Influence of Culture Medium on Susceptibility Testing with BAY n 7133 and Ketoconazole

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The effect of four culture media (two complex and undefined [Sabouraud glucose and Kimmig] and two synthetic and defined [synthetic amino acid medium, fungal, and modified yeast nitrogen base]) on the activity in vitro of two newer azole compounds (BAY n 7133 and ketoconazole) was assessed with five strains each of Candida albicans, Candida parapsilosis, and Cryptococcus neoformans. Also, the nutritional adequacy of the four media was evaluated with the same 15 strains of yeasts while the MICs of BAY n 7133 were higher in the complex media, the activity of ketoconazole was little affected. The Candida spp. grew least well and the C. neoformans grew best in yeast nitrogen base.

Previously, we showed that five kinds of yeasts grew with less susceptibility to inhibition by two imidazole derivatives, clotrimazole and miconazole, when tested in complex, undefined media than in synthetic, defined media (6, 7). We now report additional observations on the effect of culture media on the results of susceptibility testing ofazole antifungal antimicrobial agents. We assessed (i) the influence of four culture media (two complex and undefined, i.e., Sabouraud glucose medium [SAB] and Kimmig medium [KIM], and two synthetic and defined, i.e., synthetic amino acid medium, fungal [SAAMF], and modified yeast nitrogen base [YNB]) on the susceptibility in vitro of three kinds of yeastlike fungi against two newer azole derivatives and (ii) the nutritional adequacy of the four culture media for these yeastlike fungi. The latter is of concern because the antifungal activity of a drug might be exaggerated by testing in a nutritionally suboptimal milieu.

MATERIALS AND METHODS

Antifungal azoles. BAY n 7133 (B33), 1-(4-chlorophenoxyl)-3,3-dimethyl-2-(1,2,4-triazole-1-yl) methylbutan-2-01, an investigational triazole compound with a molecular weight of 309.5, is insoluble in water; it was kindly provided by George Arcieri, Miles Pharmaceuticals, West Haven, Conn. Stock solutions were prepared by dissolving 0.5 g of B33 in 40 ml of dimethylformamide (Spectramalyzed reagent; Fisher Scientific Co., Fair Lawn, N.J.) and diluting the solution to 100 ml in distilled water.

Ketoconazole, an established antifungal imidazole derivative with a molecular weight of 531.4, is relatively soluble in water (11); it was kindly supplied by Roger Aspelling, Janssen Pharmaceutica, Piscataway, N.J. Stock solutions were prepared by dissolving 0.5 g of ketoconazole in 4 to 5 ml of 0.1 M NaCl and diluting the solution to 25 ml with distilled water.

The stock solutions were filtered through membranes (0.45 μm, average pore size; Millipore Corp., Cambridge, Mass.). Working dilutions of 80 nmol/ml were prepared with sterile distilled water. Series of snap-topped, polycarbonate, tubes (11 by 75 mm; BD Labware, Oxnard, Calif.) were prepared that contained 0.1-ml portions of doubling dilutions in the four media, providing concentrations from 0.07 to 80 nmol, i.e., 0.022 to 24.760 μg/ml for B33 and 0.037 to 42.512 μg/ml for ketoconazole. The tubes were stored at −40°C until used.

Culture media. Of the two undefined media, SAB was purchased (Difco Laboratories, Detroit, Mich.), and KIM (9) was formulated from commercially available ingredients (per liter: 13 g of nutrient broth base [Difco]. 8.6 g of Bacto-Peptone [Difco], 9 g of sodium chloride [Sigma Chemical Co., St. Louis, Mo.], 5 g of glycerol [Sigma], and 10 g of glucose [Difco]).

Of the two synthetic, defined media, SAAMF (8) was purchased as a dry powder from Gibco Laboratories, Grand Island, N.Y. YNB (10) was compounded from the commercial base product (Difco) plus additions as described by Shadomy (10).

Yeastslike fungi. Originally isolated from patient specimens submitted to the Clinical Microbiology Laboratory at the University of California Davis Medical Center, five strains each of Candida albicans, Candida parapsilosis, and Cryptococcus neoformans were stored at 4°C on Sabouraud glucose agar slants. For testing, each isolate was grown out in 10 ml of liquid SAAMF at 35°C, for 24 h for the Candida spp. and for 48 h for C. neoformans. After collection by centrifugation, one wash in 10 ml of sterile 0.9% NaCl solution, and suspension in 5 ml of sterile distilled water (Vortex mixer), dilutions of the fungal cells in Gram iodine were counted in a hemacytometer. A dilution of 10^5 cells per ml was then prepared in sterile distilled water.

Susceptibility testing. MICs and minimal lethal concentrations were determined as described previously (6). Briefly, to each tube containing 0.1-ml portions of either dilution of the antimicrobial agents or drug-free media was added 0.9 ml of culture medium that contained either no inoculum or 10^4 fungal cells. After being mixed, the contents of the tubes were incubated without further agitation at 35°C. MICs were read after 24 h for the Candida spp. and after 48 h for the C. neoformans. Interpretation of MICs was based on comparison of growth in drug-free (positive) controls, inoculum-free (negative) controls, and the tests. At the time of the MIC readings, 0.1-ml portions were removed (after mixing) from tubes without apparent growth and spread on one half of

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TABLE 1. Geometric means and ranges of MIC of B33 and ketoconazole against five strains each of C. albicans, C. parapsilosis, and C. neoformans

<table>
<thead>
<tr>
<th>Medium</th>
<th>C. albicans</th>
<th>C. parapsilosis</th>
<th>C. neoformans</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B33</td>
<td>KET*</td>
<td>B33</td>
</tr>
<tr>
<td>SAB</td>
<td>0.80</td>
<td>0.07</td>
<td>5.74</td>
</tr>
<tr>
<td>(0.06-1.25)</td>
<td>(0.07)</td>
<td>(5-10)</td>
<td>(0.075-0.30)</td>
</tr>
<tr>
<td>KIM</td>
<td>0.34</td>
<td>0.07</td>
<td>3.40</td>
</tr>
<tr>
<td>(0.3-0.6)</td>
<td>(0.07)</td>
<td>(1.25-5.00)</td>
<td>(0.075-0.30)</td>
</tr>
<tr>
<td>SAAMF</td>
<td>0.30</td>
<td>0.07</td>
<td>2.14</td>
</tr>
<tr>
<td>(0.30)</td>
<td>(0.07)</td>
<td>(0.6-10.0)</td>
<td>(0.075-0.30)</td>
</tr>
<tr>
<td>YNB</td>
<td>0.13</td>
<td>0.07</td>
<td>1.22</td>
</tr>
<tr>
<td>(0.075-0.30)</td>
<td>(0.07)</td>
<td>(0.075-5.00)</td>
<td>(0.075-0.30)</td>
</tr>
</tbody>
</table>

*Triplicate determinations. Tube dilution tests were carried out in SAB, KIM, SAAMF, and YNB.

RESULTS

The geometric mean MICs and ranges of test values are given in Table 1. With B33, there was a wide variation of MICs in the different media: for Candida spp., SAB > KIM > SAAMF > YNB; for C. neoformans, YNB > SAAMF > SAB > KIM. The MICs of ketoconazole against Candida spp. were little affected by the medium of testing, but with C. neoformans, YNB > (SAAMF = SAB) > KIM. Neither B33 nor ketoconazole exerted a lethal effect at any concentration tested against any of the 15 strains of yeastlike fungi.

The arithmetic means of the CFU per milliliter are plotted as a function of time of incubation in Fig. 1, 2, and 3 (respectively, C. albicans, C. parapsilosis, and C. neoformans). There were no significant differences from one me-
dium to another in the CFU per milliliter at the terminal observations (24 h for Candida spp., 52 h for C. neoformans). However, the growth of Candida spp. in YNB lagged from 4 to <24 h; the other three media were indistinguishable. In contrast, C. neoformans did best in YNB and worst in SAB, with SAAMF and KIM indistinguishable in a middle ground.

**DISCUSSION**

By tube dilution, we found that the susceptibility of three kinds of yeastlike fungi to inhibition by ketoconazole was little affected by culture media, whereas the MICs of B33 were greatly influenced, as occurred with clotrimazole and miconazole (6). Perhaps the difference we observed is related to the finding of Beggs (2, 3) that the mechanism of activity of ketoconazole against C. albicans and C. parapsilosis differs from that of other azoles such as miconazole. However, Ahearn and McGlohn (1) reported higher MICs in YNB and SAB than in SAAMF with miconazole and ketoconazole. Their experimental conditions were different from ours; they used a microtiter system with recording of MICs after 48 h of incubation, comparing results with 22 strains of Candida spp. other than C. albicans and C. parapsilosis.

For valid testing of susceptibility in vitro, the culture medium must be free of antagonists to the drugs under study and must also be nutritionally adequate. While YNB and SAAMF generally fulfill the first requirement, we were concerned about nutritional adequacy with YNB. By casual observation, the growth of occasional strains of fungi has appeared to be slow and meager in YNB. Hence, we were not surprised to find that our 10 strains of Candida spp. grew less well in YNB than in the other media from 4 to between 12 and 24 h after inoculation. However, with C. neoformans, the situation was reversed; YNB supported better growth than the other media from 6 to 40 h after inoculation; a phenomenon for which we have no explanation.

In YNB buffered with any one of four systems, Calhoun and Galgiani (4) found that the doubling time for C. albicans was 6 h, whereas it was 2.5 h in unbuffered YNB and in SAAMF. Because we used YNB buffered with phosphate (a system also examined by Calhoun and Galgiani), it may be that the slower rates of growth of our Candida spp. in YNB reflected a similar phenomenon. There was a correlation in lower MICs of B33 in YNB than in SAAMF (Table 1), but inexplicably, there was no such effect with ketoconazole.

Taking into account both our MIC and CFU data, SAAMF may be preferable for susceptibility testing in vitro with Candida spp. and YNB may be preferable with C. neoformans.

**LITERATURE CITED**