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In vivo Efficacy of Posaconazole (POS) against Voriconazole Resistant (VCZ-R) *Aspergillus flavus* in an Inhalational Neutropenic Murine Model of Invasive Pulmonary Aspergillosis

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Authors' contributions

This work was carried out in collaboration between both authors. Author SKN designed the study, did the literature search, performed the statistical analysis and wrote the protocol and the manuscript. Author JLC performed majority of the experiments, did a literature search and contributed to the final analyses of the study. Both authors read and approved the final manuscript.

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ABSTRACT

Invasive aspergillosis (IA) is a life-threatening infection in patients with cancer. Recent studies have reported that non-*fumigatus Aspergillus* spp., including *Aspergillus flavus*, are emerging as predominant pathogens in various transplant and cancer centers in the USA and around the world. Clinical and environmental isolates of Aspergillus species showing reduced susceptibility to VCZ have been reported. Mortality, despite therapy, remains high, and drug resistance might partly account for treatment failures. In this in *vivo* study, the virulence of a VCZ-R *cyp*51A mutant of *A. flavus* and the efficacy of POS against this mutant were evaluated using a neutropenic inhalational murine model of invasive pulmonary aspergillosis. VCZ-R *A. flavus* mutant was virulent in *vivo*, and had similar infectivity as the VCZ-S parent. Posaconazole had superior activity to that of VCZ in reducing fungal burden ($p < 0.05$) and mortality ($p < 0.05$) in this experimental model of VCZ-R *A. flavus* murine infection. This study demonstrated that POS may be a viable option for certain strains of VCZ-R *A. flavus.*

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1. INTRODUCTION

Invasive aspergillosis (IA) continues to be associated with a high mortality despite timely and appropriate therapy in immunocompromised patients [1]. *Aspergillus flavus* is the second most common pathogen associated with IA in the United States and the most common pathogen isolated from IA in several arid regions and tropical countries [2]. Although majority of therapeutic failures are often attributed to various host factors (poor immunity, prolonged neutropenia, graft versus host disease, high dose steroid therapy, cancer, and other concurrent infections), azole-resistance cannot be ignored [3-5]. The major mechanism of high-level azole-resistance in Aspergillus species reported so far is mutation [6-11] and/or overexpression of target site [12,13], namely cyp51A, that encodes lanosterol demethylase of the fungal cell wall.

Environmental isolates of *A. fumigatus* and *A. flavus* have demonstrated azole-resistance which is a direct consequence of azole and benzimidazole pesticide exposure [14,15].

Voriconazole is currently the drug of choice for management of IA. Other antifungal agents that have demonstrated good in *vitro* and in *vivo* activity against invasive aspergillosis include other triazoles (itraconazole-ITZ, posaconazole, isavuconazole-ISZ), echinocandins (anidulafungin, caspofungin and micafungin; not indicated for primary therapy of IA) and the polyenes (amphotericin formulations; usually deferred due to drug toxicities). Given that the mechanism of action of azoles is via inhibition of 14α-lanosterol demethylase of Aspergillus cell wall, azole cross resistance is a major concern [16,17].

Interestingly, studies in *A. fumigatus* have shown that VCZ-R isolates may retain susceptibility to POS and ITZ, depending on the specific site of *cyp*51A mutation [18,19]. Azole-resistance studies in *A. flavus* are scarce and hence not much information is available in literature. Although newer agents such as ISZ have been introduced, it may not be an option in various countries with limited resources. Understanding the pattern of azole-resistance in *A. flavus* is essential in order to decide on the best therapeutic options for IA caused by azole-R *A. flavus*. Posaconazole needs to be tested as a viable option for VCZ-R *A. flavus* infections.

In this in *vivo* study, the virulence of a laboratory-selected VCZ-R isolate of *A. flavus* isolate (*cyp*51A mutant-K197N) and the efficacy of POS against this isolate were evaluated using a neutropenic murine model of invasive pulmonary aspergillosis by assessing the pulmonary fungal burden and mortality in various groups of mice.

2. MATERIALS AND METHODS

2.1 Murine Pulmonary Aspergillosis Model

Voriconazole-resistant *A. flavus* isolate AFLW4 (*cyp*51A mutant; K197N), selected in our lab [11] and its isogenic parent AFL188, were used for this study (Table 1). Cultures were grown on Sabouraud dextrose agar for 6 days at 35°C, fresh conidial suspensions were prepared (2 X 10^8 conidia per ml) and aliquots of this conidial suspension were used for infection.

Female ICR mice (Harlan, Indianapolis, Indiana) weighing 20-25 grams (6 weeks old), (n=30; 10 mice per group) were made neutropenic by four successive intraperitoneal injections (0.2 ml/ dose) of cyclophosphamide (200 mg/kg/dose) on days -3, -1, 1 and 4 where day 0 was the day of infection. Mice were fed grapefruit juice to inhibit the gut cytochrome P450 enzymes which (Tropicana 100% Pure Premium Ruby Red) markedly increased the blood level of VCZ. Serum levels of voriconazole were measured at specified time intervals to measure peak (2 hrs post therapy) and trough levels 1hr prior to next dose), using a previously described bioassay using *Candida kefyr* as the standard [20].

The neutropenic mice were anesthetized by exposure to isoflurane and were infected with 1 X 10^7 *A. flavus* conidia (0.05 ml of either VCZ-S or VCZ-R) delivered to the nares from a micropipette. Treatment with either VCZ or POS (25 mg/kg/d) (voriconazole: Pfizer Pharmaceuticals, NY, NY; posaconazole, Schering Plough Research Institute, Kenilworth, NJ) orally was initiated 24 h post-infection and was continued for 6 days. Control groups received comparable amounts of sterile water.

Table 1. Details of voriconazole-resistant *A. flavus* isolate AFLW4 (*cyp*51A mutant; K197N), and its isogenic parent AFL188

Susceptibility	*A. flavus* isolate	VCZ MIC (mcg/ml)	POS MIC (mcg/ml)	*cyp*51A mutation
VCZ-S	AFL0188	0.25	0.0625	NO
VCZ-R	AFLW4	4	0.0625	K197N

At the end of the experiment (Day 6), lungs from deceased and the surviving mice (after sacrificing) were surgically harvested, weighed, homogenized, serially diluted 10-100 fold and 0.1 ml aliquots were plated on Sabouraud dextrose agar plates, (supplemented with 100 µg/ml of piperacillin and amikacin) incubated at 35°C for 48 h and the number of colony forming units (CFU) per total weight of lung tissue was calculated. The efficacy of the antifungal treatment was defined in terms of increased survival rate at day 5 and by decreased fungal burden in the lungs of treated mice.

3. RESULTS

The lung fungal burden (FB) in AFL0188-infected controls or in mice treated with VCZ or POS was 27300, 612, and 340 CFU/lung respectively, resulting in ~2 log10 reduction in the drug-treated groups; FB in AFLW4 infected controls or in mice treated with VCZ or POS was 74000, 44000 and 3300 CFU/lung respectively, resulting in 0.2 and ~2 log10 reduction in VCZ or POS-treated mice respectively (Fig. 1). No survival benefit was seen between VCZ and POS-treated mice infected with AFL188. However, in mice treated with AFLW4, mortality was 60%, 30% and 0 in controls, VCZ or POS -treated groups respectively (Fig. 2). Serum levels of VCZ measured over a 24 hour period ranged from 2 mcg/ml (trough) to 16 mcg/ml (peak) (Fig. 3).

Lab-selected VCZ-R isolate of *A. flavus* had similar infectivity as the VCZ-S parent. Posaconazole demonstrated a survival benefit over VCZ in mice infected with VCZ-R *A. flavus*; survival was improved in VCZ or POS treated mice as compared to untreated controls in mice infected with VCZ-S *A. flavus.* Posaconazole had superior fungicidal activity to that of VCZ in reducing the fungal burden in mice infected with VCZ-R *A. flavus.* Our study demonstrated an in *vitro*-in *vivo* correlation between azole-susceptibility and antifungal drug efficacy in mice infected with VCZ-R *A. flavus.*

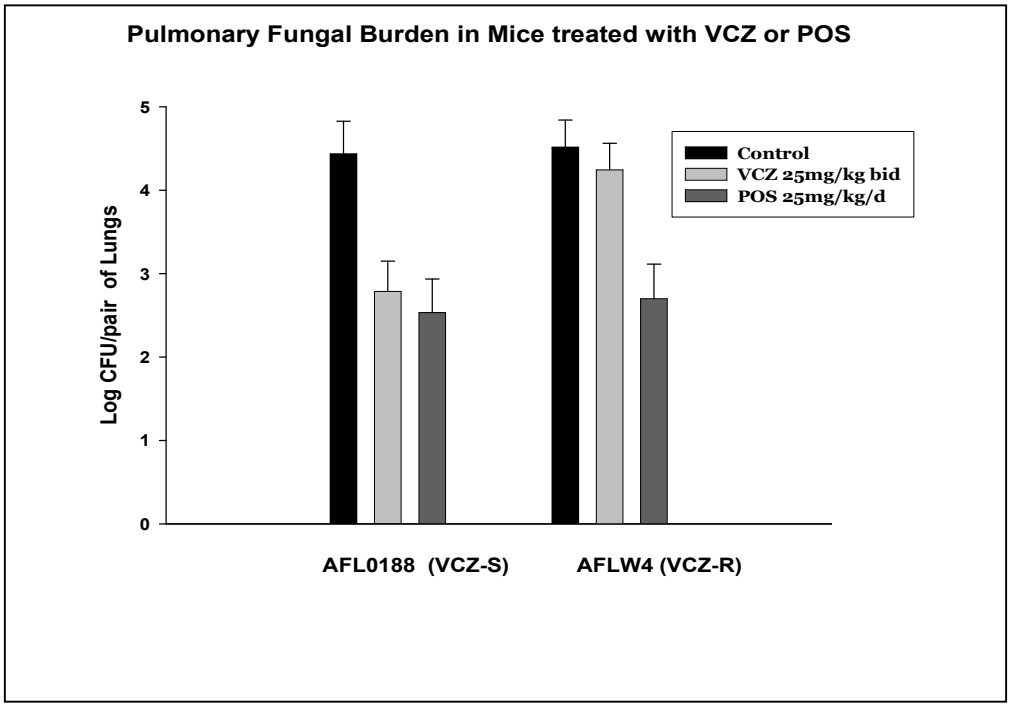

Fig. 1. Pulmonary fungal burden in mice treated with VCZ or POS

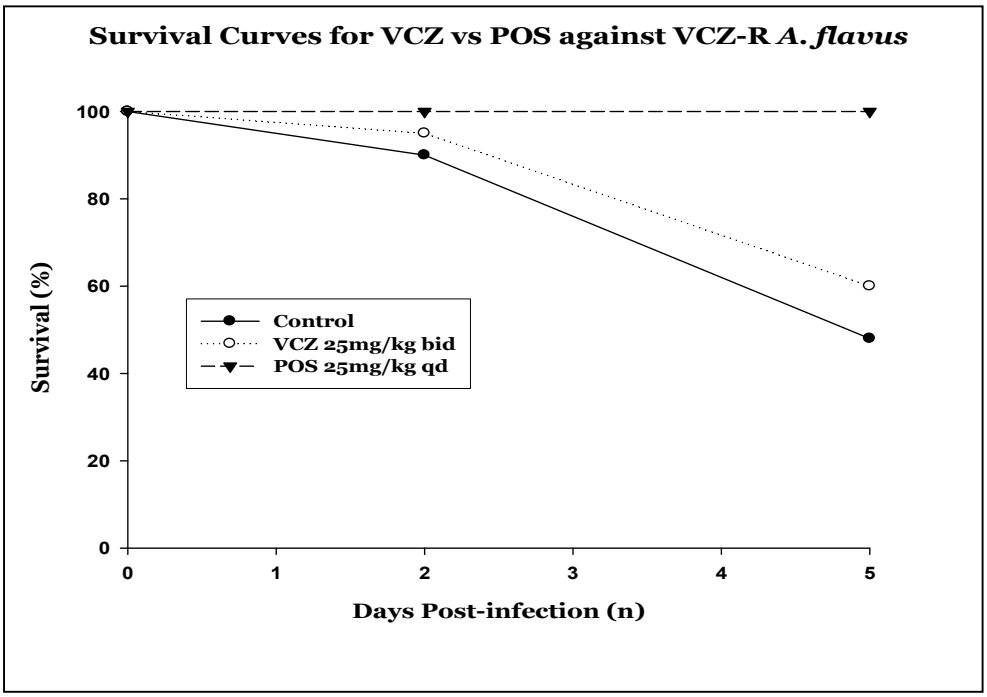

Fig. 2. Survival curves for VCZ vs POS against VCZ-R *A. flavus*

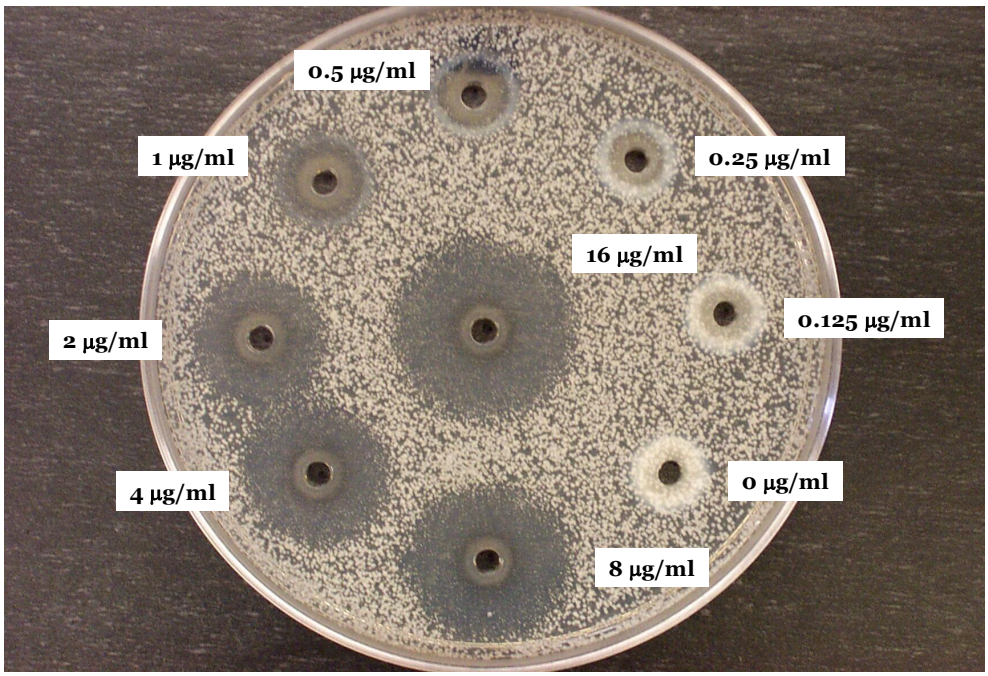

Fig. 3. *C. Kefyr* bioassay for measuring VCZ levels in mouse serum

4. DISCUSSION

Research on the molecular mechanisms of antifungal resistance in *A. flavus* is in its early infancy, consequently very little is known. Only few reports of clinical isolates of *A. flavus* resistant to antifungal drugs are available in the literature. Krishnan et al. have selected

voriconazole-resistant strains of *A. flavus* from a drug susceptible clinical isolate in the laboratory. These strains showed higher MIC (MIC ≥16 μg ml^{-1}) to voriconazole. Some of these laboratory-selected isolates also showed higher MICs to other triazoles such as itraconazole, posaconazole and ravuconazole in *vitro* [11]. Therefore, cross resistance to newer generation of triazoles (posaconazole and isavuconazole) remains a significant concern.

However, in this study we noted that azole-resistance in *A. flavus* is dependent on the location of specific mutations in *cyp*51A. Isolates of VCZ-R *A. flavus* that demonstrated the K197N mutation, were resistant to VCZ, but retained susceptibility to POS. Hence POS continues to be a valuable option for certain isolates of VCZ-R *A. flavus.*

As the study of IA caused by *A. flavus* has become an area of investigation only recently, very little is known about the frequency and the mechanisms of resistance to antifungal drugs in *A. flavus*. Azole-resistance secondary to cyp51 mutations and efflux pumps have been reported in literature, although scarce. Molecular studies and continued surveillance for azole-resistance from clinical isolates of *A. flavus* is imperative [21]. An understanding of the mechanisms of azole- resistance and establishing a correlation between specific *cyp*51 mutations and azole-resistance may have therapeutic implications [22-26]. The recent introduction of phenotypic and genotypic assays for rapid and timely identification of specific *cyp*51 mutations from clinical specimens will serve as a valuable tool in this field.

Importantly, the characterization and publication of the entire genome of *A. flavus*, by the United States Department of Agriculture (USDA), marks a new era in research on azole-R in *A. flavus*. It also paves the way to understand the pathogenesis of IA, fungal-host cell- immune interactions and will lead to the development of novel therapeutic modalities to control these lethal infections.

5. CONCLUSION

With respect to the above discussion, there are a few important points to consider. This is one of the very few studies reported in literature, that has used an inhalational model of invasive aspergillosis, which replicates human infection (the mode of infection in humans is via inhalation of Aspergillus spores). It uses two end points namely survival and fungal burden in lungs as a measure of drug efficacy. Based on our observations and data collected, we conclude that our model supports the view that VCZ-R in *vitro* does not necessarily mean that the *A. flavus* strain is pan-azole resistant. Newer generation of azoles such as POS and ISZ may still be valuable options in the treatment of VCZ-R invasive aspergillosis. However, it has to be a clinical decision based on genomics, specific mutation data and serum drug levels. Further research in this field is imperative to understand the quantitative and qualitative relationship between azoles and Aspergillus and would be an advancement in the management of these infections.

ETHICAL APPROVAL

This study was approved by the Animal Investigation Committees at Wayne State University (Detroit, MI) and by the John D. Dingell VA Medical Center and conformed to all relevant federal guidelines and institutional policies for the use of vertebrate animals in research.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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