

## Inhibition of *Candida parapsilosis* Mitochondrial Respiratory Pathways Enhances Susceptibility to Caspofungin

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**Among the *Candida* species, *Candida parapsilosis* has a unique mitochondrial respiratory network. The addition of inhibitors of the respiratory pathways in three clinical isolates of *C. parapsilosis* with high ( $\geq 2$ - $\mu\text{g/ml}$ ) 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 *Candida* and *Aspergillus* species. Because CAS is fungicidal against *Candida* species, including most azole-resistant non-*Candida albicans* species, it has become a preferred drug for invasive candidiasis (11).

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

Interestingly, *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 *Candida* spp., which have a classical respiratory chain (CRC), *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<sub>2</sub> 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 *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 *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 *C. parapsilosis* with high CAS MICs ( $\geq 2$   $\mu\text{g/ml}$ ) collected from patients with cancer and hematogenous candidiasis. We obtained FLC (Pfizer Inc., New York, N.Y.), CAS (Merck, Rahway, N.J.), 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 *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  $\mu\text{g/ml}$ ) and CAS (0.03 to 32.00  $\mu\text{g/ml}$ ) 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  $\mu\text{M}$ ) or BHAM (4 mM) to each well to selectively inhibit the classical and alternative respiratory pathways, respectively, of *C. parapsilosis* (7). We added standard concentrations of AA (2  $\mu\text{M}$ ) plus BHAM (4 mM) 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  $\mu\text{M}$ ), BHAM (4 mM), or AA (2  $\mu\text{M}$ ) plus BHAM (4 mM) 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

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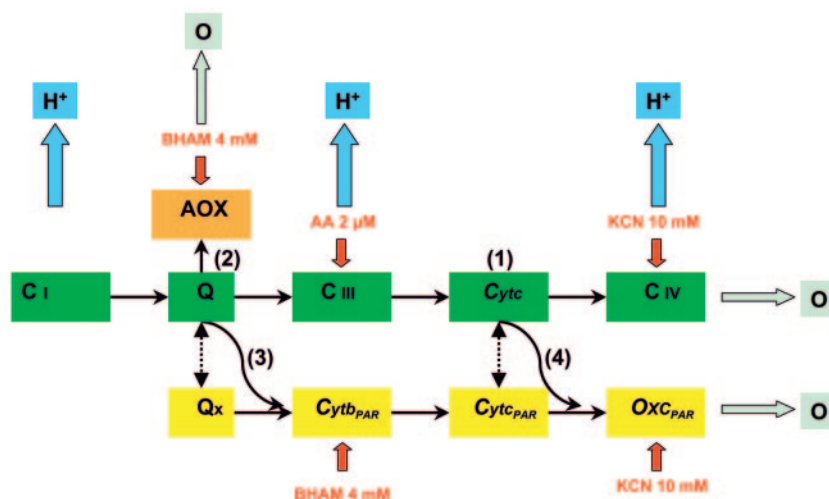


FIG. 1. Simplified figure (adapted from the work of Milani et al. [7]) of the respiratory network in *C. parapsilosis* mitochondria, which includes three terminal oxidases and four electron transport pathways. The CRC consists of Q, complex I (C I), complex III (C III), and complex IV (C IV; cytochrome *c* [Cyt<sub>c</sub>] oxidase), as shown in the green boxes. A secondary PAR comprises alternative ubiquinone (Qx), cytochrome *b* (Cyt<sub>b</sub> PAR), cytochrome *c* (Cyt<sub>c</sub> PAR), and secondary terminal oxidase (Ox<sub>c</sub> PAR) insensitive to AA but inhibited by BHAM and cyanide, as shown in the yellow boxes. A cyanide-resistant AOX branches at the Q level, as shown in the orange box. The numbers in parentheses show the four electron transport pathways, the blue arrows indicate sites of H<sup>+</sup> pumping, the red arrows indicate targets of the inhibitors (AA, BHAM, and cyanide), and the dotted lines depict putative interactions.

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 10<sup>6</sup>-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 MIC<sub>50</sub>, 2 μg/ml; range, 1 to 2 μg/ml) in all media. All *C. parapsilosis* isolates but one were susceptible to FLC (mean MIC<sub>50</sub>, 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 (MIC<sub>50</sub>, 0.06

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<sup>a</sup>

Isolate	Median MIC <sup>b</sup> (μg/ml) of the following drug with:							
	No inhibitor		AA (2 μM)		BHAM (4 mM)		AA + BHAM (2 μM + 4 mM)	
	CAS	FLC	CAS	FLC	CAS	FLC	CAS	FLC
<i>C. parapsilosis</i> 2537	2	>64.0	2	32.0	2	>64.0	0.06	32.00
<i>C. parapsilosis</i> 2582	2	0.5	2	0.5	1	0.5	0.06	0.25
<i>C. parapsilosis</i> 2587	2	0.5	1	0.5	2	0.5	0.06	0.50

<sup>a</sup> CLSI broth microdilution method M27-A2.

<sup>b</sup> The range of MICs was less than 1 dilution in all experiments.

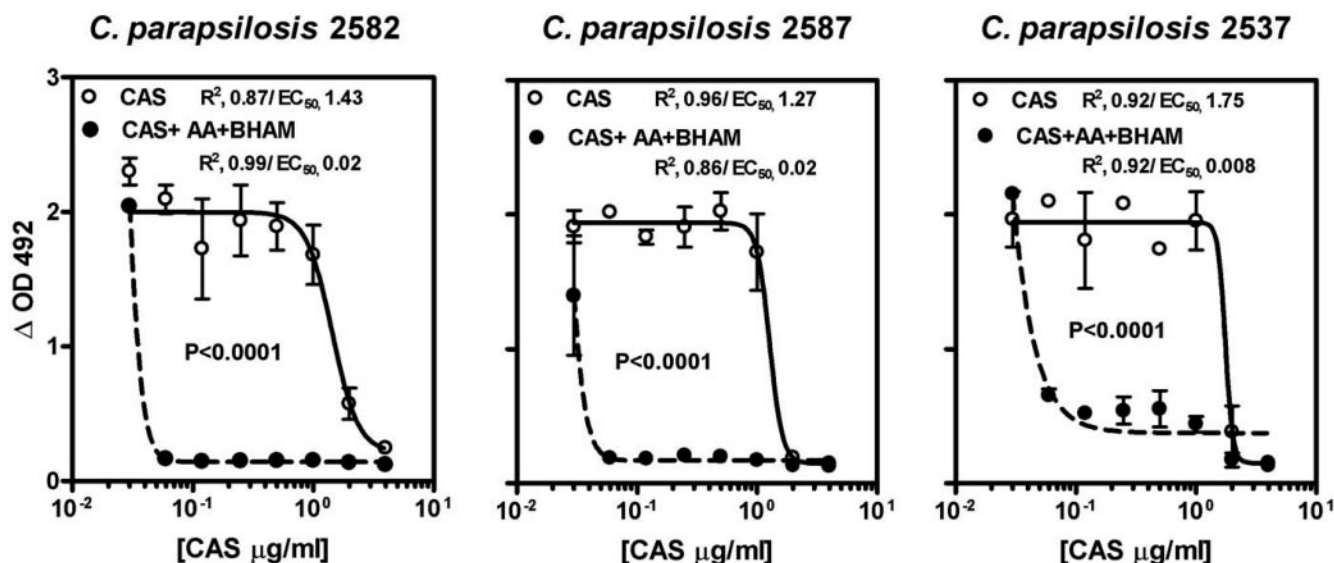


FIG. 2. XTT-based analysis of in vitro activities of CAS alone (solid lines) and of CAS in combination with the mitochondrial inhibitors AA and BHAM at standard concentrations (2  $\mu$ M and 4 mM, respectively; dotted lines) in RPMI medium against three clinical isolates of *C. parapsilosis*. Sigmoid concentration inhibitory-effect curves were generated by fitting data to a four-parameter logistic regression model (Hill equation). The symbols represent the means  $\pm$  standard deviations from experiments performed in triplicate in each case.  $\Delta$ OD492, optical density at 492 nm.

$\mu$ g/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  $\pm$  standard deviation radius of the zone of inhibition for CAS ( $8.286 \pm 1.069$  mm) increased significantly in the presence of both mitochondrial inhibitors ( $16.4300 \pm 0.9376$  mm;  $P = 0.002$ ) (Fig. 3).

Although susceptibility breakpoints for CAS have not been established, a provisional MIC of  $\geq 1$   $\mu$ g/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 *Candida* have begun to emerge (4). The mechanisms of *Candida* resistance to echinocandins remain obscure. In vitro resistance in laboratory-selected *Candida albicans* 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 *Candida* 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)

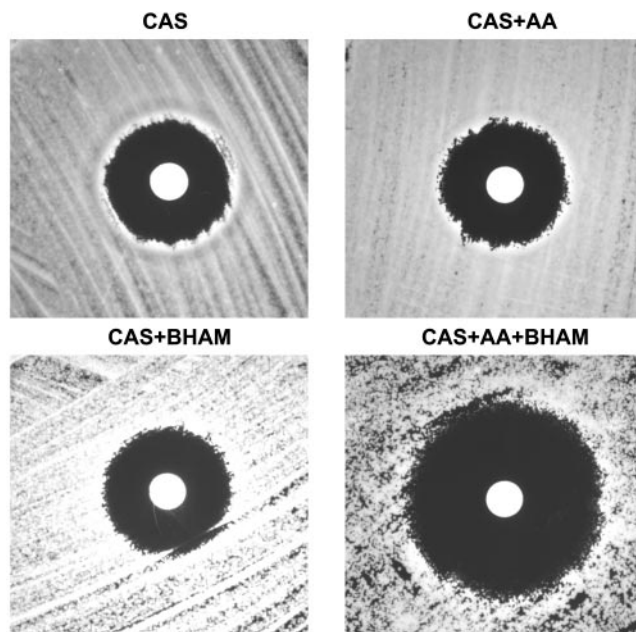


FIG. 3. Effects of the mitochondrial inhibitors AA (1  $\mu$ M), BHAM (2 mM), and AA plus BHAM (1  $\mu$ M and 2 mM, respectively) on CAS activity against a representative clinical isolate of *C. parapsilosis* (2537) as seen in disk diffusion susceptibility testing. Each disk contained 25  $\mu$ l of CAS, resulting in a final CAS concentration in each plate of 2  $\mu$ g/ml from a stock solution of 1 mg/ml.

might also be involved in the decreased susceptibility of *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 *C. parapsilosis* in response to CAS.

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