Impact of *cyp51A* Mutations on the Pharmacokinetic and Pharmacodynamic Properties of Voriconazole in a Murine Model of Disseminated Aspergillosis[▽]

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The *in vivo* efficacy of voriconazole against 4 clinical *Aspergillus fumigatus* isolates with MICs ranging from 0.125 to 2 mg/liter (CLSI document M38A) was assessed in a nonneutropenic murine model of disseminated aspergillosis. The study involved TR/L98H, M220I, and G54W mutants and a wild-type control isolate. Oral voriconazole therapy was started 24 h after intravenous infection of mice and was given once daily for 14 consecutive days, with doses ranging from 10 to 80 mg/kg of body weight, using survival as the endpoint. Survival for all isolates was dependent on the voriconazole dose level (R^2 value of 0.5 to 0.6), but a better relationship existed for the area under the concentration-time curve over 24 h in the steady state divided by the MIC (AUC/MIC ratio) or the AUC for the free, unbound fraction of the drug divided by the MIC ($fAUC/MIC$ ratio) (R^2 value of 0.95 to 0.98). The 24-h $fAUC/MIC$ ratio showed a clear relationship to effect, with an exposure index for amount of free drug required for 50% of maximum effectiveness (fEI_{50}) of 11.17 at day 7. Maximum effect was reached at values of around 80 to 100, comparable to that observed for posaconazole and *A. fumigatus*. Mice infected with an isolate having a MIC of 2 mg/liter required an exposure that was inversely correlated with the increase in MIC compared to that of the wild-type control, but due to nonlinear pharmacokinetics, this required only doubling of the voriconazole dose. The efficacy of voriconazole for isolates with high MICs for other triazoles but voriconazole MICs within the wild-type population range was comparable to that for the wild-type control. Finally, we used a grapefruit juice-free murine model of aspergillosis and concluded that this model is appropriate to study pharmacokinetic/pharmacodynamic relationships of voriconazole.

Aspergillus fumigatus is a ubiquitous fungus and is the most common cause of invasive fungal infections in severely immunocompromised patients. Invasive aspergillosis remains associated with significant morbidity and mortality, but the outcome of this disease in hematopoietic stem cell recipients has improved significantly in recent years (22). Although improved survival coincided with multiple changes in transplantation practices, the introduction and use of voriconazole were independently associated with protection from invasive aspergillosis-related death (22). Voriconazole is recommended for primary therapy for most clinical manifestations of invasive aspergillosis, including central nervous system aspergillosis (28). However, the efficacy of voriconazole might be hampered by the development of azole resistance in aspergilli. Several studies have indicated that acquired resistance is an emerging problem (20) and that *in vitro* resistance is associated with treatment failure (8, 20, 23, 24, 27). Most of the recently reported mechanisms of resistance to azoles include mutations in the fungal mitochondrial *cyp51* genes, leading to alterations in the target enzyme 14 α -sterol demethylase. The identified

different mutations in the fungal *cyp51A* gene are associated with distinct phenotypes *in vitro*. These phenotypes are commonly characterized by partial or complete loss of susceptibility to one or more of the mold-active azoles. For instance, a phenotype for amino acid substitutions at glycine 54 (G54) is expressed *in vitro* by resistance to itraconazole and posaconazole (10, 14), but the activity of voriconazole remains comparable to that of isolates without *cyp51A* mutations. Isolates harboring the M220I substitution are resistant to itraconazole, while the MIC of posaconazole is elevated compared to that for wild-type isolates. The susceptibility of M220I mutant isolates to voriconazole is comparable to that of wild-type isolates. (12). Another *A. fumigatus* resistance mechanism involves an amino acid substitution in *cyp51A*, at codon 98, in combination with the duplication in tandem of a 34-bp sequence in the *cyp51A* promoter (TR/L98H) (13). The phenotypic profile for this mechanism shows *in vitro* resistance to itraconazole and reduced susceptibility to posaconazole. The activity of voriconazole in strains with a TR/L98H mutation is variable, ranging from susceptibility of isolates to a complete lack of activity of voriconazole.

Given the prominent role of voriconazole in the primary therapy of invasive aspergillosis, it is important to understand the impact of the presence of resistance mechanisms on the efficacy of the drug. Furthermore, it is important to investigate the efficacy of voriconazole in those isolates that are resistant

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TABLE 1. Origins, *in vitro* susceptibilities, and underlying azole resistance mechanisms of *A. fumigatus* isolates

Isolate	Origin	Prior azole exposure	Cyp51A substitution	MIC (mg/liter) ^a				
				AMB	ITZ	VCZ	POS	CAS
AZN 8196	Proven invasive aspergillosis	No		0.5	0.125	0.25	0.031	0.25
V28-77	Aspergilloma	ITZ	M220I	0.5	>16	0.25	0.5	0.25
V59-73	Clinical, disease entity unknown	Unknown	G54W	0.5	>16	0.125	>16	0.25
V52-35	Proven invasive aspergillosis (24)	No	TR/L98H	0.5	>16	2	0.5	0.25

^a AMB, amphotericin B; ITZ, itraconazole; VCZ, voriconazole; POS, posaconazole; CAS, caspofungin. For caspofungin, the minimum effective concentration was determined.

to other azole compounds but remain susceptible to voriconazole *in vitro*. Using a nonneutropenic murine model of disseminated aspergillosis, we recently showed that the efficacy of posaconazole was dependent on the drug exposure and MIC of the *A. fumigatus* isolate (11), underscoring the hypothesis that the presence of *cyp51* mutations is clinically relevant.

The aim of our research was to investigate the pharmacodynamic and pharmacokinetic properties of voriconazole against clinical *A. fumigatus* isolates with *cyp51A* mutations and to determine whether the efficacy of the drug was attenuated in isolates with a mutation in the *cyp51A* gene but a voriconazole-susceptible phenotype.

MATERIALS AND METHODS

Isolates. Four clinical *A. fumigatus* isolates were used in this study. Clinical isolates with non-wild-type susceptibility had the following substitutions in CYP51A: M220I (isolate V28-77), G54W (isolate V59-73), and TR/L98H (isolate V52-35). A clinical isolate without substitutions in CYP51A was used as a wild-type control (AZN8196). For all isolates, the *cyp51A* substitutions and strain identification were confirmed by sequence-based analysis as described previously (Table 1) (20). In addition, microsatellite typing of the four isolates by short tandem repeat typing was performed to confirm that they were genetically distinct (20). The isolates had been stored in 10% glycerol broth at -80°C and were revived by subculturing on Sabouraud dextrose agar (SDA) supplemented with 0.02% chloramphenicol for 5 to 7 days at 35°C .

Susceptibility testing was performed based on the M38A method of the Clinical and Laboratory Standards Institute (CLSI), using a broth microdilution format (15).

Infection model. A total of 143 outbred CD-1 (Harlan, Horst, Netherlands) female mice (20 to 25 g; 4 to 5 weeks old) were randomized into 13 groups ($n = 11$ per group) for the four different *A. fumigatus* strains. Before the experiment, the isolates were cultured once on SDA for 7 days at 35°C and subcultured twice on Takashio slants (15-cm slants) for 5 days at 35°C . The conidia were harvested in 20 ml of sterile phosphate-buffered saline (PBS) plus 0.1% Tween 80 (Boom B.V., Meppel, 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 hemacytometer. After the inoculum was adjusted to the required concentration, the conidial suspension was stored overnight at 4°C .

Mice were infected via injection in the lateral tail vein with an inoculum corresponding to the 10% survival dose of each isolate, as determined before (11). Postinfection viability counts of the injected inocula were determined to ensure that the correct inoculum had been injected. Therapy was begun 24 h after infection and continued for 14 days. Voriconazole (Pfizer, Capelle a/d IJssel, Netherlands) oral solution was administered in doses of 10, 40, and 80 mg/kg of body weight by oral gavage once daily. The placebo groups were infected with one of the aforementioned isolates and received single doses of saline by oral gavage. On day 15 postinfection, all remaining mice were euthanized humanely under isoflurane anesthesia.

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 86/609/EEC, 24 November 1986), and all animal procedures were approved by the Animal Welfare Committee of Radboud University (procedures RU-DEC 2007-30 and RU-DEC 2008-105).

The infected mice were examined at least three times daily. Clinical inspec-

tions were carried out in order to ensure that there were no cases of desiccation, torticollis, staggering, rapid weight loss (15% decrease within 48 h or 20% decrease within 24 h), or body temperature drop to below 33°C . Mice demonstrating these signs of disease were terminated humanely.

Pharmacokinetic analysis. A total of 72 outbred CD-1 (Charles River, Netherlands) female mice (20 to 22 g; 4 to 5 weeks old) were used for separate pharmacokinetic experiments. On day 0, mice were infected with the wild-type *A. fumigatus* isolate through the lateral tail vein, and after 24 h, treatment was initiated as described above, with a dosage of 10, 40, or 80 mg/kg. On day 2 of voriconazole treatment (day 3 after infection), blood samples were drawn from the orbital vein into lithium-heparin-containing tubes at 8 predefined time points: immediately before administration of voriconazole and at 0, 0.5, 1, 2, 4, 6, 9, and 24 h postdosing. Blood samples were centrifuged for approximately 10 min at $1,000 \times g$ within 30 min of collection. Plasma was aspirated, transferred to two 5-ml plastic tubes, and stored at -70°C . Total and unbound voriconazole samples were measured by a validated (for human and mouse matrices) high-performance liquid chromatography (HPLC) method with fluorescence detection (Thermo Scientific, Breda, Netherlands), using a SymmetryShield RP18 column (Waters, Ettenleur, Netherlands). Samples were pretreated using a protein precipitation procedure. The dynamic range of the assay was 0.05 to 10 mg/liter. The assay had an accuracy range ($n = 15$), depending on the concentration, of 96.7% to 101.4%. Intraday precision varied between 0.8% and 3.2%, and interday precision was between 0 and 2.0%. To determine the free fraction of voriconazole, we pooled plasmas from identical time points ($n = 3$ samples per time point; time points were 1, 2, and 4 h after oral gavage) for all three dosing regimens to obtain at least 0.5 ml voriconazole-containing plasma. The recovery from mouse plasma of the ultrafiltrate was 83.1% (standard deviation [SD], 3.2%). No loss of voriconazole to the filter occurred during ultrafiltration. The plasma was then transferred to YM-30 Centrifree centrifuge tubes (Millipore, Carrigtwohill, Ireland). Samples were centrifuged for 10 min at a relative centrifugal force (RCF) of 2,000 (3,310 rpm) (Rotante 46R centrifuge with radius of 164 mm and angle of 45° ; temperature, 25°C). The ultrafiltrate was injected directly onto the HPLC system. For very low concentrations, the method was slightly modified to obtain a lower limit of quantitation, 0.01 mg/liter, without a loss of accuracy and precision. The assay was validated for mouse plasma and was validated externally by an international proficiency testing program (4).

Arithmetic mean plasma concentrations for three mice per time point were calculated. Peak plasma concentrations (C_{max}) were observed directly from the data. Pharmacokinetic parameters were derived using noncompartmental analysis with WinNonLin 5.2 (Pharsight Inc., Mountain View, CA). The area under the plasma concentration-time curve from time zero to 24 h postinfusion (AUC_{0-24}) was determined by use of the log-linear trapezoidal rule. The elimination rate constant was determined by linear regression of the terminal points of the log-linear plasma concentration-time curve. The terminal half-life was defined as the $\ln_2$ divided by the elimination rate constant. Clearance (CL) was calculated as dose divided by AUC_{0-24} .

Statistical analysis. All data analyses were performed by using the software package GraphPad Prism, version 5.0, for Windows (GraphPad Software, San Diego, CA). A regression analysis was conducted to determine linearity between dose and AUC. Mortality data were analyzed by the log rank test. The survival data were plotted against the dose/MIC ratio, and the Hill equation with variable slope was fitted to the data, both for each individual strain and for pooled survival data. The goodness of fit was checked by determination of the R^2 value and visual inspection. Moreover, the E_{max} (Hill) model was used to evaluate the exposure index for free drug (fEI) required for 20% (fEI_{20}), 50% (fEI_{50}), 80% (fEI_{80}), and 90% (fEI_{90}) of maximum effectiveness and outcome for all isolates.

Statistical significance was defined as a P value of <0.05 (two-tailed). Dose/MIC, AUC/MIC, and $f\text{AUC}/\text{MIC}$ ratios were calculated by dividing the dose

FIG. 1. Efficacy of voriconazole monotherapy at 10, 40 (all isolates), and 80 (TR/L98H isolate) mg/kg, depicted by strain. Placebo groups received saline. For all groups, $n = 11$.

(mg/kg of body weight), AUC, or AUC for the free, unbound fraction of the drug ($fAUC$) by the MIC. Dose/MIC, AUC/MIC, and $fAUC/MIC$ ratio data were transformed to $\log_{10}$ values to approximate a normal distribution prior to statistical analysis.

RESULTS

In vitro activity. The *in vitro* susceptibility profiles of the four isolates are presented in Table 1. Voriconazole showed reduced *in vitro* activity against the TR/L98H isolates, with a MIC of 2 mg/liter. The G54W isolate was resistant to itraconazole and posaconazole, and the M220I isolate was resistant to itraconazole. However, the MICs of voriconazole for these isolates were 0.125 mg/liter and 0.25 mg/liter, respectively. These MIC values are comparable to that of the wild-type control isolate.

Survival curves. The survival curves for all 4 control groups receiving saline by oral gavage showed a mortality rate of 90 or 100% and a median survival time of 2 to 3 days. Figure 1 shows the survival curves for the 4 infection groups against time. The survival study indicated that for all 4 groups, the outcome was significantly improved when the voriconazole concentration increased from 10 to 40 mg/kg or from 40 to 80 mg/kg ($P \leq$

0.0001). A comparative analysis of the 10-mg/kg dose of voriconazole between groups showed no difference in survival between all four groups (log rank test; $P = 0.37$). For the dose of 40 mg/kg, the comparative analysis showed no significant difference in survival for the wild-type and M220I and G54W mutant groups ($P = 0.22$). In contrast, mice infected with the TR/L98H mutant showed a significant difference in therapeutic response compared to the 3 other isolates ($P = 0.0007$): a 2-fold increase of voriconazole dosage was required for mice infected with the TR/L98H isolate to achieve a maximum response similar to that obtained with the wild-type isolate.

Pharmacokinetic analysis. Table 2 shows the results of the pharmacokinetic experiments. A total of 72 mice (3 mice per time point, 8 time points, 3 different dosages) were available for pharmacokinetic assessment. All 72 mice were alive at the time of sample collection. The AUC normalized to a dose of 10 mg/kg resulted in ratios of 0.31, 1.75, and 3.68 for dosages of 10, 40, and 80 mg/kg, respectively. Clearance decreased with increasing doses, showing nonlinearity.

Plasma voriconazole concentrations after administration of oral doses of 10, 40, and 80 mg/kg are shown in Fig. 2. The unbound fractions of voriconazole in the three different dosing

TABLE 2. Pharmacokinetic parameters of voriconazole after doses of 10 to 80 mg/kg by oral gavage

Treatment dose (mg/kg)	AUC (h · mg/liter)	Dose-normalized AUC (mg · h/liter · kg)	$fAUC$ (mg · h/liter · kg)	CL_{ss}/F (liters/h · kg)	C_{min} (mg/liter)	C_{max} (mg/liter)	T_{max} (h)	$t_{1/2}$ (h)
10	3.05	0.31	0.911	2.53	0.36	1.61	0.5	1.75
40	70.12	1.75	20.94	0.39	4.58	11.81	2.0	5.01
80	294.61	3.68	88.00	0.10	23.01	35.86	2.0	16.21

FIG. 2. Plasma voriconazole concentrations after administration of oral doses of 10, 40, and 80 mg/kg to immunocompetent infected mice. Each symbol corresponds to the geometric mean standard deviation of plasma levels for three mice.

arms ($n = 3$ per dosing arm) were 26.51%, 33.28%, and 30.59% for the 10-, 40-, and 80-mg/kg groups, respectively. The geometric mean unbound voriconazole fraction for all groups combined ($n = 9$) was 29.97% (95% confidence interval [95% CI], 27.56 to 32.59%). No comparison between the dosing regimens was performed because the numbers were too small to perform a valid statistical analysis and draw firm conclusions. These data indicate that there was no concentration-dependent protein binding over the range measured. There was no significant loss of voriconazole in the filter system.

Dose-response analysis. The dose-response curves for the four voriconazole dosing regimens and controls for all four strains are shown in Fig. 3 for days 7 and 14. Voriconazole treatment improved the survival of the mice in a dose-dependent manner for all four isolates. The curve for the TR/L98H mutant was shifted to the right because this isolate had a higher MIC than the other three isolates, and thus a higher dose was required to obtain a similar efficacy (100% survival). Dose/MIC ratio-survival plots for days 7 and 14 for all four strains are depicted in Fig. 4. As can be observed, there was significant scatter around the line of best fit by the Hill model (R^2 values of 0.60 and 0.54, respectively) for days 7 and 14. Since voriconazole displays nonlinear pharmacokinetics, this could be the reason for the observed scatter. The 50% effective dose (ED_{50}) for day 7 was 42.82 mg/kg, and that for day 14 was 59.84 mg/kg.

Exposure-response analysis. The AUC and $fAUC$ for each dose, as determined from the pharmacokinetic experiments,

were used to calculate the AUC_{0-24}/MIC and $fAUC_{0-24}/MIC$ ratios for each strain. The relationships between exposure and response on days 7 and 14 for all groups are shown in Fig. 5 and 6 (AUC_{0-24}/MIC and $fAUC/MIC$ ratios, respectively).

The relationships of AUC/MIC and $fAUC/MIC$ ratios with response were significantly better for both time points (R^2 values of 0.96 and 0.98) than for the dose/MIC ratio (R^2 values of 0.60 and 0.54), as expected for a drug with nonlinear clearance. The estimates of the fEI_{50} s on day 7 were similar to those obtained on day 14 (11.2 and 10.5, respectively) (Fig. 6), but the Hill slope was much steeper on day 14. The confidence intervals on day 14 for both the Hill slope and the EI_{50} were relatively wide, despite the high R^2 values and seemingly good fit. Inspection of goodness-of-fit criteria indicated that the covariance between these two parameters was very high, mainly because only one data point was present above 10% and below 100% survival, resulting in large standard errors (SE).

DISCUSSION

Our animal model indicated that the efficacy of voriconazole was significantly reduced in mice infected with the TR/L98H isolate, which had a MIC of 2 mg/liter. According to recently proposed breakpoints, a MIC of 2 mg/liter should be interpreted as intermediate susceptibility (26). In a previous study, we investigated the efficacy of posaconazole against the same four clinical *A. fumigatus* isolates as those used in our present study (11). The posaconazole MICs ranged from 0.063 to >16 mg/liter, and we showed that the MIC had a major impact on posaconazole efficacy (11). Our model indicated that for voriconazole, the MIC had a major impact as well, and a significant dose increase was required in mice infected with the TR/L98H isolate (MIC of 2 mg/liter) to achieve a similar voriconazole efficacy to that in mice infected with a wild-type isolate (MIC of 0.25 mg/liter). Because of the nonlinear pharmacokinetics of voriconazole (see below), the dose required to obtain a similar effect was only twofold higher for the TR/L98H mutant-infected animals.

The exposure-response relationship of voriconazole indicated that improvement of survival for all four clinical isolates was dependent on the voriconazole dose, but a much better relationship existed with the total AUC/MIC or $fAUC/MIC$ ratio. The significantly improved $fAUC/MIC$ relationship, as opposed to the dose/MIC relationship, can be explained by the

FIG. 3. Voriconazole dose-survival relationships for four *A. fumigatus* isolates. Voriconazole treatment improved the survival of the infected mice in a dose-dependent manner for all four isolates. The curves indicate fits with the Hill equation for each isolate.

FIG. 4. Survival as a function of voriconazole dose/MIC ratio for mice infected with four *A. fumigatus* isolates at two different time points. In mice infected with the TR/L98H isolate (MIC of 2 mg/liter), doubling of the dose to 80 mg/kg was required in order to achieve a similar outcome (100%) to that for the groups infected with a wild-type isolate (MIC of 0.25 mg/liter). The curve is the model fit with the Hill equation for pooled data.

nonlinear clearance of voriconazole. Thus, as opposed to posaconazole, the effect of voriconazole cannot readily be predicted by dose alone.

Our animal model furthermore showed that voriconazole remained effective against isolates with a MIC that was within the wild-type range despite the presence of a Cyp mutation and high MICs of other triazoles (26). The efficacy of voriconazole against these isolates was similar to that of the wild-type isolate. We recently investigated the relationship between Cyp mutations and the Cyp51A protein by using a homology model (2). This model indicated that there are two ligand access channels which are probably used by azole compounds for docking. Amino acids G54 and M220 are located in loops in close proximity to the opening of channel 2. It was suggested that changes close to the entrance of channel 2 disturb the docking of large azole molecules, such as itraconazole and posaconazole. Voriconazole, however, is a much smaller molecule and can access channel 2 or even channel 1 of the G54W and M220I mutants in modeling experiments (Fig. 7B and C), which corresponds with the MIC being similar to that of a wild-type isolate. In contrast, the change of leucine to histidine in the L98H mutant can disturb both channels 1 and 2 through a change at position Y107 (Fig. 7A), resulting in attenuated voriconazole susceptibility. The protein model therefore supports our observed phenotypes *in vitro* and the efficacy of voriconazole *in vivo*. Our aspergillosis model suggests that pa-

tients with an infection due to an *A. fumigatus* isolate that is resistant to one or more azoles but susceptible to voriconazole could be treated with this azole.

Voriconazole exposure-response relationships have been investigated in different experimental models of fungal infections. These studies seemed to suggest that the mouse is a poor model for investigating the efficacy of voriconazole *in vivo* because of the rapid elimination of the drug, whereas guinea pigs, with their slower metabolism and clearance, are able to retain the drug in their system longer (9, 19, 21). However, mice are preferred over guinea pigs for efficacy studies because they offer advantages in cost, animal handling, and housing. To improve the murine model for study of voriconazole, the use of grapefruit juice has been adopted by some authors for its property of inhibiting intestinal CYP3A4 and, as a consequence, enhancing systemic exposure (25). However, the use of grapefruit juice has several disadvantages. The first is a lack of standardization of the contents of the juice. The second is possible yet unknown interactions of grapefruit juice with voriconazole metabolism and activity. Furthermore, Graybill et al. suggested that grapefruit juice may exhibit antifungal activity or inhibit fungal cytochrome enzymes (7). Given that there are not sufficient data about the effects of the juice on the intrinsic factors of the CYP genes of the fungus or even the host immune system, we employed the experimental mouse model without grapefruit juice application. By carrying out a timed

FIG. 5. Statistical timed analysis of survival as a function of voriconazole AUC/MIC ratio for four *A. fumigatus* isolates. Increased voriconazole exposure was required to obtain maximum efficacy in mice infected by isolates with attenuated susceptibility. The curve is the model fit with the Hill equation for pooled data.

FIG. 6. Survival as a function of $fAUC/MIC$ ratio for four *A. fumigatus* isolates. Based on the voriconazole exposure data after oral administration of 10, 40, and 80 mg/kg, an $fAUC/MIC$ ratio of 80 to 100 might protect against azole-resistant *A. fumigatus* species with MICs reaching 2 mg/liter. The curve is the model fit with the Hill equation for pooled data.

analysis of the exposure-response data, we were able to determine the relationship between these two factors, confirming that these parameters can be investigated in a grapefruit juice-free model.

Besides rapid clearance of voriconazole, another potential drawback of the mouse model is the decrease of systemic exposure of voriconazole during multiple dosing experiments, probably due to autoinduction of voriconazole metabolism (19). This phenomenon is more pronounced in mice and rats than in guinea pigs (19). We found that the pharmacodynamic analysis could be performed on day 7 of the study, which reduces the impact of autoinduction and reduces animal suffering. Although we did not measure the voriconazole exposure on day 14, the survival-AUC/MIC relationships were similar for both time points in that the EI_{50} values were similar. However, the Hill slope was steeper on day 14. This effect could be due to the autoinduction phenomenon because of the lower voriconazole plasma levels on day 14 than on day 7. This phenomenon would result in lower drug exposure, and therefore a lower percent survival, on day 14 than on day 7, as shown in Fig. 5. However, the confidence interval for the EI_{50} estimate was large, mainly because only one data point was present above 10% and below 100% survival, resulting in large SE.

Statistical analysis at two time points of therapy demonstrated that the $fAUC/MIC$ ratio is the critical pharmaco-

netic-pharmacodynamic index associated with treatment efficacy of voriconazole against aspergillosis. As early as day 7, as depicted in Fig. 6, the 24-h $fAUC/MIC$ ratio showed a clear relationship to effect, and a maximum effect was reached at values of around 80 to 100. This corresponds to values found for *Candida* infections (1, 18) and for Gram-negative microorganisms (6). This indicates that our model is robust and useful for investigating clinically relevant pharmacokinetic and pharmacodynamic relationships, despite the fact that it is a nonneutropenic model and that infection is established through intravenous inoculation of mice rather than following the natural route of infection, via the airways. It also corresponds with a 2-log reduction of CFU of *Candida* after 2 h, as described by Andes et al. (1). For most triazoles, effect is governed only by the concentration of free drug (1, 5). Our aspergillosis model showed a voriconazole protein-free fraction of 29.87% (standard deviation, 2.9%). This was similar for all doses and for three different time points. Previous studies of humans showed that standard-dose oral voriconazole results in a total AUC_{0-24} value of 18 to 23 mg · h/liter (17). An oral dose of 200 mg twice daily would result in a free drug AUC of 8.4 $\mu\text{g} \cdot \text{h/ml}$ (16). For hematopoietic stem cell transplant (HSCT) recipients, the median total AUC_{0-24} was found to be approximately 70 mg · h/liter on study day 7 and 50 mg · h/liter on day 14 (3), which would correspond to an $fAUC$ of approx-

FIG. 7. Channels 1 and 2 of the G54W and M220I α -demethylase mutants are accessible for voriconazole (B and C), whereas they are closed through a position change of Y107 by the L98H mutation (A). The two ligand access channels are indicated in green. The heme cofactor is indicated in black.

imately 21 and 15 mg · h/liter, respectively, based on a free fraction of 29.87%. A previous study (1) reported a voriconazole protein binding level of 78%. The reasons for these discrepant findings include possible experimental variation, use of other animals, or use of different methods of determining protein binding.

Our model indicates that the highest MIC at which infections can be treated with maximum efficacy is 0.25 mg/liter. In our opinion, taking the variation in pharmacokinetic parameters in the population into account, as determined by Monte Carlo simulations (EUCAST voriconazole rationale document, version 2.0 [www.eucast.org]), the MIC would be even lower, i.e., 0.125 mg/liter, which is similar to the EUCAST breakpoint for *Candida* spp.

We concluded that our model supports the view that the efficacy of voriconazole treatment against *A. fumigatus* is dependent both on drug exposure and on the MIC for the isolate, like the case for other antifungal drugs (11). Although voriconazole has a nonlinear pharmacokinetic profile, the quantitative relationship between exposure and effect was comparable to that found for posaconazole. We have shown that increased voriconazole exposure is required to obtain maximum efficacy in mice infected by isolates with attenuated susceptibility, while the drug remains effective against those isolates with high MICs for other azoles but MICs for voriconazole that are within the wild-type population range.

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