

BRIEF COMMUNICATION

In vitro susceptibility of *Aspergillus* and *Fusarium* associated with equine keratitis to new antifungal drugs

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Abstract

Objective: To determine minimum inhibitory concentrations (MICs) of four fungal species isolated from horses presented with equine fungal keratitis (EFK) in the southeastern United States to previously untested azole, echinocandin, and carboxamide antifungal drugs.

Methods: In vitro assays were performed to determine the susceptibility of *Aspergillus flavus*, *A. fumigatus*, *Fusarium falciforme*, and *F. keratoplasticum* to five antifungal drugs representing three modes of action.

Results: Luliconazole exhibited increased growth inhibition against both *Aspergillus* and *Fusarium* compared to commonly used, standard antifungal drugs. MIC values for luliconazole at 0.001–0.002 µg/mL were at least 25-fold lower than all other antifungal drugs tested, including voriconazole.

Conclusions: The increased antifungal activity of luliconazole observed in this study warrants further investigation for its potential as an antifungal drug for equine fungal keratitis.

KEYWORDS

Aspergillus, *Fusarium*, luliconazole, voriconazole

1 | INTRODUCTION

Equine fungal keratitis (EFK) is a severe, progressive, inflammatory ocular disease resulting from invasive growth of fungi into the cornea. Fungal keratitis is one of the most common causes of blindness in horses of the Southeastern USA and is a widespread disease in horses from all states east of the Rocky Mountains.^{1,2} The most common causal organisms of EFK are the filamentous fungi *Aspergillus* and *Fusarium*.^{1–3} These fungi represent a component of the normal ocular surface microbiome and are common soil saprobes and plant pathogens, and opportunistic when invading the cornea in EFK.² Once EFK is diagnosed, treatment is generally the same for all horses regardless of fungal species present. Using this approach, treatment

results and outcome in EFK remain unacceptable. In two recent studies of EFK, 60% of horses did not respond to antifungal medical therapy and required surgery, with up to 37% of eyes resulting in enucleation.^{1,2} Available antifungal drugs for EFK are few and limited primarily to natamycin, voriconazole, amphotericin B, itraconazole, and fluconazole. No new antifungal drugs have been introduced for treatment of EFK since the use of voriconazole was described nearly 15 years ago.⁴ Voriconazole is now used worldwide for EFK, but recent studies have indicated development of resistance to voriconazole by filamentous organisms, including species of *Fusarium*.^{2,5,6} Therefore, new effective antifungal drugs and improved selection of these drugs for clinical cases of EFK are desperately needed.

In this study, we evaluated in vitro sensitivity of antifungal drugs of different chemistries and modes of action, including an imidazole (luliconazole) that has been shown to have a low MIC against fungal isolates from human keratitis patients. Chemical classes of drugs studied were triazoles (prothioconazole, voriconazole), imidazoles (luliconazole), carboxamides (pydiflumetofen), and echinocandins (caspofungin).

The objective of this study was to evaluate new antifungal drugs (caspofungin, luliconazole, prothioconazole, pydiflumetofen,) by determining MIC's for four fungal species isolated from EFK in comparison with the standard EFK therapeutic drug voriconazole.

2 | MATERIALS AND METHODS

2.1 | Antifungal drug preparation

In vitro susceptibility to caspofungin, luliconazole, and voriconazole (positive control), (Sigma-Aldrich), prothioconazole (Bayer), and pydiflumetofen (Posterity, Syngenta) were assayed using the 96-well microplate modified protocol of the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method (M38-A2 protocol) for filamentous fungi, as described previously.^{2,7} Briefly, antifungal drugs were prepared in a 2x dilution series with concentrations of: 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, and 32 µg/mL; as well as a 5x dilution series with concentrations of 0.01, 0.05, 0.25, 1.25, 6.25, 31, 70, and 156 µg/mL. Additionally, luliconazole was prepared in a 2x dilution series with concentrations of: 0.00003, 0.00006, 0.00012, 0.00024, 0.00048, 0.00096, 0.00192, 0.00384, 0.00768, and 0.0154 µg/mL; including a 5x dilution series of with concentrations of: 0.00016, 0.00008, 0.0004, 0.002, 0.01, 0.05, 0.25, 1.25, 6.25, and 31 µg/mL. With the exception of pydiflumetofen, all dilutions were formed using dry technical ingredient dissolved in DMSO. Pydiflumetofen was prepared from a 18.3% suspension concentration diluted with sterile deionized water.

2.2 | Fungal preparation

Four fungal isolates from clinical cases of EFK that had multi-locus sequence analysis performed in a previous study to identify genus, species, and genetic lineage² were used in this study (Table 1). These isolates also had in vitro susceptibility testing previously performed using common antifungal drugs, including voriconazole, terbinafine, amphotericin B, natamycin, and thiabendazole.² The EFK isolates, which included one each of *Aspergillus flavus*, *A. fumigatus*, *Fusarium falciforme*, and *F. keratoplasticum*, were inoculated onto Potato Dextrose Agar (PDA) (Becton Dickinson) Petri plates and incubated in the dark at 30°C for 7 days. Conidial suspensions were prepared by flooding plates with Potato Dextrose Broth 50% (PDB50) (Becton Dickinson) and filtering with cheesecloth. Concentrations of conidial suspensions were determined using a hemocytometer and adjusted to a concentration of 1 000 000 spores per mL for *Aspergillus* isolates and a concentration of 200 000 spores per mL *Fusarium* isolates before storage. Spore suspensions were frozen in 2 mL aliquots at -80°C. To prepare for use, isolates were quickly thawed in a 40°C water bath and then diluted using PDB50 to a concentration of 200 000 spores per mL for each *Aspergillus* isolate and 40 000 spores per mL for each *Fusarium* isolate. Each inoculated well in the 96-well microplate received 50 µL of the appropriate spore suspension, resulting in a final spore count of 10 000 conidia per well for *Aspergillus* and 2000 conidia per well for *Fusarium*. Inoculation of the plates followed addition of the chemical treatments.

2.3 | Microplate preparation

All wells received 149 µL of PDB50. Each treatment well received a 1 µL aliquot of the appropriate stock concentration of the drug and 50 µL of the appropriate conidial suspension. All edge wells served as control wells, receiving 50 µL of conidial suspension with no drug. All corner

TABLE 1 Fungal isolates for minimum inhibitory concentration testing

Strain	Species	MLST ^a (Lineage)	Sample date	Breed	Horse state of origin
IC25603	<i>Aspergillus flavus</i>	IC	1/6/18	Quarter Horse	Wake Forest, NC
IC22917	<i>Aspergillus fumigatus</i>	-	9/27/13	Arabian	Hillsborough, NC
IC22927	<i>Fusarium falciforme</i>	4hhhh, 4ffff	12/23/16	Warmblood	Reidsville, NC
IC24027	<i>Fusarium keratoplasticum</i>	2u	4/15/17	Percheron	Sedley, VA

^aMulti-locus sequence type (MLST) designations described by Cullen et al.⁵

wells served as uninoculated controls, receiving PDB50 only. Microplates were incubated in the dark at 20°C for 72 hours. MICs were determined visually using a magnifying reading mirror, with the lowest concentration which exhibited complete growth inhibition being the MIC value. Each assay was repeated in triplicate and the median MIC was calculated.

2.4 | Statistical analysis

Statistical analysis of MIC values was performed as previously described.² Using Minitab 18 ANOVA analyses were conducted. If the effect of interest (eg, Isolate) was significant at the $P \leq .05$ level, then Tukey mean separation was conducted with $\alpha = .05$.

3 | RESULTS

3.1 | In vitro antifungal susceptibility

In vitro antifungal MIC values for voriconazole, prothioconazole, pydiflumetofen, caspofungin, and luliconazole against *Aspergillus fumigatus*, *A. flavus*, *Fusarium falciforme*, and *F. keratoplasticum* are presented in Tables 2 and 3.

3.1.1 | Voriconazole

Aspergillus fumigatus had an MIC of 0.25 µg/mL while *A. flavus* had an MIC of 0.5 µg/mL for voriconazole. *Fusarium falciforme* had an MIC of 1 µg/mL, and *F. keratoplasticum* had an MIC of 8 µg/mL for voriconazole. These MIC values for the standard voriconazole were consistent with previous research.⁵

3.1.2 | Prothioconazole

Minimum inhibitory concentrations values for prothioconazole ranged from 32 µg/mL for both species of *Fusarium* to 6.25 µg/mL for *A. fumigatus*. *A. flavus* did not show inhibition of vegetative mycelial growth to any prothioconazole concentration tested.

3.1.3 | Pydiflumetofen

Aspergillus fumigatus had an MIC of 0.25 µg/mL for pydiflumetofen. *Fusarium keratoplasticum* had an MIC of 2 µg/mL while *F. falciforme* had MICs ranging from 1.25-6.25 µg/mL. *Aspergillus flavus* did not show inhibition at any pydiflumetofen concentration tested.

3.1.4 | Caspofungin

Fusarium MIC values for caspofungin ranged from 8 µg/mL for *F. falciforme* to 16 µg/mL for *F. keratoplasticum*. Neither species of *Aspergillus* was completely inhibited at any concentration tested. For all four species, ~80% growth inhibition was achieved at lower concentrations (0.01 µg/mL for *Aspergillus*, 0.5 µg/mL for *Fusarium*); but no further inhibition was achieved for *Aspergillus*, while complete inhibition of *Fusarium* was not achieved until concentrations increased to greater than 8 µg/mL. To ensure this limited growth at middle concentrations was not indicative of a delayed effect resulting in some growth in each well prior to the drug becoming fully inhibitive, all 200 µL of the treated conidia suspensions were removed from selected wells of one *F. falciforme* and one *A. fumigatus* microplate after incubation, inoculated onto PD agar plates, and grown in the dark at 37°C for 6 days. All conidia suspensions that exhibited limited growth during the microplate assay also exhibited growth after agar plating. For both isolates, solutions with higher antifungal concentrations resulted in a high quantity of small (~0.2 cm) fungal colonies, while lower antifungal concentrations resulted in fewer but larger (~1.5 cm) colonies.

3.1.5 | Luliconazole

Minimum inhibitory concentrations values for luliconazole ranged from 0.001 µg/mL for both *Aspergillus* species to 0.002 µg/mL for both *Fusarium* species. These MIC values were at least 25-fold lower than compared to the other antifungal drug evaluated including the standard voriconazole.

TABLE 2 Minimum inhibitory concentration of fungal isolates from equine keratitis

Fungal Species	Voriconazole	Pydiflumetofen	Prothioconazole	Caspofungin	Luliconazole
<i>Aspergillus flavus</i>	0.5	>156	>156	>156	0.001
<i>Aspergillus fumigatus</i>	0.25	0.25	6.25	>156	0.001
<i>Fusarium falciforme</i>	1	1.25-6.25	32	8	0.002
<i>Fusarium keratoplasticum</i>	8	2	32	16	0.002

TABLE 3 Minimum inhibitory concentration of fungal isolates from equine keratitis

MIC (µg/ml)	0.001	0.002	0.004	0.008	0.016	0.031	0.062	0.125	0.25	0.5	1	2	4	8	16	32	>156
<i>Aspergillus flavus</i>	LUL										VOR				CAS, PRO, PYR		
<i>Aspergillus fumigatus</i>	LUL								PYD, VOR		PRO				CAS		
<i>Fusarium falciforme</i>	LUL										VOR		PYD CAS		PRO		
<i>Fusarium keratoplasticum</i>	LUL										PYD		VOR CAS		PRO		

Antifungal drugs within a box do not have significantly different MIC's. Agents in different boxes have significantly different MIC's. (ANOVA, 2-factor with Log10 transformation of MIC values to improve normality. $P < .001$ for fungus x antifungal agent interaction. Mean separation: Tukey with $\alpha = .05$. Antifungal names positioned at Log2 MIC value closest to their mean for that fungal species.

Abbreviations: CAS, caspofungin; LUL, luliconazole; PRO, prothioconazole, PYD, pydiflumetofen; VOR, voriconazole.

4 | DISCUSSION

In this study, luliconazole exhibited increased growth inhibition against both *Aspergillus* and *Fusarium* compared to other commonly used antifungal drugs. It was at least 25-fold lower than all other antifungal drugs tested, including voriconazole. The increased antifungal activity of luliconazole observed in this study warrants further investigation for its potential as an antifungal drug for equine fungal keratitis.

The antifungal drugs used in this study represent three classes of antifungals: azoles, echinocandins, and carboxamides. Voriconazole, prothioconazole, and luliconazole belong to the azole class. Azole drugs inhibit fungal growth by inhibiting 4 α -lanosterol demethylase, disrupting the synthesis pathway of ergosterol, a key component in maintaining cell wall integrity and osmolality.⁸ Inhibiting 4 α -lanosterol demethylase also causes accumulation of toxic 14 α -methylated sterols in the fungal cell membrane. Caspofungin is of the echinocandin class, which inhibits fungal growth by preventing synthesis of β -1-3-d-glucan, a key structural component in fungal cell walls.⁸ Pydiflumetofen is of the carboxamide class which inhibits succinate dehydrogenase, which interferes with cellular respiration and prevents fungal cell growth.⁹ Of these classifications, echinocandins have been shown to cause both paradoxical growth (PG) and trailing effect (TE) in some *Aspergillus* isolates.¹⁰ PG is defined as when fungal growth resumes upon exposure to antifungal concentrations higher than the MIC, while TE is defined as when fungal growth

is largely reduced (~80%) but not completely inhibited at concentrations above the MIC. This effect is thought to be highly specific to both the echinocandin and fungal isolate and is caused by an upregulation in chitin synthesis in response to the echinocandin.¹⁰ The *Aspergillus flavus* and *A. fumigatus* isolates tested in this study exhibited TE consistent with previously published observations, with maximal inhibition levels of ~80% following the MIC. The species of *Fusarium* used in this study did not exhibit classical TE as complete inhibition was eventually accomplished; however, the high MIC values are consistent with previously published results suggesting possible resistance of *Fusarium* species to echinocandins. In addition to the lack of response from *A. flavus* to caspofungin, growth of *A. flavus* also was not inhibited at any concentration tested against prothioconazole or pydiflumetofen.

Luliconazole has been shown to exhibit MICs as low as 0.002 µg/mL for human-derived and environmental *Aspergillus* species and as low as 0.001 µg/mL for human-derived *Fusarium* species.¹¹ It has also been used as a successful treatment of topical dermatophytosis in humans.¹² This study demonstrated high in vitro efficacy of luliconazole against EFK-derived fungal isolates. These results suggest luliconazole's potential to be a highly effective and improved treatment against EFK; further evaluation of this drug for EFK therapy is warranted.

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