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In Vitro Activities of Investigational Triazoles against *Fusarium* Species: Effects of Inoculum Size and Incubation Time on Broth Microdilution Susceptibility Test Results

Niki I. Paphitou,* Luis Ostrosky-Zeichner, Victor L. Paetznick, Jose R. Rodriguez, Enuo Chen, and John H. Rex

Laboratory for Mycology Research, Center for the Study of Emerging and Re-Emerging Pathogens, Division of Infectious Diseases, University of Texas-Houston Medical School, Houston, Texas 77030

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We studied the effects of inoculum size and incubation time on the susceptibility testing results for various antifungal agents against 22 *Fusarium* isolates by the NCCLS microdilution method. Increased inoculum size and extended incubation time resulted in elevated MICs. Posaconazole and voriconazole exhibited promising antifungal activities.

Disseminated infection due to the emerging fungal pathogen *Fusarium* is a significant disease for the immunocompromised host (4, 9, 10, 13, 15). Its incidence appears to be increasing over the last 15 years (10), and local clusters of disease have been encountered (9). Mortality rates as high as 80% are seen and are in part due to the relative resistance of this genus to present antifungal agents (4, 10, 11, 13, 15). *Fusarium solani* and *Fusarium oxysporum* are the most frequently described causes of human infection (9, 10, 13).

As newer antifungal agents become available, efforts to standardize susceptibility testing methods for fungi have been undertaken. For filamentous fungi, these have led to the proposed method for in vitro antifungal susceptibility testing described in the M38-P document of the National Committee for Clinical Laboratory Standards (NCCLS) (16). Due to an enhanced clinical correlation for *Aspergillus fumigatus* (6), NCCLS M38-P proposes a higher inoculum (0.4×10^4 to 5×10^4 CFU/ml) than the 0.5×10^3 to 2.5×10^3 CFU/ml recommended in the NCCLS M27-A method for yeasts (17). As variations in testing conditions such as temperature, inoculum size, and time of incubation can have a significant impact on the results (8, 14, 20), we sought to determine the effect of these variables on the in vitro activities of three investigational triazoles against *Fusarium* spp.

Twenty-two clinical isolates of *Fusarium* spp. (*F. solani*, $n = 18$; *F. oxysporum*, $n = 4$) were evaluated. Identification of the isolates was performed by standard microbiological procedures. Four quality control strains (two intralaboratory quality control strains, *Candida lusitaniae* 5W31 and *C. lusitaniae* CL524, and two American Type Culture Collection quality control strains, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019) were also tested. Voriconazole (VRC) and fluconazole (FLC) (Pfizer Central Research, Sandwich, United Kingdom), amphotericin B (AMB) and ravuconazole (RVC) (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, N.J.), and posaconazole (PSC) (Schering-Plough Research Institute, Bloomfield, N.J.) were obtained from the manufacturers as defined powders. The NCCLS M38-P microdilution broth-based method (16) was used, with additional testing using an adaptation of the inoculum preparation method described in the NCCLS M27-A document (17). Drugs were prepared in dimethyl sulfoxide, except for FLC, which was prepared in sterile distilled water. Drug concentrations ranged from 64 to 0.125 μg/ml for FLC and 16 to 0.031 μg/ml for all other drugs. Testing media were RPMI 1640 (Sigma Chemical Co.) buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid for testing of the azoles and antibiotic medium 3 buffered to pH 7.0 with 0.01 M phosphate for AMB. Isolates were cultured on potato dextrose agar slants at 35°C for 72 h and then at 25°C for another 4 days. Fungal colonies were covered with 1 ml of sterile 0.85% saline and gently scraped with a sterile pipette. The resulting suspensions were transferred to sterile tubes, and heavy particles were allowed to settle.

We prepared two different final inocula, made from the same starting suspension of a conidial density adjusted to a 70% transmittance at a wavelength of 530 nm. (i) Following the M38-P microdilution method, the starting suspension was diluted 1:50 and then 1:2 when inoculated into the wells containing an equal volume of drug. The final inoculum concentration was 0.4×10^4 to 5×10^4 CFU/ml. (ii) By an adaptation of the M27-A microdilution method, the starting suspension was diluted 1:100, then 1:10, and finally 1:2 when inoculated into the wells containing an equal volume of drug. The final inoculum concentration was 0.2×10^3 to 2.5×10^3 CFU/ml, which was 20 times more diluted than the above inoculum in the first method.

Colony counts were performed on Sabouraud dextrose agar plates. MICs were visually determined at 24, 48, and 72 h of incubation at 35°C. There was adequate growth for MIC determination by 24 h, and no trailing was observed with either inoculum. MICs were defined as the lowest drug concentration at which there was complete absence of growth (AMB) or a prominent growth reduction (azoles, corresponding to approximately 50% growth reduction). MICs against the American Type Culture Collection quality control strains were within the

* Corresponding author. Present address: 1 Iona Nicolaou St., Aglanjia, Nicosia 2108, Cyprus. Phone: (357) 22335565. Fax: (357) 22769225. E-mail: GMIX@msn.com.

TABLE 1. Geometric means (ranges) of MICs against *Fusarium* spp. with two different inocula[a]

Organism and drug	MIC					
	24 h		48 h		72 h	
	M27-A	M38-P	M27-A	M38-P	M27-A	M38-P
F. solani (n = 18)						
FLC	128 (>64)	128 (>64)	128 (>64)	128 (>64)	128 (>64)	128 (>64)
AMB	4.3 (1–16)	10.4 (2–>16)	8.9 (2–>16)	14.8 (2–>16)	14.8 (2–>16)	20.1 (4–>16)
RVC	20.9 (4–>16)	27.4 (4–>16)	28.5 (8–>16)	30.7 (16–>16)	30.7 (16–>16)	32 (>16)
PSC	6.8 (0.5–>16)	19.3 (1–>16)	20.9 (2–>16)	25.3 (4–>16)	27.4 (4–>16)	32 (>16)
VRC	2.5 (0.25–>16)	3.4 (0.5–8)	4 (1–8)	5.8 (1–8)	5.4 (1–8)	8.9 (2–>16)
F. oxysporum (n = 4)						
FLC	128 (>64)	128 (>64)	128 (>64)	128 (>64)	128 (>64)	128 (>64)
AMB	6.7 (4–8)	19 (16–>16)	9.5 (8–16)	16 (16)	16 (16)	32 (>16)
RVC	0.9 (0.03–8)	5.6 (4–8)	9.5 (8–>16)	19 (16–>16)	9.5 (4–16)	26.9 (16–>16)
PSC	1 (0.5–2)	1 (1)	1 (1)	1.4 (1–2)	1 (1)	2.8 (2–4)
VRC	0.5 (0.25–1)	1.6 (1–2)	2 (1–4)	2.3 (2–4)	1.6 (1–2)	4 (2–8)

[a] MICs are expressed in micrograms per milliliter. For calculation of geometric means, off-scale values were increased to the next dilution. For M27-A, according to NCCLS method M27-A, testing was done with conidia at a final concentration of 0.2×10^3 to 2.5×10^3 CFU/ml. For M38-P, according to NCCLS method M38-P, testing was done with conidia at a final concentration of 0.4×10^4 to 5×10^4 CFU/ml.

limits proposed by Barry et al. (3). Experiments were repeated in duplicate, and MICs for the same drug-organism combinations were generally within 1 to 2 dilutions of each other; when discordant MICs were found, the higher one was reported.

Geometric means of MICs of the different antifungal agents and for different testing conditions are shown in Table 1. All of the isolates tested were universally resistant to FLC, with MICs of >64 μg/ml across all conditions. Resistance of *Fusarium* spp. to FLC is well recognized, and our findings confirm those of previous investigators (7, 19). AMB MICs were also high, but *F. solani* appeared somewhat more susceptible to this drug than was *F. oxysporum*, even though the small number of *F. oxysporum* isolates tested makes these results difficult to interpret. Other investigators have found somewhat lower AMB MICs; however, smaller numbers of isolates (2, 7, 18) or different methods (19) were used in these studies. Nevertheless, our findings are in concordance with the accumulated clinical experience of frequent treatment failures in using AMB for invasive fusariosis (4, 10, 11, 15).

For the investigational triazoles and *F. solani*, VRC appeared most active, followed by PSC and then RVC. For *F. oxysporum*, the MICs were generally lower than for *F. solani*. With the exception of the results obtained at the 24-h–M27-A inoculum conditions (at which all three triazoles appeared equally active against *F. oxysporum*), RVC exhibited the least activity and PSC exhibited slightly more activity than did VRC against these isolates. Previous in vitro studies reported relative resistance of *Fusarium* to RVC (7) and promising activities of VRC (1, 5) and PSC (12). The latter correlated with a successful in vivo outcome in a murine model of infection (12). However, other investigators utilizing a smaller number of isolates (n = 7) have reported high MICs (MICs at which 50% of the isolates tested were inhibited were 8 to >8) of all three triazoles (18). This can be explained by the wide variation in the MICs shown in our data. The promising role of VRC and PSC in the treatment of fusariosis is also supported by successful clinical outcomes when these agents were used to treat refractory disease (21; R. Y. Hachem, I. I. Raad, C. M. Afif, R. Negroni, J. Graybill, S. Hadley, H. Kantarjian, S. Adams, and G. Mukwaya, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1009, 2000; J. R. Perfect, I. Lustar, and A. Gonzalez-Ruiz, 38th Annu. Meet. Infect. Dis. Soc. Am., abstr. 303, 2000).

Both increased inoculum and extended time of incubation resulted in increased MICs. The importance of establishing the optimum inoculum and incubation time for susceptibility testing of filamentous fungi has long been recognized. Moore et al. (14) reported that use of a higher inoculum when testing itraconazole versus *Aspergillus flavus* was associated with trailing endpoints which prevented clear-cut endpoint determination. This effect can possibly be attributed to more floating spores in the wells with the use of higher inocula and was avoided by using lower inocula. Gehrt et al. also studied the effect of inoculum size on filamentous fungi susceptibility testing (8) and concluded that the magnitude of such an effect depends upon the organism-antifungal combination, with the greatest effect exhibited by *Pseudallescheria boydii* and *Rhizopus arrhizus* when tested with itraconazole. Moreover, Pujol et al. (20) found that both increased inoculum and extended time of incubation can increase AMB MICs against *Fusarium*, with inoculum effects being the most prominent, giving rise to 10- to 19-fold increases in geometric mean MICs.

One of the isolates tested in this work, *F. solani* isolate FS 1184, was previously found to be responsive to PSC in a murine model of fusariosis (12). In that study, PSC therapy produced prolongation of animal survival and enhanced organ clearance. In the present work, the PSC MIC for this isolate was 2 μg/ml for the lower-inoculum–24-h-incubation conditions and >16 μg/ml for all other conditions tested. Hence, the only in vitro reading correlating with the observed in vivo response was the lower-inoculum–24-h reading for PSC.

In conclusion, it appears that optimal testing conditions for antifungal susceptibility testing of filamentous fungi may very well vary with each drug-organism combination. VRC exhib-

ited promising antifungal activity against the *Fusarium* isolates tested, PSC showed promising activity against *F. solani*, and VRC also appeared active against *F. solani* under the 24-h–M27-A testing conditions. Data from a prior in vivo study with one of our isolates suggest that testing *Fusarium* at a lower inoculum and reading after 24 h may be the most relevant conditions for the azole antifungal agents. This study, however, used a single strain-drug combination; hence, further studies focusing on in vitro-in vivo correlations are needed to determine which in vitro results are clinically relevant.

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