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Determination of CD101 Spontaneous Mutation Frequencies and Underlying Resistance Mechanisms in *Candida* spp.

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ABSTRACT

Background: CD101 is a novel, extended half-life echinocandin under development for the treatment of candidemia and invasive candidiasis. The purpose of this study was to investigate the frequency of and genetic basis for spontaneous, single-step mutations in *Candida* spp. which confer reduced susceptibility to CD101.

Methods: CD101 and comparator echinocandins anidulafungin (ANI) and caspofungin (CAS) were tested in parallel vs. *C. albicans* (x1), *C. glabrata* (x2), *C. parapsilosis* (x1) and *C. krusei* (x1) strains. Spontaneous mutation frequencies were determined in triplicate by plating ~1 x 10^{-8} CFU on large-format Sabouraud dextrose agar plates (245 x 245 mm) containing 1X the agar inhibitory concentration of each drug. Following 48 h incubation, all putative mutant colonies were confirmed by restreaking on plates containing an equivalent amount of drug as was used in their initial selection. Mutation frequencies were calculated and all CD101-selected strains and a subset of those selected with ANID and CAS were then evaluated by MIC (CLSI) and sequencing of *FKS* gene hot spot (HS) regions.

Results: Median spontaneous mutation frequencies for CD101 among the *Candida* spp. tested were low, ranging from 5.00 x 10^{-8} to 3.86 x 10^{-9}. These values were within the ranges generated for ANI and CAS. Of the 4 *Candida* spp. evaluated, the *C. glabrata* strains had the highest mutation frequencies and MIC fold-shift increases for all three drugs. All CD101-selected mutants with MIC values increasing ≥2-fold also demonstrated cross-resistance to ANID and/or CAS. Eight different *fks* HS mutations (Fks1 HS1: S645P; Fks2 HS1: ΔF659, S663F, R665G, D666H, D666Y, D666N; Fks2 HS2: R1378S) were identified among 25 strains out of the 82 sequenced, and were typically found in mutants with the largest MIC shifts for all three drugs. Many of the "spontaneous mutant" colonies selected at 1X the agar inhibition level demonstrated insignificant changes in MIC values (i.e., ≤2-fold) suggesting that spontaneous mutations conferring >2-fold MIC shifts to all 3 echinocandins are even less frequent than the values derived in these plating experiments.

Conclusions: Single-step mutations conferring reduced susceptibility to CD101 in *Candida* spp. are rare. These mutants occur at frequencies comparable to and share common mutations with those selected with other echinocandins.

INTRODUCTION

Echinocandins target the 1,3-β-D-glucan synthase enzyme complex. Mutations in the *FKS* genes, which encode the catalytic subunit of this complex, have been associated with reduced susceptibility to echinocandins and increased clinical failure rates. Two specific "hot spot" (HS) regions within *FKS* genes ("HS1" and "HS2", encoding 9 and 8 amino acids, respectively) are most often associated with reduced susceptibility to echinocandins.

CD101 is a novel, long half-life echinocandin undergoing clinical development for the treatment of candidemia and invasive candidiasis. A front-loaded treatment paradigm may have advantages from an efficacy standpoint and could help prevent or slow the development of resistance as well. The purpose of this study was to investigate the frequency of and genetic basis for spontaneous, single-step mutations in *Candida* spp. which confer reduced susceptibility to CD101, genetically characterize the underlying resistance mechanisms and compare cross-resistance trends with anidulafungin and caspofungin.

METHODS

Reagents. CD101, caspofungin (CAS), anidulafungin (ANID), and amphotericin B (AMB) stocks were prepared freshly in 100% DMSO prior to use in MIC assays and spontaneous resistance experiments.

Strains. Representative wild-type strains of *C. albicans* (NRRL Y-477), *C. glabrata* (ATCC 2001, ATCC 90030), *C. parapsilosis* (CP02), and *C. krusei* (ATCC 6258) were chosen following prescreening on Sabouraud dextrose agar (SDA) plates containing each echinocandin to ensure that they had non-paradoxical agar growth phenotypes.

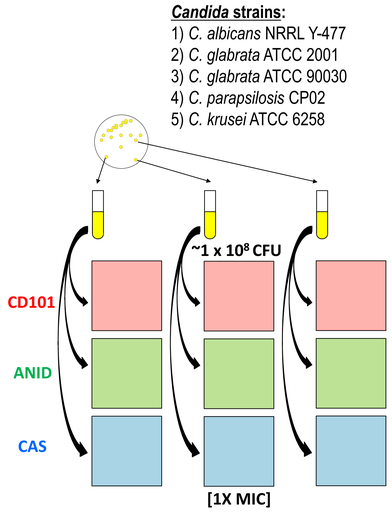
Antifungal susceptibility testing. *Candida* strains were cultured aerobically at 35˚C on SDA plates or in RPMI 1640 broth (pH 7.0). MIC assays were performed via broth microdilution in accordance with Clinical and Laboratory Standards Institute (CLSI)[1,2] with the exception that test compounds were made up at 50X final assay concentration (2 µL added to 98 µL of broth containing cells at 0.5 - 2.5 x 10^3 CFU/mL). MIC plates were read following a 24-hour incubation at 35˚C and MIC values were reported as concentrations resulting in prominent growth inhibition (~50%) per CLSI guidance for echinocandins.[1] MIC assays were performed in triplicate.

Spontaneous mutant selection. Large-format 245 x 245 mm assay dishes (Corning cat. no. 431272) were prepared with 150 mL SDA containing CD101, ANID, or CAS at the minimum concentration required to cleanly inhibit growth for each *Candida* strain. Three individual colonies from each strain were used to start cultures in RPMI. When cultures reached ~1.0 OD_{530} they were pelleted and resuspended in phosphate-buffered saline to a cell density of ~1 x 10^8 CFU/mL. One milliliter aliquots were spread onto SDA plates containing drug with sterile glass beads. Starting viable count was enumerated by triplicate plating of serial dilutions of the starting inoculum. Plates were incubated at 35˚C for 48 hours. Glycerol stocks of putative mutant colonies were stored at -80˚C. Mutant resistance phenotypes were confirmed by subculturing on SDA plates containing an equivalent amount of drug as was used in their initial selection. Spontaneous mutation frequencies were calculated by dividing the number of resistant colonies on a given plate by the starting inoculum plated. A subset of mutants (weighted heavily towards those selected with CD101) were then evaluated by MIC and sequencing of *FKS* gene hot spot regions.

Sequence analysis of *FKS* gene hot spot regions. *FKS1* hot spot 1 (HS1) and hot spot 2 (HS2) regions were amplified by PCR as previously described.[3] For *C. glabrata* strains, *FKS2* HS1 and HS2 regions were also amplified. PCR products were sequenced using upstream forward primers for each HS region and analyzed along side wild-type *FKS* sequences using Vector NTI® software (Life Technologies).

METHODS (cont'd)

Experimental scheme



RESULTS

Table 1. Echinocandin broth and agar inhibition values

Strain	Broth MIC (µg/mL)			Plate concentration (µg/mL)		
	CD101	ANID	CAS	CD101	ANID	CAS
C. albicans NRRL Y-477	0.03	0.015	0.25	0.06	0.015	0.25
C. glabrata ATCC 90030	0.06	0.06	0.25	0.25	0.125	0.5
C. glabrata ATCC 2001	0.125	0.06	0.25	0.25	0.125	0.5
C. parapsilosis CP02	2	4	0.5	8	16	1
C. krusei ATCC 6258	0.06	0.06	0.5	0.25	0.25	0.5

- Agar drug concentrations required to provide complete background growth inhibition were between 1X and 4X the corresponding broth microdilution values.

RESULTS (cont'd)

Table 2. Spontaneous mutation frequencies

a) Replicate mutation frequencies

Strain	Plate #	CD101 Col. #	CD101 Freq.	ANID Col. #	ANID Freq.	CAS Col. #	CAS Freq.
C. albicans NRRL Y-477	1	4	5.00E-08	15	1.88E-07	1	1.25E-08
	2	2	2.27E-08	14	1.59E-07	1	1.14E-08
	3	7	5.47E-08	20	1.56E-07	1	7.81E-09
C. glabrata ATCC 90030	1	3	1.35E-08	2	9.01E-09	57	2.57E-07
	2	8	3.52E-08	1	4.41E-09	110	4.85E-07
	3	3	1.26E-08	5	2.10E-08	82	3.45E-07
C. glabrata ATCC 2001	1	9	3.16E-08	20	7.02E-08	9	3.16E-08
	2	13	3.79E-08	23	6.71E-08	10	2.92E-08
	3	3	1.17E-08	28	1.09E-07	9	3.50E-08
C. parapsilosis CP02	1	2	2.08E-08	0	<1.04E-08	0	<1.04E-08
	2	1	9.62E-09	0	<9.62E-09	0	<9.62E-09
	3	2	2.08E-08	0	<1.04E-08	0	<1.04E-08
C. krusei ATCC 6258	1	4	1.54E-08	0	<3.38E-09	0	<3.86E-09
	2	1	3.51E-09	0	<3.51E-09	1	3.51E-09
	3	1	3.86E-09	0	<3.38E-09	0	<3.86E-09

b) Median mutation frequencies

Strain	CD101	ANID	CAS
C. albicans NRRL Y-477	5.00E-08	1.59E-07	1.14E-08
C. glabrata ATCC 90030	1.35E-08	9.01E-09	3.45E-07
C. glabrata ATCC 2001	3.16E-08	7.02E-08	3.16E-08
C. parapsilosis CP02	2.08E-08	<1.04E-08	<1.04E-08
C. krusei ATCC 6258	3.86E-09	<3.38E-09	<3.86E-09

- A total of 472 spontaneous mutants were recovered following selection of CD101, ANID and CAS vs. 5 *Candida* strains (63 for CD101, 128 for ANID, and 281 for CAS).
- Spontaneous mutation frequencies were lower for *C. krusei* and *C. parapsilosis* than for *C. albicans* and *C. glabrata*.
- CD101 median mutation frequencies ranged from 5.0 x 10^{-8} to 3.86 x 10^{-9}, comparable to those for CAS and ANID.

Table 3. Summary of mutants possessing *fks* mutations

Background	MIC (µg/mL) CD101	ANID	CAS	AMB	Fks amino acid substitutions Fks1 HS1	Fks1 HS2	Fks2 HS1	Fks2 HS2
C. albicans NRRL Y-477	0.03	0.015	0.25	0.5	WT	WT	WT	WT
	0.25	0.25	0.5	0.25	S645P†	WT	NS	NS
C. glabrata ATCC 90030	0.06	0.06	0.25	0.5	WT	WT	WT	WT
	2	2	4	0.5	WT	WT	ΔF659	WT
	0.25	1	1	0.5	WT	WT	D666H	WT
C. glabrata ATCC 2001	0.125	0.06	0.25	0.5	WT	WT	WT	WT
	0.25	0.25	0.25	0.25	WT	WT	WT	R1378S
	2	4	16	0.25	WT	WT	ΔF659	WT
	0.5	0.5	0.5	0.5	WT	WT	D666Y	WT
	1	1	1	0.25	WT	WT	S663F	WT
	0.5	0.5	0.5	0.25	WT	WT	R665G	WT
	0.5	0.5	0.25	0.5	WT	WT	D666N	WT

WT, wild-type; HS1, hot spot 1; HS2, hot spot 2; NS, not sequenced; † heterozygous mutation

- Seven different *fks* hot spot mutations were identified in 19 strains which typically had the most shifted MIC values.
- *fks* mutations were identified in residues with clinical precedence for echinocandin resistance.[4]
- Echinocandin cross-resistance was observed for all mutants.

CONCLUSIONS

- Spontaneous mutation frequencies for CD101 in *Candida* spp. is low, with median values in the 10^{-8} to 10^{-9} range @ 1X agar MIC.
- CD101 mutation frequencies were within the ranges generated for comparator echinocandins anidulafungin and caspofungin.
- All CD101-selected mutants with broth MIC values increasing ≥2-fold also demonstrated cross-resistance to anidulafungin and/or caspofungin.
- *fks* hot spot mutations were only identified in mutants possessing the largest MIC shifts.
- Consistent with clinical trends,[4] the *C. glabrata* strains had the highest resistance incidences of all the *Candida* spp. evaluated.
- Spontaneous mutations conferring >2-fold MIC shifts to all three echinocandins are even less frequent than the values derived in these plating experiments.
- Because of CD101's unique front-loading dosing paradigm and extended half-life, the potentially higher achievable C_{max} and AUC values may help prevent the emergence of spontaneous resistance during the course of therapy

REFERENCES

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