Voriconazole pre-exposure selects for breakthrough mucormycosis in a mixed model of *Aspergillus fumigatus-Rhizopus oryzae* pulmonary infection

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Mucormycosis is an uncommon fungal infection that has been increasingly reported in severely immunocompromised patients receiving Aspergillus-active antifungals. Although clinical studies and pre-clinical animal models have suggested a unique predisposition for breakthrough mucormycoses in patients receiving voriconazole, no study has specifically evaluated the selection dynamics of various Aspergillus-active antifungal classes in vivo. We utilized an *Aspergillus fumigatus-Rhizopus oryzae* (10:1) model of mixed fungal pneumonia in corticosteroid-immunosuppressed mice to compare the selection dynamics of daily liposomal-amphotericin B (L-AMB), micafungin (MCFG) and voriconazole (VRC) therapy. *A. fumigatus* and *R. oryzae* lung fungal burden were serially monitored in parallel using non-cross-amplifying quantitative real-time PCR assays for each fungal genus. Additionally, experiments were performed where the *R. oryzae* component of the mixed inoculum was serially passed on VRC-containing agar before animal infection. We found prior exposure to voriconazole in vitro, consistently resulted in a 1.5-2 log₁₀ increase in *R. oryzae* fungal burden by day +5 in vivo relative to animals infected with the non-VRC preexposed inoculum, irrespective of the antifungal-treatment administered in mice (p ≤ 0.02 all treatment groups). Mice infected with the VRC-preexposed inoculum and subsequently treated with saline or VRC had the highest mortality rates (82-86%), followed by MCFG (55%) then L-AMB (39%, p = 0.04 vs. control). However, in vivo treatment alone with voriconazole alone did not consistently increase the virulence of non-voriconazole preexposed *R. oryzae* vs. controls. We conclude that exposure of *R. oryzae* sporangiospores to voriconazole in vitro modulates the subsequent growth rate and/or virulence of the fungus in vivo, which reduces effectiveness of Mucorales-active antifungals. The mechanisms underlying this phenotypic change are unknown.

Introduction

Mucormycosis is a life-threatening infection caused by fungi of the order Mucorales that has increased in incidence over the last decade, especially among allogeneic hematopoietic stem cell transplantation (HSCT) recipients. In many transplant centers, the surge in mucormycosis cases has been temporally linked with the introduction and widespread use of voriconazole. However, a recent large prospective study comparing fluconazole and voriconazole for the prevention of invasive fungal infections (IFIs) in lower-risk allogeneic HSCT patients did not find an association between voriconazole prophylaxis and mucormycosis. Additionally, prospective IFI surveillance programs have noted that only 45% of mucormycosis cases in the HSCT population are associated with prior voriconazole prophylaxis, suggesting that antifungal exposure is unlikely to be the sole explanation for the rising incidence of this notoriously aggressive mold. Because of its availability in oral formulations, voriconazole is often administered for extended periods in heavily immunocompromised patients. HSCT patients receiving voriconazole may avoid earlier death due to aspergillosis, only to survive longer with continuous immunosuppression and intensifying “late risk factors” that favor mucormycosis, including polytransfusion-associated iron overload and corticosteroid-associated hyperglycemia.

These explanations do not exclude the possibility that voriconazole may directly influence the pathobiology of Mucorales in vivo. Our laboratories previously demonstrated that serial passage of *Rhizopus oryzae* on voriconazole-containing agar increased the virulence of the fungus when harvested sporangiospores were inoculated into fruit flies as well as corticosteroid-immunosuppressed mice. *R. oryzae* virulence reverted to baseline when voriconazole exposure was stopped, suggesting an unstable epigenetic modification rather than a genetic change.
Development of a mixed Aspergillus-Rhizopus pulmonary infection model. Invasive pulmonary mucormycosis (IPM) frequently presents as a progressive infection in patients receiving antifungal therapy for documented or suspected Aspergillus infection. Therefore, we wanted to explore whether antifungal selective pressure against Aspergillus species was a key element of breakthrough IPM. Protocols for murine models of invasive pulmonary aspergillosis (IPA), and IPM were adapted to establish a mixed infection in hydrocortisone-immunosuppressed mice with two well-characterized clinical isolates—A. fumigatus 293 and Rhizopus oryzae 557969 (Fig. 1). The goal was to develop a non-acutely lethal mixed infection model that allowed simultaneous analysis of Aspergillus- and Rhizopus-specific DNA in animal lungs by quantitative real-time PCR (qPCR) at serial timepoints during antifungal treatment. In preliminary studies, we explored a wide variety of A. fumigatus:R. oryzae inoculum ratios ranging from 1:1 to 1,000:1 (data not shown). However, a 10:1 inoculum of A. fumigatus (1.75 x 10⁶ conidia):R. oryzae (1.75 x 10⁵ sporangiospores) administered intranasally (35 μL) under anesthesia was found to produce the best combination of a non-acutely lethal mixed infection in corticosteroid-immunosuppressed mice that could be accurately monitored within the range of the standard curve of the qPCR assay.

Interestingly, this switch to a hypervirulent phenotype in vivo following serial passage was specific to voriconazole, because increased mortality was not observed when R. oryzae was passed on agar containing other triazoles. However, the influence of other antifungal classes in selecting mucormycosis in vivo, remains unknown. Herein, we explore this question further by comparing the early in vivo Mucorales selection potential of different antifungal classes (triazoles, polyenes and echinocandins) in the presence or absence of prior voriconazole exposure using a mixed Aspergillus fumigatus:Rhizopus oryzae pulmonary infection model.

Results

Figure 1. Experimental approach used to assess impact of antifungal treatment on breakthrough pulmonary mucormycosis. Rhizopus oryzae was serially passed four times on yeast-extract glucose (YAG) agar plates alone, or YAG plates supplemented with voriconazole 4 μg/mL before collection of sporangiospores. A. fumigatus 293 was grown on YAG plates without voriconazole supplementation before collection of conidia. Sporangiospores and conidia were combined in phosphate-buffered saline at a final concentration of 5 x 10⁵ R. oryzae: 5 x 10⁶ A. fumigatus (1:10) and inoculated intranasally (35 μL) in hydrocortisone-immunosuppressed mice. Antifungal therapy was then administered as intraperitoneal saline (control); intravenous L-AMB 10 mg/kg/day, intraperitoneal micafungin 1 mg/kg/day; or intravenous voriconazole 40 mg/kg every 12 h until day +5. Animals were euthanized at predetermined timepoints during antifungal treatment (t = 1, 72 or 120 h) or when moribund and lungs were analyzed for A. fumigatus and R. oryzae fungal burden using qPCR.
Although mice are frequently used for the in vivo evaluation of antifungal compounds, they are generally considered to be unacceptable species for studies with voriconazole (VRC) due to their high rates of drug clearance and extensive P450 metabolism resulting in low or undetectable serum concentrations. Several investigators have shown that the pharmacokinetics of VRC in mice can be improved with the administration of grapefruit juice—a known inhibitor of cytochrome P450 and P-glycoprotein in mammals. Alternatively, higher-doses of VRC administered intravenously via the lateral tail vein (i.e., 20–40 mg/kg twice daily) can also achieve serum drug exposures similar to humans, presumably due to saturation of CYP P450 clearance mechanisms. To confirm this observation in our animal model, we measured VRC serum trough concentrations at 72 and 120 h in groups of five mice administered VRC 40 mg/kg IV twice daily (Table 1). Median VRC trough concentrations were detectable (0.12–0.36 μg/mL) within the first 72 h when serum concentrations were analyzed by high-performance liquid chromatography and accumulated to concentrations of 2.91 μg/mL (range 0.35–5.82 μg/mL) by 120 h, which is similar to trough concentration range reported in humans with good clinical responses to VRC therapy. Next, we explored the impact of VRC-pre-exposure on the R. oryzae component of the mixed inoculum and whether pre-exposure similarly influenced in vivo growth of R. oryzae in animals treated with micafungin (MCF) or liposomal amphotericin B (L-AMB). Previous studies had failed to show an influence of VRC pre-exposure at subinhibitory concentrations on the subsequent growth of A. fumigatus in vivo. Additionally, our previous work demonstrated that increases in R. oryzae virulence were specific to voriconazole as serial passage of spores on yeast agar glucose (YAG) containing sub-inhibitory concentrations of amphotericin B, caspofungin, itraconazole or fluconazole did not influence virulence in fly model of mucormycosis that has excellent concordance with murine models utilized in our laboratories. R. oryzae sporangiospores were collected after four serial passages every 48 h on YAG plates or YAG plates containing 4 μg/mL of voriconazole. The sporangiospores were then combined 1:10 with the inoculum to VRC diminshed the antifungal effects of either L-AMB 10 mg/kg or MCFG 1 mg/kg in an equivalent DNA over 5 d. However, the increase in fungal burden was significantly greater for both A. fumigatus (2.2 log10; p = 0.04) and R. oryzae (3.1 log10; p = 0.009) when animals were infected with the VRC-passaged mixed inoculum (Fig. 3A and E). Unlike control animals, A. fumigatus or R. oryzae fungal burden did not significantly increase in mice infected with the non-preexposed inoculum and treated with either L-AMB 10 mg/kg or MCFG 1 mg/kg/day (Fig. 3B and C). Preexposure of the inoculum to VRC diminished the antifungal effects of both L-AMB and MCFG even though the mean fungal burden on day +5 in both groups (4.1–4.6 log10) was significantly lower than controls (6.3 log10; p = 0.001) (Fig. 3F and G).

VRC treatment was associated with a modest reduction in A. fumigatus lung fungal burden in animals infected with the non-passaged vs. VRC-passaged inoculum (Fig. 3D and H).
was also included. Untreated control animals survived an average of 6 d with an 81% mortality rate at day +7. Survival was significantly worse in controls infected with the non preexposed inoculum (41%, p = 0.02). L-AMB significantly prolonged animal survival versus controls with mean survival time of ≥7 d and 38% mortality at day 7 (p = 0.04). MCFG was less effective with a median survival time of 6 d and 55% mortality rate (p = 0.17 vs. control).

*R. oryzae* fungal burden was significantly higher non-survivors (median 6.5 log10 range 5.2–7.3) vs. survivors (median 5.8, range 3.6–6.3); p = 0.001.

**Discussion**

The results of this study build upon previous work that demonstrated serial passage of *R. oryzae* sporangiospores on VRC-containing agar significantly enhances the virulence of the fungus in an invertebrate and murine model of invasive
mucormycosis. In the current study, however, we developed mixed infection model of \textit{R. oryzae}-\textit{A. fumigatus} pneumonia in corticosteroid-immunosuppressed mice to explore whether in vivo exposure with other representative antifungal classes used in the treatment of invasive aspergillosis provoke similar selection pressure for breakthrough mucormycosis during infection. Although we found that VRC-treated mice consistently experienced more rapid growth of \textit{R. oryzae} in their lungs compared with mice treated with L-AMB and MCFG, it is notable that all of the antifungal regimens were less effective in animals infected with the VRC-preexposed inoculum, suggesting that breakthrough mucormycosis in the setting of VRC may be inherently less amendable to treatment than de novo infection.

It is also notable that in our mixed infection model, suppression of the higher \textit{A. fumigatus} fungal burden did not appear to be essential for the emergence of \textit{R. oryzae}, as the \textit{R. oryzae} “outcompeted” \textit{A. fumigatus} in control animals when administered at a 1-log lower inoculum than \textit{A. fumigatus}. The eventual domination of \textit{R. oryzae} over \textit{A. fumigatus} is consistent with the more rapid growth characteristics of \textit{R. oryzae} in vitro and in vivo, which were accelerated when the fungus was passaged on VRC. Hence, concepts of antimicrobial selection, where suppression of more susceptible populations presages the emergence of resistant pathogens with reduced fitness cost, may be less applicable opportunistic molds.

Virulence factors in \textit{R. oryzae} have only just begun to be explored, but recent studies have identified the importance of iron acquisition through high-affinity iron permeases and the glucose regulated protein receptor 787 (GRP78) in host endothelium as factors modulating the virulence of experimental \textit{R. oryzae} infection diabetic ketoadipotic mice. It is unknown whether VRC influences the expression of high affinity permeases in \textit{R. oryzae}, or interactions of the fungus with the GRP78 receptor. Several investigators have also reported that exposure of \textit{Candida} and \textit{Cryptococcus} spp. to subinhibitory concentrations of triazole antifungals increases extracellular release of aspartate proteinase virulence factors via upregulation of multidrug resistance (MDRI) efflux pumps. Therefore, it is possible that VRC exposure results in increased extracellular secretion of comparable virulence factors such as proteases, toxins or iron-scavenging siderophores in \textit{R. oryzae}. Alternatively, VRC may weakly inhibit \textit{R. oryzae} P-450 demethylase resulting in accumulation of alternative non-ergosterol sterols in the fungal cell membrane, which may enhance the adherence of the fungus to host endothelial tissue or the extracellular matrix.

The recent sequencing of the 45.3 Mb genome of \textit{R. oryzae} revealed a surprising degree of repetitive gene families and plasticity compared with other sequenced fungi that may have resulted from an ancestral whole-genome duplication event followed by a massive gene loss. This evolutionary path appears to have dramatically enriched the \textit{R. oryzae} genome with the capabilities for rapid adaptation to hostile environments or insults, and production of virulence factors including secreted aspartic proteases, subtilases, and capabilities for accelerated fungal cell wall synthesis and remodeling, as well as iron assimilation from host hemoglobin. Whatever the mechanism of voriconazole-associated increased \textit{R. oryzae} virulence, it is evident that these changes are inherited across successive mitotic generations. Exposure of germinating sporangiogospores to voriconazole resulted in increased virulence of the next generations of asexual spores that germinated in mammalian host in the absence and presence of antifungal therapy, including voriconazole. Although we did not specifically study the reversibility of this phenotype in the current study, previous work demonstrated that the hypervirulent phenotype is lost when exposure to voriconazole is terminated, suggesting an unstable epigenetic adaptation rather than a genetic mutation associated with voriconazole exposure.

Several limitations of this study include our use of a single mode of immunosuppression in a single mouse species, and testing a single clinical isolate of \textit{R. oryzae} to study the phenomena of breakthrough infection. Although our mixed infection model was reproducible, we did observe a modestly higher (0.25–0.5 log\textsubscript{10}) \textit{A. fumigatus} inoculum at t = 1 h in animals infected with the non-preexposed vs. the preexposed mixed inoculum. This difference could not be explained based on inoculum preparation procedures or qPCR assay. However, we believe it is unlikely that this small difference in \textit{Aspergillus} (10% CV) systemically biased results in favor of \textit{R. oryzae} breakthrough based on experience from inoculum ranging studies with both the mixed and single isolate inocula. It is also unclear whether patterns of breakthrough infection observed in this study can be extrapolated to hosts with other types of immune suppression.
predisposed to mucormycosis (i.e., neutropenia, diabetic ketoacidosis) or other Mucorales remains to be elucidated. Similarly, the inoculum and ratio of Aspergillus:Rhizopus adopted for this study was selected to allow serial simultaneous monitoring of A. fumigatus and R. oryzae fungal burden by qPCR, and may not represent environmental exposures or lung fungal burden encountered in patients receiving antifungal prophylaxis. Finally, other drugs commonly used in the transplant population (i.e., chemotherapy, calcineurin inhibitors, etc.) may directly modulate the virulence of these eukaryotic molds.

In conclusion, we found that exposure of R. oryzae sporangiospores to voriconazole in vitro fundamentally alters the growth rate and/or virulence of the fungus in vivo, which is attenuated by echinocandin or L-AMB treatment, but not voriconazole. In our experimental model, in vivo exposure alone was less effective at inducing virulence of R. oryzae during the experimental period. Given the increasing incidence and poor outcome of these aggressive fungi, the mechanisms underlying modulation of R. oryzae virulence by VRC should be further studied.

Materials and Methods

Fungal isolates and inoculum preparation. A clinical isolate of Rhizopus oryzae 557969 obtained from a patient at The University of Texas MD Anderson Cancer Center with invasive mucormycosis and a clinical reference strain of Aspergillus fumigatus 293 were used for all experiments. The isolates were grown from stocks maintained at -80°C and subcultured on yeast-extract agar plates containing 0.5% yeast extract, 1.0% dextrose, 0.2% vitamin mix, 0.1% trace elements, 1.5% agar and 1% MgSO₄. R. oryzae was grown on YAG containing 4 µg/mL of VRC for four passages before use (Fig. 1). In each passage for R. oryzae sporangiospores were harvested after 48 h incubation at 37°C, washed with sterile phosphate buffered saline (PBS), and immediately spread on fresh YAG plates. Susceptibility testing of isolates by broth microdilution testing did not demonstrate a change in the minimum inhibitory concentration of amphotericin B, voriconazole or the minimum effective concentrations of micafungin (Mycamine; MCFG, Astellas Inc., Deerfield, IL) were administered as i.p. injections. The human clinical formulation of liposomal amphotericin B (Ambisome; L-AMB, Astellas Inc.) was diluted according to the manufacturer’s recommendations and administered as single daily 10 mg/kg intravenous (IV) injection via the lateral tail vein. The human clinical formulation of voriconazole (Vfend; VRC, Pfizer Inc., New York, NY) was also prepared according to the manufacturer’s recommendations and administered as 40 mg/kg IV injection every 12 h. Including the day of infection, antifungal therapy was continued for a total of 5 d. At predetermined timepoints, groups of 5–8 mice were euthanized by CO₂ narcosis and lungs were excised and stored in 5 d. At predetermined timepoints, groups of 5–8 mice were euthanized by CO₂ narcosis and lungs were excised and stored in
in duplicate with an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) with PCR primers and a dual-labeled fluorescent hybridization probes specific for *A. fumigatus* and *R. oryzae* 18SrRNA genes. The conidial equivalent (CE) fungal concentrations of each fungal species were interpolated from two six-point standard curves prepared by spiking uninfected mouse-lungs with known concentrations (10^2–10^7) of *A. fumigatus* or *R. oryzae* spores. PCR reactions were performed in both sets of spiked lungs using both Aspergillus and Rhizopus probe-primer pairs to ensure no cross-reactivity. The plasmid internal standard was amplified in separate reactions to correct for the percent differences in DNA recovery. The results are reported log_{10} conidial equivalent (CE) fungal concentrations of each fungal species.

**Voriconazole assay.** VRC plasma concentrations were confirmed in groups of five immunosuppressed and infected mice at 72 and 120 h using a stability-indicating, validated high-performance liquid chromatography (HPLC) assay developed in our laboratory. Briefly, blood was collected from animals after CO2 narcosis and plasma harvested, then stored at -80°C until analysis. Thawed samples were spiked with an internal standard, 3-Nitrophenol and further processed by solid phase extraction. Following extraction, the residue was reconstituted with mobile phase. Samples were processed on a reverse-phase C8 column eluted by 40% acetonitrile-60% water (pH = 3.0 by formic acid) at a flow rate of 1.0 mL/min with UV detection at 260 nm. The range of concentrations (0.15–20 mg/L) tested was linear (r^2 = 0.999) with an intra-day and inter-day coefficient of variation less than 5%.

**Statistical analysis.** All graphical data were expressed as mean ± standard error of the mean and compared pairwise by Mann-Whitney Test or Kruskal-Wallis Test with Dunn’s post-test for multiple samples when appropriate. Fungal burdens were log-transformed before plotting and statistical analysis. Survival curves were compared by the Mantel Cox (log-rank) Test. Differences were considered statistically significant when p values were < 0.05. All analysis and plotting was performed using Graphpad Prism 5.0 (Graphpad Software Inc., La Jolla, CA).

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