$_{0-24}$/MIC$_{CLSI}$ ratio for ISA total drug was 50.48 (95% confidence interval, 44.90 to 56.74).

The relationship between the in vivo efficacy and other PDIs, such as the cumulative percentage of a 24-h period that the drug concentration exceeded the MIC under steady-state PK conditions and the peak-level $C_{max}$/MIC, was also determined (data not shown). However, AUC$_{0-24}$/MIC appeared to be the most important pharmacodynamic index correlating with efficacy.

(iv) Dose fractionation studies. Figure 7 shows the results of the dose fractionation study. There is no difference between exposure-response relationships of the groups treated with various dosing intervals.

DISCUSSION

In the present study, the efficacy of isavuconazole was demonstrated against $A. fumigatus$ wild-type and $cyp51A$ azole-resistant isolates in an immunocompetent murine model of disseminated aspergillosis. Efficacy was dependent both on the drug exposure time and on the isavuconazole MIC of the resistance phenotype of the isolates. The loss of efficacy was completely or partly compensated by increasing the doses of the prodrug isavuconazonium sulfate for the azole-resistant strains. However, for mice infected with the TR$_{34}$/L98H isolate, which had a high MIC (8 $\mu$g/ml), 

![FIG 3 Efficacy of isavuconazole against 4 A. fumigatus isolates. Survival curves are depicted by strain. Therapy by oral gavage once daily was begun 24 h after infection and continued for 14 days. The isavuconazonium sulfate (BAL8557) doses of 0.25, 1, 4, 16, 64, and 256 mg/kg correspond to ISA-equivalent doses of 0.12, 0.48, 1.92, 7.68, 30.7, 61.4, and 122.9 mg/kg, respectively. The highest dose (256 mg/kg) was used two times per day, corresponding to 512 mg/kg prodrug isavuconazonium sulfate (BAL8557)/day, in groups of animals for which 100% efficacy was not achieved with a once-daily dose of 256 mg/kg. Placebo groups received saline. For all groups, n was 11.](http://aac.asm.org/attachment/images/FIG3.png)
Isavuconazole was not efficacious. Importantly, a maximal effect of 100% survival was achieved in all strains tested except for the strain containing the TR34/L98H mutation. This is of significant importance, since the prevalence of azole resistance in *A. fumiga*-tus is increasing, and cross-resistance is a growing concern (12, 21, 32, 45, 46).

A possible limitation of the experimental design used to explore the PK/PD relationships of isavuconazole in our study is that the effects were observed in nonneutropenic animals and the route of infection was dissemination rather than inhalation (the normal route of infection). In addition, a significant difference between tail and intrapulmonary infection is the difference in inoculum size. A lower inoculum size is needed in immunosuppressed models. The effects observed could therefore be an under- or overestimation of the exposure required. However, in our previous studies with azoles, we showed that using survival as a gold standard endpoint in our model provides useful exposure-response relationships. Studies with azoles in neutropenic (27, 47, 48) and nonneutropenic (23, 49, 50) models have shown that the exposure–response relationships are of the same order of magnitude; in fact, a slightly lower drug exposure target may be required in the neutropenic model. This could possibly be because of the lower inoculum used in this model.

Isavuconazole is an investigational broad-spectrum triazole developed for the treatment of severe invasive and life-threatening fungal diseases (25, 51–54). This compound demonstrated *in vitro* activity and *in vivo* efficacy against a broad range of yeasts and molds, including *Aspergillus* spp., *Fusarium* spp., *Candida* spp., the *Mucorales*, *Cryptococcus* spp., and black yeasts and their filamentous relatives (25, 55–63).

In the current study, our model indicated that the primary driver of efficacy appears to be AUC/MIC. For a survival rate of 50%, the effective AUC_{0–24}/MIC_{CLSI} ratio for isavuconazole total drug was 50.48 (95% confidence interval, 44.90 to 56.74). Similarly, the exposure-response relationships of isavuconazole have been defined in a recent experimental immunosuppressed murine model of invasive pulmonary aspergillosis (IPA), for which a very strong relationship was observed between the PD index AUC/MIC ratio and treatment outcome (27). In that study, 10 *A. fumigatus* isolates were used, including four wild-type isolates and six cyp51 mutants. The MIC_{CLSI} range was 0.125 to 8 mg/liter. Following infection, groups of mice were treated orally with the prodrug BAL8557 at 40 to 640 mg/kg/12 h.

**FIG 4** Efficacy of isavuconazole against 4 *A. fumigatus* isolates expressed as *A. fumigatus* DNA load (copies/nanogram of DNA) in kidneys at 72 h postchallenge. For all groups, *n* was 3. Doses were administered as the prodrug isavuconazonium sulfate (BAL8557). The highest dose (256 mg/kg) was used two times per day, corresponding to 512 mg/kg prodrug isavuconazonium sulfate (BAL8557)/day, in groups of animals for which 100% efficacy was not achieved with a once daily dose of 256 mg/kg.
for 7 days. A dose-response relationship was observed for each isolate, with higher doses of isavuconazole achieving a larger microbiologic effect. The static-dose range was 65 to 617 mg/kg/12 h, for which the median total- and free-drug 24-h AUC/MIC ratio PD targets for net stasis were 503 and 5, respectively. The 1-log10 killing-dose range was 147 to 455 mg/kg/12 h, and the corresponding median free-drug AUC/MIC ratio was 11.1 (27). Since the AUC of isavuconazole given 200 mg once daily (q.d.) was reported as approximately 90 mg · h/liter in healthy subjects approaching steady state (64), we therefore conclude that the A. fumigatus strains with MICs of 0.5 mg/liter would be covered, and attainment is most likely reached for strains with MICs of up to 2 or potentially 4 mg/liter.

The efficacy of isavuconazole has also been investigated in an immunosuppressed murine model of disseminated Aspergillus flavus infection (65). Isavuconazole demonstrated impressive antifungal activity against A. flavus infection, leading to prolonged survival, equivalent to similar doses of itraconazole and voriconazole and superior to either drug administered at 10 mg/kg/dose. The excellent efficacy of isavuconazole occurred despite much lower exposure as demonstrated by 4-fold-lower AUCs. Isavuconazole was at least as effective as voriconazole or voriconazole at reducing organ burden and was able to clear all burden in 33 to 83% of mice treated with >15 mg/kg/dose (65).

Three previous studies have examined drug exposure and the efficacy of isavuconazole in a murine model of invasive candidiasis (48, 66–68) and showed a very strong relationship between the PD index AUC/MIC ratio and treatment outcome. One study investigated the efficacy of isavuconazole using a neutropenic mouse model of disseminated C. krusei and Candida tropicalis infections. Isavuconazole was as effective as voriconazole and much more effective than fluconazole at reducing brain burden. All doses of isavuconazole (6, 15, 30, 60, 90, 120, or 150 mg/kg equivalent active compound) reduced brain burden (P < 0.05) in the C. krusei model and kidney burden in the C. tropicalis model (48). Another study investigated the PK/PD properties of isavuconazole in a neutropenic murine model of invasive candidiasis (IC) against clinical isolates of Candida species, including Candida albicans, Candida glabrata, and C. tropicalis, with both a 24-h and a 96-h treatment duration (48). This study has shown that the pharmacodynamic index most closely correlated with efficacy is the ratio of the 24-h area under the concentration-time curve (AUC) to the MIC, and a target 24-h free-drug AUC/MIC ratio near 25 was associated with 50% of maximal microbiologic efficacy (48).

![Figure 5](http://aac.asm.org/) Fourteen-day survival as a function of prodrug isavuconazonium sulfate (BAL8557) dose against 4 A. fumigatus isolates. Shown are data for the isolate without substitution in the Cyp51A gene (AZN8196; isavuconazole [ISA] MIC, 0.5 mg/liter) and for the G54W (isolate V 59-73; ISA MIC, 0.5 mg/liter) and M220I (isolate V 28-77; ISA MIC, 4 mg/liter) isolates. MICs are according to EUCAST methodology. The curves indicate fits with the Hill equation for each isolate.

### TABLE 4 Comparison of efficacies of isavuconazole among four A. fumigatus isolates based on ED_{50}

<table>
<thead>
<tr>
<th>A. fumigatus strain</th>
<th>ISA MIC (mg/liter)</th>
<th>ISA ED_{50} (mg/kg)</th>
<th>Comparison with wild type</th>
<th>P value</th>
<th>F test (df_{n}, df_{d})^a</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.5</td>
<td>24.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G54W strain</td>
<td>0.5</td>
<td>28.93</td>
<td>0.16</td>
<td>2.88(1,4)</td>
<td>Nonsignificant</td>
<td></td>
</tr>
<tr>
<td>M220I strain</td>
<td>4</td>
<td>109</td>
<td>&lt;0.0001</td>
<td>89.89(1,7)</td>
<td>Significant</td>
<td></td>
</tr>
<tr>
<td>TR_{54/L98H} strain</td>
<td>8</td>
<td>438.8</td>
<td>&lt;0.0002</td>
<td>47.33(1,7)</td>
<td>Significant</td>
<td></td>
</tr>
</tbody>
</table>

^a df_{n}, degrees of freedom numerator; df_{d}, degrees of freedom denominator.
Similarly, using a nonneutropenic murine model of disseminated *Candida albicans* infection, it has been shown that the pharmacodynamic driver most likely to predict the outcome of itraconazole treatment is the AUC/MIC ratio (68).

In addition, in a neutropenic mouse model of intratracheal infection, Luo et al. investigated the efficacy of isavuconazole against a brain isolate of *Rhizopus delemar* (isavuconazole MIC<sub>100</sub> and minimum fungicidal concentration [MFC] values of 0.25 μg/ml) (69). Isavuconazole was effective against isolates with MIC and MFC values ranging between 0.125 and 1.00 μg/ml. A high dose of isavuconazole (215 mg/kg of isavuconazonium sulfate three times daily [t.i.d.] prolonged the survival time and lowered the tissue fungal burden of cyclophosphamide-cortisone acetate-treated mice. In addition, isavuconazole was as effective as a high-dose liposomal amphotericin B (15 mg/kg, given once daily through tail vein injection) treatment (69).

In the present study, we also found that the dosing frequency did not have an impact on survival. Given the half-life of the drug in mice, which is much shorter than that in humans, a once-daily dose should therefore suffice and be adequate to treat infections in humans. Of note, in clinical studies to date, positive efficacy and safety data have been reported in patients with invasive aspergil-

**FIG 6** Percentage of survival as a function of the prodrug isavuconazonium sulfate (BAL8557) AUC<sub>0–24</sub>/MIC<sub>EUCAST</sub> ratio against 4 *A. fumigatus* isolates (MIC in milligrams per liter according to EUCAST methodology). The curve is the model fit with the Hill equation for each data set. MIC values are in milligrams per liter.

**FIG 7** Impact of dose fractionation on the *in vivo* efficacy of prodrug isavuconazonium sulfate (BAL8557) against wild-type *A. fumigatus* (MIC, 0.5 mg/liter). Groups of 11 mice were treated q24 (once daily), q12 (fractionated into two doses), and q8 (fractionated into three doses) with the prodrug isavuconazonium sulfate (BAL8557) administered orally for 14 days. The curve is the model fit with the Hill equation for each data set. The dashed line is the curve fit for all data sets combined. There was no significant difference in exposure-response relationships among dosing q8, q12, or q24.
lasis (26). Isavuconazole achieved predictable drug levels supporting reliable dosing and a switch from once-daily intravenous to oral administration (26, 70, 71). In addition, no significant food effect has been found with oral administration of isavuconazole (72), which in contrast is a potential advantage of isavuconazole over the oral solution of posaconazole.

In conclusion, our data are an additional promising support for isavuconazole in the treatment of IA. As with the other azole antifungal drugs, the efficacy of isavuconazole treatment in A. fumigatus relies both on drug exposure and on the antifungal susceptibility of the etiological agent. Therefore, the quantitative relationship between exposure and effect (AUC0 –24/MIC) can be used to optimize the treatment of human infections by A. fumigatus, including strains with decreased susceptibility.

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