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Association Between In Vitro Susceptibility to Natamycin and Voriconazole and Clinical Outcomes in Fungal Keratitis

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Abstract

Purpose—To assess the association between minimum inhibitory concentration (MIC) and clinical outcomes in a fungal keratitis clinical trial.

Design—Experimental study using data from a randomized comparative trial.

Participants—Of the 323 patients enrolled in the trial, we were able to obtain MIC values from 221 patients with monocular fungal keratitis.

Methods—The Mycotic Ulcer Treatment Trial I (MUTT I) was a randomized, double-masked clinical trial comparing clinical outcomes of monotherapy with topical natamycin versus voriconazole for the treatment of fungal keratitis. Speciation and determination of MIC to natamycin and voriconazole were performed according to Clinical and Laboratory Standards Institute guidelines. The relationship between MIC and clinical outcome was assessed.

Main Outcome Measures—The primary outcome was 3-month best spectacle-corrected visual acuity. Secondary outcomes included 3-month infiltrate/scar size, corneal perforation and/or therapeutic penetrating keratoplasty (TPK), and time to re-epithelialization.

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Results—A 2-fold increase in MIC was associated with a larger 3-month infiltrate/scar size (0.21 mm, 95% confidence interval [CI] 0.10–0.31, *P* <0.001) and increased odds of perforation (odds ratio [OR] 1.32, 95% CI 1.04–1.69, *P*=0.02). No correlation was found between MIC and 3-month visual acuity. For natamycin-treated cases, an association was found between higher natamycin MIC with larger 3-month infiltrate/scar size (0.29 mm, 95% CI 0.15–0.43, *P*<0.001) and increased perforations (OR 2.41, 95% CI 1.46–3.97, *P*<0.001). Among voriconazole-treated cases, the voriconazole MIC did not correlate with any of the measured outcomes in the study.

Conclusion—Decreased susceptibility to natamycin was associated with increased infiltrate/scar size and increased odds of perforation. There was no association between susceptibility to voriconazole and outcome.

INTRODUCTION

Fungal keratitis is an important cause of visual loss worldwide.[1] The causative organisms tend to differ by geographic location.[2–6] In hot and humid environments such as India, filamentous fungi tend to predominate and are associated with poor outcomes.[7] Currently, treatment options for fungal keratitis are limited and empirical treatment is standard practice. Although a number of systemic antifungals with demonstrated in vitro activity have been suggested as treatments, the role of susceptibility testing in guiding therapy for fungal keratitis has not been well defined.

In systemic bacterial infections, in vitro susceptibility is thought to predict clinical outcomes by the "90–60 rule."[8] The rule states that infections caused by susceptible isolates respond to appropriate therapy about 90% of the time, and infections caused by resistant isolates respond about 60% of the time.[8, 9] Previous studies in fungal infections suggest that in vitro susceptibility may be correlated with clinical outcomes.[8, 10–12] Many of those studies used non-ocular isolates[13] or focused on yeast, with limited reports on filamentous fungi, especially against natamycin and voriconazole.[14] Natamycin has long been considered the standard of care for fungal keratitis and is the only topical ocular antifungal approved by the US Food and Drug Administration. Voriconazole, a newer azole, is reported to have good in vitro activity against most isolates from fungal ulcers, though there is mixed evidence regarding activity against *Fusarium* species.[14, 15] In a previous paper by our group, we demonstrated that voriconazole had poor in vivo outcomes compared to natamycin, particularly among *Fusarium* species.[16]

The Clinical and Laboratory Standards Institute (CLSI) has not yet established minimum inhibitory concentration (MIC) clinical breakpoints for filamentous fungi against natamycin and voriconazole. Without a defined guideline for classifying organisms as susceptible, intermediate, or resistant, it is currently challenging to assess the association between antifungal susceptibility and clinical outcomes in fungal keratitis. Here, we used data from the Mycotic Ulcer Treatment Trial I (MUTT I),[16] where natamycin and voriconazole MICs were determined at baseline prior to patient randomization to an antifungal treatment arm, and analyzed MIC as a continuous variable. In this way, we were able to prospectively assess the effect of MIC on clinical outcomes during the course of treatment with a single agent administered by a standardized protocol. In this report, we investigated the association

between organism, MIC, and clinical outcomes, including visual acuity, infiltrate/scar size, corneal perforation or therapeutic penetrating keratoplasty (TPK), and time to re-epithelialization.

METHODS

MUTT I is a multicenter, randomized, double-masked clinical trial investigating the optimal antimicrobial treatment of filamentous fungal keratitis. Detailed methods for MUTT I have been reported previously.[16] In brief, 323 smear-positive fungal ulcer cases with enrollment visual acuity of 20/40 (0.3 logarithm of the minimum angle of resolution [logMAR]) to 20/400 (1.3 logMAR) presenting to the Aravind Eye Care System (Madurai, Pondicherry, and Coimbatore) in India were randomized to receive 5% topical natamycin (Natacyn, Alcon, Fort Worth, TX) or 1% topical voriconazole (VFEND IV, Pfizer, New York, NY). In the two treatment arms, the dosing schedules were identical and consisted of 1 drop to the affected eye every 1 hour while awake for 1 week, then every 2 hours while awake until 3 weeks from enrollment.[16] Continuation of the masked treatment was then at the discretion of the physician. For ethical reasons, physicians were allowed to add or change medications if deemed medically necessary. The primary outcome for MUTT I was 3-month best spectacle-corrected visual acuity (BSCVA) in logMAR. Secondary outcomes included 3-week BSCVA, 3-week and 3-month infiltrate or scar size, corneal perforation and/or TPK, and time to re-epithelialization. The MUTT I trial (ClinicalTrials.gov number, NCT00996736) obtained informed consent from all patients, adhered to the Declaration of Helsinki, and received approval from the Institutional Review Boards (IRB) at Aravind, Dartmouth, and University of California San Francisco (UCSF).

Microbiology

Detailed microbiological methods of MUTT have been previously described.[11] At the screening visit, corneal scrapings were obtained for fungal cultures from all patients who were eligible for the trial. Antifungal susceptibility testing for natamycin and voriconazole were performed on all samples with a positive fungal culture using broth microdilution according to standardized methods outlined in the Clinical and Laboratory Standards Institute (CLSI) document M38-A2.[17] MIC was defined as the lowest concentration that exhibited a 100% visual reduction in turbidity when compared with the control tube for natamycin at 48 hours, and an 80% reduction in turbidity for voriconazole.[17] Only natamycin and voriconazole were analyzed since these were the treatments used in the clinical trial.

Statistical Analyses

MIC_{50} and MIC_{90} were estimated as the median and 90^{th} percentile using the PERCENTILE function in Microsoft Excel (Microsoft Inc, Redmond, Washington). The MIC_{90} was estimated for organisms with at least 9 observations, the smallest number where extrapolation would not be necessary. The 95% confidence intervals (CI) for the MIC_{50} and MIC_{90} were estimated as bootstrap percentile CI in Mathematica 8 (Wolfram, Champaign, IL) for genus and species with at least 9 observations.

A $\log_2$-transformation of MIC was used for all statistical models. Differences in MIC across groups of organism were analyzed with a one-way analysis of variance (ANOVA). For each species or genus, differences between outcomes per treatment arm were analyzed using t-test for visual acuity and infiltrate/scar size, Fisher's exact test for perforation, and log-rank test for time to re-epithelialization. The relationship between susceptibility and outcome was analyzed by multivariable logistic regression modeling for dichotomous outcomes, multiple linear regression modeling for continuous outcomes, or a multivariable Cox proportional hazards model for time to re-epithelialization, using the corresponding baseline measure and the treatment arm as covariates. Time to re-epithelialization was right-censored at 21 days after enrollment. Sensitivity analyses of the outcome models were performed by controlling for organism as a fixed effect and a random effect.

An additional analysis examined the proportion of successful treatment at each MIC value by treatment and organism, using only the most common organisms (*Fusarium* species and *Aspergillus flavus*). Treatment success was defined as improvement in BSCVA from baseline, and no corneal perforation and/or TPK, as in the pre-specified outcomes of MUTT I. All statistical analyses were conducted using Stata 10.0 (StataCorp, College Station, Texas) unless otherwise specified.

RESULTS

Of the 323 patients enrolled in the trial, 256 (79%) had ulcers with a positive fungal culture, and 221 (68%) had MIC results available and were included in the analysis. The 35 isolates that were fungal culture positive but missing MIC values had no growth during susceptibility testing. In Table 1, we report the MICs to natamycin or voriconazole for genus and species based on which treatment arm they were assigned. For natamycin-treated organisms, *Aspergillus flavus* had the highest MIC_{50} and MIC_{90} of 32 μg/ml (95% CI 32–64 μg/ml) and 64 μg/ml (95% CI 32–64 μg/ml), respectively. For voriconazole-treated organisms, *Fusarium* species had the highest MIC_{50} and MIC_{90} of 4 μg/ml (95% CI 2–4 μg/ml) and 8 μg/ml (95% CI 8–16 μg/ml), respectively.

We analyzed clinical outcomes for organism by treatment arm (Table 2, available at http://aaojournal.org). For *Fusarium* species, natamycin-treated ulcers had significantly better outcomes compared to voriconazole-treated ulcers, including visual acuity, infiltrate/scar size, corneal perforation and/or TPK, and time to re-epithelialization. There were no other significant differences in outcome between the treatment arms for other organisms, although the sample size for other genera and species were smaller than that of *Fusarium* species.

The association between MIC and outcomes are listed in Table 3. A 2-fold increase in MIC was significantly associated with a larger 3-month infiltrate/scar size (0.21 mm, 95% CI 0.10–0.31, $P<0.001$) and increased odds of perforation (OR 1.33, 95% CI 1.04–1.69, $P=0.02$). No significant association was found between MIC and 3-month visual acuity (0.02 logMAR, 95% CI −0.03 – 0.06, $P=0.53$). In natamycin-treated cases, a 2-fold increase in natamycin MIC correlated with a 0.29 mm larger 3-month infiltrate/scar size (95% CI 0.15–0.43, $P<0.001$) and an approximately 2.5-fold increase in odds of perforation (95% CI 1.46–3.97, $P<0.001$). When analyzing voriconazole-treated cases, the MIC did not correlate

with any of the measured outcomes in the study. Controlling for organism as either a fixed effect or random effect did not change the associations between MIC and measured outcomes.

To examine the correlation between MIC and outcome by organism and treatment arm, the proportion of successful treatment was determined at each MIC value for the most common organisms (*Fusarium* species and *A. flavus*) against natamycin or voriconazole (Table 4). For *Fusarium* species treated with natamycin, the average proportion of successful treatment was 92% for MIC ≤ 32 μg/ml, and in those treated with voriconazole, 59% for isolates with a MIC ≤ 16 μg/ml. The proportion of successful treatment for *A. flavus* isolates treated with natamycin was 100% at MIC of 8 μg/ml, 50% at MIC of 32 μg/ml, and 40% at MIC of 64 μg/ml. For *A. flavus* treated with voriconazole, the proportion of success was 82% at MIC < 1 μg/ml and 14% at MIC ≥ 1 μg/ml.

DISCUSSION

In this study, we investigated the relationship between organism, in vitro susceptibility, and clinical outcome. We found that decreased susceptibility to natamycin correlated with larger 3-month infiltrate/scar size and increased odds of perforation. However, susceptibility to voriconazole was not significantly associated with any of the measured outcomes in the study.

Our findings are similar to previous systemic fungal infection [8, 12, 13] and fungal keratitis studies[10, 11] that demonstrate a linear correlation between susceptibility and outcome. Although many of the systemic studies focused on *Candida* or *Cryptococcus* species,[8] there are data that suggest a correlation for filamentous fungi. One systemic study on aspergillosis found that susceptibility was a reliable predictor of fatal outcome in cases treated with amphotericin B.[12] In a fungal keratitis study of mostly *Aspergillus* and *Fusarium* species, poor susceptibility was associated with a decreased odds of healing.[10] In a previous exploratory study by our group on filamentous fungal keratitis treated with natamycin and voriconazole, a low susceptibility was found to correlate with higher odds of corneal perforation and/or TPK.[11] In this study, we found a significant correlation between poor susceptibility to natamcyin and larger 3-month infiltrate/scar size, as well as increased odds of perforation and/or TPK. 3-month visual acuity seemed to worsen with higher natamycin MICs, but this difference was not significant. Overall, decreased susceptibility to natamycin appeared to correlate with worse outcomes, suggesting poor corneal ulcer healing. In the future, a larger sample size may be needed to demonstrate if there is a correlation between visual acuity and MIC.

We were also interested in determining if MIC had any correlation with outcomes by genus or species and antifungal agent. We found that the mean proportion of successful treatment for *Fusarium* species treated with natamycin was 92%, suggesting that *Fusarium* isolates with an MIC ≤ 32 μg/ml were largely susceptible to natamycin. For *Fusarium* species treated with voriconazole, the mean proportion of successful outcome was low at 59%. This indicates that our study population of *Fusarium* isolates had overall decreased susceptibility to voriconazole.

For *A. flavus* species treated with natamycin and voriconazole, treatment success appeared to correlate proportionally with susceptibility. Using the proposed epidemiological cut-off value (ECV) of 1 μg/ml for *A. flavus* against voriconazole,[18] three isolates had MIC values greater than the ECV and a 33% successful outcome (Table 4). Although there are currently no MIC clinical breakpoints established for filamentous fungi using CLSI methodology, the ECV can help distinguish susceptible from resistant isolates. Our results support the current ECV, which indicates that *A. flavus* isolates with MIC values greater than the ECV may have potential resistance. Given our small sample size, these results should be confirmed with a larger dataset.

Host factors, including drug pharmacokinetics, immune status, and adherence to treatment, often play an important role in clinical outcome.[8, 19] In this study, we used data from patients enrolled in MUTT I to limit potential confounders of an in vivo-in vitro correlation.[20] Only natamycin or voriconazole were used per trial protocol and all patients received a standardized treatment regimen for a standardized period of time, decreasing the likelihood of bias due to multi-drug regimens and different treatment protocols. Furthermore, many patients were hospitalized during the course of the trial, reducing the rate of medication non-adherence.

Despite these standardizations, other potential host factors were still present. One challenge is determining the therapeutic dosage of medications in an in vivo setting. In our study, topical natamycin had a concentration of 5%, while topical voriconazole was 1%; thus, natamycin is five times as concentrated as voriconazole. However, voriconazole has been shown to have better penetration through the corneal epithelium than natamycin.[21–23] For either topical drug, the therapeutic concentration in the stroma is likely much lower than the dose delivered to the corneal surface. Little information is available regarding the relative concentrations of these agents in the mid and deep corneal stroma, but aqueous humor concentration serves as an indicator of deep corneal penetration.

Previous studies using topical 1% voriconazole reported a range of voriconazole concentrations in the aqueous humor from 0.3 to 11.06 μg/ml, depending on dosing schedule.[22–24] The mean (standard deviation) aqueous humor concentrations in these studies were 6.49 (3.04) μg/ml using 1 drop every 2 hours for 24 hours[24] and 1.90 (1.12) μg/ml using 1 drop every 1 hour for 4 hours.[23] Another study reported a median concentration of 0.95 μg/ml using 1 drop every 1 hour for 4 to 49 days.[22] In our study, the MIC_{50} and MIC_{90} for all ulcers treated with voriconazole were 2 μg/ml and 8 μg/ml, respectively, and the MIC range was 0.03 to 16 μg/ml. Our dosing schedule (1 drop every 1 hour while awake for 1 week, then every 2 hours while awake until 3 weeks from enrollment) does not perfectly match that of prior aqueous humor concentration studies, but is comparable. In our study, the MIC_{90} was less than 4 μg/ml for all organisms, except *Fusarium* species, and also less than the maximum aqueous humor concentration found in the literature (11.06 μg/ml).[22–24] *Fusarium* species had higher MIC values than other organisms, with an MIC_{90} of 8 μg/ml and a range of 0.25 to 16 μg/ml. These values are higher than the mean and median aqueous humor concentration reported in the literature. Voriconazole was likely not as effective against *Fusarium* ulcers since the drug concentration needed to inhibit isolates may have been higher than the actual aqueous humor drug concentration. It is likely we did not find a

correlation between voriconazole susceptibility and outcome because *Fusarium* isolates were largely resistant to voriconazole and these ulcers made up the majority of the cases. Often, correlation analyses that have little variation are unable to detect a relationship between susceptibility and outcome.[8]

There are a few limitations to this study. Patients were enrolled in India and geographic differences in types of fungus have been reported, decreasing the generalizability of this study. However, we compared our *Fusarium* and *Aspergillus* species susceptibility data with reports in the literature, including studies conducted in the US, China, India, Turkey, and Netherlands, and found they were largely comparable.[25] The similarity among susceptibility data in the literature suggests that there is unlikely increased resistance among *Fusarium* or *Aspergillus* species found in South India compared to other geographic locales. In addition, a larger sample size of isolates for each genus or species would allow us to confirm the associations between susceptibility and outcome determined in this study. In future studies, measurement of aqueous humor concentrations of both drugs may be helpful to compare with the MIC, though obtaining this measurement may be challenging.

In this study, we described the association between susceptibility and clinical outcomes in filamentous fungal keratitis cases in South India treated with natamycin or voriconazole. We found a correlation between decreased susceptibility and poor clinical outcome, specifically 3-month infiltrate/scar size and corneal perforation and/or TPK. When adjusted for treatment, the association remained between natamycin susceptibility and outcome. We were unable to find a significant relationship between voriconazole susceptibility and outcome. Our findings in this study, along with our in vivo results from the MUTT I primary paper,[16] support the use of natamycin for treating *Fusarium* species. Given poor susceptibility and clinical outcomes among *Fusarium* ulcers treated with voriconazole, we would recommend against using voriconazole as a first-line therapy for *Fusarium* keratitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Natamycin and voriconazole minimum inhibitory concentration (MIC) by treatment arm

Organism	Natamycin (μg/ml)				Voriconazole (μg/ml)			
	No.	MIC_{50} (95% CI)[b]	MIC_{90}[a] (95% CI)[b]	MIC Range	No.	MIC_{50} (95% CI)[b]	MIC_{90}[a] (95% CI)[b]	MIC Range
Fusarium species	60	4 (4 to 4)	8 (4 to 8)	2–32	66	4 (2 to 4)	8 (8 to 16)	0.5–16
Aspergillus species	25	32 (8 to 32)	64 (64 to 64)	2–64	27	0.5 (0.5 to 1)	2 (1 to 4)	0.25–4
A. flavus	14	32 (32 to 64)	64 (32 to 64)	8–64	18	0.5 (0.5 to 1)	2 (1 to 4)	0.25–4
A. fumigatus	5	4	ND	2–64	5	0.5	ND	0.25–4
A. niger	2	5	ND	2–8	0	NA	NA	NA
A. terreus	3	16	ND	8–16	0	NA	NA	NA
Other *Aspergillus* species	1	2	ND	2	4	0.5	ND	0.5–2
Curvularia species	9	2 (2 to 2)	2 (2 to 2)	1–2	8	0.38	ND	0.25–2
Exserohilum species	2	1.5	ND	1–2	6	1	ND	0.5–2
Alternaria species	2	2	ND	2–2	0	NA	NA	NA
Bipolaris species	3	2	ND	2–2	1	1	ND	1
Lasiodiplodia species	2	2	ND	2–2	1	0.5	ND	0.5
Other species	1	2	ND	2	1	0.5	ND	0.5
Unidentified dematiaceous	1	4	ND	4	2	1.12	ND	0.25–2
Unidentified hyaline	3	4	ND	4–8	1	0.03	ND	0.03
Total (*N*=221)	**108**	**4 (4 to 4)**	**32 (16 to 64)**	**1–64**	**113**	**2 (1 to 2)**	**8 (4 to 8)**	**0.03–16**
P-value		*<0.001*[c]				*<0.001*[c]		

Abbreviations: No., number; MIC_{50}, minimum inhibitory concentration median; MIC_{90}, minimum inhibitory concentration 90th percentile; CI, confidence interval; NA, not available; ND, not determined

[a] MIC_{90} was estimated for genus or species with at least 9 observations, the smallest number where extrapolation would not be necessary.

[b] 95% CIs for MIC_{50} and MIC_{90} were estimated as bootstrap percentile confidence intervals for genus or species with at least 9 observations.

[c] Analysis of variance test comparing MICs among genera

Table 3

Minimum inhibitory concentration (μg/ml) predicting clinical outcome

Outcome	Subgroup[a]	Estimated Effect (per 2-fold dilution in MIC)	95% CI	*P*-value
3-month best spectacle-corrected visual acuity[b]	All cases	0.02 logMAR	−0.03 to 0.06	0.53
	Natamycin treated	0.03 logMAR	−0.03 to 0.09	0.32
	Voriconazole treated	0.002 logMAR	−0.07 to 0.08	0.97
3-month Infiltrate/scar size[b]	All cases	0.21 mm	0.10 to 0.31	**<0.001**
	Natamycin treated	0.29 mm	0.15 to 0.43	**<0.001**
	Voriconazole treated	0.12 mm	−0.03 to 0.27	0.11
Corneal perforation and/or therapeutic penetrating keratoplasty[c]	All cases	1.33[e]	1.04 to 1.69	**0.02**
	Natamycin treated	2.41[e]	1.46 to 3.97	**<0.001**
	Voriconazole treated	1.04[e]	0.79 to 1.38	0.76
Time to re-epithelialization[d]	All cases	1.01[f]	0.91 to 1.12	0.86
	Natamycin treated	0.96[f]	0.83 to 1.12	0.61
	Voriconazole treated	1.09[f]	0.93 to 1.29	0.29

Abbreviations: MIC, minimum inhibitory concentration; CI, confidence interval; logMAR, logarithm of the minimum angle of resolution

[a] Regression model: $\log_2$MIC corrected for treatment arm and baseline variable (visual acuity, infiltrate size, ulcer depth, epithelial defect, respectively)

[b] Linear regression

[c] Logistic regression

[d] Cox proportional hazards regression

[e] Odds ratio

[f] Hazards ratio

Table 4

Proportion successfully treated with natamycin or voriconazole, by minimum inhibitory concentration

Organism	Treatment arm	Total No.	MIC (μg/ml)	Cases with Successful Treatment, % (No. of Cases/ Total No. of Cases)[a]
Fusarium species	Natamycin	60	2	100 (6/6)
			4	91 (42/46)
			8	83 (5/6)
			16	100 (1/1)
			32	100 (1/1)
	Voriconazole	66	0.5	0 (0/3)
			1	40 (2/5)
			2	50 (10/20)
			4	75 (18/24)
			8	60 (6/10)
			16	75 (3/4)
Aspergillus flavus	Natamycin	14	8	100 (1/1)
			32	50 (4/8)
			64	40 (2/5)
	Voriconazole	18	0.25	50 (2/4)
			0.5	100 (7/7)
			1	0 (0/4)
			2	0 (0/2)
			4	100 (1/1)

Abbreviations: MIC, minimum inhibitory concentration; No., number

[a] Success defined as improvement in visual acuity from baseline AND no corneal perforation and/or therapeutic penetrating keratoplasty