Susceptibility breakpoints and target values for therapeutic drug monitoring of voriconazole and *Aspergillus fumigatus* in an *in vitro* pharmacokinetic/pharmacodynamic model

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**Background:** Although voriconazole reached the bedside 10 years ago and became the standard care in the treatment of invasive aspergillosis, reliable clinical breakpoints are still in high demand. Moreover, this has increased due to the recent emergence of azole resistance.

**Methods:** Four clinical wild-type and non-wild-type *A. fumigatus* isolates with voriconazole CLSI MICs in the range of 0.125–2 mg/L were tested in an *in vitro* pharmacokinetic (PK)/pharmacodynamic (PD) model. Mouse PK was simulated and *in vitro* data were compared with *in vivo* outcome. Human PK was simulated and susceptibility breakpoints and trough levels required for optimal treatment were determined for the CLSI and EUCAST methods after 48 h and the gradient concentration MIC test strip (MTS) method after 24 h using the *in vitro* PK/PD relationship and Monte Carlo simulation.

**Results:** The *in vitro* PK/PD target (95% CI) associated with 50% of the maximal antifungal activity (EC₅₀) was 28.61 (16.18–50.61), close to the *in vivo* EC₅₀ of 14.67 (9.31–21.58) fAUC₀–2₄/CLSI MIC. When human PK was simulated, the EC₅₀ was 24.7 (17.9–35.6) fAUC₀–1₂/CLSI MIC and it was associated with 6 week survival in clinical studies of invasive pulmonary aspergillosis. Target attainment rates were ≤ 5% (0%–24%), 42% (16%–58%), 68% (54%–75%) and ≥ 79% (73%–86%) for isolates with CLSI MICs ≥ 2, 1, 0.5 and ≤ 0.25 mg/L, respectively. A trough/CLSI MIC ratio of 2 was required for optimal treatment. The susceptible/intermediate/resistant breakpoints were determined to be 0.25/0.5–1/2 mg/L for CLSI, 0.5/1–2/4 mg/L for EUCAST and 0.25/0.375–1/1.5 mg/L for MTS.

**Conclusions:** These susceptibility breakpoints and target values for therapeutic drug monitoring could be used to optimize voriconazole therapy against *A. fumigatus*.

**Keywords:** trough levels, *A. fumigatus*, azole resistance

**Introduction**

*Aspergillus fumigatus* is a ubiquitous saprophytic fungus that is usually harmless in healthy individuals. However, in immunocompromised patients this mould is the causative agent of severe infections such as invasive aspergillosis. In both European and American therapeutic guidelines, voriconazole is the first-line drug recommended for the treatment of invasive aspergillosis.¹² Recently, *A. fumigatus* clinical isolates exhibiting reduced susceptibility to voriconazole were associated with treatment failures, thereby complicating the clinical management of invasive aspergillosis.³⁴ The currently proposed susceptibility breakpoints for voriconazole and *A. fumigatus* are ≤ 0.5 and ≤ 1 mg/L using the CLSI and the EUCAST methodology, respectively. These breakpoints were derived based on epidemiological cut-off values and *in vitro* pharmacokinetic (PK)/pharmacodynamic (PD) studies.³⁵–⁷ Although epidemiological cut-off values are useful in detecting non-wild-type strains,³⁸ their clinical importance is uncertain since they are determined from MIC distribution data without taking into account drug exposure and efficacy data. *In vitro* PK/PD studies are very important in describing the PD of antifungal drugs using clinically relevant drug exposures. Yet, the challenges of these *in vitro* PK/PD models are their validation based on *in vivo* results and proper extrapolation of *in vitro* data to human
infections in order to determine clinically useful susceptibility breakpoints. Animal models can also be used to determine susceptibility breakpoints, although differences in PK, toxicity, immunology and pathology compared with humans may obscure PD effects.\textsuperscript{7,10}

In the aforementioned in vitro PK/PD studies upon which the voriconazole susceptibility breakpoints were generated, \textasciitilde 90\% of patients were estimated to attain the PK/PD target of maximal activity with standard dosing of voriconazole for \textit{A. fumigatus} isolates demonstrating CLSI MICs of \textasciitilde 0.5 mg/L. However, in a surveillance study where MICs were correlated with survival, isolates with MICs of 0.5 mg/L were associated with only 70\% 6 week survival.\textsuperscript{5} Furthermore, in clinical trials of aspergillosis where patients were usually infected with wild-type isolates and treated with standard dose of voriconazole, the 6 week survival rate was \textasciitilde 80\%.\textsuperscript{2,11} In addition, recent animal studies showed that voriconazole uniformly prolonged survival and reduced fungal burden in mice infected by isolates with MICs of \textasciitilde 0.25 mg/L, but not in mice infected by strains with MICs of 0.5–2 mg/L.\textsuperscript{12} These observations indicate that further investigation is required to confirm the susceptibility breakpoint of 0.5 mg/L for CLSI methodology.

Continued effort in optimizing in vitro PK/PD studies is warranted, because these models may play a pivotal role in predicting therapeutic drug levels in humans. The non-linear PK profile of voriconazole along with the reported large interpatient variability in plasma levels emphasizes the need for performing therapeutic drug monitoring (TDM).\textsuperscript{13} Accordingly, voriconazole trough levels \textasciitilde 1–2 mg/L have been associated with higher efficacy whereas trough levels \textasciitilde 4–5 mg/L were associated with higher toxicity.\textsuperscript{14–16} TDM may also be used to overcome antifungal resistance by increasing drug exposure for isolates with reduced susceptibility. However, target values of voriconazole exposure have not been determined in relation to MICs for optimal therapy against wild-type and non-wild-type \textit{A. fumigatus} isolates.

In the present study, a recently developed in vitro PK/PD model\textsuperscript{12,13} was compared with the results obtained from an animal model of experimental aspergillosis using the same \textit{A. fumigatus} strains and simulating mouse PK.\textsuperscript{13} Susceptibility breakpoints for voriconazole strains were then determined for CLSI and EUCAST methodologies simulating human PK and using four \textit{A. fumigatus} strains with reduced susceptibility to voriconazole and distinct mechanisms of resistance. Susceptibility breakpoints were also determined for a simpler and faster commercial antifungal susceptibility testing method based on gradient concentration strips within 24 h of incubation. Finally, the voriconazole AUC and trough levels in human serum required for optimal treatment and minimal toxicity were determined in relation to MICs.

Materials and methods

\textbf{Fungal isolates}

Four clinical \textit{A. fumigatus} isolates with defined azole resistance mechanisms and distinct susceptibility profiles to voriconazole were studied.\textsuperscript{19} These included a wild-type strain without substitutions in the cytP51A gene (AZN8196) and three non-wild-type strains with the following confirmed by sequence-based analysis: cytP51A mutations G54W (V59–73), M220I (V28–77) and TR2L/L98H (V52–35). Voriconazole MICs were determined as the lowest drug concentration corresponding to complete growth inhibition and they were 0.125, 0.125, 0.25 and 2 mg/L with the CLSI M38-A2 method after 48 h.\textsuperscript{10} 0.25, 0.25, 0.5 and 4 mg/L with the EUCAST method after 48 h\textsuperscript{11} and 0.125, 0.125, 0.19 and 1.5 mg/L with the gradient concentration MIC test strip (MTS) method after 24 h (Lifichemi, Italy) for AZN8196, V59–73, V28–77 and V52–35, respectively.

\textbf{Antifungal drugs and medium}

Pure powder of voriconazole (Pfizer Inc.) was dissolved in sterile DMSO (Carlo Erba Reactifs-SDS, Vol de Reuil, France) and stock solutions of 10 mg/ml were stored at \textasciitilde 70\Celsius until use. The medium used throughout was RPMI 1640 (with L-glutamine and without bicarbonate) (AppliChem, Darmstadt, Germany) buffered to pH 7.0 with 0.165 M MOPS (AppliChem, Darmstadt, Germany) and containing 100 mg/L chloramphenicol (Sigma-Aldrich, St Louis, USA).

\textbf{In vitro PK/PD model}

A previously used two-compartment PK/PD dialysis/diffusion closed model was optimized to correlate with the in vivo outcome of experimental disseminated aspergillosis as described below. The model consists of an external compartment comprising a conical flask connected to a peristaltic pump and an internal compartment (IC) comprising a 10 mL semi-permeable dialysis tube (Spectra/por\textsuperscript{16} Float-A-Lyzer\textsuperscript{16} G2, Spectrum Laboratories Inc., Breda, The Netherlands) inoculated with \textasciitilde 10\(^5\) cfu/ml Aspergillus conidia.\textsuperscript{17} Repeated sampling of 200 \textmu L was made from the IC in order to determine the drug concentration (100 \textmu L) time profile. Samples were stored at \textasciitilde 70\Celsius until tested.

\textbf{In vitro PK}

Voriconazole levels were measured using a microbiological agar diffusion assay as previously described using a voriconazole-susceptible Candida parapsilosis isolate.\textsuperscript{22} The drug concentrations correlated linearly with the inhibition zone diameters ($R^2$ \textasciitilde$0.98$) and the mean coefficient of variation ranged from 9\% at low concentrations to 12\% at high concentrations. Deviation from HPLC was \textasciitilde15\%, as demonstrated using external quality control standards (NEQAS, North Bristol, UK). A concentration–time curve was generated for each simulated dose of the in vitro PK/PD model and analysed by non-linear regression analysis using a one-compartment model described by the equation $C_t = C_0 e^{-k t}$ where $C_t$ (dependent variable) is the concentration of drug at a given time $t$ (independent variable), $C_0$ is the initial concentration of the drug at time $t=0\,$ h, $e$ is the physical constant $2.718$ and $k$ is the rate of drug removal. The half-life was calculated using the equation $t_{1\text{/2}} = \ln 2/ k$ and compared with the respective values observed in humans and mice. Finally, the area under the curve with respect to time ($\text{AUC}_{\text{total}}$) was calculated for each simulated dosage by applying the trapezoidal rule.

\textbf{In vitro PD}

To estimate fungal growth and the antifungal effect of each voriconazole dosing regimen, 100 \textmu L was sampled from inoculated dialysis tubes at regular intervals up to 72 h. The galactomannan index (GI) levels were determined using a commercially available sandwich enzyme-linked immunoassay (Platelia Aspergillus EIA, Bio-Rad Laboratories). A GI–time profile was constructed for each of the four strains. Moreover, the area under the GI curve (\text{AUC}_{\text{GI}}) was determined as a surrogate marker of fungal growth as previously described.\textsuperscript{16} The percentage of growth inhibition at each dose was calculated as $1 - \text{AUC}_{\text{GI,VR}}/\text{AUC}_{\text{GI,GC}}$, where $\text{AUC}_{\text{GI,VR}}$ is the $\text{AUC}_{\text{GI}}$ at a certain dose of voriconazole, whereas $\text{AUC}_{\text{GI,GC}}$ is the $\text{AUC}_{\text{GI}}$ of the drug-free growth control.

\textbf{PK/PD modelling}

For the determination of the exposure–response relationship, the $\text{fAUC}_t$/MIC ratio was calculated for each simulated dose and isolate. The $\log_{10}$ $\text{fAUC}_t$/ MIC values were associated with the percentage growth inhibition using
non-linear regression analysis based on the sigmoidal model with variable slope (E_{max} model) described by the equation $E = E_{\text{max}} \times C^n / (C^n + EC_{50}^n)$, where $E_{\text{max}}$ is the maximum percentage growth inhibition, $C$ is the drug concentration, $EC_{50}$ is the drug concentration corresponding to 50% of $E_{\text{max}}$, and $n$ is the Hill slope. The model was globally fitted to all data. The goodness of fit of the $E_{\text{max}}$ model was assessed by $R^2$ and runs-test analysis. All data were analysed using the statistics software package GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

**In vitro–animal correlation**

The in vitro PK/PD correlation was evaluated using the previously published in vivo results of an immuno-competent murine model of disseminated aspergillosis. Briefly, groups of mice were infected with the four strains used in the present study and treated daily with three different voriconazole doses for 15 days. The voriconazole dosages of 10, 40 and 80 mg/kg/day, which corresponded to total maximum mouse plasma concentrations (C_{max}) of 1.60, 11.09 and 36.49 mg/L and AUC of 3.05, 70.12 and 294.61 mg·h/L, respectively, with an average half-life of 6 h, were simulated in the in vitro PK/PD model. Drug concentrations were added at the corresponding C_{max} values in the in vitro model once daily for 3 days. Galactomannan production and drug levels were determined at regular time intervals as described above. The C_{50} obtained from the in vitro exposure–response relationship (percentage growth inhibition versus $AUC_{0-24}$/MIC) at 24, 48 and 72 h of incubation was compared with the C_{50} of the in vivo exposure–response curve (percentage survival versus $AUC_{0-24}$/MIC) after 7 and 14 days of treatment using the F-test. The in vivo $AUC_{0-24}$ was calculated on the basis of the unbound fraction of voriconazole previously found for the 10, 40 and 80 mg/kg dosages, which was 26.51%, 33.28% and 30.59%, respectively. Two independent experiments were conducted.

**In vitro–clinical correlation**

Previously reported steady-state voriconazole plasma PK in patients receiving 2–5 mg/kg twice daily intravenous dosages was simulated in the in vitro model with total maximum plasma concentrations of 0.83, 1.75, 3.5 and 7 mg/L and average half-life of 6 h. The percentage growth inhibition versus $AUC_{0-12}$/MIC relationship after 24, 48 and 72 h of incubation was constructed. The C_{50} and the $AUC_{0-12}$/MIC associated with near-maximal antifungal activity or 80% growth inhibition (EC_{50}) together with 95% CIs were determined using the $E_{\text{max}}$ model. Monte Carlo simulation analysis was performed by using the usual random number generator function of Excel (Microsoft Office 2007) for 1000 subjects receiving standard intravenous voriconazole dosages of 4 mg/kg twice daily, which corresponds to a total AUC_{0-12} of 50.40 ± 41.8 mg·h/L. The $AUC_{0-12}$ was calculated on the basis of the 42% unbound fraction of voriconazole in human plasma and was 21.2 ± 17.6 mg·h/L. The $AUC_{0-12}$/MIC was calculated for each simulated patient and for isolates with CLSI MICs of 0.5 and ≤ 0.5 mg/L, which correspond to the MIC_{50} of voriconazole and A. fumigatus isolates with CLSI. The proportion of patients that attained the EC_{50} and EC_{50} was compared with the 6 week survival rates previously reported in clinical studies of 70% for isolates with MIC of 0.5 mg/L and 80% for isolates with MIC of ≤ 0.5 mg/L.

**Susceptibility breakpoint determination**

The in vitro PK/PD index (EC_{50} or EC_{50}) that best correlated with the clinical outcome was then bridged with human PK and susceptibility breakpoints were determined for the EUCAST, CLSI and MTS methods. Monte Carlo simulation analysis was performed as described above and the proportion of patients that attained the PK/PD index (95% CIs) was calculated for different MICs ranging from 0.06 to 4 mg/L. Susceptibility breakpoints were determined based on the proportion of patients attaining the PK/PD target at each MIC.

**Optimization of human trough levels in relation to MICs**

Human serum trough levels required to attain the clinically relevant in vitro PK/PD index were calculated for different MICs. For that purpose, the fAUC_{0-12}/MIC ratio was calculated for the different MICs of each methodology. The fAUC_{0-12} was then associated with the C_{50} (trough levels of free drug) obtained in the in vitro PK/PD model. The MICs, at which the in vitro PK/PD index was attained, were plotted against the fAUC_{0-12} and t_{C_{50}} (AUC_{0-12} and trough levels of total drug observed in human plasma, respectively) calculated from the fAUC_{0-12} and C_{50} as fAUC_{0-12}/0.42 and C_{50}/0.42, respectively, on the basis of the 42% unbound fraction of voriconazole in human plasma. The highest MIC at which the clinically relevant in vitro PK/PD index was attained without trough levels exceeding the toxic level of 5.5 mg/L was determined.

**Results**

**In vitro–animal correlation**

The mean±SD PK values obtained in the in vitro PK/PD model were close to the mouse PK of the 10, 40 and 80 mg/kg/day voriconazole doses and corresponded to C_{max} of 1.51±0.07, 10.91±0.20 and 36.62±0.89 mg/L and AUCs of 10.71±0.50, 101.9±2.02 and 268.9±6.51 mg·h/L, and average t_{1/2} of 5.84±0.7 h. The in vitro PK/PD relationship for the four A. fumigatus isolates followed a sigmoid curve (R^2>0.98) similar to the in vivo PK/PD relationship of the animal model (R^2>0.96). The in vitro EC_{50} (95% CI) was 28.61 (16.18–50.61) and did not change over time and was similar to the in vivo EC_{50} of 14.67 (9.31–21.58) (P=0.07) (Figure 1).

**In vitro–clinical correlation**

The mean±SD PK values obtained in the in vitro PK/PD model were close to the human PK of 2–5 mg/kg/day voriconazole doses and corresponded to t_{1/2} of 6.18 h, C_{max} of 0.84±0.03, 1.67±0.04, 3.01±0.59 and 7.65±0.34 mg/L and AUC_{0-12} of 7.26±0.5, 13.97±0.83, 26.39±1.75, 53.05±3.9 mg·h/L (Figure 2). Galactomannan levels were fully suppressed for isolates with a CLSI MIC of 0.125 mg/L, despite the presence of a CYP51A mutation in one of them, whereas partial and no inhibition were observed for isolates with CLSI MICs of 0.25 and 2 mg/L, respectively (Figure 3). The percentage growth inhibition versus $AUC_{0-12}$/ MIC relationship for the four A. fumigatus isolates followed a sigmoid curve (R^2>0.87) with EC_{50} and EC_{50} (95% CI) of 24.7 (17.9–35.6) and 49.6 (34–77.5) for CLSI, 12.64 (9–17.8) and 24 (17–38.6) for EUCAST and 30.19 (21.3–42.7) and 55.3 (38.6–85.9) for MTS, respectively (Figure 4). The in vitro EC_{50} remained constant over time.

The mean±SD AUC_{0-12} value of the simulated patient population was 52.2±37.9 mg·h/L, with 12% of simulated patients having low (<0.2 mg/L) levels in accordance with previous clinical studies. The proportion of patients who attained the CLSI EC_{50} and EC_{50} was 68% and 42% for isolates with CLSI MICs of 0.5 mg/ L and 79% and 68% for isolates with CLSI MICs of ≤ 0.5 mg/L, respectively. Thus, the EC_{50} was best correlated with the 6 week survival rate of 70% and 80% previously reported for isolates with CLSI MIC=0.5 mg/L and CLSI MICs ≤ 0.5 mg/L, respectively. Of note, the proportion of patients who attained the CLSI EC_{50} was correlated with the 12 week survival rate in these studies.
The proportion of simulated patients exceeding the CLSI EC$_{50}$ (95% CI) of 24.7 (17.9–35.6) fAUC$_{0–1}$/MIC after standard voriconazole dosage of 4 mg/kg was ≥79% for A. fumigatus isolates with MICs of ≤0.25 mg/L [i.e. 79% (73%–82%) for MIC 0.25 mg/L, 84% (82%–85%) for MIC 0.12 mg/L and 86% (84%–86%) for MIC 0.06 mg/L], which could be classified as susceptible. Isolates with MICs of 0.5 and 1 mg/L were related to intermediate levels of PK/PD target attainment of 68% (54%–75%) and 42% (16%–58%), respectively. Finally, the corresponding percentage for strains with MICs of 2 and ≥4 mg/L was 5% (0%–24%) and 0% (0%–0%), respectively, indicating resistance (Figure 5). Thus, the susceptible, intermediate and resistant breakpoints were set to ≤0.25, 0.5–1 and ≥2 mg/L for CLSI, respectively. Similar analysis for EUCAST and MTS methodology resulted in breakpoints of 0.5, 1–2 and 4 mg/L and 0.25, 0.375–1, 1.5 mg/L, respectively (Table 1). The different breakpoints among the three methodologies reflect differences in the MICs obtained with each method, since CLSI methodology resulted in 1–2-fold dilution lower MICs than EUCAST and 0.75–2-fold dilution higher MICs than MTS.

**Trough levels and MICs**

The relationship between CLSI, EUCAST and MTS MICs and trough levels in human serum required in order to attain the EC$_{50}$ of 24.7, 12.64 and 30.19 fAUC$_{0–1}$/MIC, respectively, is shown in Figure 6. The highest MIC at which EC$_{50}$ was attained without trough levels exceeding the toxic level of 5.5 mg/L was 2, 4 and 1.5 mg/L for CLSI, EUCAST and MTS, respectively. A trough/MIC ratio of 2, 1 and 2.66 with CLSI, EUCAST and MTS, respectively, was associated with the EC$_{50}$.

**Discussion**

An in vitro PK/PD model was evaluated using four A. fumigatus strains with different MICs of voriconazole that were previously

**Figure 1.** In vitro and in vivo PK/PD relationship based on galactomannan production and survival rate of experimental non-neutropenic murine model of aspergillosis, respectively. The EC$_{50}$ represents the free drug concentration associated with 50% of maximal effect based on the sigmoidal E$_{max}$ model with variable slope.

**Figure 2.** Time–concentration profiles of simulated human twice daily dosing regimens of voriconazole in the in vitro PK/PD model with target C$_{max}$ 0.88, 1.75, 3.5 and 7 mg/L and C$_{min}$ 0.22, 0.44, 0.88 and 1.75 mg/L, respectively, and t$_{1/2}$ 6 h. Data represent drug levels in the internal compartment of the in vitro model (solid lines) and the respective target values observed in human plasma (broken lines).
tested in a non-neutropenic model of disseminated aspergillosis and simulating mouse PK. The in vitro \( \text{AUC}_{0–24}/\text{MIC} \) associated with 50% antifungal activity determined based on suppression of galactomannan production was 28.61 (independent of incubation period) whereas the corresponding in vivo \( \text{AUC}_{0–24}/\text{MIC} \) based on survival after 7 and 14 days of treatment of mice was 14.67.\(^{19} \) Although the observed 2-fold difference between the in vitro and in vivo \( \text{AUC}_{0–24}/\text{MIC} \) was not statistically significant, it may be due to the presence of neutrophils in the non-neutropenic animal model of experimental aspergillosis. In fact, a 2.5-fold difference in the isavuconazole \( \text{AUC}/\text{MIC} \) ratio was previously shown between a persistent and a transient neutropenia model of experimental disseminated candidiasis, indicating that neutrophils may augment the antifungal activity of azoles.\(^{28} \)

Furthermore, a similar \( \text{AUC}_{0–24}/\text{MIC} \) ratio of 24\( \pm \)17 was associated with 50% antifungal activity of voriconazole and other azoles (fluconazole, posaconazole and ravuconazole) in neutropenic models of disseminated candidiasis.\(^{29,30} \) Given the corroboration of our model with previous data, this tool may prove useful for predicting the in vivo outcome of experimental aspergillosis.

Having evaluated the in vitro model, human PK of voriconazole was then simulated and the effect of different human dosing regimens was studied against four \( \text{A. fumigatus} \) isolates with distinct mechanisms of azole resistance.\(^{3} \) When bridging in vitro PK/PD data with human PK, the correlation between the drug concentrations in plasma and at the site of infection (i.e. lung for pulmonary aspergillosis) is an important assumption before making predictions. Although there are human data on voriconazole concentration in the pulmonary epithelial lining fluid (ELF),\(^{31,32} \) there are no data for free voriconazole levels in the human lung parenchyma. The ELF is a different compartment from the interstitium of lung parenchyma where the fungus is growing and may be more important for prophylaxis than for treatment regimens.

![Figure 3](https://academic.oup.com/jac/article/69/6/1611/833540)

**Figure 3.** GI versus time curves for four \( \text{A. fumigatus} \) isolates with increasing MICs in the in vitro PK/PD model simulating human twice daily intravenous dosing regimens with \( c_{\text{max}} \) 0.88, 1.75, 3.5 and 7 mg/L and \( t_{1/2} \) 6 h. Error bars represent SDs.

![Figure 4](https://academic.oup.com/jac/article/69/6/1611/833540)

**Figure 4.** In vitro PK/PD relationship of voriconazole as a function of percentage growth inhibition and \( \text{AUC}_{0–12}/\text{MIC} \) ratio for the four \( \text{A. fumigatus} \) isolates tested in the in vitro PK/PD model simulating human PK. The EC\(_{50}\) was associated with an \( \text{AUC}_{0–12}/\text{MIC} \) of 24.7, 12.64 and 30.19 for the CLSI, EUCAST and MTS methods, respectively.
However, microdialysis assays in animals showed that the free drug levels in lungs were similar to the free drug levels in plasma. Thus, voriconazole plasma PK/PD parameters may be predictive of lung tissue PK/PD parameters. Furthermore, the selection of the appropriate endpoint (e.g. associated with 50% or 80% of maximal activity) that best correlates with clinical outcome is a dilemma constantly debated. These endpoints may markedly differ for shallow exposure–response relationships such as the one found for voriconazole in the present study. We therefore used both 50% and 80% of activity as endpoints to correlate with the survival rate previously observed in clinical trials of invasive aspergillosis and we found that the EC$_{50}$ correlated very well with the 6 week survival whereas the EC$_{80}$ was correlated with the 12 week survival rate. A 6 week rather than a 12 week survival rate was suggested as the preferable endpoint to judge the effectiveness of antifungal therapy, given that most deaths during the first 6 weeks of therapy are due to invasive aspergillosis while those beyond this time have been related to underlying disease. We therefore used the EC$_{50}$ in Monte Carlo analysis. In another study, voriconazole trough levels and MICs were correlated with clinical outcome, whereby the maximal clinical response was found to be $\approx 80\%$ even at high trough/MIC ratios and low MICs. This agrees with the plateau of $\approx 85\%$ PK/PD target.
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attainment found in Monte Carlo simulation even for isolates with low MICs as shown in Figure 5. Furthermore, in the latter study, the near maximal clinical response was found at a trough/MIC ratio of 2–3, which is in line with the results presented in Figure 6 where isolates with CLSI MICs ≤ ECV = 1 mg/L will be effectively treated with a trough voriconazole concentration of 2 mg/L. This evidence provide a clinical correlation of the present in vitro PK/PD model.

Such correlation was not observed with the previously determined susceptibility breakpoint of ≤ 0.5 mg/L based on the PK/PD target of near maximal activity of 55 %AUC/MIC using a more complex in vitro PK/PD open model simulating human alveolus. Several factors may account for the differences in the PK/PD target and the proposed susceptibility breakpoints between the two studies. For example, a higher inoculum of 2.5 × 10^5 cfu/mL was used in the alveolar in vitro model, although fungal burden in the lungs of neutropenic animal models of experimental aspergillosis (mice, guinea pigs and rabbits) is usually in the order of 10^3 cfu/mL. Furthermore, the medium contained 2% fetal bovine serum and the analysis was based on the total and not on the free fraction of voriconazole. Given the qualitative and quantitative differences between human and bovine serum, the unbound fraction of various drugs was markedly different between human and bovine serum. Such differences were found for voriconazole in the serum of different animals. It is generally accepted that only the unbound fraction of drug is pharmacologically active and therefore when in vitro concentrations are correlated with in vivo concentrations, in vivo drug exposures should be corrected for the protein binding. This can alter the PK of voriconazole depending on the protein-binding differences between 2% fetal bovine serum and 100% human serum. In vitro simulation of in vivo protein binding by adding serum proteins in the in vitro models is difficult since complex phenomena may take place. In addition, fungal growth may have been underestimated in that model since galactomannan was measured in the central compartment where fresh medium was continuously added diluting galactomannan levels. Furthermore, cell culture inserts with 3 μm pores were used in the peripheral compartment resulting in loss of mycelia through the pores as we found in our laboratory (J. Meletiadis, unpublished observation). Microbial loss from in vitro PK/PD open models has substantial influence on the results. Finally, the subset of patients (~12%) previously reported to have undetectable voriconazole plasma levels and the 2-fold variation in the estimated PK/PD parameter was not taken into account in the simulation analysis.

Another uncertainty in the PK/PD breakpoint setting process is the level of percentage target attainment (e.g. 80%, 80%–20% and 20%) that defines the susceptible, intermediate and resistant MIC ranges, respectively. The proposed susceptible/intermediate/resistant breakpoints of ≤ 0.25/0.5–1/2 mg/L for A. fumigatus are in line with the CLSI breakpoints for voriconazole and Candida spp. ≤ 0.125/0.25–0.5/1 mg/L. In fact, the percentage of patients with successful outcome for candidiasis was >77% for the susceptible isolates and 64%–66% for the intermediate isolates based on CLSI MIC after 48 h. This is in agreement with the percentage target attainment of ≥ 79% for the susceptible and 68%–42% for the intermediate A. fumigatus isolates found in the present study. Regarding the mechanisms of resistance, we found that >70% of A. fumigatus isolates with CLSI MICs of ≥ 2 mg/L harboured a cyp57A mutation whereas

Finally, by correlating trough levels with MICs, we estimated that the highest MIC of an A. fumigatus isolate that may be treated successfully attaining the PK/PD target of ECV and avoiding toxic serum trough levels of >5.5 mg/L albeit in a small proportion (<10%) of patients was 2, 4 and 1.5 mg/L for the CLSI, EUCAST and MTS methodologies, respectively, further supporting the proposed resistance breakpoints. Moreover, it was previously found that trough levels >1–2 mg/L are required for best efficacy. Considering that >90% of A. fumigatus isolates have CLSI MICs of ≤ 1 mg/L, trough levels of 2 mg/L will be required for PK/PD target attainment, as shown in Figure 6. In addition, breakthrough infections with isolates with CLSI MICs of >1 mg/L have been reported. This is in agreement with the findings of the present study since these isolates would require trough levels of >4 mg/L, which are usually avoided in clinical practice due to high toxicity. Furthermore, voriconazole failure has recently been reported with an A. fumigatus isolate with a CLSI MIC of 0.5 mg/L in a patient who probably did not attain therapeutic drug levels since he received standard voriconazole dosing regimens only for 48 h. Based on Figure 6, target values for TDM of voriconazole can be defined based on the isolate's MIC or local epidemiology, enabling individualized therapy and institutional guidelines, respectively.

In conclusion, the results of the in vitro PK/PD model were comparable to the in vivo outcome of voriconazole therapy in a non-neutropenic model of experimental aspergillosis using the same A. fumigatus strains and the same as the in vivo outcome of voriconazole therapy in neutropenic models of disseminated candidiasis thus providing an in vivo correlation of the present in vitro model. Furthermore, the percentage target attainment was in agreement with the percentage efficacy in clinical trials of aspergillosis in relation to CLSI MICs providing also a clinical correlation of the in vitro PK/PD model. Finally, the proposed susceptibility breakpoints of ≤ 0.25, ≤ 0.5 and ≤ 0.25 mg/L for CLSI 48 h, EUCAST 48 h and MTS 24 h methodologies can help the clinical diagnostic laboratory to correctly characterize isolates and the treating physician to optimize voriconazole therapy using in vitro MIC and drug exposure data. TDM could be used to optimize voriconazole therapy particularly against isolates with CLSI MICs of 0.5 and 1 mg/L and EUCAST MICs of 1 and 2 mg/L, targeting trough levels of 1 and 2 mg/L, respectively, whereas voriconazole should be avoided against isolates with CLSI and EUCAST MICs of >2 and >4 mg/L, respectively.

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Transparency declarations
None to declare.
References


