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Enfumafungin Derivative MK-3118 Shows Increased *In Vitro* Potency against Clinical Echinocandin-Resistant *Candida* Species and *Aspergillus* Species Isolates

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MK-3118 is as an orally active new antifungal in the early stage of clinical development that inhibits the biosynthesis of β-(1,3)-glucan. We evaluated the *in vitro* activity of this compound against wild-type and echinocandin-resistant (ER) isolates containing mutations in the *FKS* gene(s) of *Candida* spp. and *Aspergillus* spp. MK-3118 demonstrated enhanced efficacy for most *C. albicans* and *C. glabrata* ER isolates relative to caspofungin, with decreased MICs and half-maximal inhibitory concentrations (IC_{50}s).

The echinocandins are first-line agents for treating severe invasive fungal infections (IFIs) (1), being fungicidal against yeast and fungistatic against molds. They alter the integrity of the fungal cell wall via the inhibition of the synthesis of the β-(1,3)-glucan, its major component (2). Specifically, echinocandins target the catalytic subunit of the enzymatic complex β-(1,3)-glucan synthase, encoded by the *FKS* genes. Reduced susceptibility to echinocandins is associated with mutations in two specific regions in the *FKS* genes known as hot spots (HS) 1 and 2 that lead to clinical failure or poor response to the therapy (3). The three echinocandins approved by the Food and Drug Administration (FDA) for the treatment of IFIs (caspofungin, anidulafungin, and micafungin) are available only in intravenous formulation, which limits their use in the treatment of less-severe infections or as oral step-down agents. Enfumafungin is one among several new fungal triterpenoid glycosides isolated from the fermentation of *Hormonema* sp. (4) that present potent *in vitro* antifungal activity by inhibiting the β-(1,3)-glucan synthase (5). Recently, a semisynthetic derivative of enfumafungin, MK-3118 (Fig. 1), which is being evaluated as an oral therapy for fungal infections, was described (6). This new compound showed MIC values of ≤1 μg/ml and ≤0.015 μg/ml against 160 strains of 7 *Candida* spp. and 40 *Aspergillus* spp., respectively (7). Moreover, MK-3118 showed promising *in vivo* efficacy in murine models of candidiasis and aspergillosis (7, 8). To better understand the antifungal efficacy of MK-3118, we evaluated this new compound against a well-characterized panel of echinocandin-resistant (ER) *fks* mutants derived from patients who failed echinocandin therapy.

Antifungal susceptibility testing was performed in triplicate for a collection of 95 *Candida* strains (20 *C. albicans*, 20 *C. glabrata*, 2 *C. dubliniensis*, 15 *C. krusei*, 19 *C. parapsilosis*, and 19 *C. tropicalis*) that included 30 isolates showing an echinocandin resistance (ER) phenotype (caspofungin [CAS] MIC ≥ 0.5 μg/ml) and for a panel of 40 *Aspergillus* strains (14 *A. fumigatus*, 10 *A. flavus*, 10 *A. niger*, and 6 *A. terreus*) that included 1 isolate showing an ER phenotype (9) in accordance with the guidelines described in CLSI documents M27-A3 and M38-A2 (10, 11). In the case of the *Candida* isolates, MICs were also determined in the presence of 50% human serum (Sigma-Aldrich) (from human male blood, type AB) or mouse serum (Millipore) for *C. glabrata* isolates. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as quality control strains. Caspofungin and MK-3118 were obtained as standard powders from their manufacturer (Merck & Co. Inc., Rahway, NJ), and stock solutions were prepared by dissolving the compounds in water or 100% dimethyl sulfoxide (MK-3118).

The MIC distributions of the *Candida* isolates after 48 h of growth at 35°C for CAS and MK-3118 are shown in Table 1. MK-3118 did not show significant differences in MIC values for the wild-type (WT) isolate population, although overall it presented

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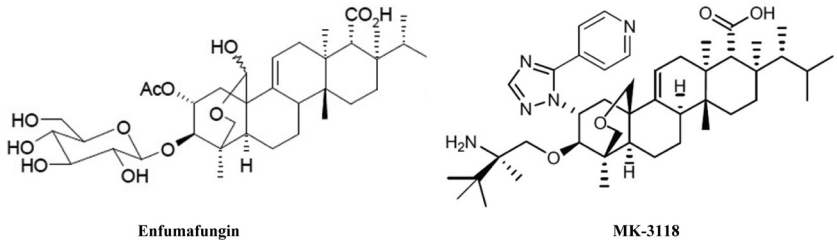

FIG 1 Structures of enfumafungin and MK-3118, a semisynthetic enfumafungin derivative.

TABLE 1 MIC distributions of CAS and MK-3118 in the presence or absence of serum for the *Candida* isolates included in this study

Species	Phenotype (no. of isolates)	Caspofungin			MK-3118		
		Mode value [MIC_{50} range (mg/liter)][a]			Mode value [MIC_{50} range (mg/liter)][a]		
		No serum	50% serum	Ratio[b]	No serum	50% serum	Ratio[b]
C. albicans	WT (10)	≤0.03 (≤0.03–0.06)	0.12 (0.12–1)	4	≤0.03 (≤0.03–0.06)	0.5 (0.5–1)	16
	ER (10)	2 (≤0.03–4)	≥16 (1-≥16)	8	1 (≤0.03–1)	0.5-≥16 (0.5-≥16)	2–16
C. glabrata	WT (9)	0.06 (≤0.03–1)	0.5 (0.5–2)	8	0.12 (0.12–0.5)	2–4 (2–8)	16–32
	ER (11)	16 (1–16)	≥16	1	0.5 (0.25–8)	≥16 (4-≥16)	32
C. dubliniensis	WT (1)	0.06	0.25	4	0.12	1	8
	ER (1)	0.06	0.5	8	0.12	1	8
C. krusei	WT (11)	0.12 (0.12–0.5)	2 (2–4)	16	0.5 (0.25–0.5)	8 (8-≥16)	16
	ER (4)	0.12–8 (0.12–8)	≥16 (2-≥16)	2–133	0.25–2 (0.25–2)	≥16 (1-≥16)	8–64
C. parapsilopsis	WT (19)	1 (0.12–16)	≥16 (4-≥16)	16	0.5 (0.25–8)	≥16 (2-≥16)	32
C. tropicalis	WT (15)	≤0.03 (≤0.03–0.06)	0.5 (0.25–0.5)	16	0.12 (0.06–0.25)	2 (1–8)	16
	ER (4)	2 (≤0.03–2)	≥16	8	0.5 (0.25–4)	8-≥16 (4-≥16)	16–32

[a] Data represent mode values and MIC ranges after 48 h of growth at 35°C. All values represent averages of the results of triplicate experiments with less than 15% variance.
[b] Data represent fold change after adding 50% of serum to the MIC plates.

enhanced *in vitro* efficacy compared to that of CAS for nearly all echinocandin-resistant isolates, especially among the *C. albicans* and *C. glabrata* isolates, where the MIC values decreased by 1- to 8-fold and 4- to 32-fold, respectively. *C. tropicalis* isolates showed a 4-fold decrease in MIC, while the fold change for *C. krusei* ER isolates was 2 to 4 times lower. Specifically, 50% of the ER isolates of *C. albicans* showed MIC values for CAS of ≥2 mg/liter whereas 70% of the ER isolates showed an MIC value of ≤0.5 mg/liter for MK-3118 after 48 h of growth (4- to 8-fold change). Moreover, only 30% of the ER strains showed MIC values of ≤0.25 mg/liter for CAS, while 60% were below this level for MK-3118. The decrease in the MIC values was genotype dependent. Thus, prominent mutations conferring modification of Ser 645 within hot spot (HS) 1 of Fks1p for *C. albicans* showed a 4- to 16-fold reduction in MIC values whereas strains containing modifications at Phe 641 showed results for CAS and MK-3118 that were similar. In addition, 64% of *C. glabrata* ER strains showed MIC values of ≤0.5 mg/liter for MK-3118 after 48 h of growth whereas all ER isolates showed MIC values of ≥1 mg/liter for CAS. The decrease in the MIC values was not genotype dependent in *C. glabrata*, as mutations in either the *FKS1* gene or the *FKS2* gene showed comparable results (8- to 32-fold reduction in both cases) (Table 2). Similar results were described by Pfaller and collaborators (12), who found that, in a cohort of wild-type clinical isolates (without *FKS* mutations), there was little or no difference in MIC values between MK-3118 and CAS by broth microdilution for *C. albicans*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*. The only exception was *C. glabrata*, where MK-3118 was 8-fold more potent than CAS. Moreover, 71% of clinical isolates harboring mutations in the *FKS* gene(s) were inhibited by MK-3118 at ≤1 mg/liter (12), which correlates well with our data.

The echinocandins are highly bound to serum proteins, with a rate of 98% reported for caspofungin (13), which alters its antifungal properties. In fact, it has been reported that the addition of 50% of human serum increased caspofungin MICs an average of 2-fold with a range of 1- to 16-fold (14). In order to ascertain if the relative *in vitro* potency of MK-3118 was affected by serum, 50% (wt/vol) human serum was added to the MIC plates, as previously described (13). In the case of *C. glabrata* isolates, 50% mouse serum was used because human serum can inhibit the growth of this organism (15). The addition of serum to the plates increased the MIC of MK-3118 an average of 16-fold with a range of 8- to 64-fold, four times higher than the values obtained for CAS (Table 1). The reduced antifungal properties of this compound in the presence of serum suggested that protein binding was having a direct effect on the drug, perhaps by altering its ability to inhibit glucan synthase, as was observed previously for the echinocandins (14, 16).

Abnormal growth morphology was used to establish a minimum effective concentration (MEC) for *Aspergillus* spp. after 24 h

TABLE 2 MIC distributions of CAS and MK-3118 for the *C. albicans* and *C. glabrata* isolates harboring mutations in the *FKS* genes included in this study

Strain	Species	Mutation(s) found		MIC (mg/liter)	
		Fks1p	Fks2p	CAS	MK-3118
DPL18	*C. albicans*	F641S		0.5	1
DPL20	*C. albicans*	S645P		4	0.5
DPL22	*C. albicans*	S645P/S		0.03	0.06
DPL1007	*C. albicans*	F641S		1	1
DPL1008	*C. albicans*	S645P		4	1
DPL1009	*C. albicans*	S645Y		2	0.12
DPL1010	*C. albicans*	S645F		2	0.12
DPL1011	*C. albicans*	S645F + R1361R/H		2	<0.03
DPL1012	*C. albicans*	D648Y		0.25	<0.03
DPL1013	*C. albicans*	P649H		0.25	0.25
DPL23	*C. glabrata*		F659del	>16	4.00
DPL26	*C. glabrata*		F659S	>16	0.50
DPL30	*C. glabrata*		S663P	>16	0.50
DPL33	*C. glabrata*		D666E	2.00	0.25
DPL34	*C. glabrata*		P667T	2.00	0.50
DPL38	*C. glabrata*	F625S		2.00	4.00
DPL39	*C. glabrata*	S629P		16.00	0.50
DPL41	*C. glabrata*	D632G		2.00	0.50
DPL42	*C. glabrata*	D632G		16.00	8.00
DPL155	*C. glabrata*		F659V	4.00	2.00
DPL236	*C. glabrata*		L664R	1.00	0.50

TABLE 3 MEC distributions of CAS and MK-3118 for the *Aspergillus* isolates included in this study

Species	Phenotype (no. of isolates)	Mode value and MEC_{50} range (mg/liter)[a]	
		Caspofungin	MK-3118
A. flavus	WT (10)	0.12 (0.06–2)	8 (2.0–16)
A. fumigatus	WT (1)	0.12	0.12
	ER (1)	>16	0.12
	WT (6 [ITR^s])[c]	0.12 (0.12–0.25)	8 (0.12–8)
	WT (8 [ITR^r])[b]	0.12 (0.06–0.25)	0.25 (≤0.03–8)
A. niger	WT (10)	0.12 (0.06–0.12)	0.12 (≤0.03–0.25)
A. terreus	WT (6)	0.06 (0.06–0.25)	0.12 (0.06–0.12)

[a] Data represent mode values and MEC ranges after 48h of growth at 35°C. All values represent averages of the results of triplicate experiments with less than 15% variance.
[b] Isolates with a WT *FKS1* gene and sensitive to azoles. ITR, itraconazole.
[c] Isolates with a WT *FKS1* gene but resistant to azoles.

and 48 h of growth at 35°C. MK-3118 and caspofungin were quite active against the four species of filamentous fungi analyzed, including eight *A. fumigatus* strains with an azole-resistant phenotype. Interestingly, the growth of all *Aspergillus* isolates was completely inhibited by treatment with high concentrations (8 to 16 μg/ml) of MK-3118. These data are in accord with those of Pfaller et al. (17), who showed that MK-3118 was active against 71 *Aspergillus* isolates, including 8 itraconazole-resistant isolates (MIC ≥ 4 μg/ml). Since echinocandin-resistant isolates from *Aspergillus* spp. have rarely been observed, MK-3118 was tested against the only ER strain of *A. fumigatus* available to date, which presents the amino acid substitution S678P, equivalent to that of the S654P of *C. albicans* (9). In this isolate, MK-3118 showed prominent increased potency with an MIC that was 133 times less than that of CAS after 24 h of growth (Table 3).

To better assess direct inhibition of MK-3118 on glucan synthase, the kinetic inhibition parameter IC_{50} (half-maximal inhibitory concentration) was determined for glucan synthases from wild-type and *fks*-containing strains. Product-entrapped 1,3-β-D-glucan synthase complexes (GS) were extracted from wild-type and ER strains containing *fks* mutations from *C. albicans* (1 WT and 3 *fks* mutant strains), *C. glabrata* (1 WT and 2 *fks* mutant strains), and *A. fumigatus* (1 WT strain and 1 *fks* mutant strain), as described previously (18, 19). As expected, evaluation of kinetic inhibition of product-entrapped enzymes isolated from ER strains yielded lower IC_{50}s for MK-3118 than for CAS in *Candida albicans* (3- to 5.5-fold) and *Candida glabrata* (3.5- to 62-fold) (Fig. 2A and Table 4). An exception was observed for glucan synthase harboring the mutation F641S, as inhibition of GS activity did not exhibit any variation in the percentage of incorporation even after exposure to high doses of the drugs (10 μg/ml); it was not possible to obtain an in-range IC_{50} for MK-3118 (Fig. 2A). In the case of *A. fumigatus*, a decrease of 28-fold was detected in the IC_{50} of a prominent ER strain (Fks1p-S678P) compared to that of the WT for MK-3118 (Fig. 2B and Table 4), indicating a potential advantage of MK-3118 over echinocandin drugs for certain echinocandin-resistant strains.

In summary, MK-3118 was highly active on most *fks*-mediated echinocandin-resistant strains, especially those from *C. albicans* and *C. glabrata*. It was also active on *Aspergillus* spp. at high con-

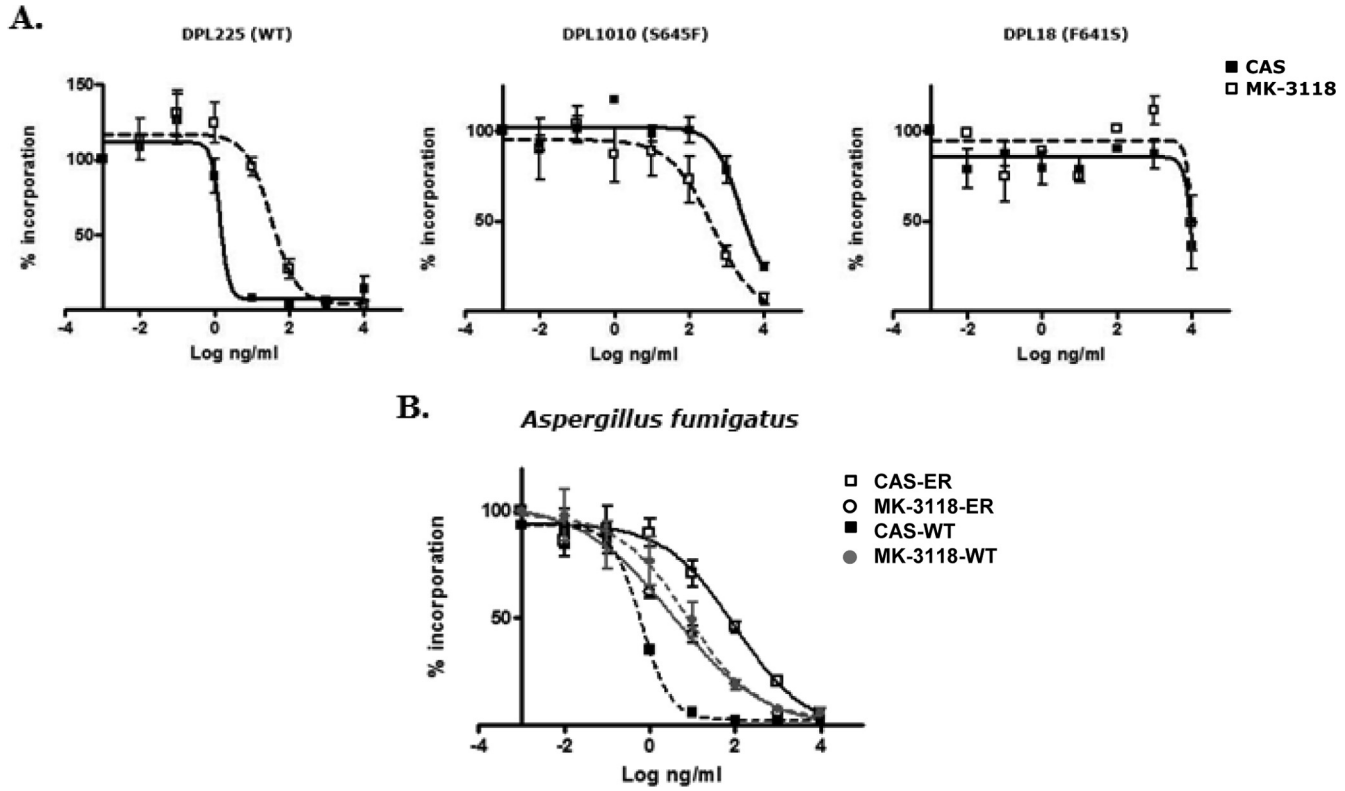

FIG 2 Antifungal inhibition profiles for product-entrapped 1,3-β-glucan synthase enzyme complexes (GS) for caspofungin (CAS) and MK-3118 for wild-type and ER clinical isolates. GS inhibition was assessed by the incorporation of [^{3}H]glucose into radiolabeled product. (A) *Candida albicans*. (B) *Aspergillus fumigatus*.

TABLE 4 *In vitro* whole-cell susceptibility and 1,3-β-glucan synthase inhibition profiles of caspofungin and MK-3118 for representative strains included in the study[a]

Strain	Organism	Fksp phenotype		MIC (mg/liter)[b]				IC_{50} (ng/ml)	
				CAS		MK-3118			
		Fks1p	Fks2p	No serum	50% serum	No serum	50% serum	CSF	MK-3118
DPL225	*C. albicans*	WT		<0.03	0.12	0.03	0.5	1.421	32.95
DPL1010	*C. albicans*	S645F		2	>16	0.12	1	2,321	423.1
DPL1012	*C. albicans*	D648Y		0.25	16	<0.03	2	149.8	51.58
DPL18	*C. albicans*	F641S		0.5	>16	1	16	>2,500[c]	>2,500[c]
DPL1021	*C. glabrata*	WT	WT	0.06	1	0.25	4	125.9	108.1
DPL235	*C. glabrata*	WT	F659V	>16	>16	0.5	8	1,945	542.1
DPL32	*C. glabrata*	WT	D666G	2	>16	0.5	4	2,273	36.25
DPL1034	*A. fumigatus*	WT		0.12	ND	0.12	ND	0.65	7.81
DPL1035	*A. fumigatus*	S678P[d]		>16	ND	0.12	ND	100.4	3.58

[a] MIC values represent whole-cell susceptibility; IC_{50} values represent 1,3-β-glucan synthase inhibition. CSF, cerebrospinal fluid; ND, not determined.
[b] MEC in the case of *A. fumigatus*.
[c] No significant inhibition at all levels.
[d] This mutation is equivalent to S645P in *C. albicans* (9).

centrations of the drug and was active against a highly ER strain. As observed previously with echinocandin drugs, serum shifted the relative efficacy of the new compound MK-3118, which was most effective against echinocandin-resistant strains.

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