Inhibition of \textit{Candida parapsilosis} Mitochondrial Respiratory Pathways Enhances Susceptibility to Caspofungin

Georgios Chamilos, \textsuperscript{1} Russell E. Lewis, \textsuperscript{1,2} and Dimitrios P. Kontoyiannis\textsuperscript{1,2}\textsuperscript{*}

\textsuperscript{1}Department of Infectious Diseases, Infection Control and Employee Health, The University of Texas M. D. Anderson Cancer Center, Houston, Texas, and \textsuperscript{2}University of Houston College of Pharmacy, Houston, Texas

Received 11 August 2005/Returned for modification 10 October 2005/Accepted 19 October 2005

Among the \textit{Candida} species, \textit{Candida parapsilosis} has a unique mitochondrial respiratory network. The addition of inhibitors of the respiratory pathways in three clinical isolates of \textit{C. parapsilosis} with high (\(\geq 2\) \(\mu\)g/ml) MICs of caspofungin significantly (fivefold) decreased caspofungin MICs but did not change fluconazole MICs.

The echinocandins are a new class of antifungal agents that target the fungal cell wall by inhibiting 1,3-\(\beta\)-D-glucan synthetase (3). Caspofungin (CAS), the first licensed echinocandin, possesses broad-spectrum activity against \textit{Candida} and \textit{Aspergillus} species. Because CAS is fungicidal against \textit{Candida} species, including most azole-resistant non-\textit{Candida albicans} species, it has become a preferred drug for invasive candidiasis (11).

\textit{Candida parapsilosis} is an important cause of neonatal and device-related infections (2, 11). In contrast to other \textit{Candida} spp., \textit{C. parapsilosis} often exhibits reduced susceptibility to CAS in vitro (8, 12). In addition, failure of CAS in the treatment of \textit{C. parapsilosis} infections has been reported previously (8). The mechanisms of the suboptimal efficacy of CAS against this species are unknown.

Interestingly, \textit{C. parapsilosis} displays natural resistance to a wide range of toxic agents (e.g., oligomycin and paromomycin), which has been largely attributed to its unique electron flux pathways (1, 7). Specifically, in contrast with the other \textit{Candida} spp., which have a classical respiratory chain (CRC), \textit{C. parapsilosis} also has two alternative respiratory electron flux pathways (1, 7): a cyanide-resistant alternative oxidase (AOX) branched with the CRC at the level of ubiquinone (Q) and a parallel respiratory chain (PAR) secondary to the CRC (Fig. 1). These two alternative respiratory pathways are capable of driving electrons to O\textsubscript{2} from both Krebs cycle and cytosolic NADH with cross talk between these pathways and the CRC (7). Notably, both AOX and PAR are insensitive to classical mitochondrial inhibitors such as antimycin A (AA) but can be inhibited by benzohydroxamate (BHAM) and cyanide at high concentrations (7).

We hypothesized that the \textit{C. parapsilosis} complex respiratory network may account for its decreased susceptibility to CAS. To that end, we inhibited the classical and alternative respiratory pathways of different clinical isolates of \textit{C. parapsilosis} and examined the effects on CAS and fluconazole (FLC; control drug) MICs. We observed a profound decrease in CAS MICs after simultaneous inhibition of all respiratory pathways in all isolates tested, whereas FLC MICs remained unchanged.

We tested three clinical isolates of \textit{C. parapsilosis} with high CAS MICs (\(\geq 2\) \(\mu\)g/ml) collected from patients with cancer and hematogenous candidiasis. We obtained FLC (Pfizer Inc., New York, N.Y.), AA (Sigma Chemical Co., St. Louis, Mo.), and BHAM (Sigma Chemical Co.) powder. We prepared drug stock solutions in distilled water (CAS and FLC, 1.28 mg/ml) or 100% methanol (AA, 10 mM; BHAM, 1 M) and stored them at \(-80^\circ\text{C}\) until use.

We determined the CAS and FLC MICs for each \textit{C. parapsilosis} isolate according to Clinical and Laboratory Standards Institute (CLSI)-approved document M27-A2 (9). We used standard RPMI 1640 medium as well as yeast-peptone-dextrose and yeast nitrogen base media (Difco, Detroit, MI). We performed susceptibility testing with microtitration plates (Corning, New York) containing serial twofold dilutions of FLC (0.06 to 64.00 \(\mu\)g/ml) and CAS (0.03 to 32.00 \(\mu\)g/ml) and a final inoculum of \(1 \times 10^3\) to \(5 \times 10^3\) CFU/ml of each isolate. We determined the CAS and FLC MICs visually and spectrophotometrically 24 and 48 h after a shaking incubation at \(35^\circ\text{C}\) as the lowest concentrations that resulted in a prominent (50%) decrease in turbidity for both drugs (9, 10). We next prepared microtitration plates for CAS and FLC as described above and added a standard concentration of either AA (2 \(\mu\)M) or BHAM (4 \(\mu\)M) to each well to selectively inhibit the classical and alternative respiratory pathways, respectively, of \textit{C. parapsilosis} (7). We added standard concentrations of AA (2 \(\mu\)M) plus BHAM (4 \(\mu\)M) to each well to simultaneously inhibit the respiratory pathways.

We also performed 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT)-based microdilution studies as described previously (6). For the XTT colorimetric assay, we prepared CLSI microtitration plates containing serial twofold dilutions of CAS, of FLC, and of both drugs in combination with either AA (2 \(\mu\)M), BHAM (4 \(\mu\)M), or AA (2 \(\mu\)M) plus BHAM (4 \(\mu\)M) at a standard concentration as described above. In pilot experiments, the combination of AA plus BHAM at higher concentrations did not alter the ability of XTT to be reduced into its colorimetric formazan derivatives (data not shown). We assessed formazan absorbance at 492...
and 690 nm (plate absorbance) by using a microplate spectrophotometer (Powerwave X; Biotech Instruments, Winooski, Vt.) and determined the change in optical density at 492 nm (6).

Additionally, we performed disk diffusion susceptibility testing of CAS against each C. parapsilosis isolate in RPMI agar plates and RPMI plates containing a standard noninhibitory concentration of AA or BHAM (1 μM or 2 mM, respectively) defined in pilot experiments. Briefly, we plated 200 μl of 10⁶-CFU/ml inoculum of each isolate, allowed the plates to dry, and then placed a sterile 1/4-inch paper disk (Schleicher and Schuell, Keene, N.H.) on the agar surface and inoculated it with 25 μl of CAS (from a stock solution of 1 mg/ml), producing a final concentration of 2 μg/ml in each plate. We incubated plates at 30°C and measured the radii of the zones of inhibition with a micrometer at 48 h. We accordingly assessed the effect of mitochondrial inhibitors on CAS activity against C. parapsilosis isolates by statistically comparing the changes in the CAS radii of the zones of inhibition.

We performed all experiments in triplicate on different days using C. parapsilosis strain ATCC 20199 as a quality control. For statistical comparisons, we used the Mann-Whitney U test where appropriate. We fitted a four-parameter logistic regression model (Hill equation) to XTT reduction data and calculated 50% effective doses by using a curve-fitting software program (Prism 4; GraphPad Software, Inc., San Diego, Calif.). We considered P values of less than 0.05 to be statistically significant.

All C. parapsilosis isolates exhibited significantly elevated CAS MICs (mean MIC₅₀, 2 μg/ml; range, 1 to 2 μg/ml) in all media. All C. parapsilosis isolates but one were susceptible to FLC (mean MIC₅₀, 0.5 μg/ml; range, 0.5 to 64.0 μg/ml). There were no significant differences in CAS or FLC MICs among the media; MICs were equal at 24 and 48 h (data not shown).

Importantly, mitochondrial inhibitors (AA and BHAM) had no effect on the growth of test isolates at the concentrations used. We found that inhibition of either the classical (with AA) or the alternative (with BHAM) mitochondrial pathway of C. parapsilosis isolates did not significantly affect CAS or FLC MICs (Table 1). However, simultaneous inhibition of all mitochondrial pathways by BHAM plus AA resulted in a profound fivefold drop in CAS MICs for all isolates (MIC₅₀, 0.06 μg/ml).

For statistical comparisons, we used the Mann-Whitney U test where appropriate. We fitted a four-parameter logistic regression model (Hill equation) to XTT reduction data and calculated 50% effective doses by using a curve-fitting software program (Prism 4; GraphPad Software, Inc., San Diego, Calif.). We considered P values of less than 0.05 to be statistically significant.

**TABLE 1. Effects of the specific mitochondrial inhibitors AA and BHAM at inhibitory concentrations on CAS and FLC MICs against three clinical isolates of C. parapsilosis in RPMI medium**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Median MIC₅₀ (μg/ml) of the following drug with:</th>
<th>No inhibitor</th>
<th>AA (2 μM)</th>
<th>BHAM (4 mM)</th>
<th>AA + BHAM (2 μM + 4 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAS</td>
<td>FLC</td>
<td>CAS</td>
<td>FLC</td>
<td>CAS</td>
</tr>
<tr>
<td>C. parapsilosis 2537</td>
<td>2</td>
<td>&gt;64.0</td>
<td>2</td>
<td>32.0</td>
<td>2</td>
</tr>
<tr>
<td>C. parapsilosis 2582</td>
<td>2</td>
<td>0.5</td>
<td>2</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>C. parapsilosis 2587</td>
<td>2</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>2</td>
</tr>
</tbody>
</table>

* CLSI broth microdilution method M27-A2.

b The range of MICs was less than 1 dilution in all experiments.
μg/ml; \( P < 0.0001 \)) but did not change FLC MICs; similar decreases in CAS MICs were observed by using an MIC endpoint of 100% inhibition of growth (data not shown). The effects of the mitochondrial inhibitors on CAS MICs were medium independent (data not shown) and further confirmed in XTT-based microdilution studies (Fig. 2). Additionally, the mean ± standard deviation radius of the zone of inhibition for CAS (8.286 ± 1.069 mm) increased significantly in the presence of both mitochondrial inhibitors (16.4300 ± 0.9376 mm; \( P = 0.002 \)) (Fig. 3).

Although susceptibility breakpoints for CAS have not been established, a provisional MIC of 1 μg/ml was proposed recently (3). We demonstrated that whereas inhibition of each of the mitochondrial pathways of \( C. \) parapsilosis had no effect on susceptibility to CAS, simultaneous inhibition of all mitochondrial pathways dramatically lowered CAS MICs but not FLC MICs. These results were also shown by the XTT assay, a viability staining method that precisely assesses the fungal biomass and is a marker for mitochondrial activity, as it is based on the reduction of XTT by mitochondrial dehydrogenase (6). Finally, our findings were confirmed by another independent disk diffusion susceptibility method.

Although rare, reports of CAS-resistant \( C. \) albidus have begun to emerge (4). The mechanisms of \( C. \) albidus resistance to echinocandins remain obscure. In vitro resistance in laboratory-selected \( C. \) albidus mutants has been associated with reduced glucan synthetase activity (5). Of interest is the fact that mitochondria have not been shown to be involved in resistance of \( C. \) albidus or other pathogenic fungi to echinocandins. Nevertheless, the ability of \( C. \) parapsilosis respiratory pathways to resist oxidative stress has long been encountered with resistance to a variety of antimicrobial agents (1). Our work implies that the unique respiratory network of \( C. \) parapsilosis partially accounts for its reduced resistance to CAS. It is tempting to speculate that the inhibition of cell wall synthesis might be associated with increased susceptibility to oxidative stress more than the inhibition of other important cellular components (ergosterol synthesis) is. However, other mechanisms of resistance (e.g., altered glucan synthetase activity)

\[ \text{Fig. 2. XTT-based analysis of in vitro activities of CAS alone (solid} \] \[ \text{lines) and of CAS in combination with the mitochondrial inhibitors AA}\] \[ \text{and BHAM at standard concentrations (2 μM and 4 mM, respectively; dotted} \] \[ \text{lines) in RPMI medium against three clinical isolates of} \] \[ \text{C. parapsilosis. Sigmoid concentration inhibitory-effect curves were generated by} \] \[ \text{fitting data to a four-parameter logistic regression model (Hill} \] \[ \text{equation). The symbols represent the means ± standard deviations from} \] \[ \text{experiments performed in triplicate in each case. ΔOD492, optical density} \] \[ \text{at 492 nm.} \]

\[ \text{Fig. 3. Effects of the mitochondrial inhibitors AA (1 μM), BHAM}\] \[ \text{(2 mM), and AA plus BHAM (1 μM and 2 mM, respectively) on CAS}\] \[ \text{activity against a representative clinical isolate of} \] \[ \text{C. parapsilosis (2537) as seen in disk diffusion susceptibility testing. Each disk} \] \[ \text{contained 25 μl of CAS, resulting in a final CAS concentration in each plate of} \] \[ \text{2 μg/ml from a stock solution of 1 mg/ml.} \]
might also be involved in the decreased susceptibility of \( \text{C. parapsilosis} \) to CAS. To that end, our preliminary observations must be explored further in studies measuring specific aspects of oxidative burst associated with sequential or simultaneous inhibition of the different respiratory pathways of \( \text{C. parapsilosis} \) in response to CAS.

We thank Nathaniel D. Albert for excellent technical assistance and Don Norwood for editorial assistance.

This study was supported by The University of Texas M. D. Anderson Cancer Center Faculty E. N. Cobb Scholar Award Research Endowment (D.P.K.).

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