

Flow Cytometry Antifungal Susceptibility Testing of *Aspergillus fumigatus* and Comparison of Mode of Action of Voriconazole vis-à-vis Amphotericin B and Itraconazole

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***Aspergillus fumigatus* isolates were tested with three antifungals by flow cytometry (FC) and fluorescence-activated cell sorting. FC results after 4 h correlated well with MICs obtained by the NCCLS M38-A method; voriconazole exhibited fungicidal activity, albeit to a lesser extent than amphotericin B, but to a greater extent than itraconazole.**

The three commonest species causing human disease are *Aspergillus fumigatus*, *A. flavus*, and *A. niger* (5). For invasive aspergillosis, amphotericin B and itraconazole have been used for treatment (5). Recently, caspofungin has been approved for salvage therapy of recalcitrant aspergillosis and voriconazole has been approved for first-line therapy (1, 6). The National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Susceptibility Tests has established a reproducible reference method (M38-A) for the antifungal susceptibility testing of filamentous fungi which requires 2 to 3 days to provide results (2, 8, 14, 17). Alternative methods such as colorimetric methods (13), glucose consumption testing (4), the Etest (7), and conidial viability testing using flow cytometry (FC) with FUN-1 dye (3) are being evaluated to overcome the limitations of the traditional testing. In the present study, *A. fumigatus* isolates were tested against three antifungals by FC and fluorescence-activated cell sorting based on propidium iodide (PI) uptake.

A. fumigatus isolates (14) were tested in parallel by FC assays and the NCCLS microdilution broth method (M38-A) (14). These cultures were maintained in sterile water at 4°C and passaged twice on potato dextrose agar at 35°C. Amphotericin B was purchased from the Sigma Biochemical Company (St. Louis, Mo.), and voriconazole and itraconazole were gifts from Pfizer Inc., New York, N.Y., and Research Diagnostics Inc., Flanders, N.J., respectively. Stock solutions of amphotericin B and voriconazole were prepared in dimethyl sulfoxide (Amresco, Solon, Ohio), while itraconazole was prepared in polyethylene glycol (Sigma Biochemical Company) (molecular weight = 8000); all of the drugs were used at concentrations of 6,400 µg/ml and stored at –70°C. The broth microdilution test was performed in accordance with M38-A protocol (14). For the FC method, amphotericin B, voriconazole, and itraconazole in serial twofold dilutions ranging from 0.03 to 32 µg/ml

were prepared as described in NCCLS protocol M38-A. The mold isolates were grown on potato dextrose agar slants for 3 to 5 days at 35°C. Inocula were prepared in 0.85% sterile saline containing 1% Tween 80, and a spectrophotometer (Pharmacia Biotech, Piscataway, N.J.) was used to adjust the suspension to an optical density at 530 nm of 0.09 to 0.11. A total of 0.5 ml of the suspension was added to each drug dilution and incubated at 35°C. The growth control tube contained cell suspension and RPMI 1640 without drugs. The suspensions were incubated for 2 h for amphotericin B and for 3 h for itraconazole and voriconazole. At the end of the incubation, 200 µl of the mixture of inoculum and drug was placed in a 12- by 75-mm tube (Falcon; Becton Dickinson, Lincoln Park, N.J.). A total of 200 µl of 0.5% sodium deoxycholate (Sigma Biochemical Company) and 10 µl of PI (1 mg/ml) was added to each dilution, and the tubes were incubated for 1 h in the dark at 35°C. The MIC was defined as the lowest concentration of drug that resulted in an increase of 50% in mean channel fluorescence (MCF) compared to the fluorescence seen with the growth control. All samples were tested twice.

TABLE 1. MICs of 14 *A. fumigatus* isolates determined by the NCCLS broth microdilution (M38-A) and FC methods

Isolate	Strain	MIC (µg/ml)					
		Amphotericin B		Itraconazole		Voriconazole	
		NCCLS	FC	NCCLS	FC	NCCLS	FC
1	41	4.0	8.0	0.5	0.25	0.5	0.12
2	42	8.0	4.0	0.5	0.5	0.5	1.0
3	43	2.0	4.0	0.5	0.5	0.5	0.5
4	44	2.0	2.0	0.5	0.5	0.5	0.5
5	45	4.0	2.0	0.5	0.25	0.5	0.25
6	46	16.0	16.0	>16.0	8.0	1.0	0.5
7	47	4.0	4.0	0.25	0.5	0.5	0.5
8	48	4.0	8.0	>16.0	16.0	0.12	0.25
9	49	>16.0	32.0	0.5	0.5	2.0	1.0
10	166-00	0.5	0.5	0.25	0.5	0.25	0.5
11	815-00	0.5	1.0	0.25	0.25	0.5	0.5
12	817-00	2.0	4.0	0.25	0.25	0.5	0.5
13	975-00	0.5	2.0	1.0	1.0	0.25	0.5
14	1122-00	0.5	0.5	0.5	0.5	0.25	0.5

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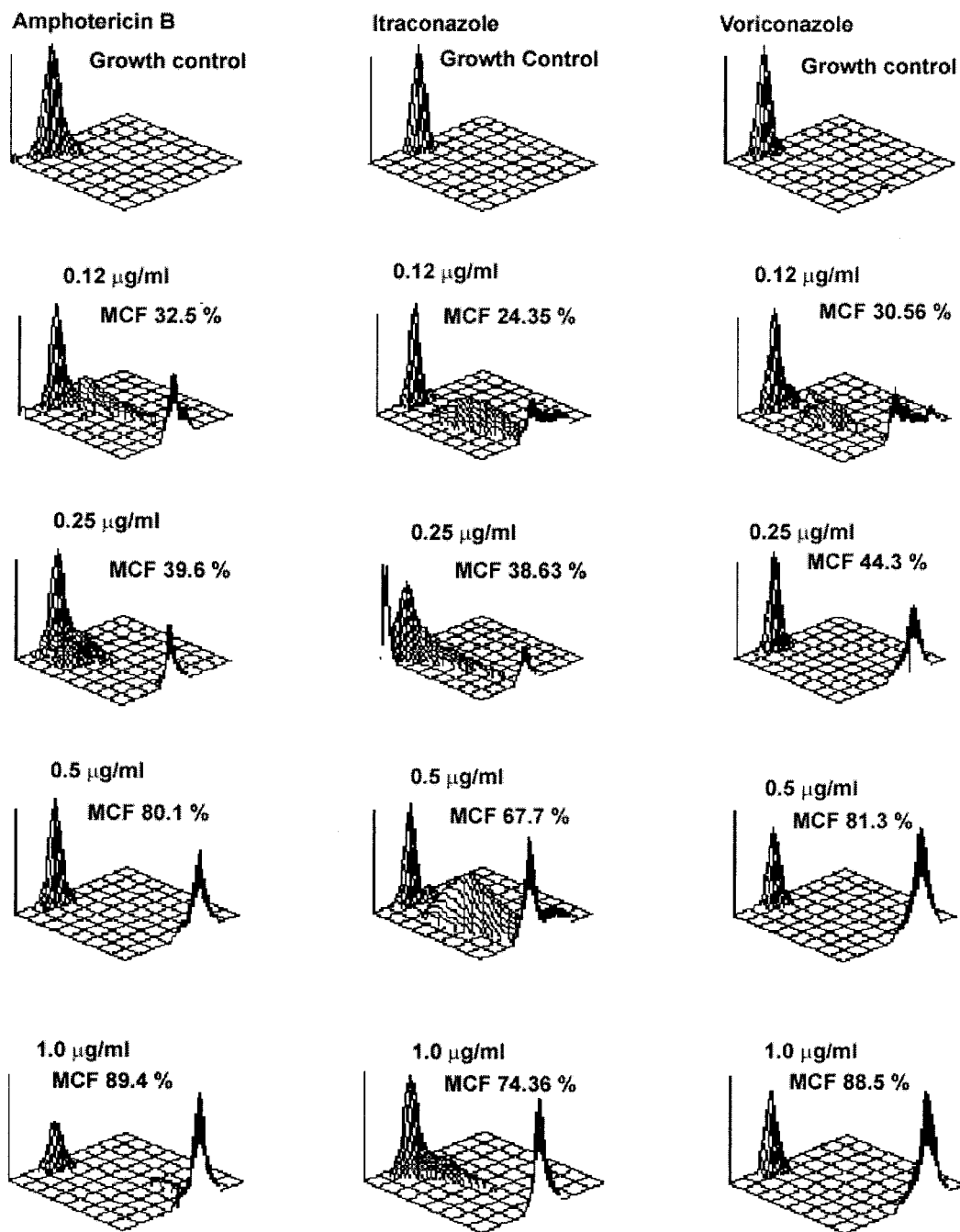


FIG. 1. FC measurements of antifungal effects on *A. fumigatus* (strain 166-00). The three-dimensional plots illustrate MCF results determined for control and drug-treated cells. There was a gradual increase in MCF, with a 50% or more increase observed between concentrations of 0.25 and 0.5 µg/ml for amphotericin B, itraconazole, and voriconazole. Therefore, 0.5 µg/ml was considered the MIC of the three drugs for this strain.

Growth control and drug-treated cell suspensions were sorted by FACS Vantage cell sorter (Becton Dickinson) into populations of PI-positive and PI-negative cells. The cells were electrostatically deflected into two tubes, plated onto potato dextrose agar plates, and incubated at 35°C for 48 h. The colonies were counted, and CFU values per milliliter were calculated and compared to those of the growth controls.

Drug MICs obtained by NCCLS broth microdilution and drug MICs obtained by the FC method for various *A. fumigatus*

isolates are summarized in Table 1. The optimal conditions included conidial suspension equivalent to a 0.5 MacFarland standard, 0.5% sodium deoxycholate, and a PI concentration of 1.0 mg/ml. An increase in MCF compared to that of the growth control was observed with an increase in the concentration of amphotericin B, itraconazole, or voriconazole (Fig. 1). The overall agreement ± 1 dilution between the M38-A (NCCLS) and FC methods for all three drugs was 93%. All PI⁺ cells collected by sorter were negative for cell viability.

TABLE 2. Comparison of MFCs obtained by FC sorter assay (48 h) and by CFU methodology (72 h) for four isolates of *A. fumigatus* for all three drugs

Strain	MFC ($\mu\text{g/ml}$)					
	Amphotericin B ^a		Itraconazole ^b		Voriconazole ^c	
	FC sorter assay	Conventional method	FC sorter assay	Conventional method	FC sorter assay	Conventional method
166-00	1.0	0.5	2.0	0.5	2.0	1.0
817-00	4.0	2.0	>16.0	>16.0	4.0	4.0
975-00	2.0	1.0	4.0	8.0	1.0	2.0
1122-00	2.0	1.0	8.0	16.0	8.0	8.0

^a Correlation coefficient, 0.95.

^b Correlation coefficient, 0.82.

^c Correlation coefficient, 0.97.

The results of viability assays by FC sorter and CFU methodologies for all four isolates at 3, 24, 48, and 72 h were compared. FC sorter samples at 3 and 24 h showed poor correlation with CFU values obtained at 72 h. However, FC sorter samples at 48 and 72 h were in good agreement with CFU values (Table 2).

In the present study, MCF values for fungal cells within 3 to 4 h of treatment proved to be a reliable indicator of a drug's antifungal activity against *A. fumigatus* isolates, and the values showed good correlation with the drug MICs obtained by the NCCLS broth microdilution method (M38-A). Our observations also indicated that voriconazole exhibited better activity (MIC-MFC) than itraconazole with *A. fumigatus* isolates, with high MICs for amphotericin B. Previous FC studies were developed to measure the antifungal susceptibility testing of pathogenic yeasts, including *Candida* species and *Cryptococcus neoformans* (10, 15, 16). FC studies have been used to describe the interactions of macrophages and *A. fumigatus* conidia (9, 12). In these studies, conidial viability was assessed using PI but the nonspecific PI positivity in metabolically active conidia inside macrophages demonstrated some limitations of this approach (12). However, in vitro no such PI positivity was observed in the present study. Recently, FUN-1 was used to measure hyphal viability after exposure to antifungal agents (11). It is noteworthy that an early drug-mediated impairment of hyphae was not detected, and 9 h of incubation was required before cell deformity could be observed (11). In contrast, a shorter incubation with amphotericin B, itraconazole, and voriconazole (3 to 4 h) was sufficient to result in a visible loss of membrane integrity (15, 16). We note that amphotericin B effects were visible earlier (at 3 h), while 4 h of incubation was necessary for the study of itraconazole and voriconazole effects. These differences could be attributed to the fact that amphotericin B binds to existing ergosterol in fungal membranes and that the two azoles act upon an inter-

mediate in ergosterol biosynthesis. The culture conditions, dilution scheme, and incubation conditions for our method were comparable to those recommended by NCCLS. This permits head-to-head comparison with previously published results determined on the basis of the NCCLS method.

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