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Enfumafungin derivative MK-3118 shows increased *in vitro* potency against clinical echinocandin resistant *Candida* spp. and *Aspergillus* spp. isolates

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Running title: MK-3118 susceptibility of *Candida* and *Aspergillus* spp.

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ABSTRACT

MK-3118 is as an orally active new antifungal in early stage 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*, *C. glabrata* ER isolates relative to caspofungin with decreased MIC and IC_{50} values.

TEXT

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 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 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 semi-synthetic derivative of enfumafungin, MK-3118 (Fig. 1) was described, which is being evaluated as an oral therapy for fungal infections (6). This new compound showed MIC values of ≤1μg/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 ER *fks* mutants derived from patients that failed echinocandin therapy.

Antifungal susceptibility testing was performed in triplicate for a collection of 95 *Candida* spp. strains (20 *C. albicans*, 20 *C. glabrata*, 2 *C. dubliniensis*, 15 *C. krusei*, 19 *C. parapsilosis* and 19 *C. tropicalis*) with 30 isolates showing an echinocandin resistant (ER) phenotype [caspofungin (CAS) MIC ≥0.5 µg/ml] and for a panel of 40 *Aspergillus* spp. strains (14 *A. fumigatus*, 10 *A. flavus*, 10 *A. niger* and 6 *A. terreus*) with 1 isolate showing an ER phenotype (9), in accordance with the guidelines described in the CLSI documents M27-A3 and M38-A2 (10, 11). In the case of *Candida* spp. isolates, MICs were also determined in presence of 50% human serum (from human male blood, type AB, Sigma-Aldrich) 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* spp. isolates after 48h 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 isolate population, although overall it presented enhanced *in vitro* efficacy compared to that of CAS for nearly all echinocandin resistant isolates, especially among *C. albicans* and *C. glabrata* where the MIC values decreased by 1-8 and 4-32 fold, respectively. *C. tropicalis* isolates showed a 4-fold decrease in MIC while the fold change for *C. krusei* ER

62 isolates was 2-4 times lower. Specifically, 50% of ER isolates of *C. albicans* showed MIC values for
63 CAS ≥ 2 mg/L while 70% of the ER isolates showed an MIC value of ≤ 0.5 mg/L for MK-3118 after 48h
64 of growth (4-8 fold change). Moreover, only 30% of the ER strains showed an MIC values ≤ 0.25 mg/L
65 for CAS, while 60% were below this level for MK-3118. The decrease in the MIC values was
66 genotype-dependent. Thus, prominent mutations conferring modification of Ser 645 within hot
67 spot (HS) 1 of Fks1p for *C. albicans* showed a 4-16 fold reduction in MIC values while strains
68 containing modifications at Phe 641 showed similar results between CAS and MK-3118. In
69 addition, 64% of *C. glabrata* ER strains showed MIC values ≤ 0.5 mg/L for MK-3118 after 48h
70 of growth while all ER isolates showed MIC values ≥ 1 mg/L for CAS. The decrease in the MIC
71 values was not genotype-dependent in *C. glabrata*, as mutations in either *FKS1* or *FKS2* genes
72 showed comparable results (8-32 fold reduction in both cases) (Table 2). Similar results were
73 described by Pfaller and collaborators (12) who found that in a cohort of wild type clinical
74 isolates (without *FKS* mutations) there was little or no difference in MIC values between MK-
75 3118 and CAS by broth microdilution for *C. albicans*, *C. krusei*, *C. parapsilosis* and *C.*
76 *tropicalis*. The only exception was *C. glabrata* where MK-3118 was 8-fold more potent than
77 CAS. Moreover, 71% of clinical isolates harboring mutations in the *FKS* gene(s) were inhibited
78 by MK-3118 at ≤ 1 mg/L (12), which correlates well with our data.
79 The echinocandins are highly bound to serum proteins with rates of 98% reported for
80 caspofungin (13), which alters its antifungal properties. In fact, it has been reported that the
81 addition of 50% of human serum increased caspofungin MICs an average of 2-fold with a range
82 of 1- to 16-fold (14). In order to ascertain if the relative *in vitro* potency of MK-3118 was
83 affected by serum, 50% w/v human serum was added to the MIC plates, as previously described
84 (13). In the case of *C. glabrata* isolates, 50% mouse serum was used because human serum can
85 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 24h and 48h of growth at 35 °C. MK-3118 and caspofungin were quite active against the four species of filamentous fungi analysed, including eight *A. fumigatus* strains with an azole-resistant phenotype. Interestingly, the growth of all *Aspergillus* spp. isolates was completely inhibited when treated with high concentrations (8-16 µg/ml) of MK-3118. This data is in accord with Pfaller *et al*. (17) where MK-3118 was shown to be active against 71 *Aspergillus* spp. isolates including 8 itraconazole-resistant isolates (MIC ≥ 4 µg/ml). Since echinocandin-resistant isolates from *Aspergillus* spp. have been rarely 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 24h 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* mutants), *C. glabrata* (1 WT and 2 *fks* mutants) and *A. fumigatus* (1 WT and 1 *fks* mutant), as described previously (18, 19). As expected, evaluation of kinetic inhibition of

product-entrapped enzymes isolated from ER strains yielded lower IC_{50} values for MK-3118 than for CAS in *Candida albicans* (3 to 5.5-fold) and *Candida glabrata* (3.5 to 62-fold) (Figure 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} value for MK-3118 (Figure 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 the WT for MK-3118 (Figure 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 concentrations 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 prominent against echinocandin resistant strains.

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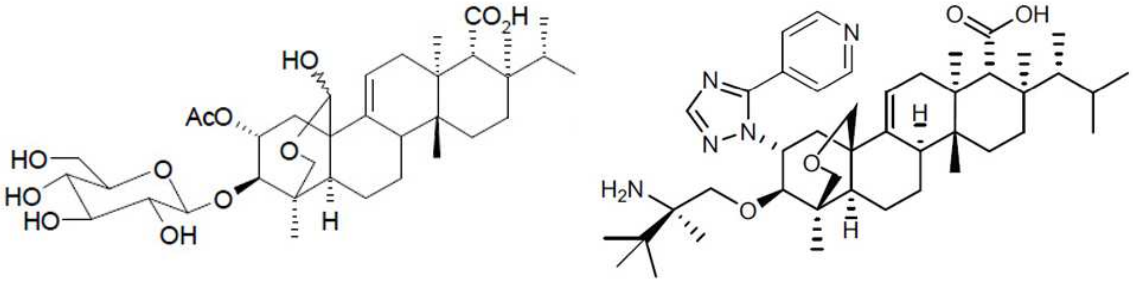

Enfumafungin **MK-3118**

Figure 1. Structures of the enfumafungin and the MK-3118, a semi-synthetic enfumafungin derivative.

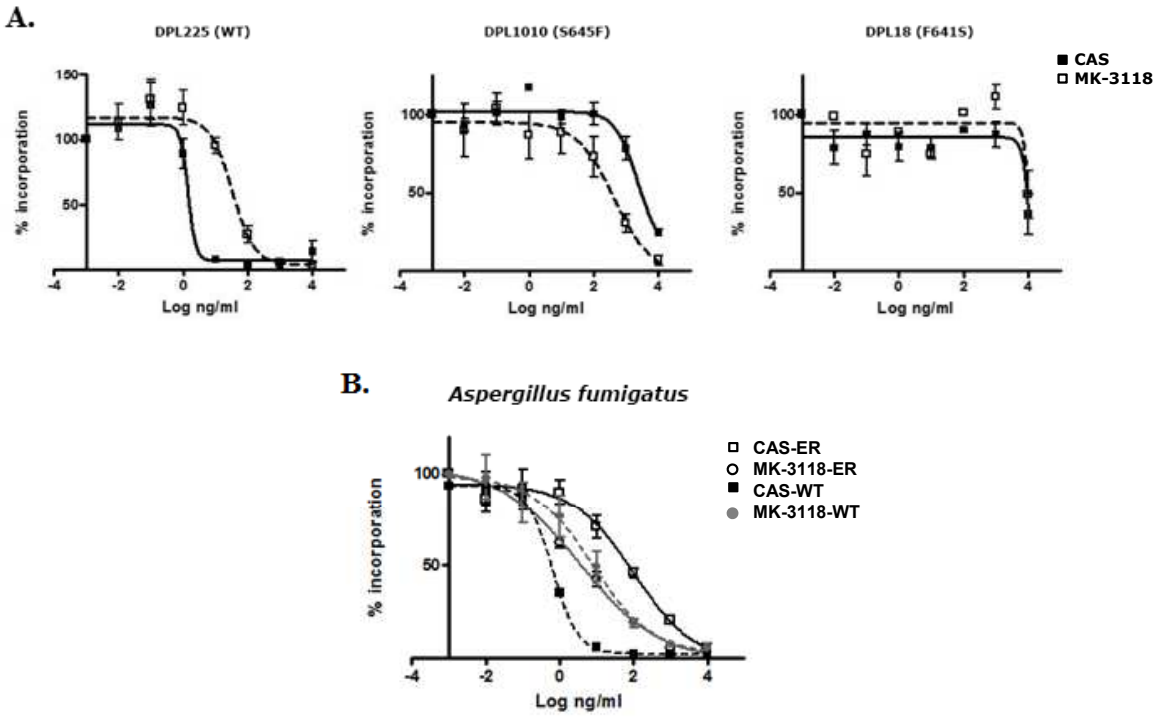

Figure 2. Antifungal inhibition profiles for product-entrapped 1,3-β-glucan synthase enzyme complexes (GS) for caspofungin (CAS) and MK-3118 for wild-type and ER clinical isolates. GS inhibition was assessed by the incorporation of [^{3}H]-glucose into radiolabeled product. A. *Candida albicans*. B.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

Table 1. MIC distributions of caspofungin (CAS) and MK-3118 in presence or absence of serum for the *Candida* spp. isolates included in this study.

		CASPOFUNGIN			MK-3118		
		MIC_{50} mg/L			MIC_{50} mg/L		
Species	Phenotype	No serum	50% serum	Ratio[c]	No serum	50% serum	Ratio
C. albicans	WT (10)[a]	≤0.03 (≤0.03-0.06)[b]	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] (n) number of isolates.

[b] Mode value and MIC range after 48h of growth at 35 °C. All values represent averages for triplicate experiments with less than 15% variance.

[c] Fold change after adding 50% of serum to the MIC plates.

Table 2. MIC distributions of caspofungin (CAS) and MK-3118 for the *C. albicans* and *C. glabrata* isolates harbouring mutations in the *FKS* genes included in this study.

Strain ID	Species	Mutation found		MIC (mg/L)	
		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. MIC distributions of caspofungin (CAS) and MK-3118 for the *Aspergillus* spp. isolates included in this study.

		MEC_{50} mg/L	
Species	Phenotype	Caspofungin	MK-3118
A. flavus	WT (10)[a]	0.12 (0.06-2)[b]	8 (2.0-16)
A. fumigatus	WT (1)	0.12	0.12
	ER (1)	>16	0.12
	WT (ITR^S 6)[c]	0.12 (0.12-0.25)	8 (0.12-8)
	WT (ITR^R 8)[d]	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] (n) number of isolates.

[b] Mode value and MIC range after 48h of growth at 35 °C. All values represent averages for triplicate experiments with less than 15% variance.

[c] Isolates with a WT *FKS1* gene and sensitive to azoles.

[d] Isolates with a WT *FKS1* gene but resistant to azoles.

Table 4. *In vitro* whole-cell susceptibility (MIC) and 1,3-β-glucan synthase inhibition profiles (IC_{50}) of caspofungin and MK-3118 for representative strains included in the study.

Strain ID	Organism	Fksp phenotype		MIC (mg/L)*				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	2321	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	>2500[a]	>2500[a]
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	1945	542.1
DPL32	*C. glabrata*	WT	D666G	2	>16	0.5	4	2273	36.25
DPL1034	*A. fumigatus*	WT	---	0.12	ND[b]	0.12	ND	0.65	7.81
DPL1035	*A. fumigatus*	S678P[c]	---	>16	ND	0.12	ND	100.4	3.58

[a] No significant inhibition at all levels

[b] ND: not determined

[c] This mutation is equivalent to S645P in *C. albicans* [9].

* MEC in the case of *A. fumigatus*.