Pharmacodynamics of Isavuconazole in an *Aspergillus fumigatus* Mouse Infection Model

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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_{EUCAST}, 0.5 mg/liter) and three azole-resistant isolates harboring substitutions in the *cyp51A* gene: G54W (ISA MIC_{EUCAST}, 0.5 mg/liter), M220I (ISA MIC_{EUCAST}, 4 mg/liter), and TR_{34}/L98H (ISA MIC_{EUCAST}, 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_{34}/L98H isolates with the highest dose of prodrug isavuconazonium sulfate (256 mg/kg q12). For a survival rate of 50%, the effective AUC_{0-24}/MIC_{EUCAST} 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_{0-24}/MIC) can be used to optimize the treatment of human infections by *A. fumigatus*, including strains with decreased susceptibility.

Invasive aspergillosis (IA) caused by *Aspergillus fumigatus* is an important opportunistic fungal infection in immunocompromised patients with an overall mortality ranging between 30 and 88% (1–4). Azole antifungals, such as voriconazole (VRC) and posaconazole (POS), are recommended drugs to manage *Aspergillus* diseases (5, 6). Voriconazole is currently recommended as a first-choice treatment for IA, and posaconazole is indicated for prophylaxis and salvage therapy (5, 6). However, the management of IA has become more complicated due to the emergence of azole resistance in *A. fumigatus* (7–16). Surveillance studies indicate that azole resistance is increasing in multiple European countries and in the Middle East, Asia, and Africa (17–21). Therefore, alternative treatment regimens need to be investigated to improve the outcome of patients with azole-resistant IA.

There are basically two alternative options with respect to the management of azole-resistant IA: treatment with a new antifungal formulation or combination therapy. Recent data suggest that combination therapy using a triazole and an echinocandin may be a beneficial treatment strategy for triazole-resistant isolates (22–24). However, for voriconazole, both *in vitro* interactions and *in vivo* studies indicated that the level of synergistic effect is lost at high voriconazole MICs (MIC of ≥8 mg/liter) (22, 23). As a consequence, this is a major drawback in the treatment of patients with azole-resistant IA. Therefore, it is important to explore the efficacy of new antifungal drugs against azole-resistant IA.

Isavuconazole (ISA) is an investigational broad-spectrum triazole currently being investigated in phase III clinical studies for the treatment of severe invasive fungal infections, including the SECURE (invasive aspergillosis and other filamentous fungi), VITAL (rare fungi), and ACTIVE (candidemia/invasive candidi-

asis) programs (http://clinicaltrials.gov). Isavuconazole is administered as a water-soluble prodrug, isavuconazonium sulfate, that is available in both intravenous (i.v.) and oral formulations. The prodrug is rapidly converted to the active moiety, isavuconazole, and nonactive metabolite upon administration. It is now granted Food and Drug Administration (FDA) fast-track status in the United States and received "orphan drug" designation in the United States and European Union for the treatment of invasive aspergillosis and mucormycosis (25). FDA also recently designated isavuconazole as a Qualified Infectious Disease Product (QIDP) for the oral and intravenous treatment of invasive aspergillosis, invasive mucormycosis, and invasive candidiasis (news release, 16 July 2014, Basilea, Basel, Switzerland). In Europe, an application also has recently been submitted to the European Medicines Agency (EMA), seeking approval for isavuconazole to be used for the treatment of invasive mold infections (news re-

lease, 17 July 2014, Basilea, 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.

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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 TR_{34}/L98H (V 52-35) 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 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 (MICs 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 (LD_{90}) 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 Takashio 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 (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 90% lethal dose (LD_{90}) was determined for each isolate, separately. The LD_{90} was 2.4×10^7 (wild-type control), 1×10^7 (G54W strain), 5×10^7 (M220I strain), and 2.5×10^7 (TR_{34}/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 isavuconazonium sulfate (BAL8557)/day in groups of animals where 100% efficacy was not achieved with a once-daily dose. The above-mentioned 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 isavuconazole. 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 endpoint 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 that 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), as previously described (36). Briefly, thermocycling conditions were 95°C for 5 s, 50 cycles of 95°C for 15 s and 60°C for 45 s, and finally 40°C for 30 s, one time. The multicopy 28S rRNA gene of *Aspergillus* sp. was detected by using primers F 5′-TCCTGCTCCTTAGTAGCCTGGT T-3′ and R 5′-GTGCTCCTTGCTTCACCTG-3′ and probe 6-carboxy-fluorescein (FAM)–AGTGACAGCCCTCAGCGACGAA-BBQ. Additionally, the PhHV isolation control was detected by using primers F 5′ TCCTGCTCCTTAGTAGCCTGGTT-3′ and R 5′-GTGCTCCTTGCTTC ACCTG-3′ and probe FAM-AGTGACAGCCCTCAGCGACGAA-BBQ (BlackBerry quencher). For the 28S rRNA detection, an 8-fold dilution series of the cloned PCR product was included to calculate the number of copies per milliliter. The ratio of copies per milliliter and total DNA isolated (nanograms per milliliter) was calculated to determine the *Aspergillus* load of each organ sample.

Pharmacokinetic analysis of isavuconazole in mice. A total of 210 outbred CD-1 (Charles River, the Netherlands) female mice, 4 to 5 weeks old, weighing 20 to 22 g, were used for the PK experiments. On day 0, mice were infected with the wild-type *A. fumigatus* isolate through the lateral tail vein, and after 24 h, treatment with the prodrug isavuconazonium sulfate was initiated at dosages of 0.25, 1, 4, 16, 64, 128, and 256 mg/kg of body weight by oral gavage (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). At day 2 of treatment (day 3 after infection), blood and bronchoalveolar lavage (BAL) fluid samples were drawn from 3 mice for each individual predefined

sampling time point (10 time points in total): immediately before administration of drugs (0.0 h) and subsequently at 0.25, 0.5, 1, 2, 4, 8, 12, 24, and 48 h postdose. The blood samples were drawn through the orbital vein or heart puncture 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 samples were obtained using a technique described previously (37). 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.

Analytical assay of isavuconazole. Isavuconazole (BAL4815) concentration in plasma and BAL fluid was measured by a validated ultraperformance liquid chromatography (UPLC) method with fluorescence detection. Details of the analytical assay are 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 [$AUC_{0–24}$], maximum concentration of drug in serum [C_{max}], the trough concentration 24 h after the start of treatment [$C_{24 h}$], half-life [$t_{1/2}$], volume of distribution [V], clearance [CL], and terminal elimination rate constant [k_{el}]) were calculated using noncompartmental analysis (Phoenix version 6.3). The area under the concentration-time curve from 0 h to infinity ($AUC_{0–\infty}$) was calculated using the linear up-log down trapezoidal rule. In addition, C_{max} and $C_{24 h}$ were directly observed from the data. Half-life was calculated by ln $2/k_{el}$, in which k_{el} was determined by linear regression of the terminal points of the log-linear plasma concentration-time curve. V was calculated using the formula $V = \text{dose}/AUC \times k_{el}$, and CL was calculated as dose/$AUC_{0–24}$.

(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 concentration$_{ELF}$ = drug concentration$_{BAL\ fluid}$ × urea$_{plasma}$/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_{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

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

| | | | | MIC or MEC (mg/liter) of drug by standard | | | | | | | | | | | | |
| | | | | AmB | | ITC | | VRC | | POS | | ISA | | AFG | |
Isolate no.	*Aspergillus* disease[a]	Prior azole exposure	Cyp51A substitution	EUCAST	CLSI	EUCAST	CLSI	EUCAST	CLSI	EUCAST	CLSI	EUCAST	CLSI	EUCAST	CLSI
AZN8196	Proven IA	No	None	0.5	0.5	0.125	0.12	0.25	0.25	0.031	0.031	0.5	0.25	0.031	0.031
V 59-73	Clinical, disease entity unknown	Unknown	G54W	0.5	0.5	>16	>16	0.25	0125	>16	>16	0.5	0.25	0.031	0.031
V 28-77	Proven IA	Yes	M220I	0.5	0.5	>16	>16	0.5	0.25	0.5	0.5	4	2	0.031	0.031
V 52-35	Proven IA	No	TR$_{34}$/L98H	0.5	0.5	>16	>16	4	2	0.5	0.5	8	8	0.031	0.031

[a] Classification according to EORTC/MSG consensus definitions.

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 (EI_{50}, EI_{80}, and EI_{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 differ-

ence 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 (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
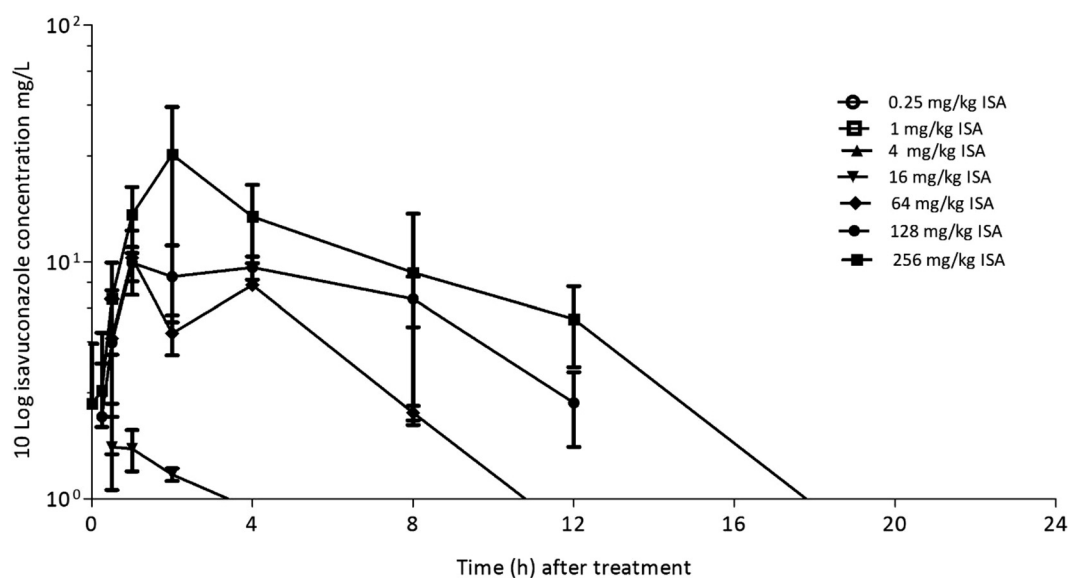
FIG 1 Plasma concentrations of isavuconazole (BAL4815) following oral administration of the prodrug isavuconazonium sulfate (BAL8557) at 0.25-, 1-, 4-, 16-, 64-, 128-, and 256-mg/kg dosages to immunocompetent infected mice. ISA-equivalent doses are 0.12, 0.48, 1.92, 7.68, 30.7, 61.4, and 122.9 mg/kg, respectively. Each symbol corresponds to the geometric mean and standard deviation of the mean plasma levels for three mice.

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)[a]

Dose group (mg/kg)	C_{max} (mg/liter)	C_{last} (mg/liter)	Half-life (h)	AUC_{INF_pred} (h · mg/liter)	$AUC_{INF\_D\_pred}$ (h · mg)/(liters · kg)	CLss_F [liters/ (h · kg)]	Vz_F (liters/kg)
0.25	0.12	0.12					
1	0.11	0.11					
4	0.54	0.08	1.12	0.97	0.24	4.09	6.62
16	2.31	0.24	2.49	9.68	0.60	1.68	6.04
64	5.69	0.52	3.00	34.70	0.54	1.87	8.11
128	9.84	2.42	6.06	107. 41	0.84	1.31	11.46
256	25.62	0.15	3.06	146.68	0.57	1.76	7.75

[a] Abbreviations: C_{last}, last observed quantifiable concentration; AUC_{INF_pred}, predicted area under the plasma concentration-time curve from time zero to infinity; $AUC_{INF\_D\_pred}$, dose-normalized AUC_{INF_pred}; CLss_F, total systemic clearance; Vz_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 $AUC_{0-\infty}$ could not be calculated reliably. Hence, the AUC_{0-12}s 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 prodrug dose. The survival curves for all control groups receiving saline by oral gavage showed a mortality of 100%. The results show that for each prodrug dose, survival decreased as the MIC increased. Similarly, when the prodrug 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 TR_{34}/L98H isolate (MIC, 8 mg/liter). For the latter isolate, the maximum dose of isavuconazole (256 mg/kg prodrug) 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 3.69×10^5 in kidneys ($n = 3$, range $= 3.5 \times 10^5$ to 3.7×10^5) 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.

(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 the azole 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.

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 prodrug 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

TABLE 3 Penetration ratio of isavuconazole (BAL4815) in ELF compared to plasma based on total drug[a]

| ISA dose (mg/kg)	AUC_{INF_pred} (h · mg/liter) Plasma	ELF	AUC_{INF_pred} ELF/ plasma ratio (%)
64	34.70	25.14	72.45
128	107.41		
256	146.68	52.57	35.84

[a] Because of variability in data and lower limit of quantification, the AUC_{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.

FIG 2 Relationship between isavuconazole (BAL4815) concentrations in plasma and ELF. The line indicates the model fit using linear regression ($r^2 = 0.86$, $P < 0.0001$).
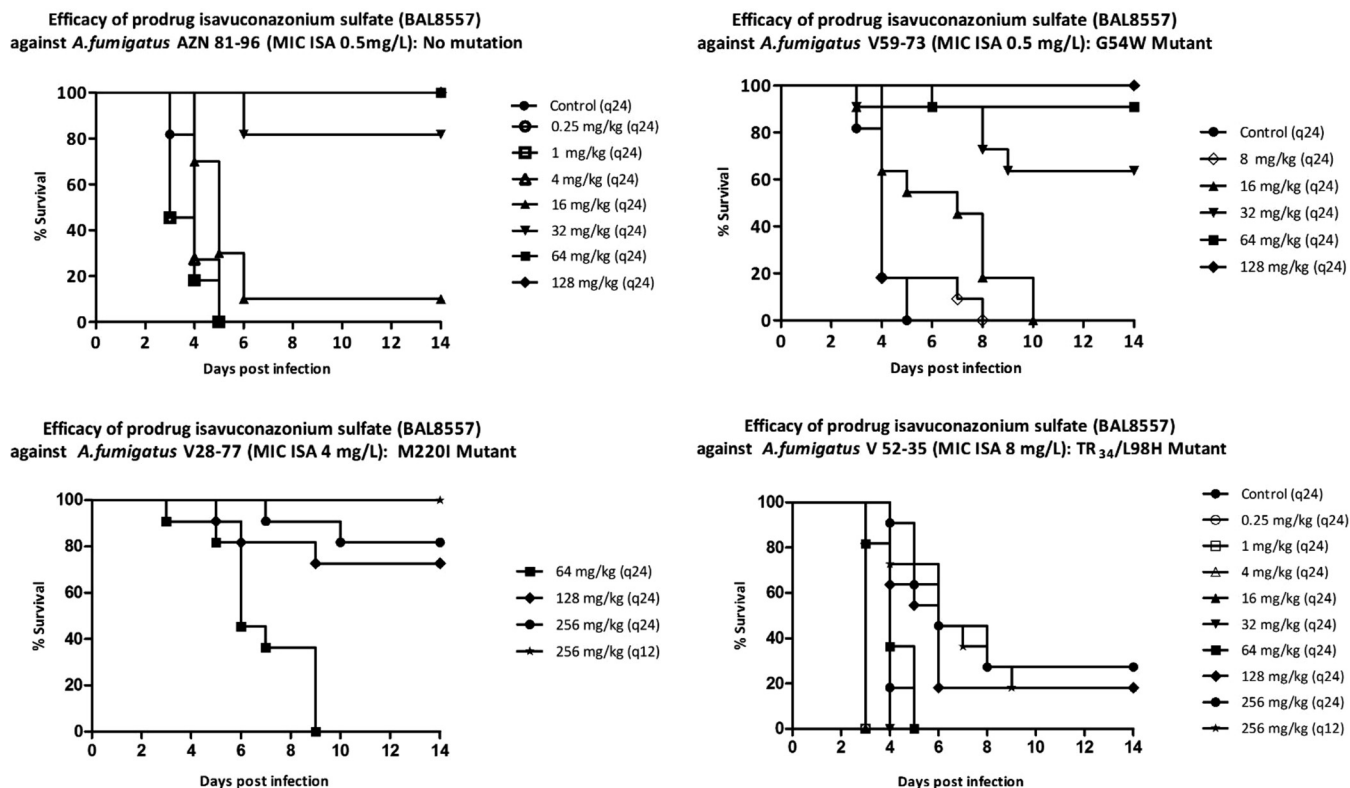
FIG 3 Efficacy of isavuconazole against 4 *A. fumigatus* isolates. Survival curves are depicted by strain. Therapy by oral gavage once daily was begun 24 h after infection and continued for 14 days. The isavuconazonium sulfate (BAL8557) doses of 0.25, 1, 4, 16, 64, 128, and 256 mg/kg correspond to ISA-equivalent 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.

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_{EUCAST}s 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 $TR_{34}/$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 μg/ml),
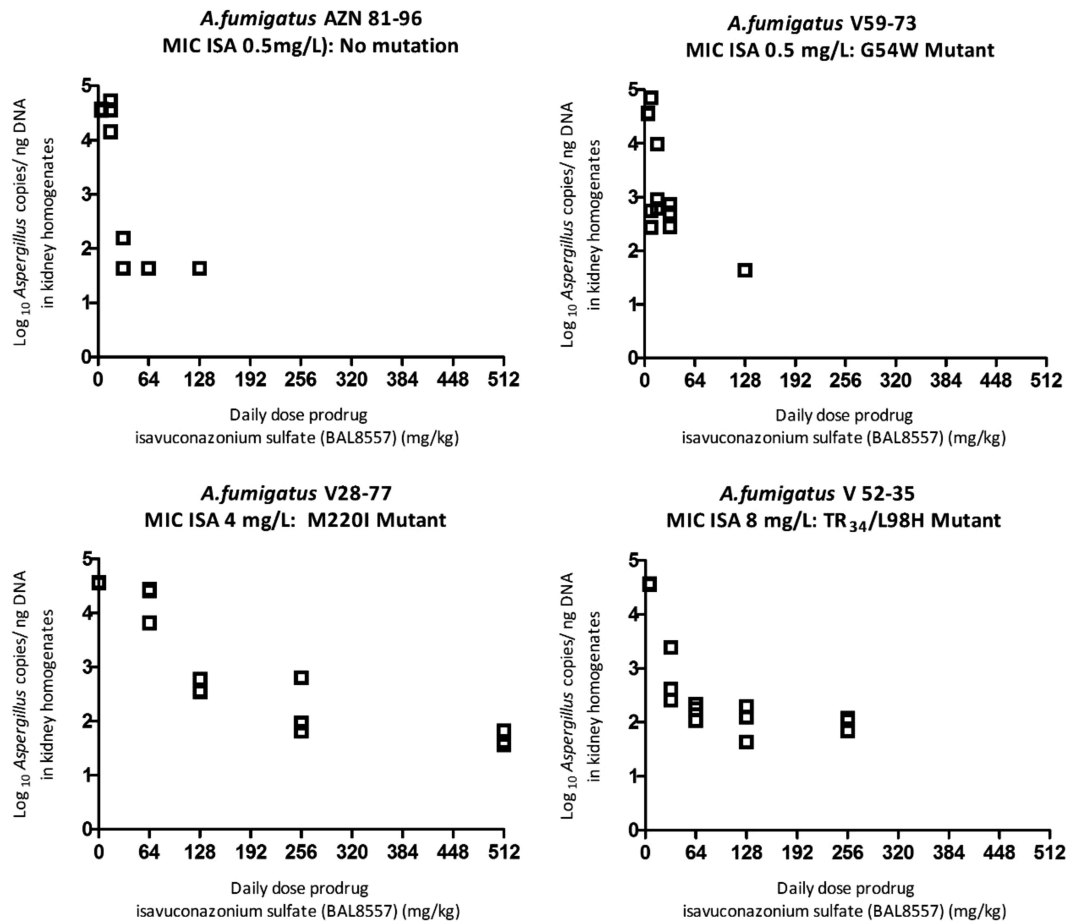
FIG 4 Efficacy of isavuconazole against 4 *A. fumigatus* isolates expressed as *A. fumigatus* DNA load (copies/nanogram of DNA) in kidneys at 72 h postchallenge. For all groups, *n* was 3. Doses were administered as the prodrug isavuconazonium sulfate (BAL8557). The highest dose (256 mg/kg) was used 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.

isavuconazole was not efficacious. Importantly, a maximal effect of 100% survival was achieved in all strains tested except for the strain containing the $TR_{34}/L98H$ mutation. This is of significant importance, since the prevalence of azole resistance in *A. fumigatus* 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

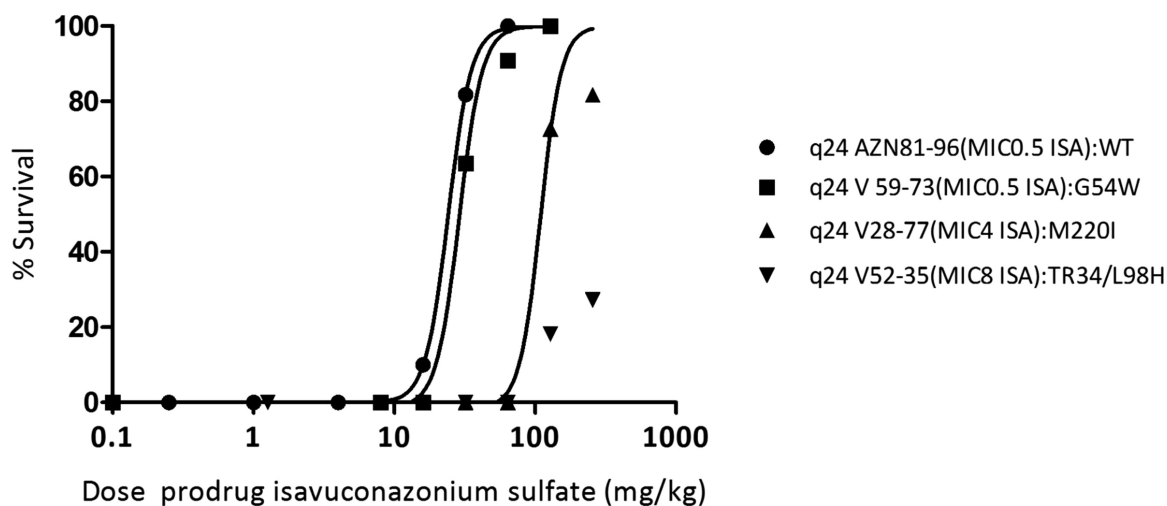|	q24 A ZN81-96(MIC0.5 ISA):WT	q24 V 59-73(MIC0.5 ISA):G54W	q24 V 28-77(MIC4 ISA):M220I	q24 V 52-35(MIC8 ISA):TR34/L98H
R square	1	0,99	0,95	0,91
ED 50	24,15	28,93	109	483,8

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.

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-$\log_{10}$ 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).

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

A. fumigatus strain	ISA MIC (mg/liter)	ISA ED_{50} (mg/kg)	Comparison with wild type		
			P value	F test (df_n, df_d)[a]	Difference
Wild type	0.5	24.15			
G54W strain	0.5	28.93	0.16	2.88 (1, 4)	Nonsignificant
M220I strain	4	109	<0.0001	89.89 (1, 7)	Significant
TR$_{34}$/L98H strain	8	438.8	<0.002	47.33 (1, 7)	Significant

[a] df_n, degrees of freedom numerator; df_d, degrees of freedom denominator.

EI50: 24.73 (95% CI, 22.50 to 27.18)
EI80: 29.89 (95% CI, 25.93 to 34.44)
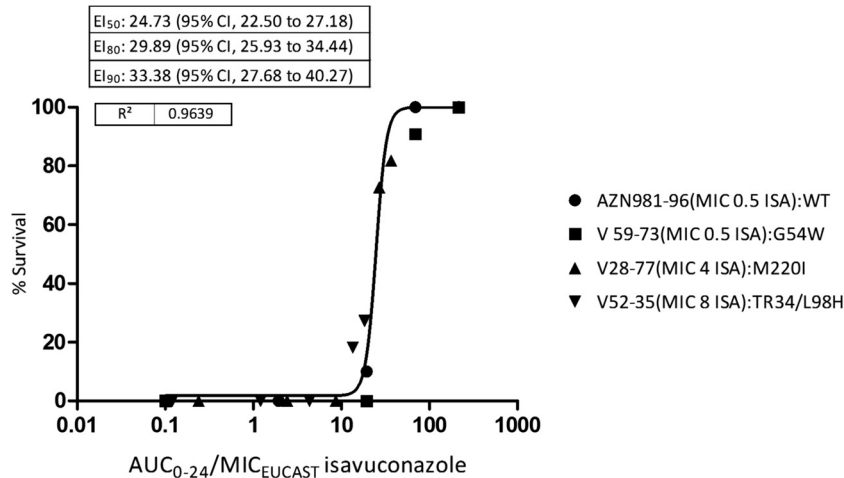EI90: 33.38 (95% CI, 27.68 to 40.27)

FIG 6 Percentage of survival as a function of the prodrug isavuconazonium sulfate (BAL8557) AUC_{0-24}/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.

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_{100} 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-

	q24 AZN81-96(MIC0.5 ISA):WT	q12 AZN81-96(MIC0.5 ISA):WT	q8 AZN81-96(MIC0.5 ISA):WT	Pooled
EC50	12.05	14.72	8.736	11.14

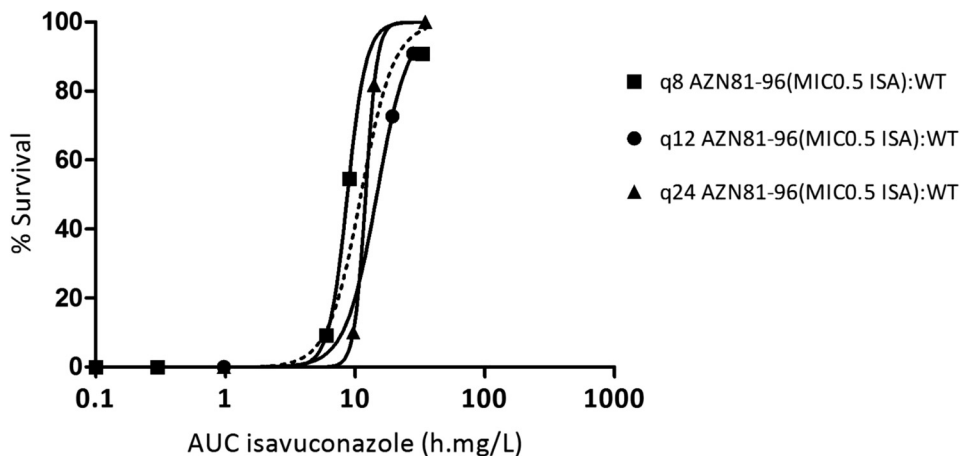|	q24 AZN81-96(MIC0.5 ISA):WT	q12 AZN81-96(MIC0.5 ISA):WT	q8 AZN81-96(MIC0.5 ISA):WT	Pooled
R^2	1.000	1.000	0.9874	0.9346

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.

losis (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 (AUC_{0-24}/MIC) can be used to optimize the treatment of human infections by *A. fumigatus*, including strains with decreased susceptibility.

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