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SUSCEPTIBILITY

Rezafungin *In Vitro* Activity against Contemporary Nordic Clinical *Candida* Isolates and *Candida auris* Determined by the EUCAST Reference Method

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ABSTRACT Rezafungin (formerly CD101) is a novel echinocandin in clinical development. EUCAST epidemiological cutoff values (ECOFFs) have not yet been established. We determined the *in vitro* activity of rezafungin and comparators against 1,293 Nordic yeast isolates and 122 Indian *Candida auris* isolates and established single-center wild-type upper limits (WT-UL). The isolates (19 *Candida* spp. and 13 other yeast species) were identified using Chromagar; matrix-assisted laser desorption ionization–time of flight (MALDI-TOF); and, when needed, internal transcribed spacer sequencing. EUCAST E.Def 7.3.1 susceptibility testing included rezafungin, anidulafungin, micafungin, amphotericin B, and fluconazole. WT-UL were established following EUCAST principles for visual and statistical ECOFF setting. *fks* target genes were sequenced for rezafungin non-wild-type isolates. EUCAST clinical breakpoints for fungi version 9.0 were adopted for susceptibility classification. Rezafungin had species-specific activity similar to that of anidulafungin and micafungin. On a milligram-per-liter basis, rezafungin was overall less active than anidulafungin and micafungin but equally or more active than fluconazole and amphotericin B against the most common *Candida* species, except *C. parapsilosis*. We identified 37 (3.1%) rezafungin non-wild-type isolates of *C. albicans* (1.9%), *C. glabrata* (3.0%), *C. tropicalis* (2.7%), *C. dubliniensis* (2.9%), *C. krusei* (1.2%), and *C. auris* (14.8%). Alterations in Fks hot spots were found in 26/26 Nordic and 8/18 non-wild-type *C. auris* isolates. Rezafungin displayed broad *in vitro* activity against *Candida* spp., including *C. auris*. Adopting WT-UL established here, few Nordic strains, but a significant proportion of *C. auris* isolates, had elevated MICs with mutations in *fks* target genes that conferred echinocandin cross-resistance. *fks1* mutations raised rezafungin MICs notably less than anidulafungin and micafungin MICs in *C. auris*.

KEYWORDS rezafungin, *in vitro* activity, MIC, *Candida*, *Candida auris*, EUCAST, antifungal resistance, antifungal susceptibility testing, echinocandin

Echinocandins are the recommended first-line treatment for candidemia and invasive candidiasis. The currently available echinocandins—anidulafungin, caspofungin, and micafungin—are dosed intravenously once daily. Echinocandins target the enzyme complex 1,3-β-D-glucan synthase, which is comprised of two subunits: a regulatory GTP-binding protein, Rho1, and a catalytic component, Fks, which is encoded by three highly homologous genes, *FKS1*, *FKS2*, and *FKS3*. Alterations in the hot spots of Fks1 and Fks2 (*Candida glabrata* only) result in reduced sensitivity to echinocandins and elevated MIC values across various *Candida* species (1).

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TABLE 1 *In vitro* activity of rezafungin against Nordic *Candida* isolates and *C. auris* isolates from India as determined by EUCAST E.Def 7.3.1

Yeast	*n*	No. with MIC (mg/liter)[a]:										MIC range (mg/liter)	MIC50 (mg/liter)	MIC90 (mg/liter)	WT-UL[b] (mg/liter)		
		0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	≥8				Visual	97.5%	99%
Nordic isolates																	
C. albicans	569	28	221	282	27	**8**	**2**	**1**				0.016 to 1	0.06	0.06	0.125	0.125	0.125
C. glabrata	328		1	87	214	16	**5**	**1**	**4**			0.03 to 2	0.125	0.125	0.25	0.25	0.25
C. krusei	82		2	26	47	6		**1**				0.03 to 1	0.125	0.125	0.25	0.25	0.25
C. tropicalis	73		2	19	43	7		**1**	**1**			0.03 to 2	0.125	0.25	0.25	0.25	0.25
C. dubliniensis	68		8	28	29	1		**1**	**1**			0.03 to 2	0.06	0.125	0.25	0.25	0.25
C. parapsilosis	61							8	40	13		1 to 4	2	4	4	4	4
C. lusitaniae	20				10	8	**2**					0.125 to 0.4	0,125	0,25	0.5	0.25	0.25
S. cerevisiae	15				6	8	1					0.125 to 0.5	0.25	0.25	0.5	0.5	0.5
Other *Candida*	55		2	16	7	1	11	11	6		1	0.03 to >4	0.5	2	ND	ND	ND
Other yeast	22		2	2	1			1		2	14	0.03 to >4	>4	>4	ND	ND	ND
Indian isolates																	
C. auris	122			3	22	63	16	**7**	**3**		**8**	0.06 to 16	0.25	1	0.5	0.5	0.5

[a]MIC values above the WT-UL are in boldface.
[b]The WT-UL was determined visually and statistically using the ECOFFinder program, including 97.5% and 99% of the wild-type population, respectively. The ECOFFinder program requires at least one isolate with an MIC at least one step above the modal MIC to calculate an upper limit. ND, not done.

Rezafungin is a novel echinocandin that is currently in phase 3 development. It has activity against *Aspergillus* and *Candida* species, including *C. auris*, an emerging pathogen with high rates of drug resistance that spreads readily in health care facilities and can cause severe nosocomial infections (2).

Rezafungin is a structural analogue of anidulafungin with a choline moiety at the C-5 ornithine position, which results in increased stability (3). The safety and pharmacokinetic (PK) profile, with a long half-life of approximately 130 h, allow for once-weekly dosing (4). The epidemiological cutoff values and clinical breakpoints for rezafungin against common *Candida* species have not yet been established due to interlaboratory variation in MIC values for the *Candida* species with the highest susceptibility to echinocandins (such as *C. albicans* and *C. tropicalis*) (5).

The objectives of this study were (i) to examine the *in vitro* activity of rezafungin against a large sample of contemporary clinical isolates of *Candida* and other yeasts from Nordic countries and clinical isolates of *C. auris* from India, (ii) to determine the pattern of rezafungin resistance mutations in contemporary clinical non-wild-type *Candida* isolates, and (iii) to examine how *fks* mutations affect susceptibility to rezafungin and the degree of cross-resistance between rezafungin and other echinocandins.

RESULTS

Rezafungin against quality control strains. *C. albicans* CNM-CL F8555, *C. parapsilosis* ATCC 22019, and *C. krusei* ATCC 6258 were tested 19, 81, and 108 times, respectively, during the study period. The MIC results for *C. albicans* CNM-CL F8555 were as follows: modal MIC, 0.03 mg/liter; MIC_{50}, 0.03 mg/liter; range, 0.03 to 0.06 mg/liter. The MICs against *C. parapsilosis* ATCC 22019 were as follows: modal MIC, 2 mg/liter; MIC_{50}, 2 mg/liter; range, 0.5 to 2 mg/liter. The MICs against *C. krusei* ATCC 6258 were as follows: modal MIC, 0.125 mg/liter; MIC_{50}, 0.125 mg/liter; range, 0.03 to 0.125 mg/liter. Thus, for all three quality control (QC) strains, the range spanned ≤3 2-fold dilutions.

Rezafungin *in vitro* activity against Nordic clinical yeast isolates. Rezafungin MIC distributions for the most common *Candida* species are shown in Table 1. Rezafungin had *in vitro* activity, with MIC_{90}s of <0.5 mg/liter for all the most common *Candia* species except *C. parapsilosis*, where the MIC range was 1 to 4 mg/liter. The modal MICs for *C. albicans* and *C. glabrata* were 0.06 and 0.125 mg/liter, respectively. The statistical wild-type upper limits (WT-UL) (99%) were 0.25 mg/liter for most *Candida* species except *C. albicans* (0.125 mg/liter) and *C. parapsilosis* (4 mg/liter). The visual WT-UL was within one dilution of the statistical WT-UL for all species.

There were 12 *Candida* species with less than 15 isolates (see Table S1 in the supplemental material). For six of them, rezafungin MICs were ≤0.25 mg/liter (*C. kefyr*

[$n = 14$], *C. inconspicua* [$n = 3$], *C. nivariensis* [$n = 2$], *C. norvegensis* [$n = 1$], *C. pelliculosa* [$n = 1$], and *C. utilis* [$n = 3$]), and for five species, rezafungin MICs were ≥0.5 mg/liter (*C. doubushaemulonis* [$n = 1$], *C. fermentati* [$n = 3$], *C. guilliermondii* [$n = 13$], *C. metapsilosis* [$n = 5$], and *C. orthopsilosis* [$n = 5$]). Finally, for two isolates, belonging to a new *Candida* species related to *C. blankii*, variable rezafungin MICs were obtained (0.06 and 8 mg/liter, respectively).

Table 2 summarizes comparisons of rezafungin MICs to those of other licensed antifungal agents for contemporary Nordic clinical yeast isolates and Indian clinical *C. auris* isolates. Rezafungin had species-specific *in vitro* activity similar to that of anidulafungin and micafungin. On a milligram-per-liter basis, rezafungin was overall less active (modal MICs were ≥2 2-fold dilutions higher) than anidulafungin and micafungin but equally or more active than fluconazole and amphotericin B, except against *C. parapsilosis*, other *Candida* spp. (amphotericin B only), and other yeasts.

The proportions of rezafungin non-wild-type isolates among the most common *Candida* species were ≤3% (Table 2). For *C. albicans*, the proportion of rezafungin non-wild-type isolates was lower than those of micafungin ($P < 0.001$) and fluconazole ($P = 0.001$) but higher than that of amphotericin B ($P < 0.001$). For *C. glabrata*, the proportion of rezafungin non-wild-type isolates was lower than that of fluconazole ($P = 0.004$) and higher than that of amphotericin B ($P = 0.001$).

Rezafungin *in vitro* activity against *Candida auris*. The rezafungin modal MIC for Indian clinical *C. auris* was 0.25 mg/liter (Table 1), which is two and one 2-fold dilutions higher than for *C. albicans* and *C. glabrata*, respectively. The rezafungin visual WT-UL and statistical WT-UL (97.5% and 99%) were 0.5 mg/liter.

The *in vitro* activity of rezafungin was similar to those of anidulafungin and micafungin (modal MIC, one 2-fold dilution higher). Rezafungin was more active than amphotericin B and fluconazole on a milligram-per-liter basis. Voriconazole and isavuconazole displayed bimodal and trimodal distributions, making comparisons with these drugs complex (Table 3).

A total of 18 of 122 *C. auris* isolates (14.8%) were non-wild type if the statistical 97.5% EUCAST epidemiological cutoff value (ECOFF) is adopted, which is higher than those for amphotericin B ($P < 0.001$) and micafungin ($P = 0.03$) but lower than those for anidulafungin ($P = 0.03$) and fluconazole ($P < 0.001$) (Table 2).

Resistance mutations in non-wild-type strains. We identified 18 (14.8%) rezafungin non-wild-type strains among the Indian *C. auris* isolates and 26 (2.1%) among the most common Nordic *Candida* strains: *C. albicans* ($n = 11$; 1.9%), *C. glabrata* ($n = 10$; 3.3%), *C. tropicalis* ($n = 2$; 2.7%), *C. dubliniensis* ($n = 2$; 2.9%), and *C. krusei* ($n = 1$; 1.2%) (Table 1). Alterations in Fks1 and/or Fks2 hot spots (or within ±3 amino acids) were found in all 26 Nordic isolates and in 8/18 *C. auris* non-wild-type isolates. The Fks1/Fks2 hot spot mutations, as well as the MICs of rezafungin and comparators, are summarized in Table 4. Rezafungin MICs for isolates with *fks* hot spot mutations were 0.25 to 2 mg/liter for the Nordic isolates but 8 to 16 mg/liter for *C. auris* isolates. The increase in rezafungin MICs (number of 2-fold dilutions) conferred by substitution of phenylalanine for serine in Fks1 or Fks2 hot spots was 2 to 4 for *C. glabrata* and *C. krusei* but 5 to 6 for *C. auris*. The increase in MIC conferred by the S639F mutation in *C. auris* was even more marked for anidulafungin and micafungin (>8 2-fold dilutions).

For 5/11 *C. albicans* isolates with Fks1 hot spot mutations and rezafungin MICs greater than the WT-UL, the anidulafungin MIC was less than or equal to the ECOFF. However, generally, *fks* hot spot mutations affected rezafungin susceptibility (as measured by the increase in the MIC relative to the modal MIC) to a similar degree as or less than anidulafungin and micafungin (Table 4).

DISCUSSION

We studied the *in vitro* activity of rezafungin and comparators against 1,293 contemporary clinical yeast isolates from Nordic countries and 122 clinical *C. auris* isolates from India. The Nordic isolates comprised isolates from Danish samples, primarily from patients with candidemia and invasive infections, as well as clinical isolates from other

TABLE 2 Comparison of rezafungin MICs to those of other licensed antifungal agents for 1,293 contemporary Nordic clinical *Candida* isolates and 122 *C. auris* isolates from India

Species	Rezafungin			Anidulafungin			Micafungin			Amphotericin B			Fluconazole		
	MIC range (mg/liter)	Modal MIC (mg/liter)	% (*n*) >WT-UL	MIC range (mg/liter)	Modal MIC (mg/liter)	% (*n*) >ECOFF	MIC range (mg/liter)	Modal MIC	% (*n*) >ECOFF	MIC range (mg/liter)	Modal MIC (mg/liter)	% (*n*) >ECOFF	MIC range (mg/liter)	Modal MIC (mg/liter)	% (*n*) >ECOFF
Nordic clinical isolates															
C. albicans	0.016 to 1	0.06	1.9 (11)	0.004 to 0.25	0.004	1.1 (6)	0.004 to 2	0.016	6.2 (35)[a]	0.06 to 0.5	0.25	0 (0)[a]	0.03 to >64	0.125	5.6 (32)[a]
C. glabrata	0.03 to 2	0.125	3.0 (10)	0.008 to 1	0.03	3.3 (11)	0.008 to 0.5	0.016	4.9 (16)	0.03 to 1	0.25	0 (0)[a]	0.5 to >64	4	8.2 (27)[a]
C. krusei	0.03 to 1	0.125	1.2 (1)	0.016 to 0.25	0.03	2.4 (2)	0.06 to 4	0.125	3.7 (3)	0.5 to 1	0.5	0 (0)	8 to >64	16	ND
C. tropicalis	0.03 to 2	0.125	2.7 (2)	0.008 to 2	0.03	2.7 (2)	0.016 to 2	0.03	2.7 (2)	0.125 to 1	0.25	0 (0)	0.125 to >64	0.5	8.2 (6)
C. dubliniensis	0.03 to 2	0.125	2.9 (2)	0.004 to 0.25	0.008	2.9[b] (2)	0.08 to 2	0.03	2.9[b] (2)	0.03 to 0.25	0.06	0[b] (0)	0.06 to 64	0.125	5.9[b] (4)
C. parapsilosis	1 to 4	2	0 (0)	0.25 to 2	1	0 (0)	0.25 to 4	2	0 (0)	0.125 to 1	0.5	0 (0)	0.25 to 64	0.5	4.9 (3)
C. lusitaniae	0.125 to 0.4	0.125	0 (0)	0.03 to 0.25	0.03	5.0[b] (1)	0.03 to 0.5	0.06	5.0[b] (1)	0.06 to 1	0.125	5.0[b] (1)	0.25 to 32	0.5	10.0[b] (2)
S. cerevisiae	0.125 to 0.5	0.25	0 (0)	0.016 to 0.125	0.06	0[b] (0)	0.06 to 0.25	0.125	0[b] (0)	0.03 to 1	0.25	0[b] (0)	0.25 to 32	8	0[b] (0)
Other *Candida*	0.03 to >4	0.06	ND[f]	0.004 to 4	0.25	ND	0.016 to 0.5	0.25	ND	0.06 to >4	0.25	ND	0.25 to >64	0.25	ND
Other yeast	0.03 to >4	>4	ND	0.004 to >4	>4	ND	0.008 to >4	>4	ND	0.03 to 2	0.25	ND	0.125 to >64	4	ND
Indian clinical isolates															
C. auris	0.06 to 16	0.25	14.8[c] (18)	0.016 to >32	0.06	27.0[c] (33)[a]	0.03 to >32	0.125	6.6[c] (8)	0.5 to 1	1	0[d] (0)[a]	0.5 to >64	>64	99.2[e] (121)[a]

[a]The proportion of non-wild-type strains is significantly different from that for rezafungin ($P < 0.05$).

[b]Percent greater than the WT-UL based upon the MICs for the contemporary Nordic clinical isolates included in this study.

[c]Percent greater than the WT-UL (statistical 97.5%) based on 122 clinical *C. auris* isolates from India (Table 3).

[d]Percent greater than the WT-UL (visual).

[e]For fluconazole, non-species-specific *Candida* sp. breakpoints have been set by EUCAST (susceptible, ≤2 mg/liter; resistant, >4 mg/liter).

[f]ND, not done.

TABLE 3 *In vitro* activities of rezafungin and comparators against 122 clinical *C. auris* isolates as determined by EUCAST E.Def 7.3.1[a]

Drug	Value for MIC (mg/liter): ≤0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	≥64	MIC range (mg/liter)	Modal MIC (mg/liter)	MIC_{50} (mg/liter)	WT-UL (mg/liter) Visual	97.5%	99%
Rezafungin					3	22	**63**	16	7	3		6	2			0.06 to 16	0.25	0.25	0.5	0.5	0.5
Anidulafungin[b]			1	11	35	**30**	12	12	11	2	1			7		0.016 to >32	0.06	0.125	0.25	0.25	0.5
Micafungin[b]				5	30	**70**	9							8		0.03 to >32	0.125	0.125	0.25	0.25	0.25
Amphotericin B[b]								14	**108**							0.5 to 1	1	1	2	NP	NP
Fluconazole[b]								1					2	10	**109**	0.5 to ≥64	≥64	≥64	NP	NP	NP
Voriconazole[b]	1			1	1	16	13	**34**	38	13	5					≤0.004 to 4	Bimodal	0.5	4	4	8
Isavuconazole[b]	20	1	1	19	9	**19**	21	21	6	5						≤0.004 to 2	Trimodal	0.125	0.016 or 2	NP	NP

[a]The shaded areas indicate that concentrations were not tested for the compound. MIC_{50}s are in boldface. The underlined values are the modal MICs for unimodal distributions but the lowest MIC peak for multimodal distributions, thus illustrating the modal MIC of the presumed wild-type distribution.

[b]The MIC distributions for comparator antifungals against *C. auris* were compiled from reference 20.

TABLE 4 *fks* hot spot mutations, MICs, and relative increases in MICs compared to modal MICs of rezafungin and comparators for *Candida* sp. isolates with rezafungin MICs above the wild-type upper limit

Organism	Mutation[a]		MIC[b] (mg/liter)					Increase in MIC[c]		
	Fks1	Fks2	RZF	ANF	MCF	AMB	FLU	RZF	ANF	MCF
C. albicans	S645P	NT	**1**	**0.25**	**2**	0.25	0.25	4	6	7
	D648Y	NT	**0.5**	**0.06**	**0.125**	0.25	0.125	3	4	3
	P1354S	NT	**0.5**	**0.06**	**0.125**	0.5	**>64**	3	4	3
	P1354S	NT	**0.25**	0.016	**0.06**	0.5	**>32**	2	2	2
	P1354S	NT	**0.25**	0.016	**0.06**	0.5	**64**	2	2	2
	P1354S	NT	**0.25**	0.016	**0.06**	0.5	**64**	2	2	2
	P1354P/S	NT	**0.25**	0.03	**0.06**	0.5	**>64**	2	3	2
	P1354P/S	NT	**0.25**	**0.06**	**0.06**	0.5	**>64**	2	4	2
	R1361R/S	NT	**0.25**	**0.06**	**0.125**	0.125	0.125	2	4	3
	R1361G	NT	**0.25**	**0.06**	**0.125**	0.125	0.25	2	4	3
	R1361G	NT	**0.25**	0.016	**0.06**	0.5	**64**	2	2	2
C. glabrata	L630Q	S663F	**2**	**1**	**0.5**	0.5	1	4	5	5
	L630Q	S663F	**2**	**1**	**0.5**	0.5	32	4	5	5
	WT	S663F	**2**	**1**	**0.5**	0.5	2	4	5	5
	WT	S663F	**1**	**0.25**	**0.125**	0.125	2	3	3	3
	WT	S663F	**0.5**	**0.25**	**0.125**	0.5	2	2	3	3
	WT	S663F	**0.5**	0.06	**0.06**	0.5	2	2	1	2
	WT	S663P	**2**	**1**	**0.5**	0.125	2	4	5	5
	WT	S663P	**0.5**	**0.125**	**0.125**	0.25	4	2	2	3
	Y1429X	Y658N/L664Q	**0.5**	**0.125**	**0.06**	0.125	**>32**	2	2	2
	WT	F659del	**0.5**	0.06	**0.06**	0.25	**>64**	2	1	2
C. tropicalis	F650S	NT	**1**	**0.25**	**1**	0.25	0.5	3	3	5
	S654P	NT	**2**	**2**	**2**	0.5	0.5	4	6	6
C. dubliniensis	S645P	NT	**2**	**0.25**	**2**	0.03	0.125	4	3	6
	S645P	NT	**1**	**0.25**	**2**	0.03	0.125	3	3	6
C. krusei	S659F	NT	**1**	**0.25**	**4**	0.5	32	3	3	7
C. auris	S639F	NT	**16**	**4**	**>32**	1	**>256**	6	6	>8
	S639F	NT	**16**	**>32**	**>32**	1	**>256**	6	>9	>8
	S639F	NT	**8**	**>32**	**>32**	1	**>256**	5	>9	>8
	S639F	NT	**8**	**>32**	**>32**	1	**>256**	5	>9	>8
	S639F	NT	**8**	**>32**	**>32**	1	**>256**	5	>9	>8
	S639F	NT	**8**	**>32**	**>32**	1	**>256**	5	>9	>8
	S639F	NT	**8**	**>32**	**>32**	1	**>256**	5	>9	>8
	S639F	NT	**8**	**>32**	**>32**	1	**>256**	5	>9	>8
	WT	NT	**2**	**2**	0.25	1	**>256**	3	5	1
	WT	NT	**2**	**1**	0.25	1	**256**	3	4	1
	WT	NT	**2**	0.03	0.03	0.5	**256**	3	−1	−2

[a]NT, not tested.
[b]MICs above the wild-type upper limit/ECOFF are in boldface. RZF, rezafungin; ANF, anidulafungin; MCF, micafungin; AMB, amphotericin B; FLU, fluconazole.
[c]The number of two-fold dilution increases in the MIC compared to the modal MIC.

Nordic countries, that were sent to the reference mycology laboratory due to clinical suspicion of antifungal resistance. Repeated testing of quality control strains was included and demonstrated high intralaboratory reproducibility during the 1-year study period, despite weekly in-house production of microdilution trays.

Rezafungin *in vitro* activity against Nordic clinical isolates. Rezafungin had species-specific *in vitro* activity similar to that of anidulafungin and micafungin and was more active than fluconazole on a milligram-per-liter basis against the most common *Candida* spp., except *C. parapsilosis*.

The proportion of *Candida* spp. with rezafungin MICs above the WT-UL was low (<3%) and, for *C. albicans* isolates, significantly lower than for micafungin. Although the proportion of *C. albicans* isolates with micafungin MICs greater than the ECOFF was higher than the proportion with rezafungin MICs greater than the WT-UL, the data did not indicate that the *C. albicans* isolates were less susceptible or had higher rates of resistance to micafungin than to rezafungin (Table 5). This difference is likely explained

TABLE 5 *In vitro* activity of rezafungin compared to anidulafungin and micafungin against 569 Nordic *C. albicans* isolates as determined by EUCAST E.Def 7.3.1

Drug	No. with MIC (mg/liter)[a]:										MIC range (mg/liter)	Modal MIC (mg/liter)	MIC_{50} (mg/liter)
	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2			
Rezafungin			28	221	**282**	27	8 (8)	2 (2)	1 (1)		0.016–1	0.06	0.06
Anidulafungin	**391**	159	12 (4)	1 (1)	5 (5)		1 (1)				0.004–0.25	0.004	0.004
Micafungin	18	221	**295**	24	6 (6)	4 (4)				1 (1)	0.004–2	0.016	0.016

[a]Modal MICs are in boldface. The numbers of isolates with mutations in *fks* target genes are in parentheses. The wild-type ranges defined by WT-UL for rezafungin and by EUCAST ECOFFs for anidulafungin and micafungin are shaded.

by technical issues, as the mode of the micafungin MIC distribution in our study was in general one 2-fold dilution higher than that in the data set that formed the basis for the EUCAST ECOFF setting, which confers a risk of misclassification of some wild-type isolates as resistant. The echinocandins are highly potent compounds with a tendency to adhere to plastic. Microdilution testing of echinocandins has previously been associated with variation, as previously reported for caspofungin, anidulafungin, and rezafungin, and the data found here suggest that may also be the case occasionally for micafungin, a challenge that needs attention (6–8).

Our findings confirm and expand those from a previous study that investigated the *in vitro* activity of rezafungin and comparators against 531 *Candida* isolates using the Clinical and Laboratory Standards Institute (CLSI) broth microdilution methods for yeasts (9). However, rezafungin MIC_{50} values were generally two 2-fold dilutions lower in that study. In contrast, a prior single-center study that assessed rezafungin MIC values for *Candida* spp. demonstrated similar results when CLSI and EUCAST methods were compared (10). The reason for these differences in MIC values between studies is not clear but may reflect method-specific differences or the interlaboratory variation mentioned above. Indeed, a multicenter study from four European mycology reference laboratories found significant interlaboratory variation in rezafungin and anidulafungin MIC values (5), which was partly explained by the use of different microtiter plates (8).

Human PK studies have demonstrated that 400 mg rezafungin administered intravenously once weekly results in an area under the concentration-time curve (AUC) from 0 to 168 h at 1,840 mg·h/liter (4). With once-weekly dosing of rezafungin at 400 mg intravenously, the estimated MIC ceiling to achieve a PK-pharmacodynamic (PD) target of >90% for the 1-log_{10} CFU reduction endpoint is 0.5 mg/liter for *C. albicans* and 4 mg/liter for *C. glabrata* (11). In the present study, the MIC_{90} values for *C. albicans* and *C. glabrata* were well below these estimated MIC ceilings (0.06 and 0.125 mg/liter, respectively). Only one of 569 (<0.2%) *C. albicans* isolates had an MIC above 0.5 mg/liter, and none of 329 *C. glabrata* isolates had an MIC above 4 mg/liter.

Rezafungin *in vitro* activity against *Candida auris*. Rezafungin had *in vitro* activity against clinical *C. auris* isolates; however, approximately 15% of the isolates were classified as non-wild type using a statistical 97.5% endpoint, and 8 (6.6%) harbored Fks1 hot spot alterations and had high MICs of 8 to 16 mg/liter. These mutant strains were also resistant to other echinocandins and fluconazole but susceptible to amphotericin B. Our results are in accordance with a study by Berkow and Lockhart showing *in vitro* activity of rezafungin against *C. auris* isolates but echinocandin cross-resistance of those harboring the S639P alteration at the same codon, using the CLSI methodology (12). However, that study did not include comparisons with azoles or amphotericin B.

In a previous study based on a neutropenic mouse model, the estimated MIC ceiling to achieve 1-log-kill target exposures against *C. auris* was 1 to 2 mg/liter, and for the stasis target it was 2 to 4 mg/liter (13). In the present study, a total of 14.8% of the tested *C. auris* isolates had an MIC of ≥1 mg/liter, and 9% had an MICof ≥2 mg/liter. Thus, a significant proportion of the clinical *C. auris* isolates had MICs that were at or above the above-mentioned estimated MIC ceilings. The MICs for anidulafungin and micafungin against these isolates were even higher, suggesting that liposomal amphotericin B may be a more appropriate choice for empirical treatment of severe *C. auris*

infections until results of susceptibility testing are available in regions where this *fks* genotype is prevalent.

Resistance mutations in non-wild-type strains. Among the Nordic *Candida* strains, only 26 non-wild-type isolates with mutations in *fks* target genes were detected. Locke et al. found that there was a low potential for resistance development for rezafungin, similar to other echinocandins, and that rezafungin-selected strains had MICs that were lower than or equal to those for the majority of strains generated under selection with anidulafungin and caspofungin (14). We found cross-resistance between rezafungin and the comparator echinocandins. Generally, the relative increase in rezafungin MICs conferred by *fks* mutations was comparable to or slightly less than those for anidulafungin and micafungin. In addition, it was codon dependent, with the most pronounced MIC elevations for alterations involving S645 in *C. albicans* and *C. dubliniensis* and the corresponding codon in the other *Candida* species (S663 in *C. glabrata*, S654 in *C. tropicalis*, and S659 in *C. krusei*). Of note, a differential impact on susceptibility was observed for the Fks1 hot spot S639F substitution in *C. auris*. Although this mutation caused significant elevation in MICs for rezafungin, in agreement with a study by Berkow and Lockhart showing a significant MIC elevation of isolates with an S639P alteration, the MIC elevation was more than three and four 2-fold-dilutions greater for the two other echinocandins. These findings suggest that the MIC elevation depends on the codon involved, the species in question, and the substitution, as previously suggested for other *Candida* spp. (15, 16).

In conclusion, rezafungin displayed broad *in vitro* activity against *Candida* spp., including *C. auris*. Adoption of center-specific WT-UL allowed a reliable categorization of isolates as wild type or non-wild type, which was supported by molecular analysis, an approach that may be helpful until official ECOFFs and clinical breakpoints are established. Few Nordic strains, but a notable proportion of the Indian *C. auris* isolates, had elevated MICs due to a mutation in the *fks1* target gene that conferred echinocandin cross-resistance. *fks1* mutations raised rezafungin MICs slightly less than the anidulafungin and micafungin MICs in most *Candida* spp., but notably less in *C. auris*.

MATERIALS AND METHODS

Isolates. A total of 1,293 clinical yeast isolates (19 *Candida* and 13 other yeast species) received at the Danish mycology reference laboratory in 2017-2018 were included in the study. Of these 1,293 isolates, 1,226 were from Denmark (841 blood isolates and 385 isolates from tissue, pus, or swabs) and 67 were isolates referred from other Nordic countries due to clinical suspicion of antifungal resistance (Norway, 32; Sweden, 32; Greenland, 1; and Faroe Islands, 2). The species distribution (number of isolates) was as follows: *C. albicans* (569), *C. dubliniensis* (68), *C. glabrata* (328), *C. krusei* (82), *C. lusitaniae* (20), *C. parapsilosis* (61), *C. tropicalis* (73), other *Candida* spp. (41), and other yeast species (36). In addition, 122 clinical isolates of *C. auris* were collected from individual patients in six tertiary-care hospitals in India from 2010 to 2015. The isolates were mainly obtained from blood from patients with candidemia ($n = 100$) and samples from patients with other invasive *Candida* infections ($n = 22$), which included tissue, pleural fluid, and pus.

Species identification. Identification methods, including the use of matrix-assisted laser desorption ionization–time of flight mass spectrometry (Bruker, Bremen, Germany), were performed as previously described (15), with the addition of DNA sequencing as described below when needed.

Susceptibility testing. Rezafungin (Cidara Therapeutics, San Diego, CA, USA) pure substance was stored in aliquots at −70 to −80°C, and stock solutions were prepared in dimethyl sulfoxide (DMSO) (5,000 mg/liter; Sigma-Aldrich, Brøndby, Denmark). EUCAST MICs were determined following E.Def 7.3.1 methodology (17). The final drug concentration range studied was 0.004 to 4 mg/liter, except for *C. auris* (range, 0.004 to 256 mg/liter). The following comparator compounds were also investigated (final concentration range and source of compound in parentheses): anidulafungin (0.004 to 4 mg/liter; Pfizer A/S, Ballerup, Denmark), micafungin (0.004 to 4 mg/liter; Astellas Pharma Inc., Tokyo, Japan), amphotericin B (0.004 to 4 mg/liter; Sigma-Aldrich), fluconazole (0.03 to 32 mg/liter for Nordic isolates and 0.5 to 256 mg/liter for *C. auris*; Sigma-Aldrich), and voriconazole (0.004 to 4 mg/liter; Pfizer A/S, Ballerup, Denmark). Cell culture-treated microtiter plates (Nunc MicroWell 96-well microplates; catalog no. 167008; Thermo Fisher Scientific) were used throughout. The microtiter plates were prepared with 2-fold drug dilutions in double-concentration medium according to the EUCAST methodology and frozen at −80°C prior to use. The EUCAST QC strains *C. albicans* CNM-CL F8555, *C. parapsilosis* ATCC 22019, and *C. krusei* ATCC 6258 were tested in parallel.

Molecular identification and *fks* gene sequence analysis. Sequencing of internal transcribed spacer regions ITS1 and ITS2 was performed, and DNA sequence analysis of echinocandin target hot spots in *fks1*, and for *C. glabrata* also in *fks2*, was performed for non-wild-type isolates as previously described (18). The sequences obtained were compared to relevant reference sequences.

Data management. MIC ranges and modal MIC (the most common MIC), MIC_{50}, and MIC_{90} values were calculated. EUCAST ECOFFs and EUCAST clinical breakpoints for fungi (version 9.0) were adopted for wild-type and susceptibility classification where available. The WT-UL, defined as the upper MIC value where the wild-type distribution ends, were determined for rezafungin following principles for setting EUCAST ECOFFs. However, as the values reported here are not formally accepted EUCAST rezafungin ECOFFs, we used the term "WT-UL" to avoid confusion. The conventional method for determining the ECOFF is a visual inspection of histograms of the MICs for single species (the eyeball method) (19). Additionally, WT-UL were determined statistically using 97.5% and 99% endpoints and the EUCAST ECOFFinder program (19). A "non-wild-type" isolate was defined as an isolate with an MIC above the statistical WT-UL (97.5%).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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We have the following potential conflicts to declare. M.C.A. has, over the past 5 years, received research grants/contract work (paid to the SSI) from Amplyx 219, Basilea, Cidara, F2G, Gilead, Novabiotics, Scynexis, and T2 Biosystems and speaker honoraria (personal fees) from Astellas, Gilead, Novartis, MSD, Seges, and Pfizer. She is the current chairman of the EUCAST-AFST. M.H. has, over the past 5 years, received speaker honoraria from GSK, CLS Behring, and Gilead. GSK and Gilead have funded her participation in scientific meetings and conferences. K.M.J. has received a meeting grant from MSD and travel grants from F2G and Amplyx. R.K.H. has received meeting grants from MSD, Pfizer, Gilead, and Astellas and a research grant from Gilead. R.D. and A.C. have nothing to declare.

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