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# Pharmacodynamics of Voriconazole for Invasive Pulmonary Scedosporiosis

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Potential conflicts of Interest

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## ABSTRACT

*Scedosporium apiospermum* is a medically important fungal pathogen that causes a wide range of infections in humans. There are relatively few antifungal agents that are active against *Scedosporium* spp. Little is known about the pharmacodynamics of voriconazole against *Scedosporium*. Both static and dynamic *in vitro* models of invasive scedosporiosis were developed. Monoclonal antibodies that target a soluble cell wall antigen secreted by *Scedosporium apiospermum* were used to describe the pharmacodynamics of voriconazole. Mathematical pharmacokinetic-pharmacodynamic models were fitted to the data to estimate the drug exposure required to suppress the release of fungal antigen. The experimental results were bridged to humans using Monte Carlo simulation. All 3 strains of *S. apiospermum* tested invaded through the cellular bilayer of the *in vitro* models and liberated antigen. There was a concentration-dependent decline in antigen with near maximal antifungal activity in all 3 strains with 10 mg/L. Similarly, there was a drug exposure dependent decline in circulating antigen in the dynamic model and complete suppression of antigen with an AUC of approximately 80 mg.h/L. A regression of the AUC:MIC versus area under the antigen time curve showed that near maximal effect was obtained with AUC:MIC of approximately 100. Monte Carlo simulation suggested that only isolates with an MIC of 0.5 mg/L enable pharmacodynamic targets to be acheived with a standard regimen of voriconazole. Isolates with higher MICs may need higher drug exposure targets than are currently recommended for other fungi.


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## INTRODUCTION

*Scedosporium* spp. are environmentally ubiquitous hyaline moulds (1). *Scedosporium apiospermum* is a medically important member of the *Scedosporium* genus that also comprises *Scedosporium boydii*, *Scedosporium angustum*, *Scedosporium minutisporum*, *Scedosporium dehoogii*, and *Scedosporium aurantiacum* (2, 3). Infections caused by these pathogens occur in a range of contexts that include near-drowning, solid organ transplantation, chronic lung disease (e.g. cystic fibrosis) and otherwise seemingly immunocompetent hosts (1). *Scedosporium* spp. cause diseases that are as disparate as mycetoma, airway colonisation in cystic fibrosis, pneumonia, skin and soft tissue infection and disseminated infection (4). The fungus is neurotropic and central nervous system infection has been demonstrated in experimental models of infection, and is apparent from a multitude of case studies and series (5–9).

Voriconazole is considered a first-line agent for the treatment of invasive infections caused by *Scedosporium apiospermum* (10). The overall treatment response for infections caused by *Scedosporium* spp. is approximately 43% (11). This disappointingly low figure raises several issues. The first is uncertainty surrounding the optimal use of voriconazole for *Scedosporium* spp. There is no specific information for this indication that informs the optimal regimen, targets for therapeutic drug monitoring or *in vitro* susceptibility breakpoints. The second is the paucity of antifungal agents that are effective for *Scedosporium* spp. The advent of newer triazoles such as posaconazole and isavuconazole do not appear to offer a significant therapeutic advance over voriconazole for the treatment of these fungi. There are no experimental pharmacodynamic models of invasive scedosporiosis that enable current antifungal agents to be optimised or newer antifungal agents to be developed.

Here, we adapt an existing in vitro cell culture model of the human alveolus that has been used for *Aspergillus fumigatus* (12) and *Aspergillus flavus* (13) infections to develop a new model of invasive pulmonary scedosporiosis. The key step in the development of the model described in this study was the use of a double- sandwich monoclonal antibody (mAb)-based ELISA for the quantification of a soluble cell wall antigen shown previously to be an accurate biomarker for tracking species in the *Scedosporium apiospermum* complex (14). The antigen provided a continuous assessment of the interaction of voriconazole with its fungal target potentially in the same way as galactomannan has been used for *Aspergillus* spp. A deep understanding of the pharmacodynamics of voriconazole against *Scedosporium* enables the design of optimised treatment regimens and is fundamental to establishing in vitro susceptibility breakpoints.

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## RESULTS

### *Provenance, Identification and In Vitro Susceptibility Testing*

Two strains were obtained from the Spanish Mycology Reference Laboratory and a further strain from the Regional Mycology Laboratory, University Hospital, South Manchester (Table 1). No details were available as to the relevant clinical details. The three isolates used in this study were identified as *Scedosporium apiospermum* based on sequencing of the ITS and ß-tubulin genes and searching within the FUNCBS.txt database. The MICs using Clinical Laboratory Sciences Institute (CLSI) methodology are shown in Table 1. Strains with voriconazole MICs of 0.5-1 mg/L were used for experiments so as to study isolates largely representative of a susceptible population according to $MIC_{90}$ values (21).

### *Static models of the human alveolus*

A previously described cellular bilayer model of the human alveolus (15) was used to study the pharmacodynamics of voriconazole against *Scedosporium apiospermum.* The bilayer is comprised of a layer of human pulmonary arterial endothelial cells and human alveolar epithelial cells. The bilayer delineates an airspace (alveolar compartment) and blood space (endothelial compartment). Previous studies of *Aspergillus* spp. have used galactomannan as a biomarker to assess fungal invasion through the cellular bilayer and quantify the response to antifungal therapy (12, 15). In the current study, a novel cell wall antigen liberated by *Scedosporium* spp. during growth was used (see below) for this purpose.

Following inoculation of macroconidia into the alveolar compartment and subsequent germination, there was progressive liberation of antigen in the endothelial compartment (Figure 1). Antigen appeared in the endothelial compartment approximately 24 hours after inoculation and plateaued thereafter.

The exposure-response relationships for voriconazole in the static model of invasive pulmonary scedosporiosis are shown in Figure 2. In these experiments, voriconazole was administered to the endothelial compartment 6-hours post inoculation from where it distributed into the cellular bilayer and into the alveolar compartment. There was more pharmacodynamic variability in the alveolar compartment than the endothelial compartment as evidenced by the larger standard deviation of the experimental replicates. Some of the exposure response relationships in the alveolar compartment were right-shifted with respect to those in the endothelial compartment. This was apparent from the data and the estimates of $EC_{50}$ from the inhibitory sigmoid Emax relationship. Maximal antifungal activity, as estimated using the release of antigen, was achieved in both the alveolar and endothelial compartments of all strains with voriconazole concentrations of approximately 10 mg/L (Figure 2). Nevertheless, there was significant strain-to-strain variability in the pharmacodynamics of voriconazole.

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*Dynamic models of the human alveolus*

A dynamic model of invasive pulmonary scedosporiosis was developed to enable the pharmacodynamics in response to human-like pharmacokinetic profiles of voriconazole to be examined. Purpose-built bioreactors have previously been described as has the circuitry that enables the simulation of human-like pharmacokinetics (12). Samples to estimate the pharmacokinetics and pharmacodynamics of voriconazole were taken from medium passing through the endothelial compartment of the dynamic in vitro model.

In untreated circuits, there was a progressive increase in antigen concentrations through the experimental period. A human-like PK profile of voriconazole was simulated in the dynamic model with average $AUC_{72\text{-}96}$ that ranged from 7.84-80.68 mg.h/L. These drug exposures were based on preliminary experiments, prior knowledge of the pharmacodynamics of voriconazole against filamentous fungi, and were also designed to encompass the entire exposure-response relationship for *Scedosporium apiospermum*.

There was a drug-exposure-dependent decline in antigen concentrations. As the AUC increased, there was progressively larger suppression of circulating antigen concentrations (Figure 3). An $AUC_{0\text{-}24}$ of approximately 80 mg.h/L resulted in complete suppression of antigen release from all three strains (Figure 3). There was no evidence of a paradoxical effect with even higher drug exposures (data not shown).

*Mathematical modelling*

A linked pharmacokinetic-pharmacodynamic mathematical model was fitted to the entire experimental dataset using the population pharmacokinetic program Pmetrics (16). The mathematical model fitted the PK-PD data well (Figure 4). The median parameter values fitted the data better than the means and are shown in Table 2. The Bayesian estimates for each arm (circuit) were used to calculate the area under the voriconazole concentration-time curve for each regimen from 72 to 96 hours post initiation of voriconazole using the trapezoidal rule. Similarly, the area under the antigen-time curve was estimated from the time of treatment initiation (i.e. time =0) to 96 hours post initiation of voriconazole using the Bayesian posterior estimates of each circuit and the trapezoidal rule.

*Pharmacodynamics of voriconazole*

The estimates for both the voriconazole $AUC_{72\text{-}96}$ and $AUC_{72\text{-}96}$:MIC versus the area under the antigen-time curve were used to establish pharmacodynamic relationships for voriconazole against *Scedosporium apiospermum*. Inhibitory sigmoid Emax models fitted to the data suggested that an AUC and AUC:MIC of 16.69 mg*h/L and 22.88, respectively was



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185 associated with half-maximal effect (Figure 5). The shape and coefficient of determination
186 for the two regressions were similar, but the AUC:MIC versus effect relationship was slightly
187 right-shifted.

188

189 *Bridging to Humans*

190 A standard licenced regimen was used for the simulation (i.e. 6 mg/kg i.v. q12h for
191 two dosages followed by 4 mg/kg q12h i.v.). Voriconazole was infused over 1 hour. The
192 $AUC_{0\text{-}24}$ at the end of 5 days of therapy was calculated using integration. Each simulated
193 patient's AUC was divided by the MIC to calculate AUC:MIC for each MIC value. The
194 proportion of simulated patients achieving (or exceeding) the pharmacodynamic target at
195 each MIC was determined. The expectation of overall target attainment across the
196 population was 34%.

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## DISCUSSION

There is only limited information on the MIC distribution of *Scedosporium apiospermum*. MICs estimated using Clinical Laboratory Sciences Institute (CLSI) M-38A methodology suggests that the $MIC_{50}$ and $MIC_{90}$ is 1 and 2 mg/L, respectively (17). Inspection of the MIC distribution of strains described in this study suggests that the epidemiological cut-off value for voriconazole is 4 mg/L. Using an AUC:MIC pharmacodynamic target of 100 (that is associated with complete suppression of circulating antigen in the *in vitro* cell culture model) the currently licenced regimen of voriconazole (i.e. 6 mg/kg q12h for two dosages followed by 4 mg/kg q12h) has an overall probability of target attainment of 34% (i.e. 34% of simulated patients are predicted to have complete suppression of the circulating antigen when assessed against a distribution of MICs). These simulations provide experimental support a breakpoint of 0.5 mg/L, which is less than the epidemiological cutoff value of 4 mg/L. These conclusions are based upon an AUC:MIC target of 100, which needs further validation using further experimental and clinical data.

Immunosuppressed murine models of disseminated infection of *Scedosporium* spp. have been developed and used to assess pathogenesis and efficacy of various antifungal agents. The primary model readouts include survival, and the fungal burden in kidneys, spleen, brain, liver and lungs. Infection is initiated by i.v. injection of conidia, rather than the usual clinically relevant route of infection (i.e. inhalation of conidia) (5, 18, 19). Disseminated models of fungal infection may not be ideal to establish pharmacodynamic relationships because they are not faithful mimics of human pathogenesis and disease. The model endpoints are also a potential issue. For example, survival in mice is confounded by mixing efficacy and drug-related toxicity. There are also well-established problems in performing quantitative counts from tissue homogenates to assess the fungal burden of moulds (see, for example, (20)). These limitations are further compounded by the complex pharmacology of many antifungal agents. For example, voriconazole is notoriously difficult to study in mice because of auto-induction of clearance, and a pharmacokinetic profile that is significantly different from humans (21). Hence, alternative model systems of invasive scedosporiosis are required to assess the pharmacodynamics of new and existing antifungal agents.

We have previously developed static and dynamic models of the human alveolus to describe the pharmacodynamics of voriconazole against *Aspergillus* spp. Outputs from these models have aligned well with the available data from laboratory animal models and clinical datasets (12, 13, 15). This has motivated the development of newer models for other medically important fungi. The use of galactomannan as a biomarker circumvents the problems in directly quantifying the burden of *Aspergillus* in tissue beds, and enables direct *in vivo*-to-clinical correlations. Galactomannan cannot however be used for fungi other than *Aspergillus* spp. For the *Scedosporium* studies described here, we used previously characterised monoclonal antibodies (HG12 and GA3), raised against a cell wall antigen present in species within the *Scedosporium apiospermum* complex, to determine *S. apiospermum* growth. The absolute identity of the antigen bound by the mAbs has yet to be

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239 determined, but they bind to a carbohydrate epitope on a major 120 kDa glycoprotein
240 secreted during active growth of *Scedosporium* spp. (14). Upon germination of conidia, the
241 antigen ceases to be expressed on the spore surface and is then produced extracellularly by
242 developing hyphae (14). The antigen is therefore a potentially useful biomarker for
243 determining active growth of *Scedosporium* spp., and for tracking response to antifungal
244 drugs.

245 The often-suboptimal clinical outcomes of patients infected with *Scedosporium*
246 *apiospermum* complex provides the impetus to develop new antifungal agents. The
247 availability of a range of pharmacodynamic models enables the acquisition of the
248 underpinning evidence for patients to be enrolled in clinical trials. While there is a general
249 paucity of compounds in development pipelines, several new compounds demonstrate *in*
250 *vitro* activity against *Scedosporium*. These compounds include F901318 (F2G, Eccles, UK) and
251 APX001 (Amplyx, San Diego, USA). These compounds could be further assessed in this
252 model.

253 There are several limitations of this study. Firstly, this is a cell culture model that is
254 representative of relatively early invasive pulmonary disease. The pharmacodynamics of
255 voriconazole may change in later phases of established disease where there is significant
256 tissue necrosis. Furthermore, the pharmacodynamic relationships specifically pertain to
257 invasive pulmonary infection and may not necessarily represent other forms of infection and
258 disease caused by *Scedosporium* spp., such as those that involve the central nervous system.
259 The *in vitro* model does not contain immunological effectors and does not enable their
260 adjunctive antifungal effect to be estimated. This may lead to an overestimate of the
261 pharmacodynamic target for immunocompetent patient populations. A novel antigen was
262 used as a biomarker to assess the pharmacodynamics of voriconazole in this experimental
263 model. The clinical relevance of this antigen remains to be determined. This will require a
264 combination of experimental and clinical research in the same manner as occurred for
265 galactomannan.

266 Despite potential limitations, this *in vitro* model provides new insights into the
267 pharmacodynamics of voriconazole against a less common, but nevertheless medically
268 important fungal pathogen. The development of new agents and therapeutic approaches will
269 require an array of experimental models that allow their pharmacodynamics to be elucidated
270 and optimised. The approach of using novel soluble cell wall antigens as biomarkers with
271 prognostic value provides a way in which the pharmacodynamics of relatively uncommon
272 pathogens and antifungal compounds with challenging pharmacological properties can be
273 elucidated.

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## METHODS

### *Identification of Isolates to Species Level*

Isolates of *Scedosporium apiospermum* were obtained from the Regional Mycology Reference Laboratory, University Hospital of South Manchester and the Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III, Madrid, Spain. Isolates were stored at -80$^{o}$C.

*Scedosporium apiospermum* was definitively identified via sequencing of the internal transcribed spacer (ITS) and β-tubulin regions. Extraction of fungal DNA was performed as follows. Approximately 20-40 acid-washed glass beads were suspended in 0.5 mL of sterile distilled water. The strains were grown on oatmeal agar for five days prior to experimentation. Mycelia and conidia were harvested by gently abrading the fungus with a sterile needle before adding to the beads and water. Samples were homogenised using a FastPrep-24 homogeniser. Filters (2 mm diameter) were cut from Whatman Flinders Technology Associates classic indicator cards (Whatman International, Kent, United Kingdom) and inoculated with 2 µL of homogenate. Filters were dried for 10 minutes before being washed twice with 100 µL sterile distilled water.

For the amplification of DNA, 700 µL of polymerase chain reaction (PCR) reaction mix was prepared. This consisted of 70 µL buffer (QIAGEN, UK), 7 µL dNTPs (2.5mM), 70 µL forward primer, 70 µL reverse primers, 420 µL PCR grade H2O, 3.5 µL HotstarTaq (QIAGEN, UK). The PCR reaction mixture was vortexed for 1 min. A total of 100 µL of PCR reaction mixture was added to each PCR tube containing the inoculated filter. The PCR reaction conditions were as follows: 15mins 94$^{o}$C for one cycle, 40 cycles of 30 sec at 94$^{o}$C, 30 sec 50$^{o}$C, 90 sec 72$^{o}$C, one cycle of 7 mins at 72$^{o}$C, 4$^{o}$C for remainder. After the PCR cycle was completed, 8 µL was transferred to a new PCR tube and 5 µL of the appropriate forward primer was added for sequencing of the ITS and β-tubulin regions.

Sequencing of the amplicons was performed by Beckman- Coulter Genomics Inc. Sequences were analysed for identification via the FUNCBS.txt database (http://www.cbs.knaw.nl/Collections/BioloMICSSequences.aspx).

### *Preparation of Inoculum for Experimental Pharmacology*

Ten days prior to experimentation, isolates were sub-cultured to oatmeal agar (Sigma-Aldrich, Poole, UK) and incubated in air at 30$^{o}$C. Conidial suspensions were prepared by flooding a flask with 20 mL of PBS and simultaneously abrading the surface of the colonies with a sterile cotton swab. The resulting suspension was filtered through sterile gauze before being centrifuged for 10 minutes at 2500 rpm and re-suspending the pellet in 10mL PBS. The washing step was performed twice. For the preparation of the final inoculum, basal cell

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culture medium,EBM-2, (Promocell, Heidelberg, Germany) was used as the diluent. A conidial suspension of 1 x $10^6$ CFU/mL was prepared using a haemocytometer and confirmed by quantitative cultures by plating to Sabouraud agar supplemented with chloramphenicol (Oxoid Ltd, Basingstoke, UK).

*In vitro Susceptibility Testing*

Minimum inhibitory concentrations (MIC) of voriconazole against *S. apiospermum* isolates were determined in five independently conducted experiments using Clinical Laboratory Sciences Institute (CLSI) methodology.

*Cell Culture Models of Invasive Scedosporiosis*

Novel cell culture models of invasive pulmonary scedosporiosis were developed to investigate the pharmacodynamics of voriconazole. These were based on similar models developed for *Aspergillus fumigatus* and *Aspergillus flavus* (12, 13, 15). Both static and dynamic models of invasive disease were developed. In the former, drug concentrations were fixed, as occurs in *in vitro* susceptibility testing. In the latter, peristaltic pumps were used to simulate human pharmacokinetics to ensure a more clinically relevant and tractable estimate of drug exposure.

Both model systems required the construction of a cellular bilayer consisting of human pulmonary arterial endothelial cells (HPAECs) (Promocell, Heidelberg, Germany) and human alveolar epithelial cells (A549s; LGC Standards, Middlesex, UK). Cells were seeded on opposing surfaces of polyester membrane cell culture inserts, as previously described (15).

*Growth of A549 and HPAECs cells*

A549 cells were cultured using endothelial basal medium-2 (EBM-2; Invitrogen, Paisley, UK) medium containing 10% foetal bovine serum (FBS). HPAECs were cultured using endothelial growth medium (EGM-2) according to the manufacturer's instructions (Promocell, Heidelberg, Germany), which consists of EBM-2 supplemented with 2% FBS (Invitrogen, Paisley, UK), ascorbic acid, heparin, hydrocortisone, human endothelial growth factor, vascular endothelial growth factor 165, human fibroblast factor B and R-3-insulinlike growth factor 1.

Once 70-80% confluence was achieved, cells were washed using Hanks Balanced Salt Solution (HBSS) (Sigma-Aldrich, Poole, UK) and harvested with 6 mL of a 0.25% trypsin–ethylenediaminetetraacetic (EDTA) solution (Sigma-Aldrich, Poole, UK). Cells were then centrifuged for 5 min. at 2,000 rpm and re-suspended in the appropriate medium to achieve final density of 5.5 x $10^5$/mL and 1 x $10^6$/mL for A549s and HPAECs, respectively.



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*Construction of the Static Model of Invasive Pulmonary Scedosporiosis*

12 mm cell culture inserts with a 3.0 µm pore polyester membrane for 24-well tissue culture plates (Appleton Woods, Birmingham, UK) were inverted. For the construction of the endothelial cell layer 100 µL of HPEAC cell suspension was pipetted onto the underside of the membrane. Inserts were then incubated (37°C, 5% $CO_2$) for 2 hours to enable cellular adhesion to the membrane. Inserts were then transferred to 24-well plates containing 0.6 mL of EGM-2 medium. One-hundred µL of EBM-2 containing 10% FBS was then added to the alveolar compartment. The cell culture plates were then incubated at 37°C in 5% $CO_2$ for 24 hours.

After 24 hours, medium from the alveolar compartments was removed and inserts were transferred to new plates containing the relevant amounts of fresh EGM-2 medium. One-hundred µL of the A549 suspension was added to the alveolar compartment. After 2 hours, medium from the alveolar compartment was removed to create an air-liquid interface. The EGM-2 medium in the bottom compartment was replaced every 48 hours and medium that had accumulated in the alveolar compartments was removed and discarded. The bilayers were ready for experimentation after 5 days.

*Construction of the Dynamic Model of Invasive Pulmonary Scedosporiosis*

The same process was used for the dynamic models except larger 12-well cell culture plate inserts were used (Greiner Bio-One, Stonehouse, UK). Larger volumes were required because of the larger surface areas, hence, 400 µL of the HPAEC suspension, and 400 µL of the A549 suspension was used to seed the membrane. Inserts were immersed in 1.5 mL of EGM-2 medium in 12-well tissue culture plates. The protocols for establishing the bilayer were the same as for the static model.

*Pharmacodynamic Experiments Using the Static in Vitro Model of the Human Alveolus*

Cell culture inserts were transferred to a 24 well plate containing 0.6 mL of EBM-2 medium supplemented with 2% FBS. A conidial suspension of 1 x $10^6$ of *Scedosporium apiospermum* was prepared and warmed to 37°C for 20 minutes. One-hundred µL of the conidial suspension was inoculated into the alveolar compartment of each cell culture insert and incubated at 37°C in 5% CO2 for 12 hours. The remaining (spent) inoculum was then removed and discarded to enable an air-liquid interface to re-establish.

Pure voriconazole was dissolved in DMSO (Sigma- Aldrich, Poole) to produce a stock solution of 3 mg/ml. The desired final concentration of voriconazole for each insert was achieved by serial dilution of the stock in EBM-2 medium that was supplemented with 2%

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FBS. Medium containing different concentrations of voriconazole was placed in each well 12 hours post inoculation in order to replicate an early invasive infection. Plates were then incubated at 37°C, 5% CO2 for 48 hrs. Samples from the endothelial and alveolar compartments were obtained and stored at −80°C for measurement of drug concentrations and detection of the cell wall antigen.

*Construction of dynamic model*

Cell culture inserts were housed in custom-designed stainless steel bioreactors. Bioreactors were specifically engineered to house individual cell culture inserts and allowing media to flow past the endothelial layer. All components were autoclaved and circuits were assembled in a class II safety cabinet.

Each bioreactor was connected to a circuit using Marprene® thermoplastic elastometer tubing (Watson Marlow, Cornwall, UK). All connections were made using polypropylene barbed luer adapters (Cole Palmer, London UK; West Group, Hants, UK). A central reservoir containing 200 mL of warmed media was connected to the circuit using 1.5-mm bore polytetrafluoroethylene (PTFE) semi-rigid tubing (Diba Labware, Kinesis Ltd., Cambridge, UK) fitted into Omni-Fit Q-series bottle caps (Diba Labware, Kinesis Ltd., Cambridge, UK). The central reservoir contained a magnetic stirring bar to ensure continuous mixing of drug. Duran bottles containing fresh medium and empty bottles for the removal of waste were connected to the central reservoir using Silastic®, 1.6 mm bore tubing (Dow Corning) to enable the generation of first-order decay in voriconazole concentrations. Two 205-U multichannel cassette peristaltic pumps fitted with 1.52 mm bore Marprene manifold tubing (Watson Marlow, Cornwall, UK) were used to circulate medium through the circuit and bioreactor.

Once assembled, circuits were transferred to a 37°C incubator for the remainder of the experiment. Dulbecco's modified Eagle medium (DMEM) containing 4500 mg/L D-Glucose, L-Glutamine and HEPES buffer (Invitrogen, Paisley, UK) was supplemented with 2% FBS and a penicillin- streptomycin solution (Sigma-Aldrich, Poole) to give a final concentration of 100 U/mL penicillin and 0.1 mg/mL streptomycin.

*Pharmacokinetic and Pharmacodynamic Experiments Using the Dynamic in Vitro Model of the Human Alveolus*

Pure voriconazole powder was dissolved in DMSO. Drug was administered directly into the central compartment as a bolus. Dosages were selected from previously published data demonstrating the achievement of voriconazole exposures that ranged from sub-optimal to successful in treating organisms of similar susceptibilities in this model (13). Amounts of 0 mg, 0.1 mg, 0.2 mg, 1 mg and 3 mg were administered every 12 hours to the



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central compartment to achieve initial concentrations of 0, 0.5, 1.5, 5 and 15 mg/L respectively. The pump connecting the Duran containing fresh medium and the waste was run to achieve a flow of 10 mL/hour. This was previously demonstrated to generate human-like concentration- time profiles for voriconazole (13). Voriconazole therapy commenced 12 hours post inoculation (as was the case with the static model described above).

*LC/MS/MS*

Voriconazole concentrations were measured using a validated ultrahigh-performance liquid chromatography tandem mass spectrometry method comprising of a Agilent 6420 Triple Quad Mass spectrometer and a Agilent 1290 infinity LC system (Agilent Technologies UK Ltd, Cheshire, UK). Voriconazole was extracted by protein precipitation; 300 µl of acetonitrile containing the internal standard (IS) Phenacetin 0.1 mg/L (Sigma Aldrich, Dorset, UK) was added to 100 µl of matrix. The samples were then vortexed for 5 seconds and centrifuged at 13000 g for 3 minutes. Two hundred µl of the supernatant was removed and placed in 96 well plate, 3 µl were injected on an Agilent a Zorbax Eclipse Plus C18 (2.1 by 50 mm, 1.8-m particle size) (Agilent Technologies UK Ltd, Cheshire, UK).

Chromatographic separation was achieved using a gradient with the starting conditions of 70:30 (0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B). Starting conditions were held for 0.2 minutes, then mobile phase B was increased to 100% over 1.5 minutes. This was held at 100% mobile phase B for 0.5 minutes, and then reduced to starting conditions for 1 minute of equilibration. The mass spectrometer was operated in multiple reaction monitoring (MRM) scan mode in positive polarity. The precursor ion for voriconazole was 350.0m/z, and it was 180.1 m/z for the IS. The product ion for voriconazole was 281.1 m/z and it was 110.0 m/z for the IS. The source parameters were set as 4000 V for capillary voltage, 350°C for gas temperature, and 60 lb/in2 for the nebulizer gas. The standard curve for voriconazole encompassed the concentration range of 0.025-20.0 mg/L and was constructed using blank matrix. The data were acquired and processed using the Mass Hunter Quantitation B.6.0 software package. For voriconazole in media the limit of detection was 0.0025 mg/L and the limit of quantitation was 0.0025 mg/L. The CV% was 8.9% over the concentration range 0.0025-20.0 mg/L and the intra and inter-day variation was < 7.2%.

*Enzyme linked immunosorbent assay*

Elucidation of the pharmacodynamics of voriconazole against *S. apiospermum* was enabled by measuring a soluble cell wall antigen released during active growth of the pathogen using a double-antibody-sandwich (DAS)-ELISA. The HG12 and GA3 monoclonal antibodies used in the DAS-ELISA are specific to *Scedosporium* spp., and bind to an extracellular 120-kDa antigen secreted by hyphae following spore germination (14). Fifty µL volumes of mAb HG12 (IgG1) tissue culture supernatant (TCS) were used to coat the wells of



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Maxisorp microtiter plates (Fisher Scientific, Loughborough, UK). The plates were incubated for 16 hours at 4°C before washing four times with PBS containing 0.05% (v/v) Tween 20 (PBST). The plates were then washed once with PBS and once with distilled water before being placed in a drying cabinet at 23°C for 1 hour. Plates were stored at 4°C in sealed plastic bags until used.

Working volumes were 50 µL; all incubation steps were conducted in sealed plastic bags at 23°C, and wells were washed three times with PBST (5 minutes each time) between incubations. Wells containing immobilised HG12 were blocked for 10 minutes with 100µL of PBS containing 1% (w/v) of bovine serum albumin (BSA) to prevent non-specific protein binding. Blocked wells were then incubated with experimental solutions for 2 hours to allow antigen capture by mAb HG12. Unbound antigen was removed by washing with PSBT and the wells then incubated with mAb GA3 (IgM) TCS for 1 hour. Washed wells were then incubated for 1 hour with goat anti-mouse IgM (µ-chain specific) biotin conjugate (Sigma Aldrich, Poole, UK) diluted 1:1000 in PBST containing 0.5% (w/v) BSA. After removal of unbound biotin conjugate by washing with PBST, wells were incubated for a further hour with Extravidin-peroxidase (Sigma-Aldrich, Poole, UK) diluted 1:1000 in PBST containing 0.5% (w/v) BSA. After washing with PBST and a single rinse with PBS, bound antibody-antigen complexes were visualized by incubating wells in tetramethyl benzidine (TMB) substrate solution (Sigma-Aldrich, Poole, UK) for 30 minutes. Reactions were stopped by the addition of 3M $H_2SO_4$ and absorbance values determined at 450 nm.

*Mathematical Modelling*

A population methodology was used to fit a mathematical model to the data from each isolate. For *Scedosporium* datasets the structural mathematical model consisted of the following two inhomogeneous ordinary differential equations:

$$dX1/dt = B(1)-(SCL/V)*X1 \qquad \text{Equation 1}$$

$$dN/dt = Kgmax*(1-(N/POPMAX))*N \qquad \text{Equation 2a}$$

$$*(1-(X1/Vc)^{Hg}/(X1/Vc)^{Hg}+C50g^{Hg})) \qquad \text{Equation 2b}$$

with output equations

$$Y(1)=X(1)/V \qquad \text{Equation 3}$$

$$Y(2)=X(2) \qquad \text{Equation 4}$$

Where: B(1) represents a bolus input of voriconazole into the central compartment, SCL is the clearance of drug from the circuit, Vc is the volume of the circuit, N is the antigen value,



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Kgmax is the maximal rate of growth; POPMAX is the theoretical maximal antigen concentration within the circuit; Hg is the slope function for the suppression of growth; and, $C_{50}g$ is the concentration of drug in the circuit where there is half-maximal suppression of growth.

Equation 1 describes the rate of change of the mass of voriconazole in the circuit. Equation 2 describes the rate of change of antigen in the circuit and contains terms that describe fungal growth in the absence of drug (Equation 2a) and the drug induced suppression of growth (Equation 2b).

*Pharmacodynamic Target Associated with Maximal Antifungal Activity*

The mathematical model fitted to each strain was used to estimate the voriconazole area under the concentration-time curve and the area under the antigen-time curve. Each circuit contributed one summary estimate of the effect of voriconazole.

The relationship between drug exposure and the effect on the biomarker was modelled using an inhibitory sigmoid Emax function that took the form:

$$Effect = Econ - \frac{Emax * Drug\ Exposure^{H}}{EC_{50}{}^{H} + Drug\ Exposure^{H}}$$

Where: Econ is the antigen in controls, Emax is the maximum antifungal effect induced by voriconazole, Drug exposure is the AUC or AUC:MIC of voriconazole, H is the slope (Hill) function and $EC_{50}$ is the voriconazole drug exposure where the effect is half maximal. The model was fitted to the data using the pharmacokinetic program ADAPT5.

*Monte Carlo Simulation and Bridging Studies*

Monte Carlo simulations were performed in ADAPT 5 (22) using a previously published population pharmacokinetic model for voriconazole (23). This model was fitted to data from healthy volunteers and patients receiving voriconazole therapy for proven or probable invasive aspergillosis. The mean parameter values and their associated variances were used and embedded in subroutine PRIOR of ADAPT 5. A log normal distribution was used and the ability of the simulation to reproduce the original parameter estimates and their dispersions was assessed. A standard licenced regimen was used for the simulation (i.e. 6 mg/kg i.v. q12h for two dosages followed by 4 mg/kg q12h i.v.). Voriconazole was infused over 1 hour. The $AUC_{0\text{-}24}$ at the end of 5 days of therapy was calculated using integration. Each simulated patient's AUC was divided by the MIC to calculate AUC:MIC for each MIC value. The proportion of simulated patients achieving (or exceeding) the pharmacodynamic target at each MIC was determined. The MICs used for these calculations were determined using CLSI methodology and provided by Professor Jacque Meis.



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604 Table 1. Challenge Strains of *Scedosporium apiospermum*

| | Antifungal Agent[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| Strain[b] | AMB | ITRA | VORI | POSA | ISA | ANI | MICA |
| 8353 | 16 | >16 | 0.5 | 1 | 4 | 8 | 0.5 |
| CNM-CM6322 | 8 | >16 | 1 | 1 | 4 | 4 | 0.5 |
| CNM-CM6386 | >16 | >16 | 1 | 2 | 8 | 8 | 8 |

605

606 [a]Strains of *Scedosporium apiospermum* used in this study and their minimum inhibitory
607 concentrations (mg/L) determined using Clinical Laboratory Sciences Institute (CLSI)
608 methodology. [b]Antifungal agents are as follows: AMB (amphotericin B), ITRA (itraconazole),
609 VORI (voriconazole), POSA (posaconazole), ISA (isavuconazole), ANI (anidulafungin) and MICA
610 (micafungin).

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628 Table 2. Parameters from the pharmacokinetic-pharmacodynamic model fitted to the
629 experimental data from each strain.

630

| Parameter[a] | Strain 1 (8353) | Strain 2 (6322) | Strain 3 (6386) |
|---|---|---|---|
| SCL (L/h) | 0.021 | 0.019 | 0.030 |
| V (L) | 0.646 | 0.850 | 1.036 |
| Kgmax ($OD_{450}$/h) | 0.036 | 0.013 | 0.021 |
| POPMAX ($OD_{450}$) | 1.808 | 2.068 | 2.999 |
| $C_{50}$ (mg/L) | 0.469 | 0.455 | 0.902 |
| Hg | 5.582 | 9.932 | 2.155 |
| Initial Condition ($OD_{450}$) | 0.046 | 0.121 | 0.091 |

631

632 [a]The median parameter values are shown and are as follows: SCL (L/h) is the clearance of
633 voriconazole from the circuit; V (L) is the volume of the central compartment; Kgmax is the
634 maximum rate of fungal growth as estimated by the increase in fungal antigen liberated into
635 the circuit; POPMAX is the maximum theoretical fungal density; $C_{50}$ is the concentration of
636 voriconazole where there is a 50% reduction in the rate of growth; Hg is the slope function
637 and the Initial Condition is the estimated fungal density at time of treatment initiation (which
638 is 12 hours postinoculation).

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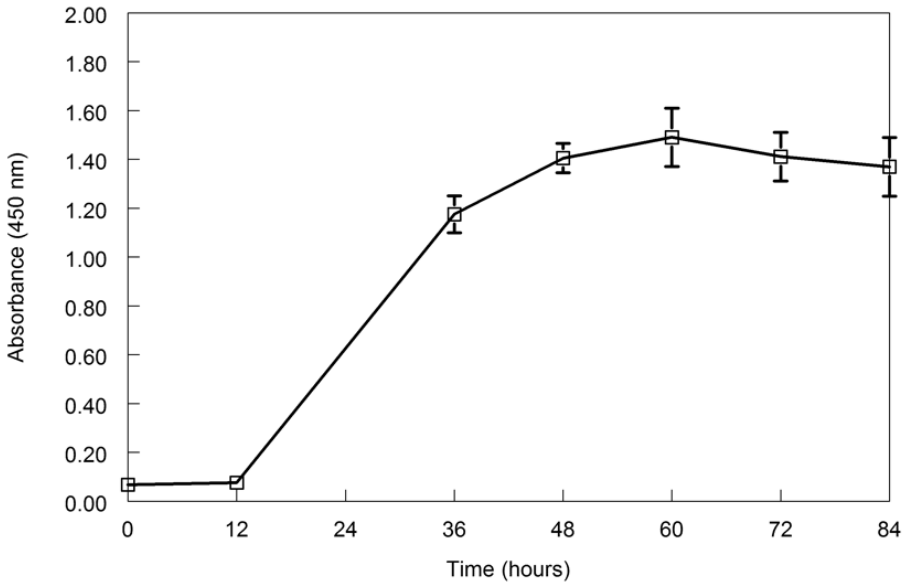


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643 Figure 1. The time-course of antigen release in the *in vitro* static model. Data are the mean ±
644 standard deviation of three inserts. Data were generated with strain 8353.
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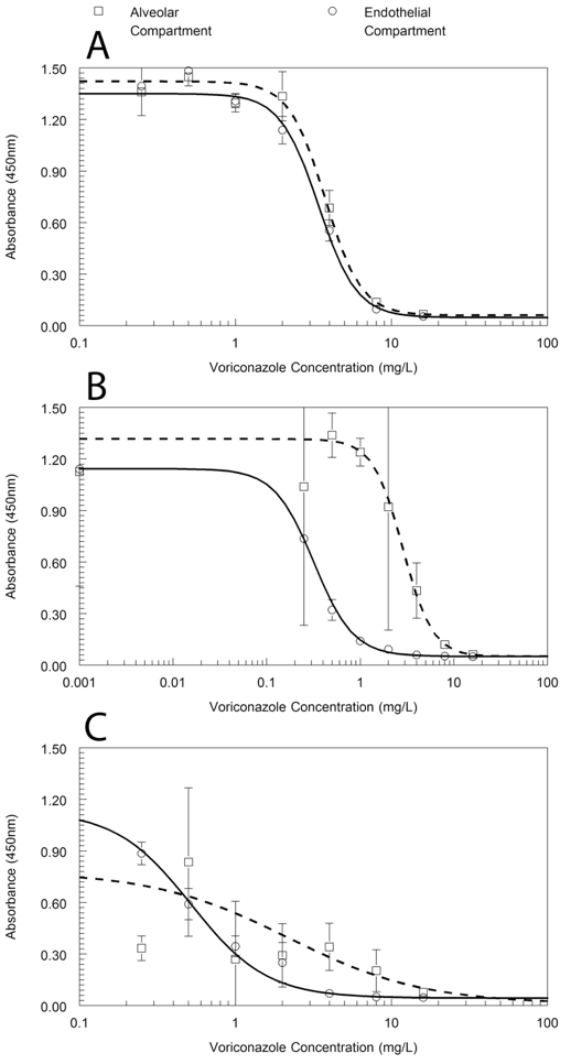


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649

650 Figure 2. The concentration-response relationships for the three challenge strains of
651 *Scedosporium apiospermum*. Data are mean ± standard deviation. The broken and solid
652 lines are the fit of an inhibitory sigmoid Emax model to the data from the alveolar and
653 endothelial compartments, respectively. Panel A, strain 8353; Panel B,6386; Panel C 6322.

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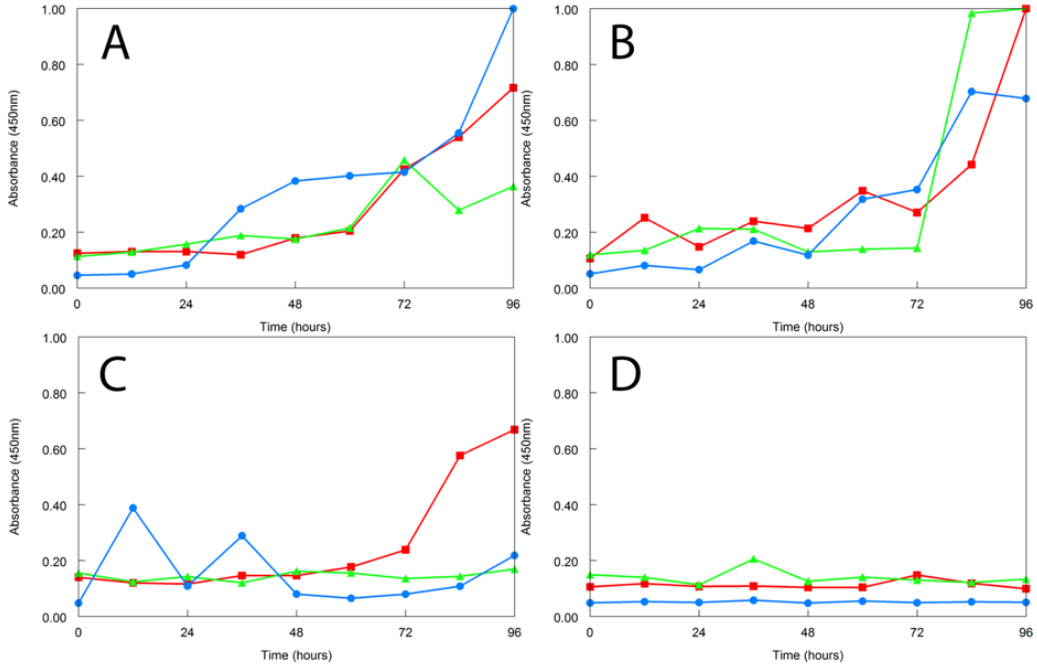


Figure 3. Pharmacodynamics of voriconazole against *Scedosporium apiospermum* isolates from dynamic models of invasive pulmonary scedosporiosis. Time zero is the time of treatment initiation and is 12 hours post inoculation. Each line represents data from a single strain. Voriconazole was administered at time = zero and every 12 hours thereafter. Drug was administered as a bolus. Pharmacodynamic samples were obtained from the endothelial compartment of the model. The challenge strains are as follows: 6386 (red squares), 6322 (green triangles) and 8353 (blue circles). Panel A, Vehicle-treated control; Panel B, average AUC = 7.84 mg.h/L inducing negligible effect; Panel C, average AUC = 15.62 mg.h/L inducing submaximal antifungal activity, Panel D, average AUC = 80.68 mg.h/L inducing near maximal antifungal activity.

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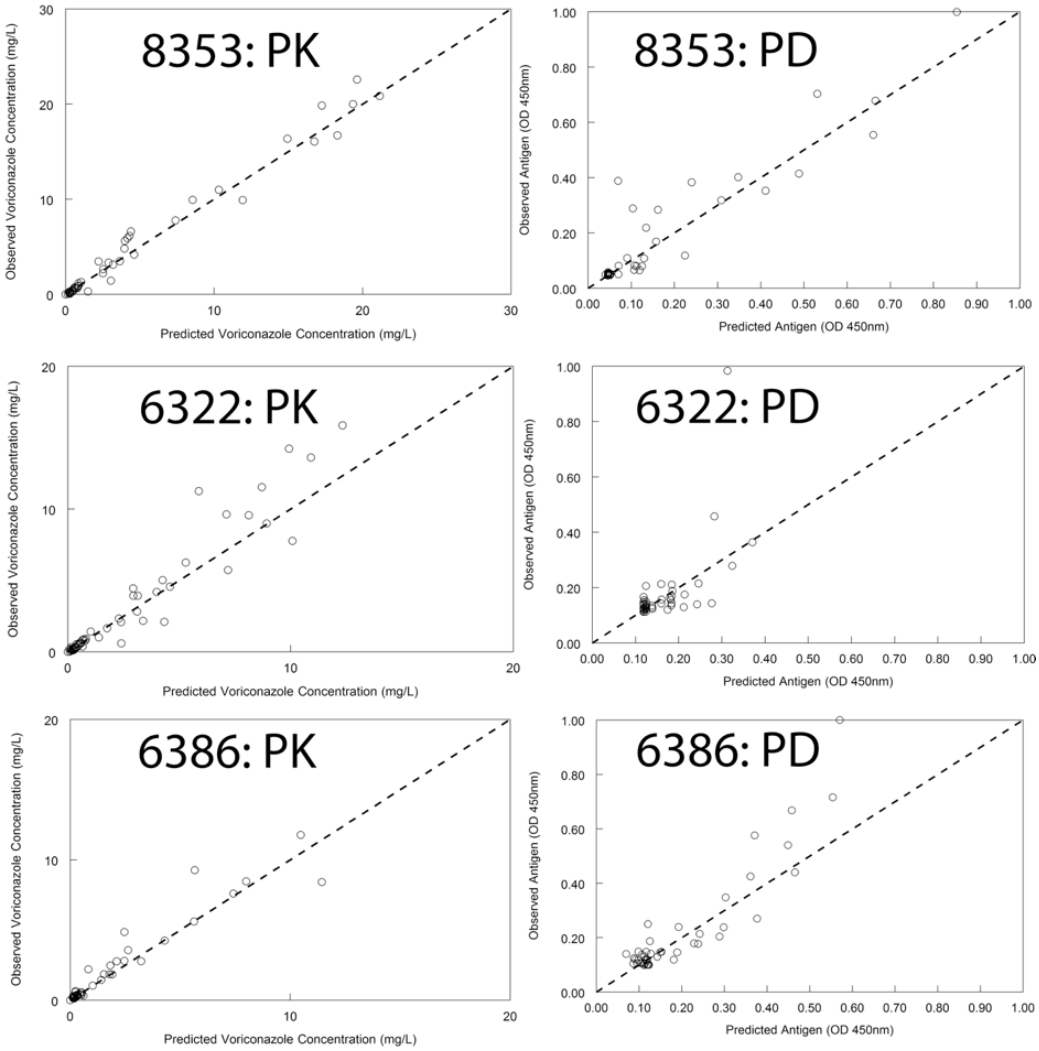


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676 Figure 4. Observed-predicted values from the mathematical models fitted to the
677 pharmacokinetic and pharmacodynamic data from each strain. The broken line is the line of
678 identity (observed = predicted).

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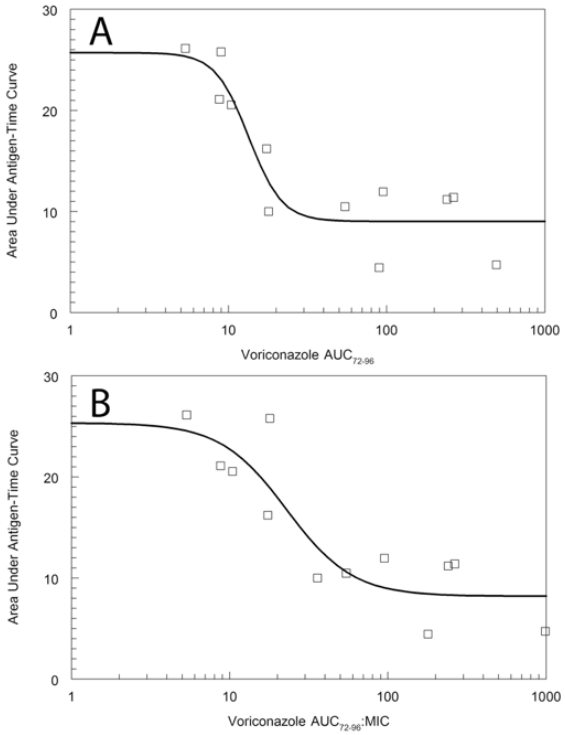


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684 Figure 5. Panel A, The relationship between voriconazole $AUC_{72\text{-}96}$ and the antifungal effect
685 quantified in terms of the area under the antigen-time curve. The inhibitory sigmoid Emax
686 model is given by Area Under Antigen-Time Curve=25.71-
687 $(16.69*(AUC)^{4.15}/(13.42^{4.15}+(AUC)^{4.15}))$; $r^2$ 0.86; Panel B, The relationship between
688 voriconazole $AUC_{72\text{-}96}$:MIC and the antifungal effect quantified in terms of the area under the
689 antigen-time curve. The inhibitory sigmoid Emax model is given by Area Under Antigen-Time
690 Curve=25.34-$(17.14*(AUC:MIC)^{2.09}/(22.88^{42.09}+(AUC:MIC)^{2.09}))$; $r^2$ 0.82.

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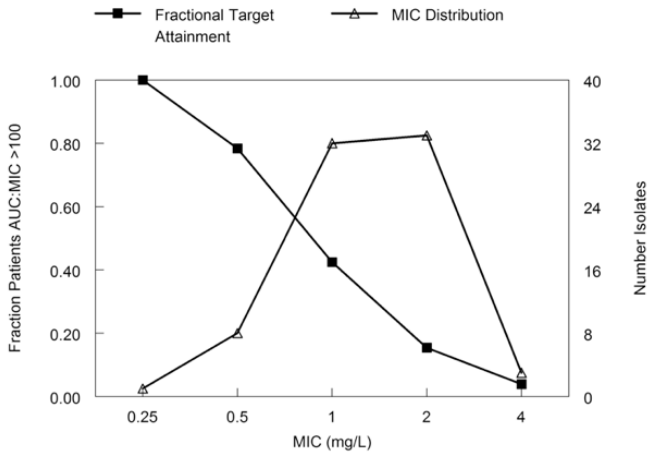


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695 Figure 6. The probability of target attainment for a regimen of i.v. voriconazole 6 mg/kg q12h
696 for two dosages followed by 4 mg/kg q12h thereafter. Drug was infused over 1 hour. A
697 weight of 75 kg was used for the simulations and the $AUC_{0-24}$ was determined at the end of
698 day 5. The fraction of 5,000 simulated patients that achieved an AUC:MIC of >100 for each
699 MIC value is shown by the solid squares. The MIC distribution of *S. apiospermum* from 77
700 strains is shown by the open triangles. The overall expectation of target attainment for the
701 population is 34%.

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