F-754

Characterization of Resistance Following Serial Passage of *Candida* spp. in the Presence of CD101

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ABSTRACT

Background: CD101 is a novel, extended half-life echinocandin under development for the treatment of candidemia and invasive candidiasis. In this study we investigated the potential for and genetic mechanisms underlying the development of resistance to CD101 in *Candida* spp. through serial passage.

Methods: Serial passage studies were conducted using CD101 and comparator echinocandins anidulafungin (ANID) and caspofungin (CAS) vs. *C. albicans* (x1), *C. glabrata* (x2), *C. parapsilosis* (x1) and *C. krusei* (x1) strains. An agar (SDA) drug gradient plate methodology was used in which drug concentrations above and below the inhibitory levels for each strain at each passage were present and were increased as strains developed reduced susceptibility over time. Following each 24 h passage, the leading edge of growth (i.e. most resistant cells) was scraped off the plate and resuspended in 0.85% NaCl to an OD_{530} of ~1.0 and 100 µL was spread onto a fresh gradient plate (~1 x 10^6 CFU). Twenty consecutive passages were completed and total population MIC values (CLSI broth microdilution) were determined every 5th passage. *FKS* gene hot spot (HS) regions were sequenced in representative passage #20 (P20) colonies.

Results: Maximal P20 CD101 MIC values for CD101-selected strains were 0.25 µg/mL for *C. albicans*, 1 µg/mL for both *C. glabrata* strains, 4 µg/mL for *C. parapsilosis*, and 0.125 µg/mL for *C. krusei*, corresponding to MIC fold shift increases equivalent to or lower than the majority of those generated under selection with ANID and CAS. Cross-resistance was broadly observed among the three echinocandins evaluated and there were no CD101-selected mutants that conferred reduced susceptibility to CD101 but not also to ANID and/or CAS. Of the 15 selection groups, 6 of them had P20 strains possessing a total of 5 different *fks* HS mutations (Fks1 HS1: S645Y, D632Y; Fks1 HS2: I1366S; Fks2 HS1: D666I, F659I/D666Y), all of which were homozygous. With the exception of *C. krusei*, only P20 strains with the largest MIC shifts possessed *fks* HS mutations. *C. glabrata* demonstrated the highest potential for echinocandin resistance development.

Conclusions: The potential for resistance development to CD101 among 4 clinically-relevant *Candida* spp. was low over the course of 20 serial passages and generally comparable to 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 potential for clinically relevant *Candida* spp. to develop reduced susceptibility to CD101 following serial exposure, 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 serial passage 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 clean, 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 are reported as concentrations resulting in prominent growth inhibition (~50%) per CLSI guidance for echinocandins.[1] MIC assays were performed in triplicate.

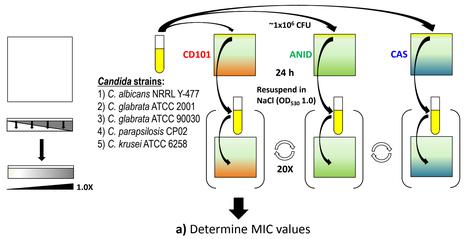
Serial passage. SDA drug gradient plates were created by pouring two overlapping layers of media as previously described[3] using 90x90 mm square petri dishes. As reduced susceptibility developed, drug concentrations were increased to maintain the leading edge of growth within the drug gradient. Following each passage the leading edge of growth (i.e., most resistant cells) was resuspended in 0.85% NaCl to an absorbance of ~1.0 OD_{530} and a 100 µL aliquot (~1.0 x 10^6 CFU) was spread onto a fresh passage plate using sterile glass beads. As strains developed reduced susceptibility to selecting drugs and were able to grow past the halfway point on the gradient plate, drug concentrations were increased 2-fold for subsequent passages. A glycerol stock was made from the total cell population for each culture condition for each passage. Twenty serial passages were completed for each drug/strain combination. MIC testing was performed on total cell populations for each group every 5th passage.

Analysis of individual passage 20 colonies. Passage #20 (P20) total populations were streaked to isolation on SDA and three colonies were selected. All three colonies were assessed via MIC, and a representative colony of the total population MIC was selected for further analysis.

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.[4] 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 WT *FKS* sequences using Vector NTI® software (Life Technologies).

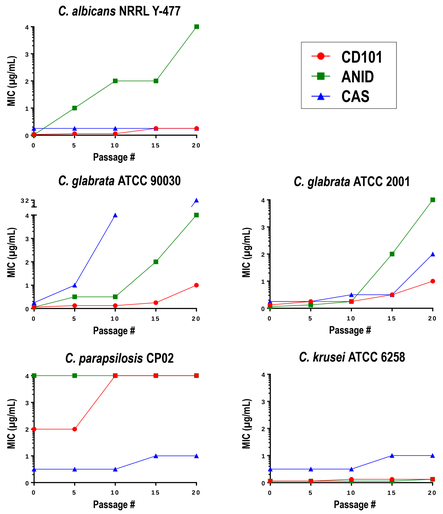
METHODS (cont'd)

Gradient plate selection methodology



RESULTS

Fig. 1. Total population serial passage MIC plots



- Reduced susceptibility was observed for all 3 echinocandins vs. most of the 5 *Candida* strains following 20 serial passages.
- *C. glabrata* strains had the most consistently high MIC shifts at P20 for all drugs, followed by *C. albicans*, and finally *C. krusei* and *C. parapsilosis* had the lowest resistance potential of all strains tested.

RESULTS (cont'd)

Table 1. MIC fold shift increases over 20 serial passages

Background	Selecting drug	MIC (µg/mL) WT	P20	MIC fold-shift
C. albicans NRRL Y-477	CD101	0.03	0.25*	8
	ANID	0.015	4*‡	256
	CAS	0.25	0.25	1
C. glabrata ATCC 90030	CD101	0.06	1	16
	ANID	0.06	4*‡	64
	CAS	0.25	32*‡	128
C. glabrata ATCC 2001	CD101	0.125	1	8
	ANID	0.06	4‡	64
	CAS	0.25	2‡	8
C. parapsilosis CP02	CD101	2	4	2
	ANID	4	4	1
	CAS	0.5	1	2
C. krusei ATCC 6258	CD101	0.06	0.125	2
	ANID	0.06	0.125	2
	CAS	0.5	1‡	2

*required 48 h of incubation to read MIC values; ‡ indicates CLSI "resistant" MIC value; MIC breakpoints for CD101 have not yet been established; WT, wild-type.

- Serial passage generated strains that were CAS-resistant for both *C. glabrata* strains and *C. krusei* and ANID-resistant for both *C. glabrata* strains and for *C. albicans* following 20 passages.

Table 2. MIC and *FKS* gene hot spot sequence analyses of individual P20 strains

Background	Selecting drug	Strain	MIC (µg/mL) CD101	ANID	CAS	AMB	Fks amino acid changes Fks1 HS1	Fks1 HS2	Fks2 HS1	Fks2 HS2
C. albicans NRRL Y-477	-	WT	0.03	0.015	0.25	0.5	WT	WT	WT	WT
	CD101	P20-1*	0.25	0.125	0.5	0.5	WT	WT	NS	NS
	ANID	P20-1*	2	4	2	0.5	S645Y‡	WT	NS	NS
	CAS	P20-1	0.03	0.015	0.25	0.25	WT	WT	NS	NS
C. glabrata ATCC 90030	-	WT	0.06	0.06	0.25	0.5	WT	WT	WT	WT
	CD101	P20-2	1	1	1	0.5	WT	WT	WT	WT
	ANID	P20-1*	2	4	2	0.5	WT	WT	D666I	WT
	CAS	P20-1*	8	8	32	0.25	WT	WT	F659I, D666Y	WT
C. glabrata ATCC 2001	-	WT	0.125	0.06	0.25	0.5	WT	WT	WT	WT
	CD101	P20-2	1	1	1	0.25	WT	WT	WT	WT
	ANID	P20-2	2	4	2	1	D632Y	WT	WT	WT
	CAS	P20-2	2	2	2	0.5	WT	WT	WT	WT
C. parapsilosis CP02	-	WT	2	4	0.5	0.5	WT	WT	WT	WT
	CD101	P20-1	4	8	1	0.5	WT	WT	NS	NS
	ANID	P20-1	4	4	0.5	0.5	WT	WT	NS	NS
	CAS	P20-1	2	4	1	0.5	WT	WT	NS	NS
C. krusei ATCC 6258	-	WT	0.06	0.06	0.5	1	WT	WT	WT	WT
	CD101	P20-2	0.125	0.25	1	1	WT	I1366S†	NS	NS
	ANID	P20-2	0.125	0.125	0.5	1	WT	WT	NS	NS
	CAS	P20-2	0.25	0.25	1	1	WT	I1366S†	NS	NS

* required 48 hours of incubation to read MIC values; † homozygous mutation; WT, wild-type; HS1, hot spot 1; HS2, hot spot 2; NS, not sequenced.

- Cross-resistance with at least one other echinocandin was observed for all P20 strains with any level of reduced susceptibility to the selecting drug.
- With the exception of *C. krusei*, only strains with highly shifted MIC values possessed mutations in *FKS* hot spot regions.
- Fks residues mutated at hotspot positions S645 (*C. albicans*), D632, D666, and F659 (*C. glabrata*) have clinical precedence while the substitution at I1366 in P20 *C. krusei* has not been observed.[5]

CONCLUSIONS

- The potential for resistance development vs. CD101 among 4 key clinically-relevant *Candida* species was low.
- CD101 had the smallest overall MIC fold-shift increases at passage #20
- Consistent with clinical observations and its haploid nature, *C. glabrata* demonstrated the highest potential for echinocandin resistance development.
- Cross-resistance was broadly observed among the three echinocandins evaluated.
- With the exception of *C. krusei*, only P20 strains with the largest MIC shifts possessed *fks* hot spot mutations.
- Although CD101 resistance trends were similar to CAS and ANID in vitro, its long half-life and front-loading dosing paradigm (i.e., high C_{max} and AUC) may help prevent the selection of resistant strains in a clinical setting.

REFERENCES

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ACKNOWLEDGEMENTS

ATCC 2001, ATCC 90030, and ATCC 6258 were obtained form the American Type Culture Collection. The *C. parapsilosis* CP02 isolate was kindly provided by Dr. Jack Sobel. *C. albicans* NRRL Y-477 was acquired from the United States Department of Agriculture (USDA) Agricultural Research Service (ARS) culture collection.