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Evaluation of CD101 Glucan Synthase Inhibition, MIC Values and Mutant Prevention Concentrations Against Echinocandin-Susceptible and -Resistant *Candida* spp.

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ABSTRACT

Background: The aim of this study was to characterize the *in vitro* activity of CD101, a novel long half-life echinocandin, through assessment of enzymatic inhibition of wild-type and mutant β-(1,3)-glucan synthase (GS), MIC values against well-characterized echinocandin-susceptible and -resistant isolates and mutant prevention concentration (MPC) in *Candida* spp.

Methods: The kinetic inhibition parameter IC_{50} (half-maximal inhibitory concentration) was determined for GS extracted from wild-type and *fks* mutant strains by measuring incorporation of radiolabeled glucose from UDP-[^{14}C]-glucose into ethanol-insoluble polymeric products. 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*) that included 30 isolates displaying echinocandin resistance (ER). To determine the MPC, susceptible *C. albicans* and *C. glabrata* strains with wild-type Fks were cultured in RPMI 1640 medium for 24 h in the presence of 0 - 32 mg/L of CD101 or micafungin (MCF). Serial dilutions of the samples were plated on YPD and incubated at 37°C for 1-2 days prior to colony counting.

Results: Wild-type GS purified from *C. albicans* and *C. glabrata* were as sensitive to CD101 as MCF, with mean IC_{50} values of 2.57-14.25 and 0.45-17.65 ng/ml for CD101 and MCF, respectively. GS from echinocandin-resistant strains of *C. albicans* and *C. glabrata* with well-defined amino acid substitutions in either Fks1p or Fks2p showed a 24- to >3890-fold decrease in sensitivity to CD101 similar to MCF. CD101 MIC values for all *Candida* spp. (range <0.03-4 mg/L) were comparable to and demonstrated cross-resistance with MCF. For the MPC assay, a 3- to 5-log decrease in CFUs was seen around the MICs for CD101 and MCF for *C. albicans* and *C. glabrata*. An adapted cell population persisted through 8 mg/L and decreased at 16 – 32 mg/L. Thus, 16 mg/L was identified as the MPC for CD101 and MCF.

Conclusions: Overall, CD101 demonstrates comparable *in vitro* inhibitory activity to MCF at the enzyme and cellular levels, as well as in its MPC values for *Candida* spp. and warrants further analysis as a promising new glucan synthase inhibitor with unique pharmacokinetic properties.

INTRODUCTION

Echinocandins are members of the leading class of antifungal agents for the treatment of systemic fungal infections [1]. These compounds target the cell wall by preventing the production of β-(1,3)-glucan via inhibition of the catalytic subunit of the β-(1,3)-glucan synthase complex encoded by the *FKS* genes [2]. Resistance to echinocandins is associated with mutations in two hot spot (HS) regions in the *FKS* genes that correlate with clinical failure or poor response to therapy [3]. The three echinocandins approved by the Food and Drug Administration (FDA) for the treatment of invasive fungal infections (caspofungin, anidulafungin, and micafungin) are available only in intravenous formulation. While the incidence of resistance remains low, the daily administration of these antifungal agents may contribute to the rise in reports of breakthrough infections. CD101 is a novel long-acting echinocandin being developed as an intravenous as well as topical formulation for the treatment of invasive fungal infections. CD101 displays long-acting pharmacokinetics with a long half-life and slow clearance [4,5]. CD101 has shown MIC/MEC_{90} values of ≤1 mg/L and ≤0.015 mg/L against 106 strains of 5 *Candida* spp. and 67 *Aspergillus* spp., respectively [6]. In this study, we evaluated the antifungal efficacy of CD101 against a well-characterized panel of echinocandin-resistant (ER) *fks* mutants derived from patients who failed echinocandin therapy.

MATERIALS AND METHODS

Glucan Synthase (GS) purification and assay. Three *C. albicans* strains (DPL1002, DPL18, DPL20) and three *C. glabrata* strains (DPL50, DPL23, DPL30) were grown with vigorous shaking at 37°C to early stationary phase in YPD (1% Yeast extract, 2% Peptone, 2% Dextrose) broth, and cells were collected by centrifugation. Cell disruption, membrane protein extraction and partial β-(1,3)-glucan synthase purification by product-entrapment were performed as previously described [7]. Sensitivity to MCF and CD101 was measured in a polymerization assay using a 96-well 0.65-µm multiscreen HTS filtration system (Millipore Corporation, Bedford, MA) in a final volume of 100 µl, as previously described [8]. Serial dilutions of the drugs (0.001–10,000 ng/ml) were used to determine IC_{50} values. MCF (Astellas Pharma, Inc.) was dissolved in water and CD101 (Cidara Therapeutics, Inc.) was dissolved in 100% dimethyl sulfoxide (DMSO). Reactions were initiated by addition of glucan synthase. Inhibition profiles and half maximal inhibitory concentrations (IC_{50}s) were determined using a normalized response (variable-slope) curve fitting algorithm with GraphPad Prism, version 5.04, software (Prism Software, Irvine, CA).

Antifungal susceptibility testing. 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 mg/L) in accordance with the guidelines described in CLSI document M27-A3. MCF was dissolved in water and CD101 was dissolved in 100% DMSO. Stock solutions of the drugs were kept at −86°C. Microtiter plates were read visually at 24 hr and the MIC values were determined using prominent inhibition [corresponding to fifty percent (50%)] as endpoint.

Mutant prevention concentration (MPC) determination. Clinical isolates DPL225 and BAD55 [9] were grown overnight in YPD (1% Yeast extract, 2% Peptone, 2% Dextrose) broth with vigorous shaking at 37°C. Cells were collected by centrifugation and washed 1X with distilled water. Samples were diluted to 1 x 10^8 CFU/ml in a total volume of 1.5 ml. One hundred microliters of fungal suspension was added to 0.9 ml of RPMI 1640 medium buffered with MOPS to pH 7.0 with or without drug, providing the starting inoculum of approximately 1 x 10^7 CFU/ml. The range of CD101 or MCF concentrations tested was 0.03 – 32 mg/L. The culture vials were incubated with agitation at 37°C for 24 hr. A 100 µl sample was removed from each culture vial and serially diluted with sterile water. Subsequently, 100 µl aliquots of several dilutions were plated on YPD. When colony counts were suspected to be low, 100 µl was taken directly from the culture vials and plated without dilution. Plates were incubated at 37°C for 1-2 days prior to colony counting.

RESULTS

IC_{50} values for *C. albicans* and *C. glabrata* isolates. Inhibition curves for CD101 and MCF against the wild-type (WT) *C. albicans* isolates showed the typical pattern of β-(1,3)-glucan synthase echinocandin susceptibility (Fig. 1 and Table 1). The F641S mutant exhibited a 100-fold and 24.3-fold increase in IC_{50} values for MCF and CD101, respectively, compared to the WT. The S645P mutant exhibited 144-fold and 185.3-fold increases for MCF and CD101, respectively. Mean IC_{50} values for the WT *C. glabrata* enzyme were 0.447 and 2.57 ng/ml for MCF and CD101, respectively. The *C. glabrata* F659del mutant GS did not exhibit significant reductions in activity after treatment with a high dose (10,000 ng/ml) of either MCF or CD101. The S663P mutant exhibited a lower IC_{50} for MCF compared to CD101.

Figure 1: Echinocandin inhibition profiles of enriched GS complexes from susceptible and resistant *C. albicans* and *C. glabrata* isolates.

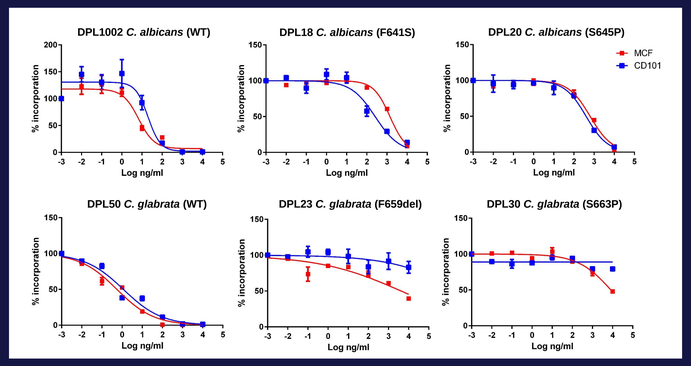


Table 1: Half maximal inhibitory concentrations (IC_{50}s) of β-(1,3)-glucan synthases from susceptible and resistant *C. albicans* and *C. glabrata* isolates used in the study.

DPL#	Organism	Fks1 Phenotype	Fks2 Phenotype	IC_{50} (ng/ml)[a] MCF	IC_{50} (ng/ml)[a] CD101
1002	*C. albicans*	WT	WT	17.65	14.25
18	*C. albicans*	F641S		1782	347.4
20	*C. albicans*	S645P		2555.77	2641.4
50	*C. glabrata*	WT	WT	0.447	2.57
23	*C. glabrata*		F659del	>10,000	>10,000
30	*C. glabrata*		S663P	6772.33	>10,000

[a]IC_{50} values represent the arithmetic mean of results from three independent experiments.

MIC distributions. CD101 did not show significant differences in MIC values for the WT isolate population relative to MCF with the exception of *C. krusei* isolates, which were 2- to 4-fold lower for CD101 than MCF. Although MIC values were comparable for both CD101 and MCF for the different *Candida* ER isolates, 60% of *C. albicans* ER isolates had an MIC of ≤0.5 mg/L for CD101 compared to 40% for MCF (Table 2). However, only 18% (2/11) of *C. glabrata* ER isolates had an MIC of ≤0.5 mg/L for CD101 compared to 63% (7/11) for MCF. This finding does not appear to be genotype-dependent, as mutations in either *FKS1* or *FKS2* showed comparable results with MCF. CD101 MICs were 1- to 2-fold lower compared to MCF for *C. krusei* ER isolates.

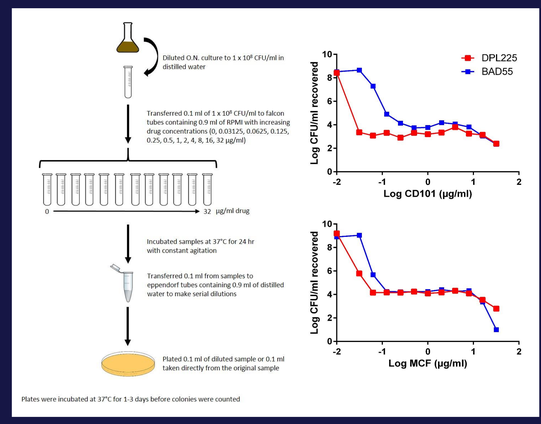
Table 2: *In vitro* whole-cell susceptibility (MIC) distributions of MCF and CD101 for the *Candida* isolates included in this study.

Species	Phenotype (no. of isolates)	MIC median (range), mg/L[a] MCF	MIC median (range), mg/L[a] CD101
C. albicans	WT (10)	<0.03 (<0.03)	<0.03 (<0.03)
	ER (10)	0.50 (0.03-4)	0.50 (0.06-2)
C. glabrata	WT (9)	<0.03 (<0.03)	0.06 (0.03-0.12)
	ER (11)	0.50 (0.06-4)	1.00 (0.12-4)
C. dubliniensis	WT (1)	0.03	0.03
	ER (1)	0.03	0.03
C. krusei	WT (11)	0.12 (0.03-0.25)	0.03 (<0.03-0.06)
	ER (4)	1.00 (0.03-8)	0.50 (<0.03-4)
C. parapsilosis	WT (19)	4 (1-4)	2 (2-4)
C. tropicalis	WT (15)	0.03 (0.03)	0.03 (0.03)
	ER (4)	2.00 (1-4)	2.00 (0.5-2)

[a]Data represent median MIC values and ranges after 24 hr of growth at 37°C. MIC values at 48 hr were the same or within a 2-fold range of the 24 hr value. All values represent averages of the results of triplicate experiments.

Mutant prevention concentration (MPC). The MPC represents a threshold above which the selective proliferation of resistant mutants is expected to occur rarely. It is based on the concept that mutants are selected within a concentration range that extends from the MIC up to the MPC [10]. Using a modified version of the original method, we observed a 3- to 5-log decrease in CFUs around the MICs for MCF and CD101 and a second reduction at around 16-32 mg/L. We determined the MPC for both MCF and CD101 to be 16 mg/L against *C. albicans* and *C. glabrata*.

Figure 5: MCF and CD101 MPC determination for wild-type *C. albicans* and *C. glabrata* isolates used in the study.



CONCLUSIONS

✓CD101 is a potent inhibitor of glucan synthase with comparable activity to other echinocandins against *Candida* spp.

✓CD101 presented similar IC_{50} values as MCF against the WT and *fks* mutant strains of *C. albicans* and *C. glabrata* analyzed. However, it showed enhanced activity relative to MCF against the common Fks1-F641S mutant enzyme from *C. albicans*.

✓*In vitro* susceptibility testing of CD101 against isolates of *Candida* spp. provided results comparable to MCF.

✓The significance of MPCs for CD101 and MCF to overcome resistance is intriguing and warrants further investigation in an *in vivo* model.

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