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Influence of DMSO on antifungal activity during susceptibility testing in vitro

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ABSTRACT

Dimethylsulfoxide (DMSO) binds within the plasma membrane of cells and increases membrane permeability. DMSO is used in antifungal (AF) susceptibility assays for water insoluble agents. DMSO was observed to cause *Candida albicans* to express enhanced, diminished, or no change in growth compared to control medium, suggesting DMSO could influence the efficacy of water-insoluble AF agents. The activity of 4 water soluble AF agents against 6 yeast species was tested under conditions similar to the CSLI M27-A3 method. Growth response to 0.5% and 1% DMSO was variable. In 15 of 67 AF assays, DMSO resulted in a different MIC-2 (substantial inhibition of growth) value compared to control. Two of these involved differences of two doubling dilutions. The results imply that, in some cases of water-insoluble AF drug—yeast combinations, the MIC-2 may be skewed from the more clinically relevant MIC, providing one reason for possible discordance between MIC results and clinical outcomes.

1. Introduction

The ability to determine the relative susceptibility of fungi to antifungal agents has been made possible by the development of standard reference methods (CLSI, 2008a; CLSI, 2008b; CLSI, 2008c; Evan et al., 1985). While initially only available for fluconazole against *Candida* species, the standards have now been expanded to include echinocandins, other triazoles, and other antifungal agents, and for testing moulds including the dermatophytes (CLSI, 2008a; CLSI, 2008b; CLSI, 2008c). Depending on the antifungal agent and the organism, susceptibility is based on minimal concentration causing complete inhibition of growth (MIC-0), substantial inhibition of growth (MIC-2), or the minimal effective concentration.

Preparation of stocks and intermediate stocks for the assays differs based on whether the drug is water soluble or water insoluble. In the case of water-insoluble drugs, the current reference methods indicate that those drugs should be prepared in DMSO and that the final concentration of DMSO in the final test solution should be 1% (v/v) for the Clinical and Laboratory Standards Institute (CLSI) standard and 0.5% for the European Union Committee for Antimicrobial Susceptibility Testing (EUCAST) standard (CLSI, 2008a; CLSI, 2008b; CLSI, 2008c; Evan et al., 1985). The growth control wells or tubes also contain the same concentration of DMSO.

The choice of DMSO over other solvents for the susceptibility assays has an unclear history, although DMSO offers relative convenience because it can be used for all the water-insoluble drugs that are currently described in CLSI documents M27-A3 and M38-A2. Only a few studies have investigated whether DMSO affects the growth of pathogenic fungi, but none was conducted under the conditions used for the CLSI or EUCAST assays (Oh et al., 1995; Pottz et al., 1967; Randhawa, 2006, 2008; Rodriguez-Tudela et al., 2001).

I noted that during the course of other investigations that, when compared to growth in DMSO-naïve conditions, low concentrations (<4%) of DMSO variably affected the growth of *C. albicans* isolates (K. C. Hazen, in preparation). That is, DMSO caused cells to grow at similar, higher, or lower rates than cells in medium without DMSO. These observations suggested that DMSO could potentiate or lessen the inhibitory activity of water-insoluble drugs in the standard susceptibility assays. This possibility is investigated here by comparing MIC results and growth turbidity of various *Candida* species exposed to 4 water soluble drugs in the presence and absence of 1% DMSO.

2. Materials and methods

2.1. Yeast strains

Two to 3 strains of each of the following *Candida* species were used in this study: *C. albicans, C. dubliniensis, C. glabrata, C. parapsilosis, C. orthopsilosis*, and *C. tropicalis.* The strains were originally obtained as clinical isolates from clinical microbiology laboratories in Louisiana and Virginia.

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2.2. Growth conditions

Isolates were stored as either room temperature water stocks or frozen at −80 °C. In preparation for experimentation, 3–5 μL of the stock suspension was dispensed onto a Sabouraud glucose agar (SGA) plate and the plate was incubated at 35 °C for 24 to 48 h in a humidified incubator. These dot cultures were used to start first transfer cultures on SGA agar plates which were incubated at 35 °C for 22 to 25 h. The first transfer cultures were subcultured onto SGA and the second transfers, which served as the inoculum for the DMSO and drug assays, were incubated under the same conditions as the first transfer cultures. For the first and second transfer cultures, the SGA plates were inoculated in a manner to result in isolated colonies.

2.3. Preparation of inoculum for assays

Colonies from second transfer growth plates were suspended in 3 mL of RPMI 1640 containing 0.2% glucose (w/v) and buffered with morpholinepropanesulfonic acid (herein designated as RPMI) to achieve a visual turbidity equivalent to a McFarland 0.5 turbidity standard (CLSI, 2008a; CLSI, 2008b; CLSI, 2008c). The suspension was diluted 1:50, and this dilution was further diluted 1:20 in medium with and without 2% DMSO to achieve a total dilution of 1:1000. Once diluted, the cell suspension was used within 15 min to inoculate microwell plates.

2.4. DMSO titer assay plates

DMSO (Alfa Aesar, Ward Hill, MA, USA) was added to RPMI to achieve concentrations between16% and 0.25% (v/v) inclusive. Sterile deionized water (diH_2O) was used to compensate for decreased volume of DMSO necessary to achieve the concentrations below 8% and ensure the RPMI 1640 in each test solution was similarly diluted. The positive growth control medium also received water. For DMSO, 100 μL of each solution was added to designated wells of 96-well microtiter plates (U-shaped bottom, Corning plate #3755, Corning, NY, USA). The plate sides were sealed with an air permeable sealing film (Parafilm M, Pechiney Plastic Packaging, Chicago, IL, USA), and each plate was returned to its original packaging which was then closed with tape and placed in a −20 °C freezer. Plates were used within 4 weeks of their preparation. The EtOH titer plates were prepared similarly to the DMSO plates except that they were used within 2 h of preparation.

2.5. Antifungal agents

Four water soluble drugs were used: fluconazole (FLUC, MP Biomedicals, Santa Ana, CA, USA), 5-fluorocytosine (5-FC, Sigma, St. Louis, MO, USA), nikkomycin Z (NKZ, Sigma), and micafungin (MFN, AstellasPharma US, Deerfield, IL, USA). Terbinafine (TRB), a water-insoluble drug, was also included. All antifungal agents, except MFN, were obtained as pure powders. The pharmaceutical formulation of MFN (Mycamine®) was obtained from the University of Virginia Health Systems pharmacy. This formulation contains 200 mg of lactose for each 100 mg of MFN. Previous studies demonstrated that the presence of lactose did not affect the MIC results of the antifungal susceptibility assays (K.C. Hazen, unpublished results). Stock solutions of FLUC, 5-FC, NKZ, and MFN were prepared in diH_2O. The TRB stocks were prepared in DMSO and in ethanol (EtOH, Aaper Alcohol and Chemical, Shelbyville, KY, USA). The concentration of the FLUC, 5-FC, MFN, and TRB stocks was 5120 μg/mL, and that of the NKZ stock was 5000 μg/mL. Twenty-five-microliter aliquots (i.e., total drug amount of 128 μg/aliquot) of the MFN, FLUC, and 5-FC stock solutions and 40-μL aliquots of NKZ (200 μg/aliquot) were prepared and frozen at −20 °C.

2.6. Antifungal plates

Antifungal plates containing 2× the final drug concentration in each well were prepared no more than 4 weeks prior to testing. Drugs were diluted in RPMI or RPMI containing 2% DMSO or EtOH (for TRB) to achieve a concentration range of 64–0.125 μg/mL for FLUC and 5-FC, 64–0.25 μg/mL for TRB, 80–0.156 μg/mL for NKZ, and 2–0.0039 μg/mL for MFN. One hundred microliters of drug solution and RPMI alone (for negative and positive growth controls) or RPMI with 2% DMSO or EtOH was transferred to designated wells of microtiter plates. The plates were sealed as described above and frozen until use at −20 °C.

2.7. Microbroth dilution assay

Frozen drug plates were allowed to thaw for 30 to 60 min prior to inoculation with organism. Diluted organism suspensions (100 μL) were added to each drug and positive growth control well. The medium used to suspend cells was also added to each negative control well. Each organism–drug concentration with and without DMSO or EtOH was tested in triplicate wells. The plates were placed into a light-tight humidified incubator set at 35 °C and incubated for 23–25 h for MFN, FLUC, and NKZ, unless growth in the controls was insufficient, in which case the plates were incubated for 46–48 h. All 5-FC plates were incubated for 46–48 h.

2.8. Assessment of MIC-0 and MIC-2

After incubation, the cell pellets in each well were scored on a 0 to 4+ scale with growth in the positive growth control well always representing 4+ growth (CLSI, 2008a; CLSI, 2008b; CLSI, 2008c). Once scored, the cells in each well were suspended by biologic trituration (4–6 cycles of withdrawing the medium into a micropipette and dispensing back into the well). To minimize the time between biologic trituration of each well, a multichannel pipette was used. Plates were immediately scanned by a multiwell plate reader (Model 550, Bio-Rad, Hercules, CA, USA) at $\lambda = 595$ nm and the resulting absorbances were stored in an Excel spreadsheet (Microsoft, Redmond, WA, USA). MIC-0 and MIC-2 were determined as described in Clinical and Laboratory Standards Institute (CLSI, 2008a). A change in growth was recorded when the average absorbance ± SD of the DMSO or EtOH wells did not overlap with average absorbance ± SD of the respective wells without DMSO or EtOH.

3. Results and discussion

3.1. Effect of DMSO on growth

DMSO was added to RPMI 1640 to achieve a concentration range of 0.125% to 4% (v/v). Of the 14 isolates tested, 10 (71%) demonstrated reduced growth when exposed to 1% DMSO and 9 of these were also affected by 0.5% DMSO (Table 1). Five of 14 isolates showed reduced growth in medium containing only 0.125% DMSO. These included both *C. tropicalis* isolates, 2 of 3 *C. orthopsilosis* isolates, and 1 of 2 *C. dubliniensis* isolates (strain Y2.850).

Sixty-seven MIC assays were performed in which the yeast isolates were tested in the presence and absence of 1% DMSO (representing the positive growth controls). Unlike the DMSO titer experiments, 1% DMSO enhanced growth of yeasts in 28 instances. One strain each of *C. albicans, C. parapsilosis*, and *C. tropicalis* consistently demonstrated enhanced growth by DMSO in each MIC assay. The range of increase for the 28 occurrences was 5.3% to 91.7% (average 28.7, SD 18.8%). Decreased growth occurred in 9 instances with a range of 5.3% to 23.6% (average 10.6, SD 5.6%). In general, increases or decreases in growth of 20% or more were visually evident in the size of the cell buttons.