Use of Fluorescent Probes To Determine MICs of Amphotericin B and Caspofungin against *Candida* spp. and *Aspergillus* spp.

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We investigated the utility of mechanism-based fluorescent probes for determination of MICs (FMICs) of amphotericin B and caspofungin against *Candida* spp. and *Aspergillus* spp. Amphotericin B was selected as a membrane-active antifungal agent, and caspofungin was selected as a cell wall-active agent. FMICs were also compared to the MIC determined by CLSI (formerly NCCLS) methods. Five isolates per species of *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Aspergillus fumigatus*, and *Aspergillus terreus* were studied with either amphotericin B or caspofungin. The fluorescent probes, carboxyfluorescein diacetate (CFDA) for cytoplasmic esterase activity and dihexyloxacarbocyanine iodide (DiOC₆) for cell membrane potential, were each added to their respective plates. MICs and FMICs were determined in at least three separate experiments (in duplicate). Fluorescence was measured using a 96-well plate fluorometer. For amphotericin B and caspofungin, the FMIC end point was the lowest concentration of drug at which the percent growth inhibition from treated organisms versus control organisms displayed 80% inhibition for amphotericin B and 50% inhibition for caspofungin as measured by a fluorescent signal. The MIC for amphotericin B was defined as the lowest concentration of antifungal displaying no visible growth for both *Aspergillus* and *Candida* spp. The MIC for caspofungin was the lowest concentration of drug that displayed a minimum effective concentration for *Aspergillus* spp. For *Candida* spp., the MIC for caspofungin was defined as the concentration at which the antifungal agent significantly inhibits the organism. The FMICs of both antifungals, as measured by the DiOC₆ membrane probe, showed good agreement (83% to 100%), within one well dilution, with the MICs against amphotericin B and caspofungin for all species. Also, the FMICs measured by the CFDA cytoplasmic esterase probe reflecting damage due to cell wall or cell membrane showed strong agreement (79 to 100%) with the MICs of both amphotericin B and caspofungin for all species. There was no significant difference in comparisons of MIC and FMIC values ($P \geq 0.05$). The use of fluorescent probes provides a mechanism-based method of determination of MICs of amphotericin B and caspofungin against *Candida* spp. and *Aspergillus* spp. that correlates well with standard methods.

Recent advances in fluorescent-probe technology may permit the use of these reagents in assessing the alteration of physiologic function due to antifungal agents (1, 5, 6, 11, 12, 23). Therefore, we investigated the utility of a fluorometric assay using the fluorescent probes carboxyfluorescein diacetate (CFDA) and dihexyloxacarbocyanine iodide (DiOC₆) to assess the in vitro antifungal activities of amphotericin B deoxycholate representing the class of membrane-active polyenes and caspofungin representing the class of cell wall-active echinocandins (7).

The CFDA probe is taken up by viable cells and upon hydrolysis by intracellular esterases, forms carboxyfluorescein. The carboxyfluorescein is retained in the cell, and its fluorescent signal can be measured fluorometrically. The process of hydrolysis is disrupted when the cell is damaged. As a marker for cytoplasmic and cell membrane integrity, the CFDA probe may be used to assess fungal cell injury induced by cell membrane- and cell wall-active antifungal agents. DiOC₆ is an alkyl-carbocyanine that accumulates on cellular membranes and is transported into the lipid bilayer. The loss of cell membrane

electrical potential due to membrane damage results in the decreased emission of fluorescence. The membrane-specific probe DiOC₆ may be best applicable to assess fungal cell injury due to membrane-active antifungal agents, such as amphotericin B. Given the different cell targets and mechanisms of action of the cell wall-active echinocandins and membrane-active polyenes, we investigated the utility of determining antifungal susceptibility using these mechanism-based fluorescent probes and compared these results to standard CLSI (formerly NCCLS) methods (3, 9, 10, 16, 20).

MATERIALS AND METHODS

Organisms. All strains were obtained from patients at the National Institutes of Health Warren Grant Magnuson Clinical Center. Five species were studied: *Candida albicans* (NIH 8621, ATCC MYA1237), *Candida parapsilosis*, *Candida glabrata*, *Aspergillus fumigatus* (NIH 4215, ATCC MYA1163), and *Aspergillus terreus*. A total of three clinical isolates of *Candida albicans*, *Candida parapsilosis*, and *Aspergillus fumigatus* and five clinical isolates of *Candida glabrata* and *Aspergillus terreus* were examined. Cultures were stored on the surface of potato dextrose agar slants (Remel, Lenexa, KS) at -70°C .

Antifungal drugs. Solutions of caspofungin (MK 0991) (Merck Sharp and Dohme, West Point, PA) and amphotericin B (Bristol-Myers Squibb, Princeton, NJ) were prepared daily by weighing the antifungal powder and dissolving it in 0.165 M morpholinopropanesulfonic acid (MOPS)-buffered RPMI 1640 (Bio-Whittaker, Walkersville, MD) at pH 7.0. A 100- μl aliquot of concentrated antifungal compound was diluted twofold into 100 μl of inoculum suspension and incubated at 35°C for 48 h. The final drug concentration ranged from 0.015 $\mu\text{g}/\text{ml}$ to 8 $\mu\text{g}/\text{ml}$ for caspofungin and from 0.03 to 16 $\mu\text{g}/\text{ml}$ for amphotericin B.

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TABLE 1. Median MICs and FMICs of amphotericin B^a

Organism	MIC	FMIC	
		CFDA	DiOC ₆
<i>C. albicans</i>	0.5 (0.5–0.5)	0.5 (0.25–1.0)	0.25 (0.25–1.0)
<i>C. glabrata</i>	1.0 (0.5–64)	2.0 (0.5–64)	1.0 (0.5–64.0)
<i>C. parapsilosis</i>	1.0 (0.5–1.0)	0.5 (0.25–1.0)	0.5 (0.25–2.0)
<i>A. fumigatus</i>	0.5 (0.5–1.0)	1.0 (0.5–2.0)	0.5 (0.5–1.0)
<i>A. terreus</i>	4.0 (1.0–8.0)	2.0 (1.0–16.0)	4.0 (1.0–8.0)

^a Each species was tested at least 18 times using the fluorescent probes CFDA and DiOC₆. Values are in micrograms per milliliter. Values in parentheses are ranges.

Antifungal susceptibility testing. Susceptibility testing was performed using the NCCLS M27-A2 method for yeast isolates (13) and CLSI M38-A method for the filamentous fungi (14). All experiments were performed at least 18 separate times.

Briefly, *Aspergillus* spp. were grown on potato dextrose agar slants for 3 to 5 days. The slants were then flooded with 7 ml of saline and gently scraped with a sterile transfer pipette. Heavy particles were allowed to settle before the supernatant was transferred to a sterile tube. The *Aspergillus* inoculum was adjusted spectrophotometrically to a final concentration of 0.4×10^4 to 5.0×10^4 CFU/ml.

Candida spp. were grown for 24 h on Sabouraud glucose agar. The inoculum for *Candida* spp. was prepared by adjusting a suspension of the organism to a turbidity of a 0.5 McFarland standard for a stock concentration of 1×10^6 to 5×10^6 CFU/ml. A working suspension was then prepared by a 1:100 dilution followed by a 1:20 dilution with RPMI 1640 with L-glutamine, without sodium bicarbonate, and buffered with 0.165 M MOPS.

Each drug-containing well and each drug-free control well was inoculated with 100 μ l of the working inoculum of either *Aspergillus* or *Candida* spp. The plates were incubated at 35°C for 48 h in ambient air. The MIC was determined according to the NCCLS M27-A2 document for *Candida* spp. or the NCCLS M38-A document for *Aspergillus* spp. The MIC of amphotericin B for *Candida* spp. and *Aspergillus* spp. was defined as the well with the lowest concentration that inhibits discernible growth compared to the drug-free control well. For caspofungin, the MIC was defined as the minimum effective concentration compared to the drug-free control well for *Aspergillus* spp. The MIC of caspofungin for *Candida* spp. was defined as the lowest concentration of drug that significantly inhibits the organism compared to the drug-free growth control (15).

Fluorescent-probe assay. CFDA (Molecular Probes, Eugene, OR) and DiOC₆ (Molecular Probes, Eugene, OR) were studied in separate plates in conjunction with each antifungal, amphotericin B and caspofungin. The same 96-well plates used to determine the MIC were centrifuged, and the fluorescent probes, CFDA or DiOC₆, were added to the different plates. A separate plate was prepared for each probe. The FMIC (the MIC found by using a fluorescent probe) was then determined as described further below.

CFDA probe. The CFDA probe was weighed and diluted in 100% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) for a stock concentration of 5 mg/ml. A 0.1 M MOPS buffer solution was prepared by dissolving the MOPS powder (Fisher Biotech, Fair Lawn, NJ) in water. The solution was adjusted to a pH of 3.0 using hydrochloric acid. This probe was further diluted in the buffered MOPS-HCl solution to a final concentration of 50 μ g/ml. After the conventional MIC was recorded, the same 96-well plates were centrifuged at $784 \times g$ for 10 min, and RPMI 1640 was removed by inverting the plates. The organisms were rinsed with the MOPS-HCl solution, centrifuged again for 10 min, and buffer was then removed. CFDA was added to the centrifuged wells and incubated at 37°C, in CO₂, for 45 min in the dark. The plates were rocked gently for the entire incubation period. No additional washing was performed. The plates were read at 485 nm (excitation wavelength) and 538 nm (emission wavelength) using a Spectra Max Gemini XS fluorometer (Molecular Devices, Sunnyvale, CA). A numeric relative fluorescence unit was assigned by the fluorometer for each well.

DiOC₆ probe. DiOC₆ powder was diluted in 100% dimethyl sulfoxide to a concentration of 200 μ g/ml. The probe was further diluted in MOPS and potassium hydroxide (pH 10) to a final concentration of 2 μ g/ml. As for the CFDA probe, the 96-well plates used to determine the MIC were also used to test the fluorescent probe DiOC₆. The plates were spun at $784 \times g$ for 10 min, RPMI 1640 was removed, and the organisms were rinsed with the buffer. DiOC₆ was added to the centrifuged wells, and plates were incubated with gentle rocking at room temperature for 45 min in the dark. A 10- μ l aliquot of the detergent

TABLE 2. Median MICs and FMICs of caspofungin^a

Organism	MIC	FMIC	
		CFDA	DiOC ₆
<i>C. albicans</i>	1.0 (0.5–1.0)	0.5 (0.25–2.0)	0.5 (0.5–8.0)
<i>C. glabrata</i>	1.0 (0.5–2.0)	2.0 (1.0–2.0)	1.0 (1.0–2.0)
<i>C. parapsilosis</i>	2.0 (1.0–2.0)	2.0 (0.5–4.0)	1.0 (0.5–2.0)
<i>A. fumigatus</i>	1.0 (0.5–1.0)	0.5 (0.25–2.0)	0.5 (0.125–1.0)
<i>A. terreus</i>	0.5 (0.5–64.0)	0.5 (0.5–64.0)	0.5 (0.5–64)

^a Each species was tested at least 18 times using the fluorescent probes CFDA and DiOC₆. Values are in micrograms per milliliter. Values in parentheses are ranges.

((octylphenoxy) polyethoxyethanol) IGEPAL (Sigma-Aldrich, St. Louis, MO) was diluted 1:20 in water and added to plates, and the plates were incubated for an additional 30 min. At the completion of incubation, plates were spun for 10 min and the probe was removed. All plates were rinsed with MOPS (pH 7.0) buffer, and 200 μ l was left in the wells for reading on the fluorometer at 485 and 538 nm.

FU and FMIC. Plates were read using a Spectra Max Gemini XS fluorometer. The fluorometer assigned a fluorescence unit (FU) to each well. The percent growth inhibition was generated by dividing the FU of the drug-containing well by the FU of the drug-free control well and multiplying by 100. The FMIC for amphotericin B was established at 80% (or more) growth inhibition. The FMIC for caspofungin was defined at 50% growth inhibition for both *Aspergillus* and *Candida* spp.

Statistical analysis. The MIC and FMIC were compared (as percent agreement) within one well dilution. Within-group variations were measured by the Mann-Whitney U test. All *P* values were two sided. *P* values of ≤ 0.05 were considered significant.

Quality control. ATCC strains of *Candida krusei* (ATCC 6258) and *Candida parapsilosis* (ATCC 22019) were used as controls in all experiments. All quality control isolates were within the acceptable ranges for both amphotericin B and caspofungin.

RESULTS

The median conventional MICs of amphotericin B and caspofungin were determined for each of the five *Candida* and *Aspergillus* species studied. Tables 1 and 2 demonstrate that the median FMICs were similar to the median conventional MICs. These similarities were observed for both CFDA and DiOC₆ fluorescent probes.

The conventional MIC was compared to the FMIC as a percentage of agreement within one well dilution (Table 3). The percent agreement between the MIC and FMIC for *C. albicans* ranged between 86 and 100% for amphotericin B, while for caspofungin the percent agreement ranged between 83 and 95% using DiOC₆ and CFDA, respectively. The MIC and FMIC of *C. glabrata* and *C. parapsilosis* showed 90 to 100%

TABLE 3. Comparison of the conventional MIC and FMIC as percent agreement within one well dilution^a

Organism	% Agreement within one well dilution			
	CFDA-AM	DiOC ₆ -AM	CFDA-CAS	DiOC ₆ -CAS
<i>C. albicans</i>	100	86	95	83
<i>C. glabrata</i>	100	100	96	97
<i>C. parapsilosis</i>	100	90	97	100
<i>A. fumigatus</i>	95	100	95	94
<i>A. terreus</i>	79	100	82	92

^a Each species was tested at least 18 times using the fluorescent probes CFDA and DiOC₆ and antifungal agents amphotericin B (AM) and caspofungin (CAS).

FIG. 1. Growth inhibition of *Candida albicans* as measured by fluorescence units versus log₂ amphotericin B (AmB) concentration (µg/ml) and log₂ caspofungin (Caspo) concentration (µg/ml) using the fluorescent probes CFDA and DiOC₆. The percent growth inhibition is determined by the ratio of three isolates of *C. albicans* that were studied in a total of 18 replicate experiments.

agreement for amphotericin B for both probes, while the percent agreement for caspofungin was approximately 96 to 100% for both probes. For *A. fumigatus*, the MIC and FMIC agreed 95 to 100% for amphotericin B and 94 to 95% for caspofungin using DiOC₆ and CFDA probes. The MIC and FMIC for *A. terreus* agreed 79 to 100% against amphotericin B and 82 to 92% against caspofungin for CFDA and DiOC₆, respectively.

The percentage of growth was generated for each concentration of antifungal compound for each species and isolate tested. The percent growth versus the concentration of antifungal agent (log₂ µg/ml) was plotted for *Candida albicans*. This organism displayed an exponential decline in the percentage of growth, as the concentration of antifungal agent increased (Fig. 1).

DISCUSSION

Based upon the conjugate molecule, fluorescent probes CFDA and DiOC₆ are able to accurately measure different degrees of mechanism-based cellular injury (1, 5, 6, 23). This is the first study to our knowledge that simultaneously investigates the activity of these probes using amphotericin B and echinocandin as model membrane-active and cell wall-active drugs against both *Candida* spp. and *Aspergillus* spp.

The CFDA probe is a lipophilic substrate that is easily taken up by the cell. Diacetate is cleaved from the molecule by intracellular cytoplasmic esterases, producing carboxyfluorescein, which is fluorescent. The amount of carboxyfluorescein accumulated within *C. albicans* is a direct measure of cell

viability (8, 9). Cellular injury from our study is reflected more globally by CFDA than by DiOC₆ at the cell membrane and cytoplasmic levels in *C. albicans*, *C. glabrata*, *C. parapsilosis*, *A. fumigatus*, and *A. terreus*.

By comparison, DiOC₆ measures alterations in membrane potentials in fungal and mammalian cells (9, 11). DiOC₆ is a lipophilic, cationic molecule with short alkyl chains that is also taken up by the cell membrane as well as by mitochondria in viable yeasts and molds as a function of electrical membrane potential.

Amphotericin B is a polyene that binds to ergosterol in the cell membrane. The binding forms a polyene-sterol complex that disrupts the cell membrane, resulting in efflux of potassium and magnesium ions and influx of sodium and calcium. The alteration in cellular homeostasis results in the denaturation of cytoplasmic proteins, including esterases, and consequently, fungal cell death. The diminished CFDA probe signal correlated with the diminished cytoplasmic esterases and ultimately resulted in fungal cell death.

Exposure of the cells to amphotericin B likely disrupts accumulation of DiOC₆ probe in the cell membrane. The intensity of the DiOC₆ probe signal was inversely related to increasing amphotericin B concentrations. As the intracellular potassium and magnesium contents are diminished, cellular gradients of hydrogen ions also deteriorate. As intracellular pH declines, membrane potentials are also degraded, leading to a diminution of the DiOC₆ probe signal. As these cell membrane and mitochondrial electrical gradients are degraded, membrane potential is lost, and the DiOC₆ signal is diminished in a dose-dependent manner. Overall, both probes showed strong agreement between the MIC and FMIC (within one well dilution) for amphotericin B for all species (Table 3). For caspofungin, the percent agreement was slightly lower for both probes.

These probes are widely used in the study of mammalian cellular metabolism and are applicable to the study of fungal metabolism. One should note, in this regard, that all incubations of organisms with antifungal compounds over 48 h in these studies were conducted in RPMI 1640 (without sodium bicarbonate) buffered in MOPS to a pH of 6.8 to 7.2. Following a standard incubation time of 48 h at pH 6.8 to 7.2, the pH was adjusted for 45 min at pH 3 for CFDA and for 90 min at pH 10 for DiOC₆ to allow for analysis of depleted cytoplasmic esterase and altered membrane potential, respectively. The objective of the fluorescent-probe assay is not to measure the effect on membrane potential at neutral pH; instead, the DiOC₆ is intentionally studied at a pH of 10 in order to ascertain the damage that the antifungal agent had on damaging the cell membrane during the 48-h incubation period at pH 7. Thus, the pH was never intended to measure alterations of the actual membrane potential at pH 7. Instead, the DiOC₆ probes for damage to cell membrane as evidenced by alterations to membrane potential; that analytical test is optimally performed at pH 10.

Our data would indicate that amphotericin B and, to a lesser extent, the echinocandin influence cell membrane potential. Although echinocandins do not directly target the cell membrane, ultrastructural studies from our laboratory and from others clearly demonstrate a membrane-altering effect, possibly as a secondary effect of disruption of the osmotic gradient

due to alteration of the cell wall integrity (2). By comparison, CFDA reflects cytoplasmic esterase activity that is altered by both the cell membrane- and cell wall-active compounds.

A Mann-Whitney U-test comparison analysis was done comparing the conventional MIC to FMIC (CFDA) and comparing the conventional MIC to FMIC (DiOC₆) with all species against both amphotericin B and caspofungin. There were no significant differences found between the conventional MIC and FMIC using the CFDA and DiOC₆ probes for amphotericin B and caspofungin for all five species ($P \geq 0.05$), further indicating agreement between the fluorescent-probe method and the NCCLS microdilution method.

The conventional analysis of assessing variation within one tube dilution may not necessarily have sensitivity for detection of more subtle interactions. In order to further understand the quantitative relationship between FMICs and conventional MICs, we further analyzed the data using a Pearson's correlation coefficient for each probe against each organism tested.

The FMICs measured by the DiOC₆ membrane probe correlated with the MICs of amphotericin B ($r \geq 0.70$; $P < 0.005$) but not as well with those of caspofungin ($r \geq 0.28$; $P < 0.47$). By comparison, the FMICs measured by the CFDA cytoplasmic esterase probe correlated with the MICs of both amphotericin B ($r \geq 0.59$; $P < 0.015$) and caspofungin ($r \geq 0.67$; $P < 0.023$) against the same species.

Several methods have been developed and correlate well with the NCCLS method for yeasts and filamentous fungi. Some of these methods involve broth microdilution, while others are agar based.

The YeastOne colorimetric antifungal panel (TREK Diagnostics Systems, Cleveland, OH) is a commercially available system that uses oxidation/reduction of an alamar blue colorimetric indicator. The YeastOne colorimetric antifungal panel is comparable to the reference method and provides a full range of MICs for amphotericin B, fluconazole, 5-fluorocytosine, itraconazole, and ketoconazole. Several studies have tested *Candida* spp., *Cryptococcus neoformans*, and filamentous fungi using this panel (4, 16, 17, 19). In one study of *Candida* by Pfaller et al., there was an overall agreement of 95% for antifungal triazoles compared to the NCCLS M27-A2 method (17).

An agar-based method, the Etest (AB Biodisk, Solna, Sweden), is also available commercially. This test is based on a strip containing various concentrations of antifungal agents (16, 18). The strip is placed on an agar plate while the antifungal compound diffuses through the plate. A good correlation was found between the Etest method and the reference NCCLS M27-A2 method for most *Candida* spp. and *Cryptococcus neoformans* against several antifungal agents. Moreover, it has been used to reliably detect amphotericin B resistance in several *Candida* spp., *Cryptococcus neoformans*, and *Saccharomyces cerevisiae* (18).

Flow cytometry is based on binding (or lack of binding) of fluorescent dyes to organisms. This assay assesses viability, within 2 to 6 h, after exposure to an antifungal agent. This method is rapid and has good agreement with the NCCLS M27-A2 method for yeast (22). A recent study comparing several *Candida* spp. and *Cryptococcus neoformans* found that overall flow cytometry susceptibility testing showed a strong

correlation to the NCCLS method using fluconazole and amphotericin B (21).

Using fluorescent-probe-based assays may be particularly useful for assessing the response of filamentous fungi to antifungal compounds where the end point may be subjective and difficult to determine. The use of a 96-well fluorometer may also permit high throughput of potentially large numbers of assays per unit time. Fluorescent-probe assays may further facilitate assessment of the antifungal activities of approved and investigational compounds on the basis of their mechanism of action instead of observed biomass within the well.

In summary, FMICs measured by fluorescent probes provide a practical mechanism-based approach to measuring antifungal susceptibility. The fluorescent probe CFDA is applicable to polyenes and echinocandins, while DiOC6 applies to membrane-active drugs, such as amphotericin B.

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