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Rapid Determination of Antifungal Activity by Flow Cytometry

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We have developed a rapid assay of antifungal activity which utilizes flow cytometry to detect accumulation of a vital dye in drug-damaged fungal cells. Results of these studies suggest that flow cytometry may provide an improved, rapid method for determining and comparing the antifungal activities of compounds with differing modes of action.

Methods currently used to determine the MIC and minimal fungicidal concentration (MFC) of antifungal compounds are generally not standardized and are frequently difficult to interpret (1, 5, 14, 15, 22). During the last two decades, flow cytometry has been developed as a powerful tool (11, 13, 21, 24) in many diagnostic and research laboratories. Flow cytometry is widely used for study of mammalian cells, including phenotyping (8, 10), phagocytic function (2), and discrimination of living and dead cells (19), and there is increasing interest in the use of flow cytometry in studies of bacteria (3, 23) and fungi (4, 7, 9, 12, 20, 25).

We have developed a flow cytometric assay for antifungal activity based on detection of increased permeability of the fungal cell membrane to propidium iodide (PI) (Sigma, St. Louis, Mo.), a nucleic acid-binding fluorochrome largely excluded by intact cell membranes, following drug treatment.

Organisms from stock cultures maintained at Lilly Research Laboratories (LRL) were grown overnight at 32°C, diluted to 2×10^6 cells per ml in yeast peptone dextrose (YPD) (Difco, Detroit, Mich.) broth immediately prior to use. Stock solutions (1 mg/ml) of cilofungin (CF; LRL), and LY295337 (Takara Shuzo, Shiga, Japan), were prepared in ethanol; a stock solution of fluconazole (Pfizer, Groton, Conn.) was prepared in distilled water; and amphotericin B (AMB; Sigma) was prepared in dimethyl formamide. For assay, equal volumes of dilutions of antifungal compounds in YPD broth and of the cell suspension were mixed. For controls, organisms were diluted in YPD broth alone or suspended in 70% ethanol. Tubes were incubated at 32°C with continuous shaking for the desired time, and the cells were pelleted, resuspended in PI solution (25 μg/ml in phosphate-buffered saline), and incubated for 30 min at 32°C.

For sample analysis, cell size (forward scatter of incident laser light) and PI fluorescence intensity data were collected for 10,000 cells with a Coulter EPICS Profile II flow cytometer. In each experiment, untreated control cells were sampled first and analyzed by plotting the data in a 2-parameter histogram, with the y axis being the size (forward scatter) and the x axis being the log of the mean PI fluorescence intensity. The histogram was divided into four quadrants, with quadrant boundaries set so that 95% ± 1% of the untreated control cells were contained in quadrant 1 (Fig. 1A). When treated cells were analyzed, cell membrane damage was evidenced by increased PI staining, resulting in increased cellular fluorescence intensity. Damaged cells, such as those incubated in 70% ethanol, moved to the right on the x axis and shifted into quadrant 2 (Fig. 1B). Figure 1C to 1F are representative histograms from typical experiments in which *Candida albicans* A26 cells were stained with PI following a 3-h treatment with AMB, at a concentration approximately five times the MIC; CF, at two times the MIC; LY295337, at two times the MIC; or fluconazole, at two times the MIC. Approximately 30 to 40% of the cells treated with AMB or LY295337 (Fig. 1C and 1E) incorporated PI, whereas 90% of CF-treated cells incorporated PI (Fig. 1D). The histogram of cultures treated with fluconazole was distinct from those of cultures treated with the fungicidal drugs owing to the absence of highly PI-permeable (dead) cells. There was, however, a partial (30%) shift of cells into quadrant 2. The difference with the fungistatic drug can be seen best by comparing the fluorescence intensity values (mean log of the mean PI fluorescence intensity) (Table 1).

MICs of the antifungal agents for the various organisms were determined by broth microdilution using previously described methods (6), and the MFC was the lowest concentration of drug which allowed no growth when 100 μl from wells of the completed broth microdilution experiments was incubated for an additional 48 h at 35°C in 5.0 ml of antibiotic-free medium. To establish MFCs by flow cytometry, cells were incubated with serial twofold dilutions of the drug and percentages of PI-stained cells at each concentration were compared. The MFC was defined as the lowest drug concentration in the dilution series that did not produce a 25% or greater decrease in PI-positive cells compared with the next higher concentration. Determination of the flow cytometric MFCs of CF and LY295337 for *C. albicans* A26 is shown in Table 1. The MFC of CF was 0.4 μg/ml, which is within the ranges of MICs and MFCs (0.3 to 0.6 μg/ml and 0.3 to 1.2 μg/ml, respectively) obtained at LRL by broth microdilution studies during the past 2 years. The MFC of LY295337 was 0.02 μg/ml, which is close to the MIC and MFC (0.02 and 0.04 μg/ml, respectively) determined by broth microdilution procedures. Good agreement was also shown between flow cytometric and broth microdilution MFCs for five additional *Candida* strains having various susceptibilities to LY295337 (Table 2) and for one strain each of *Saccharomyces cerevisiae* and *Cryptococcus neoformans*. More studies are needed to establish MIC criteria for fluconazole; however, increased PI staining compared with that of nontreated controls was consistently observed when cells were treated with fluconazole at concentrations of 1.0 μg/ml or greater for 3 h (Table 1). The range of MICs of fluconazole for *C. albicans* A26 determined by broth microdilution assay at LRL during the past 2 years is 0.5 to 1.0 μg/ml. There was no increase in cellular PI staining when two fluconazole-resistant strains, CA-3 and 7.220, each having a broth microdilution MIC of fluconazole of ≥64 μg/ml, were

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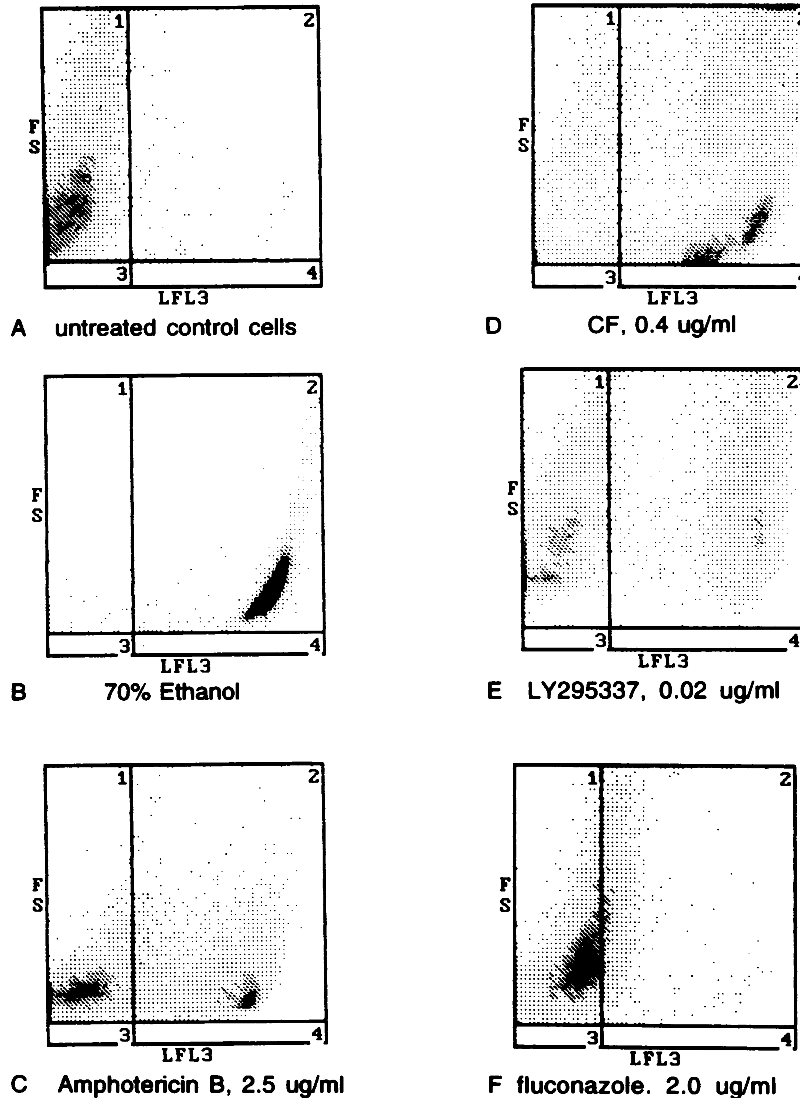

FIG. 1. Profile II two-parameter histograms of control and drug-treated *C. albicans* cultures stained with PI. FS, forward scatter or size. LFL3, log of the mean PI fluorescence intensity.

assayed after treatment with fluconazole at concentrations of up to 128 μg/ml. The flow cytometric MFCs of AMB for *C. albicans* A26, 0.63 μg/ml, and for *Cryptococcus neoformans*, 0.125 μg/ml, were also comparable to the values determined by standard techniques (data not shown).

To determine correlation of PI uptake and cell viability, aliquots of cultures treated with the antifungal agents were removed just prior to staining for flow cytometry, serially diluted, and plated in duplicate on YPD agar to obtain CFU-per-milliliter data. The flow cytometric and plate count data from representative experiments using *C. albicans* A26 treated with CF or LY295337 are shown in Table 3. PI uptake in cells treated with CF for 3 h correlated with cell death as determined by plate count data ($r = 0.996$). The percent PI staining of cells was almost identical to the percent reduction in CFU per milliliter, except at the sub-MIC. With LY295337, however, the percent reduction in CFU per milliliter was greater (90% reduction) than the percentage of PI-stained cells after 3 h of drug treatment (40% PI positive). Regression analysis demonstrated a strong correlation ($r = 0.991$) between the percent PI positive and the percent reduction in CFU. This indicates that a 3-h exposure to LY295337 was sufficient to cause eventual cell death but not maximal PI incorporation.

Similar results were obtained with AMB. Plate counts showed that greater than 90% of the cells were killed after 3 h of incubation in AMB but that only 30 to 40% of cells incorporated PI (data not shown).

Comparison of PI assay and CFU-per-milliliter data showed that the level of PI uptake seen in fluconazole-treated *C. albicans* A26 was indicative of growth inhibition but not cell death. Treatment with fluconazole (4.0 μg/ml) for 6 h produced 83% PI-positive cells with a log mean fluorescence value of 3.7, compared with 4% PI-positive cells in the untreated control culture (log mean fluorescence value of 1.6), whereas the number of CFU per milliliter for aliquots plated after 6 h of drug treatment (9.6×10^5) was 10-fold less than that of the

TABLE 1. Susceptibility testing of *C. albicans* A26 with a Profile II flow cytometer

Treatment	*n*	Mean % PI positive (± SEM)[b]	Mean log of the mean PI fluorescence intensity (± SEM)[c]
Control			
None	8	3.7 (0.8)	1.5 (0.09)
Ethanol	8	94 (2.5)	23.8 (2.0)
CF (μg/ml)			
1.6	3	85 (1.9)	11.4 (1.1)
0.8	5	84 (1.9)	9.7 (0.5)
0.4	5	82 (2.8)	9.2 (0.8)
0.2	5	38 (7.7)	5.2 (0.5)
0.1	5	12 (3.4)	2.9 (0.4)
0.05	5	7 (1.8)	2.1 (0.4)
LY295337 (μg/ml)			
0.08	6	37 (4.2)	7.1 (0.6)
0.04	6	40 (3.8)	6.7 (0.7)
0.02	6	32 (4.1)	3.9 (0.3)
0.01	6	8 (1.1)	1.7 (0.1)
Fluconazole (μg/ml)			
2.0	3	31 (3.3)	3.6 (0.2)
1.0	3	21 (0.5)	2.8 (0.1)
0.5	3	11 (2.5)	1.4 (0.1)
0.25	3	3 (1.2)	1.3 (0.1)
0.0		5 (0)	1.4 (0.2)

[a] Cells were incubated with CF, LY295337, or fluconazole for 3 h and stained with PI for 30 min.
[b] Mean percentage of PI-positive cells (three to eight experiments) in quadrant 2.
[c] Mean (three to eight experiments) of the log mean PI fluorescence of analyzed cells.

untreated control culture at 6 h (1.0×10^7) but virtually identical to that of the untreated control culture at 0 h (1.1×10^6).

In vitro antifungal activity of compounds with different modes of action can be accurately measured by analysis of PI-stained cells by flow cytometry. The flow cytometric technique offers several advantages, including (i) short incubation time (3 to 4 h); (ii) higher precision—standard deviations are reduced by analysis of 10,000 or more cells; (iii) greater accuracy, by decreasing the number of dilution steps required and eliminating colony counting errors and difficulties in reading turbidometric endpoints; and (iv) speed of analysis. In our experiments, flow cytometry provided a unique way to compare the kinetics of drug action and subsequent changes in membrane permeability. Compared with two previously reported methods for flow cytometric detection of antifungal activity (15–18), our assay is more rapid (3.5 h versus 7 or 9 h).

TABLE 2. MFC of LY295337 for five *Candida* strains and one strain each of *Cryptococcus neoformans* and *S. cerevisiae* by flow cytometry and broth microdilution

Strain	MFC (μg/ml) by:	
	Broth microdilution	Flow cytometry
C. albicans A26	0.02	0.02
C. albicans SC5314	0.02	0.02
C. tropicalis CT5	0.02	0.02
C. parapsilosis CP5	0.1	0.08
C. parapsilosis CP8	0.8	0.8
Cryptococcus neoformans M1-106	0.5	0.63
S. cerevisiae DBY746	0.4	0.4

TABLE 3. Correlation of flow cytometric and plate count susceptibility testing data for *C. albicans* A26 incubated with CF of LY295337 for 3 h[a]

Treatment	Flow cytometry (% PI positive)[b]	Plate count (% reduction in CFU)[c]
CF (μg/ml)		
0.8	88	90
0.4	88	89
0.2	55	54
0.1	18	6
0	3	0
LY295337 (μg/ml)		
0.16	41	90
0.08	43	91
0.04	41	92
0.02	40	81
0.01	11	1
0	4	0

[a] Regression analysis of percent PI positive versus percent reduction in CFU for CF ($r = 0.996$; $y = 7.44 + 0.899X$) and LY295337 ($r = 0.991$; $y = 7.386 + 0.382X$).
[b] Percentage of PI-positive cells in quadrant 2.
[c] Mean of two separate plate counts.

Also, we used a higher concentration of fluorochrome (25 μg/ml versus 1 or 2 μg/ml), added after, not during, drug incubation, enhancing our ability to detect fungistatic activity and eliminating uncertainties concerning growth-inhibiting effects of the dye or possible synergistic effects of the dye and various drugs on test organisms.

In summary, the flow cytometric antifungal activity assay we have described can be used to rapidly determine MFCs and MICs and provides additional information. The assay is simple, reliable, and adaptable for research and clinical applications. It can be used in drug development to screen candidate compounds and compare them to each other and to commonly used drugs and may be useful in clinical trials of new antifungal compounds.

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