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Escape of *Candida* from Caspofungin Inhibition at Concentrations above the MIC (Paradoxical Effect) Accomplished by Increased Cell Wall Chitin; Evidence for β-1,6-Glucan Synthesis Inhibition by Caspofungin

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Concentrations above the MIC of caspofungin allow growth of some *Candida* isolates. A strain demonstrating paradoxical growth was grown in the presence and absence of caspofungin, and the cell wall content was analyzed. β-1,3-Glucan declined 81% in the presence of caspofungin, as expected. β-1,6-Glucan declined 73%. Chitin increased 898%, demonstrating a mechanism for paradoxical growth—a rapid shift in the key polymer.

The inhibition of fungi by caspofungin, an echinocandin, is attributed to interference with synthesis of the cell wall polymer β-1,3-glucan. The principal target of caspofungin is the protein Fks1, and binding leads to reduced activity of the enzyme glucan synthase and reduced formation of and extrusion of the glucan polymer through the cell membrane and into the wall (5). With *Candida* species, the result is a low MIC (typically <0.5 μg/ml), and fungicidal activity at or very close to the MIC (5). However, many isolates of *Candida* are able to grow at some caspofungin concentrations higher than the MIC (11), which we have termed the paradoxical effect. The frequency of this phenomenon is 21% of 76 *Candida albicans* isolates (4; D. A. Stevens, unpublished). The cause of the paradoxical growth was not mutations in resistance-associated regions of the β-1,3-glucan synthase complex, selection of a resistant subpopulation, or destruction of the drug, but the mechanism remains unelucidated (12). We proposed that the gene(s) is promptly activated by the presence of high caspofungin concentrations (11, 12), and speculation on the mechanism included "compensatory upregulation of synthesis of another wall component" (11).

A stimulus for the present studies was our observation (10) that an echinocandin and a chitin synthesis inhibitor acted in a highly synergistic manner in vitro, which was corroborated in vivo (3). This suggests that if one cell wall polymer is inhibited, the other might compensate, and that if both were blocked, the fungus would be defenseless.

The present studies examined cell wall content in an isolate (*C. albicans* strain 95-68) that we reported previously (11) as showing a prominent paradoxical effect. The paradoxical growth was compared to that in the absence of caspofungin.

Growth in the presence of high (12.5 μg/ml) concentrations of caspofungin and its absence was as described previously (11, 12). The cells were disrupted by >150 cycles of freezing and thawing followed by glass bead bombardment until no viable cells could be demonstrated by subculture, and the disruption was verified by microscopy (12). The cell walls were separated from cytosol and glass beads by centrifugation (12), and the walls were dried, lyophilized, and frozen. Two separate preparations were made, 7 months apart, in the presence and absence of caspofungin. Each preparation was studied, two or three 30-mg samples of each culture condition in each time period, and each sample was studied in duplicate. Student's *t* test was used for comparison of caspofungin with no caspofungin for each carbohydrate group analyzed.

Glucan and chitin contents were determined by published methods (2, 9) with several modifications. Each sample analyzed was a 10-mg quadruplicate sample. Lyophilized cell wall (≥20 mg) was extracted three times with 500 μl of 3% NaOH at 75°C for 1 h. The supernatants from NaOH extraction were added to 2 volumes of ethanol and incubated at −20°C for >1 h. After centrifugation at 15,000 rpm for 5 min at 4°C, precipitates were dissolved in water, and hexose contents were measured by the borosulfuric acid method (1) (alkali-soluble glucans). The NaOH extraction pellets were washed once with 100 mM Tris-HCl and 10 mM Tris-HCl (pH 7.5). The pellets were digested with 4 mg/ml Zymolyase 100T (prepared from *Arthrobacter luteus*, primarily β-1,3-glucan hydrolase; Seikagaku Corporation, Tokyo, Japan) in 10 mM Tris-HCl overnight at 37°C and centrifuged for 10 min at 15,000 rpm at 4°C. The supernatants were divided into two portions. One was used for measurement of hexose content (alkali-insoluble β-1,3- plus 1,6-glucans). The other was dialyzed against distilled water using a Spectra/Por membrane with a molecular weight exclusion of 6,000 to 8,000 (Spectrum Medical Industries, Inc., Rancho Dominguez, CA), and hexose contents were measured (alkali-insoluble β-1,6-glucans). Zymolyase-insoluble pellets were washed once with 10 mM Tris-HCl and water. The pellets were suspended in water and divided into two portions. One was used for measurement of hexose content (Zymolyase-indigestible pellets). The other was centrifuged, and the pellet was used for measurement of hex-

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TABLE 1. Glucan and chitin content of cell walls in the presence and absence of caspofungin[a]

Preparation	Presence of caspofungin	Hexose Content					
		Alkali insoluble				Alkali-soluble glucan	Chitin
		β-1,3-plus β-1,6-glucan	β-1,6-Glucan	β-1,3-Glucan	Zymolyase-indigestible pellet		
1	No	134.9 (8.7)	34.0 (5.4)	100.9	7.6 (1.2)	104.5 (12.4)	8.7 (0.7)
	Yes	8.0 (4.3)[b]	3.4 (0)[b]	4.6	5.5 (0.6)[c]	51.1 (3.7)[d]	98.8 (6.5)[b]
2	No	86.1 (6.3)	29.2 (2.8)	56.9	8.4 (1.4)	98.8 (7.2)	7.5 (5.3)
	Yes	31.5 (10.5)[b]	5.4 (5.4)[d]	26.1	5.9 (0.6)[c]	54.2 (2.8)[b]	46.7 (3.5)[b]

[a] Results are means (standard deviations) of quadruplicate assays in two separate experiments/row. The isolate was grown in the presence and absence of caspofungin twice (two separate preparations). Glucan and chitin contents are expressed as μg/mg dried cell wall. The hexose content of the Zymolyase-indigestible pellet is shown. The β-1,3-glucan value was derived by subtraction of β-1,6-glucan from the combination determined previously (14). Alkali-soluble glucan is almost completely mannoproteins, with small amounts of glucan (those glucans not cross-linked to chitin). *P* values compare data in the presence and absence of caspofungin.

[b] $P \leq 0.0001$.

[c] $P = 0.02$.

[d] $P = 0.0002$.

osamine content to determine chitin concentrations. The pellets were digested in 6 N HCl at 100°C for 6 h. After evaporation, the pellets were suspended in water, and the hexosamine content was measured by colorimetry (13). D-Glucosamine (0 to 200 μg/ml) (Wako Pure Chemical Industries, Japan) was used as a standard.

The results (means of duplicate results/experiment; two experiments/sample, two samples/two independent preparations) are shown in Table 1. They show a highly significant effect of caspofungin on the cell wall content of all hexoses, including glucans, and chitin. The glucan content is significantly depressed by caspofungin, and the chitin content is significantly upregulated. The depression of β-1,3- and β-1,6-glucan levels and increase of chitin levels by caspofungin was not quite as marked in the second preparation run, though the differences were highly significant with respect to caspofungin effect in the two independent runs.

These results provide an explanation for the paradoxical effect described previously (11): namely, that those isolates which are capable of surviving in caspofungin at high concentrations do so by compensatorily increasing their cell wall chitin content. At these concentrations the glucan synthesis inhibition effect of caspofungin remains highly significant. This also confirms findings (12) with the enzyme itself that upregulation of glucan synthase is not the mechanism of escape. Studies with *C. albicans* involving only brief exposure to caspofungin (15) confirmed earlier observations that there is upregulation of the intracellular protein kinase C cell wall integrity pathway. That compensatory chitin synthesis occurs in paradoxical growth is consistent with inferences from gene interaction studies of *Saccharomyces cerevisiae* of what occurs in the usual caspofungin growth inhibition (8).

We also demonstrate that caspofungin also inhibits synthesis of β-1,6-glucan, as well as β-1,3-glucan. β-1,6-Glucan synthesis and incorporation proceed through several enzyme steps (14), and Fks1p inhibition would affect a late stage. Our finding is consistent with suggestions from gene disruption studies (6) and studies of glucose linkages in *Cryptococcus neoformans* (7) and broadens our understanding of caspofungin antifungal mechanisms.

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