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 MIC<sub>EUCAST</sub> 0.5 mg/liter) and three azole-resistant isolates harboring substitutions in the cyp51A gene: G54W (ISA MIC<sub>EUCAST</sub> 0.5 mg/liter), M220I (ISA MIC<sub>EUCAST</sub> 4 mg/liter), and TR<sub>3A</sub>L98H (ISA MIC<sub>EUCAST</sub> 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 TR<sub>3A</sub>L98H isolates with the highest dose of prodrug isavuconazonium sulfate (256 mg/kg q12). For a survival rate of 50%, the effective AUC<sub>0–24</sub>/MIC<sub>EUCAST</sub> 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 (AUC<sub>0–24</sub>/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 isavuconazole 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 isavuconazole 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 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 isavuconazole for susceptible as well as azole-resistant isolates in comparison with the other azoles.

(Items 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], Barcelona, 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 (V52-35) and M220I (V28-77) isolates that have become resistant during patient azole therapy and a TR34/L98H/(V52-35) isolate that has become resistant through environmental azole exposure. Strain identification and the cyp51A gene substitutions were confirmed by sequence-based analysis as described previously (9). In addition, microsatellite genotyping of the isolates was performed to confirm that they are genetically distinct (29). The isolates were stored in 10% glycerol broth at −80°C and were cultured on Sabouraud dextrose agar (SDA) supplemented 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 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 × 10^5 to 5 × 10^5 CFU/ml (30).

In vitro antifungal susceptibility testing. In vitro antifungal susceptibility testing (MFCs and minimum effective concentrations [MECs]) was performed by using the EUCAST (European Committee for Antimicrobial Susceptibility Testing) (30) and CLSI (Clinical and Laboratory Standards Institute) (31) broth microdilution guidelines. The final concentrations of the antifungal agents ranged from 0.016 to 16 mg/liter for amphotericin B (Amb), itraconazole (ITC), voriconazole (VRC), posaconazole (POS), isavuconazole (ISA), and anidulafungin (AFG). Aliquots of 100 μl of each drug at a concentration two times the targeted final concentration were dispensed in microtiter plates (Costar, Corning, NY). Trays were maintained for a period of less than 1 month at −70°C until the day of testing. After the microtitration trays were defrosted, 100 μl of the inoculum was added to each well, corresponding to a final concentration of 2 × 10^5 to 5 × 10^5 CFU/ml for each isolate. The microtiter plates were incubated at 35 to 37°C for 48 h.

Growth inhibition was quantified by using a visual mirror. Candida parapsilosis (ATCC 22019) and Candida krusei (ATCC 6258) were used for quality control (QC) in all experiments. All incubations were performed in three independent replicates, and the breakpoints reported by 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. Isavuconazole (BAL4815) and the prodrug isavuconazonium sulfate (BAL8557) were provided by Astellas Pharma B.V. For in vitro studies, isavuconazole 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 were corrected for its 89% purity. The conversion factor for determining the equivalent isavuconazole 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 1 mg/kg of prodrug administered orally, the equivalent in vivo isavuconazole dose was considered 0.48 mg/kg.

In the pharmacokinetic study, only isavuconazole (active drug BAL4815) concentrations were quantified. The purity of isavuconazole powder (BAL4815) for in vitro susceptibility testing was >99%.

Infection model. The efficacy of isavuconazole monotherapy was determined in an immunocompetent mouse model of disseminated aspergillosis 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 to 37°C and subcultured twice on 15-cm Takahashi slants for 5 days at 35 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 adjusted to the required concentration, the conidial suspension was stored overnight at 4°C. The 90% lethal dose (LD90) was determined for each isolate, separately. The LD90 was 2.4 × 10^7 (wild-type control), 1 × 10^7 (G54W strain), 5 × 10^6 (M220I strain), and 2.5 × 10^6 (TR34/L98H strain) conidia. Confirmatory postinfection viability counts of the injected inocula were determined to ensure that the correct inoculum had been injected.

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 into two or three daily doses where applicable. The highest dose (256 mg/kg) was used two times per day, corresponding to 512-mg/kg prodrug dosages. The control group received single doses of saline. In addition, dose fractionation studies were performed to determine which pharmacokinetic/pharmacodynamic (PK/PD) index correlated with efficacy. Mice were infected with the A. fumigatus isolate through the lateral tail vein, and after 24 h, treatment 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 conducted in accordance with the recommendations of the European Community (Directive 2010/63/EU revising Directive 86/609/EEC on the pro-
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 postinfection was recorded for each mouse in each group and considered primary outcome-effect measure to assess the therapeutic efficacy. Details of the analytical assay were described elsewhere (38). Briefly, samples were pretreated using a protein precipitation procedure (acetonitrile-methanol, 50/50, and formic acid, 0.1%). A seven-point calibration curve with three quality control (QC) samples was used. All measurements were done in duplicate. The dynamic range of the isavuconazole assay was 0.0495 to 29.69 mg/liter, and the accuracy range, which was dependent on the concentration, was 96.1% to 101.5%. Interday coefficient of variation was 0.46 to 1.8%, and extraction recovery was 84.6%. The assay was validated for the mouse plasma matrix according to the FDA and EMA directive for bioanalytical methods validation with the exception of intraday precision as well as freeze-thaw cycles. These experiments were not considered necessary based on the experience with human plasma.

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 [AUC0–24], maximum concentration of drug in serum [Cmax], the trough concentration 24 h after the start of treatment [C24 h], half-life [1/2], volume of distribution [V], clearance [CL], and terminal elimination rate constant [kτ]) were calculated using noncompartmental analysis (Pho- nix version 6.3). The area under the concentration-time curve from 0 h to infinity (AUC0–∞) was calculated using the linear up-log down trapezoidal rule. In addition, Cmax and C1/2 were directly observed from the data. Half-life was calculated by In 2/kτ, in which kτ was determined by linear regression of the terminal portion of the log-linear plasma concentration-time curve. V was calculated using the formula V = dose/AUC × kτ, and CL was calculated as dose/AUC0–24.

(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 concentrationELF = drug concentrationBAL fluid × ureaBAL fluid/ureaELF 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 Cmax data were log10 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 R2 and
visual inspection. Dose/MIC and AUC<sub>0–24</sub>/MIC ratios were calculated by dividing the dose (milligrams per kilogram) or AUC by the MIC. Dose/MIC and AUC<sub>0–24</sub>/MIC ratio data were log<sub>10</sub> transformed to approximate a normal distribution prior to statistical analysis. The 50% effective PK/PD indexes (E<sub>I</sub>50, E<sub>I</sub>80, and E<sub>I</sub>90) best correlating with efficacy were determined. In addition, the 50% effective doses (ED<sub>50</sub>) of isavuconazole best correlating with efficacy were determined. For comparison between strains, an F test was performed to define whether ED<sub>50</sub> differed among the four groups. Statistical significance was defined as a <i>P</i> value of < 0.05 (two-tailed).

RESULTS

**In vitro susceptibility.** The characteristics and in vitro susceptibilities of the four <i>A. fumigatus</i> 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 MICEUCAST of 0.5 mg/liter for the wild-type isolate, isavuconazole showed similar activity against the isolate harboring the G54W resistance mechanism (MICEUCAST, 0.5 mg/liter) but reduced in vitro activity against M220I and TR<sub>34/L98H</sub> isolates, with MIC<sub>EUCAST</sub> 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) for single doses (dose expressed as the prodrug, concentration expressed as isavuconazole) ranging from 16 mg/kg to 256 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 (<i>C</i><sub>max</sub>) of isavuconazole (Table 3). A significant relationship between mean isavuconazole concentrations in plasma and ELF was noted by linear regression analysis (<i>r</i><sup>2</sup> = 0.86, <i>P</i> < 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

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**TABLE 1** Disease classification, history of previous azole exposure, underlying azole resistance mechanisms, and in vitro antifungal susceptibilities of <i>A. fumigatus</i> isolates used in isavuconazole study

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Aspergillus disease&lt;sup&gt;a&lt;/sup&gt;</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></td>
<td></td>
<td></td>
<td></td>
<td>AmB EUCAST/CLSI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ITC EUCAST/CLSI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VRC EUCAST/CLSI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>POS EUCAST/CLSI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ISA EUCAST/CLSI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AFG EUCAST/CLSI</td>
</tr>
<tr>
<td>AZN8196</td>
<td>Proven IA</td>
<td>No</td>
<td>None</td>
<td>0.5/0.5 0.125/0.12 0.25/0.25 0.031/0.031 0.5/0.5 0.031/0.031 0.5/0.5 0.031/0.031</td>
</tr>
<tr>
<td>V 59-73</td>
<td>Clinical, disease entity unknown</td>
<td>Unknown</td>
<td>G54W</td>
<td>0.5/0.5 &gt;16/16 0.25/0.25 0.031/0.031 0.5/0.5 0.031/0.031 0.5/0.5 0.031/0.031</td>
</tr>
<tr>
<td>V 28-77</td>
<td>Proven IA</td>
<td>Yes</td>
<td>M220I</td>
<td>0.5/0.5 &gt;16/16 0.25/0.25 0.5/0.5 0.5/0.5 0.031/0.031</td>
</tr>
<tr>
<td>V 52-35</td>
<td>Proven IA</td>
<td>No</td>
<td>TR&lt;sub&gt;34/L98H&lt;/sub&gt;</td>
<td>0.5/0.5 &gt;16/16 0.5/0.5 0.5/0.5 0.5/0.5 0.031/0.031</td>
</tr>
</tbody>
</table>

<sup>a</sup>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 doses ranging from 0.12 to 122.9 mg/kg)\textsuperscript{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/liters · kg)</th>
<th>Clss,$\text{F}$ (liters/h)</th>
<th>Vz,$\text{F}$ (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>0.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5.54</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>

\textsuperscript{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$_{\text{INF, pred}}$; Clss,$\text{F}$, total systemic clearance; Vz,$\text{F}$, volume of distribution.

54.1% (Table 3). For the ELF data from the 128-mg/kg dose, $k_{\text{el}}$ (terminal elimination rate constant) could not be determined with the consequence that the predicted $\text{AUC}_{0-12s}$ could not be calculated reliably. Hence, the AUC$_{0-12s}$ in plasma and ELF 27.27% survival in mice.

(ii) 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. 

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 the TR34/L98H isolate (MIC, 8 mg/liter). For the latter isolate, the maximum dose of isavuconazole (256 mg/kg produg) resulted in 27.27% survival in mice.

Isavuconazole therapy significantly reduced kidney Aspergillus copy numbers at day 3 postinfection. The mean number of genome copies detected in untreated animals was $3.69 \times 10^5$ in kidneys ($n = 3$, range = $3.5 \times 10^5$ to $3.7 \times 10^5$) at day 3 postinfection (Fig. 4; see also Fig. S1 in the supplemental material).

Further, there was a mean 3- to 4-log$_{10}$ reduction of $A.\ fumigatus$ genome copies in infected animals treated with the highest dosage of ISA.

TABLE 3 Penetration ratio of isavuconazole (BAL4815) in ELF compared to plasma based on total drug\textsuperscript{a}

<table>
<thead>
<tr>
<th>ISA dose (mg/kg)</th>
<th>AUC$_{\text{INF, pred}}$ (h · mg/liter)</th>
<th>AUC$_{\text{INF, pred}}$ ELF/plasma ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>ELF</td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>34.70</td>
<td>25.14</td>
</tr>
<tr>
<td>128</td>
<td>107.41</td>
<td></td>
</tr>
<tr>
<td>256</td>
<td>146.68</td>
<td>52.57</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Because of variability in data and lower limit of quantification, the AUC$_{\text{INF, pred}}$ (predicted area under the plasma concentration-time curve from time zero to infinity) could not be reliably determined for the 128-mg/kg dose.
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 TR34/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 TR34/L98H isolate, which had a high MIC (8 μg/ml),
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.

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**FIG 4** Efficacy of isavuconazole against 4 *A. fumigatus* isolates expressed as *A. fumigatus* DNA load (copies/μg 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 itraconazole 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).

![FIG 5 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.](http://aac.asm.org/)

<table>
<thead>
<tr>
<th>A. fumigatus strain</th>
<th>ISA MIC (mg/liter)</th>
<th>ISA ED&lt;sub&gt;50&lt;/sub&gt; (mg/kg)</th>
<th>Comparison with wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>P</em> value</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.5</td>
<td>24.15</td>
<td>0.16</td>
</tr>
<tr>
<td>G54W strain</td>
<td>0.5</td>
<td>28.93</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>M220I strain</td>
<td>4</td>
<td>109</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TR&lt;sub&gt;150&lt;/sub&gt;/L98H strain</td>
<td>8</td>
<td>438.8</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> df<sub>n</sub>, degrees of freedom numerator; df<sub>d</sub>, degrees of freedom denominator.
Similarly, using a nonneutropenic murine model of disseminated
*Candida albicans* infection, it has been shown that the pharmaco-
dynamic 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 MIC100 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](http://aac.asm.org/)

**FIG 6** Percentage of survival as a function of the prodrug isavuconazonium sulfate (BAL8557) AUC\(_{0\rightarrow24}\)/MIC 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](http://aac.asm.org/)

**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.
llosis (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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