

In Vitro Activity of Caspofungin (MK-0991) against *Candida albicans* Clinical Isolates Displaying Different Mechanisms of Azole Resistance

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Caspofungin inhibits the synthesis of 1,3- β -D-glucan, a key step in fungal cell wall biosynthesis. Here we report on its potent in vitro activity (MIC at which 90% of the isolates tested are inhibited = 1 μ g per ml of RPMI medium) against 32 *Candida albicans* fluconazole-susceptible and -resistant clinical isolates irrespective of the underlying resistance mechanism (alterations in *ERG11* and/or upregulation of *MDR* and *CDR* genes encoding efflux pumps) and provide further evidence that caspofungin is not a substrate for multidrug transporters.

The increase in occurrence of fungal infections, their changing epidemiology, the emergence of resistance, and the toxicity displayed by some of the presently used antifungal therapies have resulted in the need for an expanded arsenal of antifungal drugs. Antifungal agents at different stages in the development pipeline include new-generation azole derivatives and echinocandins. Azole agents target ergosterol biosynthesis, whereas echinocandins represent a new class of antifungal agents that act by inhibiting synthesis of 1,3- β -D-glucan, a key step in fungal cell wall biosynthesis (2). Because of their different mode of action and molecular structure, it is unlikely that cross-resistance between azole antifungal agents (targeting ergosterol synthesis) and echinocandins occurs. Thus, echinocandins, which are fungicidal against yeasts and have a different mode of action, may constitute effective prophylactic and therapeutic options for the management of a variety of fungal infections, including those that are refractory to azoles. Caspofungin acetate (Cancidas, formerly reported as MK-0991 and L-743,872) is a water-soluble, potent echinocandin with activity against a number of clinically important fungi (2).

Fluconazole has proven effective in treating mucosal candidiasis even in individuals with advanced immunodeficiency. However, resistance to fluconazole and other azole antifungal drugs has become an important clinical problem in the management of candidiasis. Mechanisms of azole resistance are multifactorial and include alterations in the target enzyme (lanosterol demethylase, encoded by the *ERG11* gene), including overexpression and point mutations and increased extrusion of drug mediated by two types of multidrug efflux transporters, the ABC transporters (encoded by *CDR* genes) and the major facilitators (encoded by *MDR* genes) (12). Due to the different mechanisms of action, alterations in the lanosterol demethylase are unlikely to affect susceptibility to caspofungin. However, multidrug efflux pumps display affinity for a wide variety of compounds (including different types of antifungals), and thus the possibility exists that their overexpres-

sion may affect susceptibility to caspofungin. Previous studies have demonstrated that caspofungin is highly active against *Candida* species, including some azole-resistant isolates (1, 3, 11), but the molecular mechanisms responsible for azole resistance in these isolates were not known.

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The *Candida albicans* clinical isolates included in this study have been described before (8). Briefly, they were obtained by direct swab or by oral saline rinses from 12 human immunodeficiency virus-infected patients with recurrent oropharyngeal candidiasis enrolled in a longitudinal study to assess the significance of fluconazole resistance (Table 1). The identity of the isolates as *C. albicans* was confirmed by standard biochemical and microbiological procedures (7). Initial susceptibility testing against fluconazole was performed following NCCLS broth macrodilution methodology (5). For each patient, matched sets (as determined by DNA-typing methods) of fluconazole-susceptible and -resistant (MIC \geq 64 μ g/ml) *C. albicans* isolates were included in this study. The molecular mechanisms responsible for azole resistance in this series of isolates have been previously reported (8). Briefly, a Northern blot technique was used to study levels of expression of *ERG11* (encoding lanosterol demethylase, the target enzyme for azole derivatives) and *MDR1* and *CDR* genes (encoding efflux pumps) implicated in the development of azole resistance. We also obtained the nucleotide sequences of *ERG11* genes from all isolates after PCR amplification from genomic DNA. Sequence data were compared to a published *ERG11* sequence in search of nucleotide changes resulting in amino acid substitutions affecting the affinity of the enzyme for azole derivatives. Fluconazole MICs and a summary of the molecular resistance mechanisms detected for each of the different isolates are included in Table 1.

Testing of antifungal susceptibility to caspofungin was determined following NCCLS procedures using a broth microdilution method (5). Caspofungin was provided by Merck Research Laboratories (Rahway, N.J.) as a standard powder and was tested at final concentrations of 0.015 to 16 μ g/ml. Both the reference RPMI 1640 and Antibiotic Medium 3 with 2% glucose (AM3) were used as test media. We used the spectro-

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TABLE 1. *C. albicans* isolates included in this study^a

Pt	Isolate ^b	Fluconazole MIC ^c	Mechanism(s) of resistance ^d	Caspofungin MICs ^e obtained with the following medium:	
				RPMI 1640	AM3
7	412S	<0.125/0.5		0.5/1	<0.015/<0.015
	2307R	16/>64	<i>ERG11</i> and <i>CDR</i>	0.25/1	<0.015/<0.015
9	1002S	0.25/0.25		0.25/1	<0.015/<0.015
	2823R	>64/>64	<i>ERG11</i> and <i>MDR1</i>	0.25/0.5	<0.015/<0.015
	3795R	>64/>64	<i>ERG11</i>	0.25/1	<0.015/<0.015
14	580S	1/4		0.25/0.5	<0.015/<0.015
	2440R	32/64	<i>ERG11</i> and <i>MDR1</i>	0.5/0.5	<0.015/<0.015
	2500R	32/64	<i>ERG11</i> and <i>MDR1</i>	0.5/1	<0.015/<0.015
15	945S	4/8	D116E and G450E	1/2	<0.015/<0.015
	1619R	32/64	D116E, G450E, and G307S, <i>ERG11</i>	1/1	<0.015/<0.015
16	3107S	2/4	Y132F	0.5/0.5	<0.015/<0.015
	3119R	64/>64	Y132F, <i>MDR1</i>	0.5/0.5	<0.015/<0.015
	3120R	32/>64	Y132F, <i>MDR1</i>	0.25/0.5	<0.015/<0.015
	3184R	64/64	Y132F, <i>CDR</i>	0.5/1	<0.015/<0.015
	3281R	32/64	Y132F, <i>CDR</i>	0.5/1	<0.015/<0.015
	5044S	4/4	D446N	0.5/1	<0.015/<0.015
28	5052R	32/64	D446N, <i>MDR1</i> and <i>CDR</i>	0.5/1	<0.015/<0.015
	5106S	4/8		0.5/1	<0.015/<0.015
42	5108R	32/64	G464S, <i>ERG11</i> , <i>MDR1</i> , and <i>CDR</i>	0.25/0.5	<0.015/<0.015
	1691S	0.25/0.25		0.25/0.25	<0.015/<0.015
	3731R	>64/>64	F126L and K143R, <i>MDR1</i>	0.25/0.25	<0.015/<0.015
43	3733R	64/64	F126L and K143R, <i>MDR1</i>	0.25/0.25	<0.015/<0.015
	1649S	0.25/0.5		0.5/0.25	<0.015/<0.015
	3034R	>64/>64	<i>MDR1</i> and <i>CDR</i>	0.5/1	<0.015/<0.015
51	2274SDD	16/16	S405F	0.5/0.5	<0.015/<0.015
	2257R	32/64	S405F	0.25/0.5	<0.015/<0.03
	2339R	16/64	S405F, <i>CDR</i>	0.25/0.25	<0.015/<0.015
59	3917S	2/4	F449S	0.25/0.5	<0.015/<0.015
	4617R	32/64	F449S and T229A, <i>MDR1</i> and <i>CDR</i>	0.25/0.25	<0.015/0.03
	4639R	64/>64	F449S and T229A	0.25/0.25	<0.015/<0.015
64	4018S	1/4		0.25/0.5	<0.015/<0.015
	4380R	32/64	<i>CDR</i>	0.5/0.5	<0.015/<0.015

^a Serial isolates (matched susceptible and resistant) of *C. albicans* from patients with oropharyngeal candidiasis. Pt, patient.

^b Letters in isolate designations signify the following: S, susceptible; SDD, susceptible (dose dependent); R, resistant.

^c Values are in micrograms per milliliter. Two MIC values are given for each isolate: the value to the left of the slash is the value obtained after 24 h of incubation and the value to the right of the slash is the value obtained after 48 h of incubation.

^d Underlying mechanism(s) (i.e., amino acid substitutions in *Erg11p* and/or genes subject to upregulation) responsible for azole resistance. Only amino acid substitutions with a demonstrated effect on azole resistance are listed. See reference 8 for a detailed analysis of molecular mechanisms of azole resistance in this series of isolates.

photometric method of inoculum preparation corresponding to a concentration of 0.5×10^3 to 2.5×10^3 cells per ml for each of the isolates prepared in the test medium. Yeast inocula (100 μ l) were added to each well of microdilution trays containing 100 μ l of antifungal drug solution (prepared at twice the final concentration). MICs were read visually as the lowest concentration at which complete growth inhibition was observed after both 24 and 48 h of incubation.

The results of the MIC readings for caspofungin against *C. albicans* fluconazole-susceptible and -resistant clinical isolates are shown in Table 1. When RPMI 1640 was used as the test medium, the caspofungin MICs measured at 24 h ranged from 0.25 to 1 μ g/ml; 48-h readings ranged from 0.25 to 2 μ g/ml. The caspofungin MICs at which 90% of isolates were inhibited at 24 and 48 h were 0.5 and 1 μ g/ml, respectively, and these values were the same for both azole-susceptible and -resistant isolates. With AM3, the MICs were all < 0.015 μ g/ml at 24 h and ranged from < 0.015 to 0.03 μ g/ml at 48 h. Again, caspofungin was equally active against fluconazole-susceptible and fluconazole-resistant isolates (the MIC at which 90% of isolates tested were inhibited was < 0.015 μ g/ml at both 24 and 48 h)

when tested using AM3. Our results confirmed previously published data showing remarkably lower MICs of caspofungin when AM3 instead of RPMI 1640 was used for the assay (6). The reason for this finding is still not clear. Although interpretative criteria have not yet been defined for caspofungin, the MICs obtained here were in the range of recently reported achievable levels of caspofungin in serum in humans that are approximately 1 μ g/ml for a 50-mg caspofungin daily dose (J. A. Stone, J. B. McCrea, P. J. Wickersham, S. D. Holland, P. J. Deutsch, S. Bi, T. Cicero, H. Greenberg, and S. A. Waldman, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 854, p. 26, 2000) and thus should be considered susceptible.

To further assess the potential role of multidrug efflux pumps in the susceptibility to caspofungin, we also performed susceptibility testing of the parental strain *C. albicans* SC5314 and the following mutants with knockouts in genes encoding multidrug efflux pumps: DS 448 (Δ *cdr1*), DS 653 (Δ *cdr2*), DS 465 (Δ *mdr1*), DS 654 (Δ *cdr1cdr2*), and DS 468 (Δ *cdr1mdr1*). These strains were a kind gift from D. Sanglard (9, 10). The caspofungin susceptibility profiles of all multidrug transporter

mutants tested were unchanged from that of strain SC5314. This was in contrast to their susceptibility to fluconazole, for which these mutants have been shown to be hypersusceptible (9, 10), and in our assay they demonstrated up to a three- or twofold-dilution decrease in fluconazole MICs compared to their parental strain. These strains were tested only against caspofungin in RPMI medium. At 24 h, the caspofungin MICs were between 0.5 and 1 $\mu\text{g/ml}$, whereas at 48 h, all MICs were the same, 1 $\mu\text{g/ml}$.

Azoles target ergosterol synthesis by blocking lanosterol demethylase, an enzyme that is encoded by the *ERG11* gene. Caspofungin, on the other hand, affects fungal cell wall synthesis by inhibiting the production of 1,3- β -D-glucan (2). It is therefore unlikely that cross-resistance between these two classes of antifungals could be explained by an overexpression of *ERG11* or a point mutation within the *ERG11* gene. Our results supported this theory. It was not yet clear if overexpression of genes encoding efflux pumps, like *CDR* or the fluconazole-specific *MDR1*, could affect susceptibility to caspofungin. Our clinical isolates demonstrated low-stable caspofungin MICs, independent of fluconazole resistance and overexpression of genes encoding these efflux pumps. Thus, these results are indicative that caspofungin is not likely to be a substrate for these types of transporters. The observation that a series of multidrug transporter mutants did not exhibit a hypersusceptible phenotype against caspofungin compared to their parental strain corroborated that caspofungin is not a substrate for these pumps that have been widely implicated in resistance to different classes of antifungal drugs and other compounds. Of note, it has been previously shown that the majority of fluconazole-resistant isolates included in this study also exhibit decreased susceptibility against other triazoles, including the new investigational azole agents to which our patients have not had any previous exposure (8). This is in line with observations indicating that cross-resistance between different azoles, even investigational substances, can occur, as demonstrated by other authors (4).

In conclusion, caspofungin seems to be highly active against azole-susceptible and -resistant isolates, regardless of the underlying molecular mechanism(s) of azole resistance. It may therefore constitute an effective therapeutic option for the

treatment of *C. albicans* infections, including those refractory to conventional treatment with triazole agents.

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