Mycopathologia (2006) 162: 103–109
DOI: 10.1007/s11046-006-0040-y
© Springer 2006

Application of fluorescent probes to study structural changes in *Aspergillus fumigatus* exposed to amphotericin B, itraconazole, and voriconazole

Madhurama Gangwar[1,5], Richard Cole[2], Rama Ramani[1], Daniel J. Sheehan[4] & Vishnu Chaturvedi[1,3]

[1]*Mycology Laboratory, Wadsworth Center, NYSDOH, 120 New Scotland Ave., Albany, NY, 12208 - 2002, USA;* [2]*Advanced Light Microscopy Core, New York State Department of Health, Wadsworth Center, New York, USA;* [3]*Department of Biomedical Sciences, School of Public Health, State University of New York at Albany, Albany, NY, USA;* [4]*Pfizer Inc., New York, NY, USA;* [5]*Department of Microbiology, Punjab Agricultural University, Ludhiana, India*

Received 30 January 2006; accepted 20 June 2006

Abstract

The broad objective of this study was to document patterns of structural changes following antifungal treatment, and to determine any relationship with minimum inhibitory concentration (MIC) of an antifungal. Three clinical isolates of *Aspergillus fumigatus*, with high, intermediate, and low amphotericin B (AB), itraconazole (IZ), and voriconazole (VZ) MICs were studied in 24-well plates with cover slips. The fluorescent probes used were Calcofluor White (cell wall), propidium iodide (nucleus), and MitoTracker Green FM (mitochondria). Fluorescent microscopy as early as 3-h after exposure revealed that AB treated hyphae had intact cell wall with deformed mitochondria and nuclei while IZ and VZ treated hyphae revealed no intact cell wall, and deformation of mitochondria and nuclei. At 48 h, AB treated cells revealed rupture of hyphae and disintegration of mitochondria, and nuclei, IZ treated hyphae were swollen with disintegration of mitochondria, and nuclei while VZ treated hyphae showed rupture and disintegration of mitochondria and nuclei. The structural changes for the three strains studied were similar in fluorescent microscopy as long as the incubation time and their respective MICs were used. Thus, AB, IZ, and VZ induced gross organelle defects in *A. fumigatus* nuclei, mitochondria, and cell wall, which were consistent with respective MICs of antifungals used.

Key words: antifungals, *Aspergillus fumigatus*, fluorescent microscopy, internal organelles

Introduction

Invasive aspergillosis (IA) is recognized as a serious opportunistic fungal infection in immunocompromised patients [1, 2]. The most common causal agents include *A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus* [1]. The long established treatment regimens for IA relied upon amphotericin B (AB) and itraconazole (IZ), but with serious limitations [2]. These include high nephrotoxicity of intravenously administered AB, and poor bioavailability with IZ. The revised Infectious Diseases Society of America (IDSA) guidelines for the treatment of IA include voriconazole (VZ), and caspofungin for refractory disease or drug intolerance in addition to AB and IZ [2]. VZ is an orally available azole drug with excellent susceptibility profile against *A. fumigatus* in the laboratory and clinical trial, while caspofungin is administered intravenously [2]. The Clinical and Laboratory

Standards Institute (CLSI; formerly known as National Committee for Clinical Laboratory Standards) has developed a reference method for antifungal susceptibility testing of filamentous fungi [3]. A number of investigators have reported the results of in vitro susceptibility testing of *Aspergillus* spp. using various methodology such as M38-A broth dilution, *E*-test, Sensititre YeastOne colorimetric, disc diffusion, precise flow cytometry (FC), rapid susceptibility assay, etc. [3–14]. Most of these methods require mycelial growth and hence long incubation time, except for flow cytometry, where the time required was 3–16 h [4, 12]. However, FC has not been standardized in multi-laboratory trials [4, 12, 15].

The fluorescent probes are being increasingly used as useful tools for the microscopic characterization of molds and yeasts [10, 15–25]. Fluorescent microscopy (FM) was previously used to study *Penicillium chrysogenum* morphology, growth, and effects of various chemicals [20]. Fluorescent dyes - 3,3′-dihexyloxocarbocyanin and Calcofluor have been used to detect the level of metabolic activity and internal organelles in filamentous organisms such as *A. oryzae* [16]. Similarly, echinocandidin-*A. fumigatus* interactions have been extensively studied microscopically [17, 22–24, 26]. These investigators used fluorescent dyes such as 5,(6)-carboxyfluorescein diacetate (CFDA) and *bis*-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC), and demonstrated that echinocandins act at the site of new cell wall synthesis, and killing the hyphae of *A. fumigatus* [17, 22–24, 26]. The broad objective of this study was to document patterns of structural changes following antifungal treatment, and to determine any relationship with minimum inhibitory concentration (MIC) of an antifungal.

Materials and methods

Antifungal agents and strains

Three *A. fumigatus* clinical isolates were selected based on their variable MICs to antifungals tested by M38-A protocol [3]. AB was purchased from Sigma Biochemical Company (St. Louis, MO), while IZ and VZ were kind gifts from Research Diagnostics Inc. (Flanders, NJ), and Pfizer Inc. (New York, NY), respectively. All antifungals were dissolved in dimethyl sulfoxide to yield stock solutions of 1,600 μg/ml. Broth microdilution susceptibility testing was performed according to CLSI (NCCLS) M38-A protocol [3]. MIC determination was by visual examination of growth inhibition as described in NCCLS document M38-A [3].

Phase-contrast microscopy (PCM)

The procedures described by Lass-Florl and coworkers with modifications were followed [10]. The *Aspergillus* strains were grown on potato dextrose agar slants for 3–5 days at 35°C. Inocula were prepared in 0.85% sterile saline containing 1% Tween 80, and spectrophotometer was used to adjust the suspension to an optical density at 530 nm of 0.13 to 0.18. One ml of the conidial solution was added to each well of the 24-well plates containing sterilized coverslips, and incubated at 35°C for 18–22 h to allow formation of hyphae. The medium was drained off, and 1 ml of dilutions ranging from 0.03 μg/ml to 16.0 μg/ml of AB, IZ, and VZ, (using the same dilution range as in susceptibility testing protocol,) was added. Plates were incubated at 35°C for varying periods of time, ranging from 3 to 72-h. Photomicrographs were taken with an Olympus AX 70 microscope in PCM mode at a magnification of 400X (Olympus USA Inc., Lake Success, NY).

Fluorescent Microscopy (FM)

Specific fluorescent probes were used to assess the structural changes in the treated and control hyphae. Calcofluor White (Sigma Biochemical, St. Louis, MO) was used to stain cell walls and septa. Propidium iodide and MitoTracker Green FM (Molecular Probes, Eugene, OR) were used for staining of nuclei and mitochondria, respectively. The methods used to grow the culture, and to determine antifungal susceptibility were the same as described for PCM. After the stipulated time of incubation, the medium was drained off from each well, and the coverslip was rinsed in 0.1M potassium phosphate buffered saline (pH 7.2) for 5 min. The fungal growth was fixed with fixative (80% methanol; 4% formamide; 1% glutaraldehyde) for 30 min. The coverslips were rinsed in PBS, and sodium borohydrate (1 mg/ml) was added to reduce the autofluorescence for 10 min, then the

coverslips were again rinsed in PBS, and they were stained with Calcofluor White (0.5 mg/ml) for 30 min. The coverslips were rinsed in PBS for 5 min, stained with propidium iodide (50 μg/ml) for 5 min, and rinsed twice in PBS for 5 min each. Finally, they were stained with MitoTracker Green FM (10 μmol/ml) for 10 min, and rinsed in PBS for 5 min. One drop of mounting medium (5 mg/ml of paraphenylenediamine) was placed on a clean glass slide and covered with a coverslip. It was then sealed with clear nail polish and stored at 4°C in the dark until observation in a fluorescence microscope at 400× (Nikon Optiphot, Nikon Inc., Melville, NY). Control hyphae were treated with 0.5% TritonX-100 for 30 min before fixation to facilitate better penetration of fluorescent dyes. Fluorescent-labeled mycelia were photographed using a Nikon Optiphot microscope (Nikon Inc.) equipped with a Quad Fluor epi-fluorescence attachment and a 100× 1.4-NA objective lens. The quad-fluor filters used were UV-2E/C for Calcofluor, B-2E/C for Mitotracker green, and B-2E/C for propidium iodide. The color images were captured digitally using a Spot CCD camera (Diagnostic Imaging, Sterling Heights, MI). Contrast enhancement and montaging were done in Adobe PhotoShop software, version 6.0 (Adobe Inc., San Jose, CA).

Results and discussion

Three *A. fumigatus* isolates with high, intermediate, and low MICs of AB, IZ, and VZ were selected for microscopy (Table 1). Initially, we compared observation periods from 60 min to 72 h to find optimal time for recording maximum structural changes using PCM and FM. This observation period was chosen as representative of reported measurement of fluorescence for *A. fumigatus* antifungal interactions between 0.5 and 9.0 h [10, 12, 15]. Final results were recorded at 3 h and 48 h as these time points were optimal for recording initial and final structural changes. The microscopic morphology of *A. fumigatus* (isolate #041-00) showed intact vegetative hyphae in PCM, and hyphae with blue cell walls and septa, well formed green mitochondria, and discrete red nuclei in FM (Figure 1 a–b). After 3 h treatment with 4.0 μg/ml of AB, PCM showed swelling of hyphae while FM showed intact hyphae with deformation of mitochondria and nuclei (Figure 1 c–d). IZ (1.0 μg/ml) caused hyphal swelling (Figure 1e), and hyphal swelling with no intact cell wall and deformations of mitochondria and nuclei (Figure 1f). VZ (1.0 μg/ml) caused shrinkage with focal dilatations in hyphae (Figure 1g) and swelling with no intact cell wall and deformation of mitochondria and nuclei (Figure 1h). Further incubation of isolate 041-00 up to 48 h with AB showed shrinkage of hyphae (Figure 2c), and shrinkage and rupture with disintegration of mitochondria and nuclei (Figure 2d). Similar incubations for 48 h with IZ and VZ caused swelling, and disintegration of mitochondria and nuclei (Figure 2e–h). *A. fumigatus* isolate 817-00 following 48 h exposures to relevant MICs of antifungals revealed rupture and disintegration of mitochondria and nuclei for AB (Figure 3 a–b), swelling and disintegrated mitochondria and nuclei for IZ (Figure. 3c) and rupture and disintegration of mitochondria and nuclei for VZ (Figure 3d). *A. fumigatus* isolate 166-00, treated for 48 h with relevant antifungal MICs, showed rupture and disintegration of mitochondria and nuclei with AB (Figure 4a–b); IZ treated hyphae showed swelling and disintegrated mitochondria and nuclei (Figure 4c), and VZ treated hyphae showed rupture and disintegration of mitochondria and nuclei (Figure 4d). Evidently, there were no notable differences in microscopy of the three strains as long as same incubation time and respective MICs of antifungals were used for testing.

Table 1. MICs (μg/ml) of the three *A. fumigatus* strains*

Isolates	Antifungals		
	Amphotericin B	Itraconazole	Voriconazole
041-00	4.0	1.0	1.0
817-00	2.0	0.25	0.5
166-00	0.5	0.25	0.25

*Values are from ten replicates

The fluorescent probes used in this study were chosen for their demonstrated usefulness for demarcation of fungal morphology. Calcofluor White (CFW) is a stilbene compound that binds to glucans and chitin in the cell walls and septa of fungi [16, 27]. Propidium iodide is positively charged, membrane-impermeable fluorochrome that can only pass through the membranes of

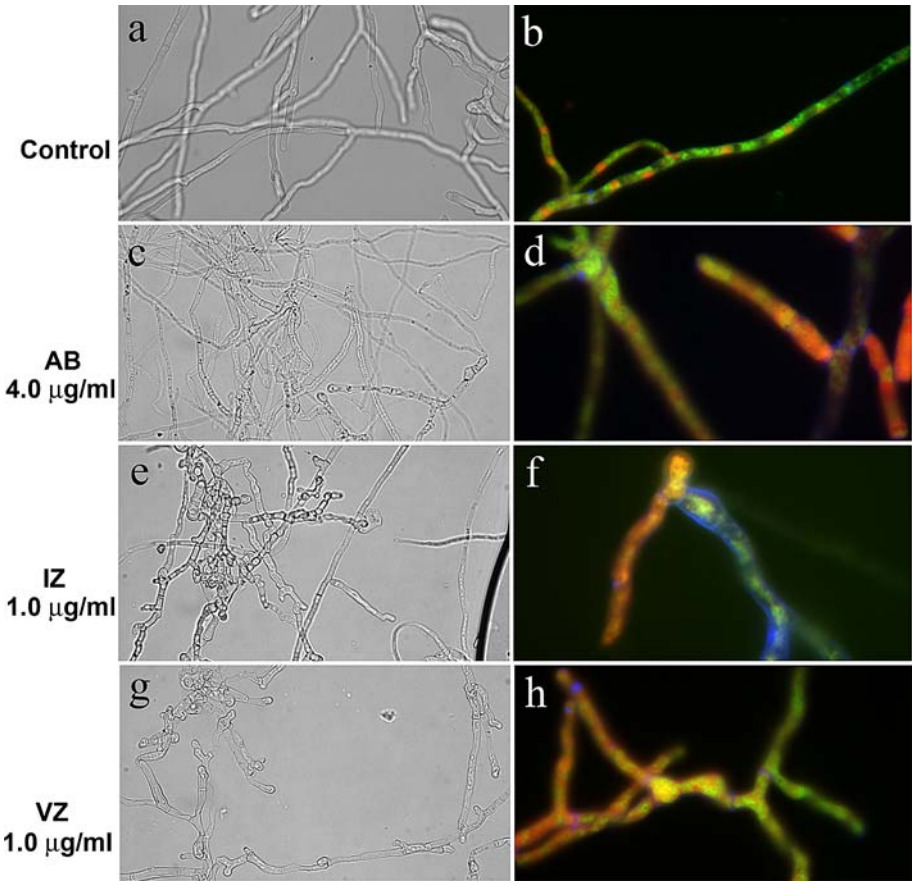

Figure 1. A. fumigatus isolate 041-00 following 3 h antifungal exposures (400×). Both phase contrast (PCM) and fluorescent microscopy (FM) images are shown in adjacent panels. Panels c–d show swelling of hyphae (PCM), and intact hyphae with deformation of mitochondria and nuclei as evident from diffusion of green and red fluorescent dyes (FM). Panels e–f show hyphal swelling (PCM), and hyphal swelling with no intact cell wall and deformations of mitochondria and nuclei (FM). Panels g–h show shrinkage with focal dilatations in hyphae (PCM) and swelling with no intact cell wall and deformation of mitochondria and nuclei (FM).

stressed, injured, or dead cells [19]. This dye binds by intercalating into DNA and RNA, where it fluoresces red. MitoTracker Green FM is typically used for visualization of mitochondrial shape, mass, or swelling by measurement of membrane potential [18, 21, 25].

FM has been successfully used to characterize internal morphology of fungi [16, 20]. *Candida albicans* treated with several imidazoles showed swollen mitochondria or amorphous bodies when visualized with carbocyanine probe $DiOC_6$ [25]. Exposure of yeast cells to higher concentrations of antifungal drug showed deleterious effect on mitochondria [25]. Fluorescent probe FUN-1 was used to evaluate the susceptibility of *Candida* isolates and the results demonstrated that this probe could be used as an alternative and rapid method for the differentiation of fluconazole – susceptible and –resistant clinical isolates [15]. Also, FUN-1 staining was used as a rapid and sensitive method for assaying the viability of antifungal treated *Aspergillus* hyphae. [10]. However, in the present study, three different fluorescent probes were used to study the changes caused by various antifungals in different organelles of *A. fumigatus*. Also, the present study uses hyphae as inocula, to demonstrate the effect of AB, IZ, and VZ on cell wall, mitochondria, and nuclei of *A. fumigatus* hyphae. Recently, the fluorescent dyes CFDA and $DiBAC_4$ were used to characterize the antifungal activity of caspofungin and micafungin against *A. fumigatus* [17, 22–24]. These studies demonstrated that the dye staining patterns revealed the active centers of the cells of *A. fumigatus* are killed when exposed to

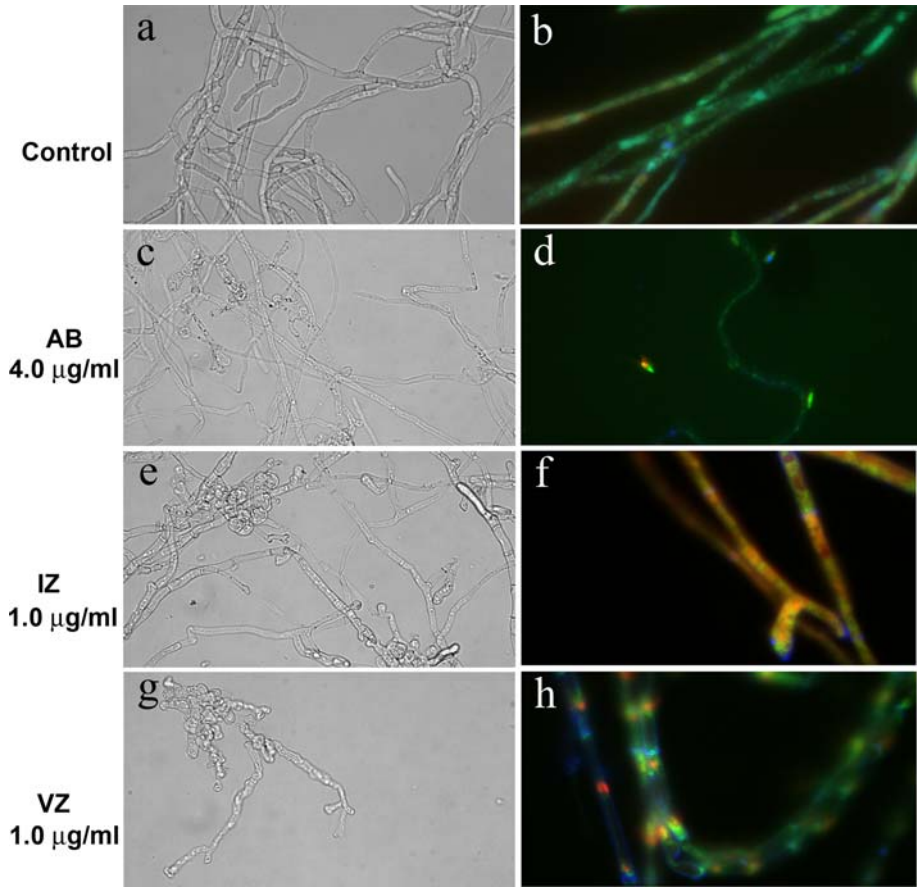

Figure 2. *A*. *fumigatus* isolate 041-00 following 48 h antifungal exposures (400×). Both phase contrast (PCM) and fluorescent microscopy (FM) images are shown in adjacent panels. Panels c–d show shrinkage of hyphae (PCM), and shrinkage and rupture with disintegration of mitochondria and nuclei (FM). Panels e–f show hyphal swelling (PCM), and hyphal swelling with disintegration of mitochondria and nuclei (FM). Panels g–h show focal swelling in hyphae (PCM) and hyphal rupture with disintegration of mitochondria and nuclei (FM).

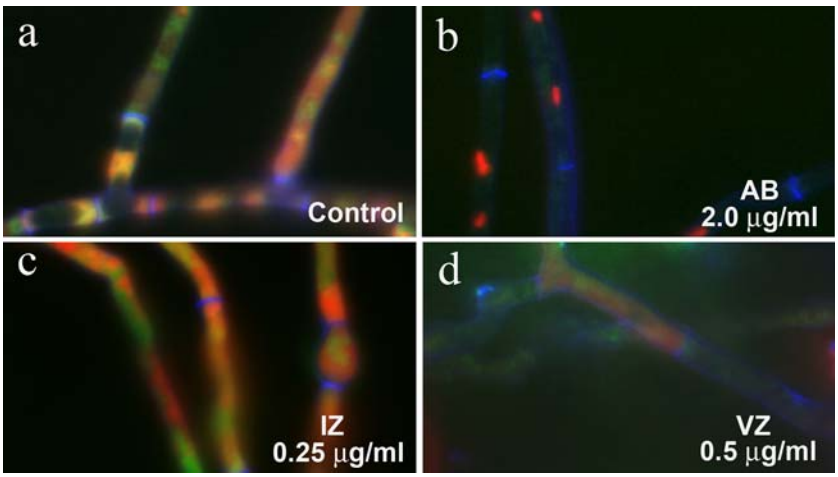

Figure 3. *A*. *fumigatus* isolate 817-00 following 48 h antifungal exposures (400×). Panel a – control hyphae; Panel b – AB treated hyphae show rupture and disintegration of mitochondria and nuclei; Panel c – IZ treated hyphae show swelling and disintegrated mitochondria and nuclei; Panel d – VZ treated hyphae show rupture and disintegration of mitochondria and nuclei.

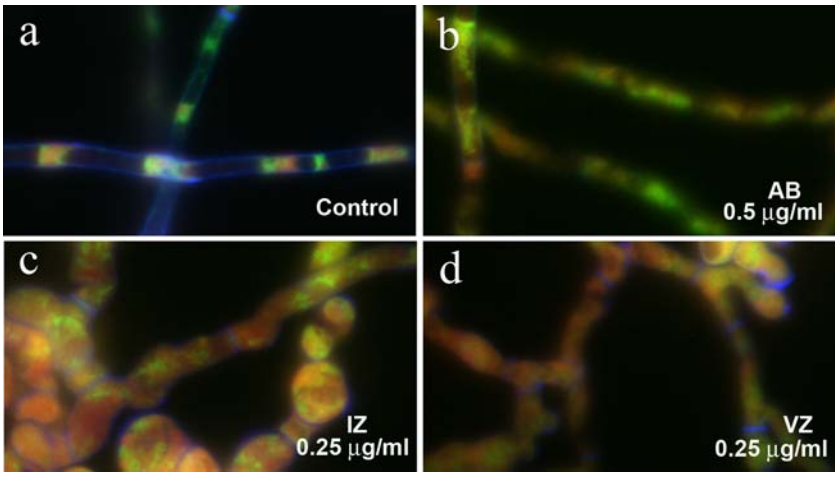

Figure 4. A. fumigatus isolate 166-00 following 48 h antifungal exposures (400×). Panel a –control hyphae; Panel b – AB treated hyphae show rupture and disintegration of mitochondria and nuclei; Panel c – IZ treated hyphae show swelling and disintegrated mitochondria and nuclei; Panel d – VZ treated hyphae show rupture and disintegration of mitochondria and nuclei.

caspofungin and micafungin [17, 22–24]. In present study, rupture of cell wall along with complete disintegration of mitochondria and nuclei were observed for AB and VZ, while swollen cell wall with disintegration of mitochondria and nuclei were observed for IZ at 48 h. Thus, AB, IZ, and VZ induced gross organelle defects in *A. fumigatus* nuclei, mitochondria, and cell wall, which were consistent with respective MIC of antifungals used. This FM method should be further evaluated as a potential laboratory tool for the study of antifungal – *A. fumigatus* interactions.

Acknowledgement

We thank Andrea Doney for performing antifungal susceptibility testing of *Aspergillus fumigatus*. This study was supported in part with an unconditional grant from Pfizer Inc., NY (VC).

References

1. Hope WW, Walsh TJ, Denning DW. Laboratory diagnosis of invasive aspergillosis. Lancet Infect Dis 2005; 5: 609–622.
2. Maschmeyer G, Ruhnke M. Update on antifungal treatment of invasive *Candida* and *Aspergillus* infections. Mycoses 2004; 47: 263–276.
3. National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi. Approved standard NCCLS document M38-A. National Committee for Clinical Laboratory Standards, Wayne, Pa, 2000.
4. Balajee SA, Imhf A, Gribskov JL, Marr KA. Determination of antifungal drug susceptibilities of *Aspergillus* species by a fluorescence-based microplate assay. J Antimicrob Chemother 2005; 55: 102–105.
5. Castro C, Serrano MC, Flores B, Espinel-Ingroff A, Martin-Mazuelos E. Comparison of the Sensititre YeastOne colorimetric antifungal panel with a modified NCCLS M38-A method to determine the activity of voriconazole against clinical isolates of *Aspergillus* spp. J Clin Microbiol 2004; 42: 4358–4360.
6. Diekema DJ, Messer SA, Hollis RJ, Jones RN, Pfaller MA. Activities of caspofungin, itraconazole, posaconazole, ravuconazole, voriconazole, and amphotericin B against 448 recent clinical isolates of filamentous fungi. J Clin Microbiol 2003; 41: 3623–3626.
7. Espinel-Ingroff A. In vitro activity of the new triazole voriconazole (UK-109, 496) against opportunistic filamentous and dimorphic fungi and common and emerging yeast pathogens. J Clin Microbiol 1998; 36: 198–202.
8. Espinel-Ingroff A, Rezusta A. *E*-test method for testing susceptibilities of *Aspergillus* spp. to the new triazoles voriconazole and posaconazole and to established antifungal agents: comparison with NCCLS broth microdilution method. J Clin Microbiol 2002; 40: 2101–2107.
9. Gomez-Lopez A, Garcia-Effron G, Mellado E, Monzon A, Rodriguez-Tudela JL, Cuenca-Estrella M. In vitro activities of three licensed antifungal agents against Spanish clinical isolates of *Aspergillus* spp. Antimicrob. Agents Chemother. 2003; 47: 3085–3088.
10. Lass-Florl C, Nagl M, Speth C, Ulmer H, Dierich MP, Wurzner R. Studies of in vitro activities of voriconazole and itraconazole against *Aspergillus* hyphae using viability staining. Antimicrob. Agents Chemother. 2001; 45: 124–128.
11. Pfaller MA, Messer SA, Hollis RJ, Jones RN (The Sentry participants group). Antifungal activities of posaconazole,

ravuconazole, and voriconazole compared to those of itraconazole and amphotericin B against 239 clinical isolates of *Aspergillus* spp. and other filamentous fungi: report from SENTRY Antimicrobial Surveillance Program, 2000. Antimicrob Agents Chemother 2002; 46: 1032–1037.
12. Ramani R, Gangwar M, Chaturvedi V. Flow cytometry antifungal susceptibility testing of *Aspergillus fumigatus* and comparison of mode of action of voriconazole vis-à-vis amphotericin B and itraconazole. Antimicrob Agents Chemother 2003; 47: 3627–3629.
13. Serrano MC, Ramirez M, Morilla D, Valverde A, Chavez M, Espinel-Ingroff A, Claro R, Fernandez A, Almeida C, Mazuelos EM. A comparative study of the disc diffusion method with the broth microdilution and Etest methods for voriconazole susceptibility testing of *Aspergillus* spp. J Antimicrob Chemother 2004; 53: 739–742.
14. Verweij PE, Te Dorsthorst DT, Rijs AJ, Vries-Hospers HGDe, Meis JF (The Dutch interuniversity working party for invasive mycoses). Nationwide survey of in vitro activities of itraconazole and voriconazole against clinical *Aspergillus fumigatus* isolates cultured between 1945 and 1998. J Clin Microbiol 2002; 40: 2648–2650.
15. Pina-Vaz C, Sansonetty F, Rodrigues AG, Costa-de-Oliveira S, Martinez-de-Oliveira J, Fonseca AF. Susceptibility to fluconazole of *Candida* clinical isolates determined by FUN-1 staining with flow cytometry and epifluorescence microscopy. J Med Microbiol 2001; 50: 375–382.
16. Agger T, Spohr AB, Carlsen M, Nielsen J. Growth and product formation of *Aspergillus oryzae* during submerged cultivations: verification of a morphologically structured model using fluorescent probes. Biotechnol Bioeng 1998; 57: 321–329.
17. Bowman JC, Hicks PS, Kurtz MB, Rosen H, Schmatz DM, Liberator PA, Douglas CM. The antifungal echinocandin caspofungin acetate kills growing cells of *Aspergillus fumigatus* in vitro. Antimicrob Agents Chemother 2002; 46: 3001–3012.
18. Browser DN, Minimikawa T, Nagley P, Williams DA. Role of mitochondria in calcium regulation of spontaneously contracting cardiac muscle cells. Biophys J 1998; 75: 2004–2014.
19. Brul S, Nussbaum J, Dielbandhoesing SK. Fluorescent probes for wall porosity and membrane integrity in filamentous fungi. J Microbiol Methods 1997; 28: 169–178.
20. Cox PW, Paul GC, Thomas CR. Image analysis of the morphology of filamentous microorganisms. Microbiology 1998; 144: 817–827.
21. Funk RH, Nagel F, Wonka F, Krinke HE, Golfert F, Hofer A. Effect of heat shock on the functional morphology of cell organelles observed by video-enhanced microscopy. Anat Rec. 1999; 255: 458–464.
22. Kurtz MB, Heath IB, Marrinan J, Dreikorn S, Onishi J, Douglas C. Morphological effects of lipopeptides against *Aspergillus fumigatus* correlate with activities against (1, 3)-ß-D-glucan synthase. Antimicrob. Agents Chemother 1994; 38: 1480–1489.
23. Nishiyama Y, Hasmui Y, Ueda K, Uchida K, Yamaguchi H. Effects of micafungin on the morphology of *Aspergillus fumigatus*. J Electron Microsc 2005; 54: 67–77.
24. Watabe E, Nakai T, Matsumoto S, Ikeda F, Hatano K. Killing activity of micafungin against *Aspergillus fumigatus* hyphae assessed by specific fluorescent staining for cell viability. Antimicrob Agents Chemother 2003; 47: 1995–1998.
25. Yang HC, Taguchi T, Nishimura K, Miyaji M. Imidazole-induced morphological abnormalities of mitochondria of *Candida albicans*. Mycoses 1998; 41: 481–486.
26. Chiou CC, Mavrogiorgos N, Tillem E, Hector R, Walsh TJ. Synergy, pharmacodynamics, and time-sequenced ultrastructural changes of the interaction between nikkomycin Z and echinocandin FK463 against *Aspergillus fumigatus*. Antimicrob. Agents Chemother. 2001; 45: 3310–3321.
27. Kasten FH. Introduction to fluorescent probes. In: Mason WT), ed. Fluorescent and Luminescent Probes for Biological Activity, Academic PressLondon, 1993: 12–33.

Address for correspondence: Vishnu Chaturvedi, Mycology Laboratory, Wadsworth Center, NYSDOH, 120 New Scotland Ave., Albany, NY, 12208 - 2002, USA
Phone: +1-518-474-4177; Fax: +1-518-486-7811
E-mail: vishnu@wadsworth.org