Vaginal Isolates of *Candida glabrata* are Uniquely Susceptible to Ionophoric Killer Toxins Produced by *Saccharomyces cerevisiae*.

Lance R. Fredericks¹, Mark D. Lee¹, Hannah R. Eckert^{1*}, Shunji Li^{1*}, Mason A. Shipley^{1*},
Cooper R. Roslund^{1*}, Dina A. Boikov², Emily A. Kizer¹, Jack D. Sobel², Paul A. Rowley^{1#}

¹Department of Biological Sciences, University of Idaho, Moscow, ID, USA

²Department of Internal Medicine, Division Infectious Diseases, Wayne State University School of Medicine, Detroit, MI, USA

*Authors contributed equally

#Correspondence: prohley@uidaho.edu

ABSTRACT

Compared to other species of *Candida* yeasts, the growth of *Candida glabrata* was inhibited by many different strains of *Saccharomyces* killer yeasts. The ionophoric K1 and K2 killer toxins were broadly inhibitory to all clinical isolates of *C. glabrata* from patients with recurrent vulvovaginal candidiasis, despite high levels of resistance to clinically relevant antifungal therapeutics.

RESEARCH FINDINGS

Vulvovaginal candidiasis (VVC) is estimated to afflict two in every three women worldwide at some point in their lives, causing significant suffering and associated economic losses (1–3). *Candida albicans* is most often isolated as the dominant species present in cases of VVC, followed by *Candida glabrata* (4). In certain diabetic patient populations, *C. glabrata* can be the dominant yeast species associated with VVC (5, 6). The main treatment for VVC is the orally administered fungistatic azole, fluconazole. During pregnancy, to relieve symptoms of VVC and to prevent *Candida*-associated complications, topical application of azoles is preferred over oral

administration due to the potential for fetal toxicity in the first trimester (7). However, drug-resistance in isolates of *C. glabrata* and other species of *Candida* yeasts is increasing and can result in long courses of suppressive treatment and treatment failure (8–10). The limited availability of effective non-toxic therapies to treat VVC warrants the exploration of novel therapeutics that are active at the normal low pH of the vagina.

Killer toxins produced by *Saccharomyces* “killer” yeasts are optimally active in acidic conditions (pH $\leq$ 4.6) that overlap the pH of the vaginal mucosa (~pH 4.2) (11). In addition, there have been many studies describing killer yeasts that can inhibit the growth of pathogenic fungi (12–17). Given the discovery of novel killer toxins produced by *Saccharomyces* killer yeasts, 16 species of the *Candida* genus were screened for their susceptibility to nine killer yeast strains known to express K1, K1L, K2, K21/K66, K28, K45, K62, K74, or Klus killer toxins encoded by double-stranded satellite RNAs (18–24). To test the ability of different killer yeasts to inhibit the growth of *Candida* yeasts, a well assay was used to inoculate killer yeasts into killer assay agar plates (YPD agar plates with 0.003% w/v methylene blue buffered to pH 4.6 with sodium citrate (25)) seeded with $\sim 1 \times 10^5$ *Candida* yeast cells (Fig. S1). After three days of incubation at room temperature, growth inhibition was identified by the appearance of yeast-free zones and/or halos of oxidized methylene blue around killer yeasts (Fig. 1 and S1). Of the 16 species of *Candida* yeasts challenged, *C. glabrata* appeared to be the most susceptible to killer yeasts and was only resistant to the killer yeast that expressed K62 (Fig. S1 and Table S1). One additional species of the *Nakaseomyces* genus (*C. nivariensis*) was also more susceptible to *Saccharomyces* killer yeasts than other *Candida* yeasts (Fig. 1A and B). The K21 killer yeast appeared to have the broadest spectrum of antifungal activity (Fig. 1C).

Fig. 1. *Candida glabrata* is more susceptible to inhibition by killer yeasts than other species of *Candida*

yeasts. (A) The number of killer yeasts that inhibit the growth of different species of *Candida* yeasts; *C. glabrata* (*C. gla*), *C. nivariensis* (*C. niv*), *C. kefyr* (*C. kef*), *C. bracarensis* (*C. bra*), *C. rugosa* (*C. rug*), *C. pararugosa* (*C. par*), and *C. albicans* (*C. alb*) ($n = 2$). (B) A schematic illustration of the effect of a killer yeast on the growth of a competing lawn of yeast. Representative well assay plates with nine different killer yeasts on agar seeded with representative species of *Candida* yeasts. (C) The percentage of *Candida* yeast species found to be inhibited by each type of killer toxin ($n = 2$).

As killer toxin susceptibility can vary widely within a species, 53 unselected clinical isolates of *C. glabrata* from the human vagina were challenged by killer yeasts. These clinical isolates were collected at the Wayne State University Vulvovaginitis clinic in Detroit, MI, between 2015 and 2019. Killer yeasts were inoculated onto the surface of killer assay agar plates seeded with lawns of *C. glabrata* and qualitatively assayed for evidence of growth inhibition. Of the 477 interactions measured between killer yeasts and *C. glabrata*, K1, K2, and K45 killer yeasts inhibited the growth of 100%, 96%, and 75% of *C. glabrata* isolates, respectively (Fig. 2A, top).

The remaining killer yeasts each inhibited less than 33% of *C. glabrata* isolates. The susceptibility of *C. glabrata* to K1 and K2 killer yeasts greatly contrasts the widespread resistance of *Saccharomyces* yeasts (Fig. 2A, bottom). To test the susceptibility of the clinical isolates of *C. glabrata* with acute killer toxin exposure, K1 and K2 toxins were partially purified from 1 mL of spent culture medium, as described previously (23). Exposure of lawns seeded with $\sim 1 \times 10^5$ of *C. glabrata* cells to K1 or K2 demonstrated a concentration-dependent growth inhibition of *C. glabrata* (Fig. 2B). All isolates of *C. glabrata* were inhibited by K1 and K2 at the highest concentration tested, with K2 exposure resulting in large halos of methylene blue ($185.68 \text{ mm}^2 \pm 28.66$; 95% CI) whereas K1 produced larger zones of growth inhibition ($92.34 \text{ mm}^2 \pm 11.24$; 95% CI) (Fig. 2B). The inhibition of *C. glabrata* is similar to the fungicidal effects of K1 and K2 toxins against susceptible strains of *S. cerevisiae* (Fig. S2). Neither K1 nor K2 were acutely cytotoxic to cultured human cells at physiological pH as measured by an alamar blue viability assay (Fig. S3). Both K1 and K2 retained measurable activity against yeast cells after a 1 hour incubation with human epithelial cells (HeLa cell line) in Dulbecco's modified eagle medium with 10% serum at pH 7 (Fig. S3).

Fig. 2. Drug resistant clinical isolates of *C. glabrata* from the vagina are most susceptible to the ionophoric killer toxins K1 and K2. (A) Cluster analysis of the susceptibility of 53 isolates of *C. glabrata* (top) and 53 strains of *Saccharomyces* species (bottom) to different types of killer yeasts as assayed on agar plates. (B) The susceptibility of 50 isolates of *C. glabrata* to partially purified K1 and K2 killer toxins showing the mean killer toxin activity based on the area of complete growth inhibition (I) or methylene blue staining (B) on agar. The 1× concentration of K1 used was 13 μg/mL as measured by Western blotting using a custom antibody raised to a K1 derived peptide. (C) The minimum inhibitory concentration of seven antifungal drugs against 27 Wayne State clinical isolates of *C. glabrata* compared to the total area (methylene blue staining and zone of growth inhibition) of cytotoxicity caused by K1 or K2 killer toxins. Correlation and significance values were calculated by Spearman's rank correlation analysis (n.s. = not significant, **p<0.05). Antifungal drugs assayed: fluconazole (FLU), clotrimazole (CLO), ketoconazole (KCA), miconazole (MIC), itraconazole (ITR), amphotericin B (AMB), and flucytosine (5FC).

Clinical isolates of *C. glabrata* were found to vary in their resistance to antifungals used to treat both VVC and invasive candidemia using the NCCLS M27-A method to calculate MIC values (Fig. 2C) and the disk diffusion assay (Fig. S2). Even when *C. glabrata* isolates were highly resistant to clinical antifungals they remained susceptible to acute K1 and K2 exposure. There was no significant correlation between drug resistance and killer toxin susceptibility (Fig. 2C and S2), except a weak correlation between K1 and K2 resistance and amphotericin B resistance ($p < 0.05$) (Fig. 2C and S2).

Killer toxins are notoriously strain- and species-specific to the point that they have been used to identify different strains of pathogenic yeasts (26). However, the data presented in this study show that most types of known *Saccharomyces* killer yeasts can inhibit the growth of *C. glabrata*. Specifically, the qualitative screening of killer yeasts identified that the ionophoric toxins K1 and K2 were broadly inhibitory to vaginal isolates of *C. glabrata* and that purified toxins inhibited growth in a concentration-dependent manner. The *C. glabrata* cell wall is structurally similar to *S. cerevisiae* and this would suggest that K1 and K2 bind the *C. glabrata* cell wall β -1,6 glucan as the primary receptor (27). *C. glabrata* also expresses a homolog of *S. cerevisiae* Kre1p, which is the GPI-anchored secondary membrane receptor used by both K1 and K2 that enables membrane attack (28). The mechanism of *C. glabrata* intoxication is likely to involve the disruption of ion homeostasis by pore formation in the plasma membrane, as has been shown for *S. cerevisiae* (29). However, cell wall binding and the presence of Kre1p are not sufficient for intoxication, as K1 is able to bind the cell wall and utilize Kre1p of *C. albicans*, which is intrinsically K1 resistant (30, 31). Furthermore, it is unclear why other species of the *Nakaseomyces* genus (*C. nivariensis* and *C. bracarensis*) that are closely related to *C. glabrata* are more resistant to inhibition by the same killer yeasts.

The alteration of ergosterol biosynthesis can cause resistance to azoles and amphotericin B in *Candida* yeasts (32–35), and we find that the latter significantly correlates with increased K1 and K2 resistance (Fig. 2C). As amphotericin resistance can be caused by a reduction in the concentration of membrane ergosterol, increased K1 and K2 resistance in *C. glabrata* could be due to alterations in the composition, fluidity, and permeability of the yeast plasma membrane. Similar protection from K1 intoxication is observed in strains of *S. cerevisiae* with defects in ergosterol biosynthesis (36). Natural K1 resistant strains of *S. cerevisiae* also have a lower expression of ergosterol biosynthesis genes and a reduced concentration of ergosterol esters (37). The depletion of membrane sterols can result in resistance to many cytotoxic proteins (38–42). Moreover, sterol-rich membrane microdomains (lipid rafts) provide a nucleation point for protein toxins that bind raft-localized receptors (43–45). The depletion or redistribution of cholesterol can disrupt raft integrity and inhibit the binding of toxins to their cognate receptor (43, 46). Thus, susceptibility of *C. glabrata* to K1 and K2 could be influenced by membrane ergosterol and the localization and function of the GPI-anchored secondary membrane receptor, Kre1p.

The screening of killer yeasts has served to identify the unique activity of K1 and K2 against *C. glabrata* suggesting that they could be useful as novel antifungal agents. Compared to azoles, these killer toxins are fungicidal, optimally active at low pH, and are non-toxic to human cells at physiological pH(28, 47–49). Therefore, we speculate that with further mechanistic studies, formulation and stabilization, K1 and K2 could be developed for topical application to combat *C. glabrata* associated with VVC.

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REFERENCES

1. Denning DW, Kneale M, Sobel JD, Rautemaa-Richardson R. 2018. Global burden of recurrent vulvovaginal candidiasis: a systematic review. *Lancet Infect Dis* 18:e339–e347.
2. Achkar JM, Fries BC. 2010. *Candida* infections of the genitourinary tract. *Clin Microbiol Rev* 23:253–273.
3. Fukazawa EI, Witkin SS, Robial R, Vinagre JG, Baracat EC, Linhares IM. 2019. Influence of recurrent vulvovaginal candidiasis on quality of life issues. *Arch Gynecol Obstet* 300:647–650.
4. Sobel JD. 2007. Vulvovaginal candidosis. *Lancet* 369:1961–1971.
5. Leon EM de, Jacober SJ, Sobel JD, Foxman B. 2002. Prevalence and risk factors for vaginal *Candida* colonization in women with type 1 and type 2 diabetes. *Bmc Infect Dis* 2:1.
6. Goswami D, Goswami R, Banerjee U, Dadhwal V, Miglani S, Lattif AA, Kochupillai N. 2006. Pattern of *Candida* species isolated from patients with diabetes mellitus and vulvovaginal candidiasis and their response to single dose oral fluconazole therapy. *J Infection* 52:111–117.
7. Zhu Y, Bateman BT, Gray KJ, Hernandez-Diaz S, Mogun H, Straub L, Huybrechts KF. 2020. Oral fluconazole use in the first trimester and risk of congenital malformations: population based cohort study. *BMJ* 369:m1494.
8. Sobel JD, Wiesenfeld HC, Martens M, Danna P, Hooton TM, Rompalo A, Sperling M, Livengood C, Horowitz B, Thron JV, Edwards L, Panzer H, Chu T-C. 2004. Maintenance fluconazole therapy for recurrent vulvovaginal candidiasis. *New Engl J Med* 351:876–883.
9. Richter SS, Galask RP, Messer SA, Hollis RJ, Diekema DJ, Pfaller MA. 2005. Antifungal susceptibilities of *Candida* species causing vulvovaginitis and epidemiology of recurrent cases. *J Clin Microbiol* 43:2155–2162.
10. Sobel JD, Zervos M, Reed BD, Hooton T, Soper D, Nyirjesy P, Heine MW, Willems J, Panzer H. 2003. Fluconazole susceptibility of vaginal isolates obtained from women with complicated *Candida* vaginitis: Clinical implications. *Antimicrob Agents Ch* 47:34–38.
11. Owen DH, Katz DF. 1999. A vaginal fluid simulant. *Contraception* 59:91–95.
12. Hodgson VJ, Button D, Walker GM. 1995. Anti-*Candida* activity of a novel killer toxin from the yeast *Williopsis mrakii*. *Microbiology* 141 (Pt 8):2003–2012.
13. Giovati L, Santinoli C, Ferrari E, Ciociola T, Martin E, Bandi C, Ricci I, Epis S, Conti S. 2018. Candidacidal Activity of a Novel Killer Toxin from *Wickerhamomyces anomalus* against Fluconazole-Susceptible and-Resistant Strains. *Toxins* 68.

14. Buzzini P, Martini A. 2001. Discrimination between *Candida albicans* and other pathogenic species of the genus *Candida* by their differential sensitivities to toxins of a panel of killer yeasts. *J Clin Microbiol* 9:3362–4.
15. Middelbeek EJ, Hermans JM, Stumm C, Muytjens HL. 1980. High incidence of sensitivity to yeast killer toxins among *Candida* and *Torulopsis* isolates of human origin. *Antimicrob Agents Chemother* 17:350–354.
16. Walker GM, McLeod AH, Hodgson VJ. 1995. Interactions between killer yeasts and pathogenic fungi. *FEMS Microbiol Lett* 127:213–222.
17. Schmitt MJ, Poravou O, Trenz K, Rehfeldt K. 1997. Unique double-stranded RNAs responsible for the anti-*Candida* activity of the yeast *Hanseniaspora uvarum*. *J Virol* 71:8852–8855.
18. Rodríguez-Cousiño N, Maqueda M, Ambrona J, Zamora E, Esteban R, Ramírez M, Rodrigues ML. 2011. A new wine *Saccharomyces cerevisiae* killer toxin (Klus), encoded by a double-stranded RNA virus, with broad antifungal activity is evolutionarily related to a chromosomal host gene. *Appl Environ Microbiol* 77:1822–1832.
19. Vepškaitė-Monstavičė I, Lukša J, Konovalovas A, Ežerskytė D, Stanevičienė R, Strazdaitė-Žielienė Ž, Serva S, Servienė E. 2018. *Saccharomyces paradoxus* K66 killer system evidences expanded assortment of helper and satellite viruses. *Viruses* 10.
20. Bostian KA, Elliott Q, Bussey H, Bum V, Smith AI, Tipper DJ. 1984. Sequence of the preprotoxin dsRNA gene of type I killer yeast: Multiple processing events produce a two-component toxin. *Cell* 36:741–751.
21. Schmitt MJ. 1995. Cloning and expression of a cDNA copy of the viral K28 killer toxin gene in yeast. *Mol Gen Genet* 246:236–246.
22. Rodríguez-Cousiño N, Gómez P, Esteban R. 2017. Variation and distribution of L-A helper Totiviruses in *Saccharomyces sensu stricto* yeasts producing different killer toxins. *Toxins* 9:313–20.
23. Fredericks LR, Lee MD, Crabtree AM, Boyer JM, Kizer EA, Taggart NT, Hunter SS, Kennedy CB, Willmore CG, Tebbe NM, Harris JS, Brocke SN, Rowley PA. 2021. The species-specific acquisition and diversification of a novel family of killer toxins in budding yeasts of the Saccharomycotina. *PLoS Gen* 2:e1009341.
24. Dignard D, Whiteway M, Germain D, Tessier D, Thomas DY. 1991. Expression in yeast of a cDNA copy of the K2 killer toxin gene. *Mol Gen Genet* 227:127–136.
25. Crabtree AM, Kizer EA, Hunter SS, Leuven JTV, New DD, Fagnan MW, Rowley PA. 2019. A rapid method for sequencing double-stranded RNAs purified from yeasts and the identification of a potent K1 killer toxin isolated from *Saccharomyces cerevisiae*. *Viruses* 11:70.

- 235 26. Buzzini P, Turchetti B, Vaughan-Martini AE. 2007. The use of killer sensitivity patterns for
236 biotyping yeast strains: the state of the art, potentialities and limitations. *FEMS Yeast Res* 7:749–
237 760.
- 238 27. Groot PWJ de, Kraneveld EA, Yin QY, Dekker HL, Groß U, Crielaard W, Koster CG de,
239 Bader O, Klis FM, Weig M. 2008. The cell wall of the human pathogen *Candida glabrata*:
240 differential incorporation of novel adhesin-like wall proteins. *Eukaryot Cell* 7:1951–1964.
- 241 28. Breinig F, Tipper DJ, Schmitt MJ. 2002. Kre1p, the plasma membrane receptor for the yeast
242 K1 viral toxin. *Cell* 108:395–405.
- 243 29. Martinac B, Zhu H, Kubalski A, Zhou XL, Culbertson M, Bussey H, Kung C. 1990. Yeast
244 K1 killer toxin forms ion channels in sensitive yeast spheroplasts and in artificial liposomes.
245 *Proc Natl Acad Sci* 87:6228–6232.
- 246 30. Zhu H, Bussey H. 1989. The K1 toxin of *Saccharomyces cerevisiae* kills spheroplasts of
247 many yeast species. *Appl Environ Microbiol* 55:2105–2107.
- 248 31. Boone C, Sdicu A, Laroche M, Bussey H. 1991. Isolation from *Candida albicans* of a
249 functional homolog of the *Saccharomyces cerevisiae* *KRE1* gene, which is involved in cell wall
250 beta-glucan synthesis. *J Bacteriol* 173:6859–6864.
- 251 32. Morschhäuser J. 2002. The genetic basis of fluconazole resistance development in *Candida*
252 *albicans*. *Biochimica Et Biophysica Acta Bba - Mol Basis Dis* 1587:240–248.
- 253 33. Young LY, Hull CM, Heitman J. 2003. Disruption of ergosterol biosynthesis confers
254 resistance to amphotericin B in *Candida lusitanae*. *Antimicrob Agents Ch* 47:2717–2724.
- 255 34. Kelly SL, Lamb DC, Kelly DE, Loeffler J, Einsele H. 1996. Resistance to fluconazole and
256 amphotericin in *Candida albicans* from AIDS patients. *Lancet* 348:1523–1524.
- 257 35. Whaley SG, Rogers PD. 2016. Azole Resistance in *Candida glabrata*. *Curr Infect Dis Rep*
258 18:41.
- 259 36. Pagé N, Gérard-Vincent M, Ménard P, Beaulieu M, Azuma M, Dijkgraaf GJP, Li H,
260 Marcoux J, Nguyen T, Dowse T, Sdicu A-M, Bussey H. 2003. A *Saccharomyces cerevisiae*
261 genome-wide mutant screen for altered sensitivity to K1 killer toxin. *Genetics* 163:875–894.
- 262 37. Gier S, Simon M, Gasparoni G, Khalifa S, Schulz MH, Schmitt MJ, Breinig F. 2019. Yeast
263 viral killer toxin K1 induces specific host cell adaptations via intrinsic selection pressure. *Appl*
264 *Environ Microb* 86:e02446-19.
- 265 38. Giddings KS, Johnson AE, Tweten RK. 2003. Redefining cholesterol's role in the
266 mechanism of the cholesterol-dependent cytolysins. *Proc Natl Acad Sci* 100:11315–11320.

39. Kodedová M, Valachovič M, Csáky Z, Sychrová H. 2019. Variations in yeast plasma-membrane lipid composition affect killing activity of three families of insect antifungal peptides. *Cell Microbiol* 21:e13093.
40. Linder R, Bernheimer AW. 1984. Action of bacterial cytotoxins on normal mammalian cells and cells with altered membrane lipid composition. *Toxicon* 22:641–651.
41. Schraw W, Li Y, McClain MS, Goot FG van der, Cover TL. 2002. Association of *Helicobacter pylori* Vacuolating Toxin (VacA) with Lipid Rafts. *J Biol Chem* 277:34642–34650.
42. Orlandi PA, Fishman PH. 1998. Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J Cell Biology* 141:905–915.
43. Waheed AA, Shimada Y, Heijnen HFG, Nakamura M, Inomata M, Hayashi M, Iwashita S, Slot JW, Ohno-Iwashita Y. 2001. Selective binding of perfringolysin O derivative to cholesterol-rich membrane microdomains (rafts). *Proc Natl Acad Sci* 98:4926–4931.
44. Nelson KL, Raja SM, Buckley JT. 1997. The glycosylphosphatidylinositol-anchored surface glycoprotein Thy-1 is a receptor for the channel-forming toxin aerolysin. *J Biol Chem* 272:12170–12174.
45. Shewell LK, Day CJ, Jen FE-C, Haselhorst T, Attack JM, Reijneveld JF, Everest-Dass A, James DBA, Boguslawski KM, Brouwer S, Gillen CM, Luo Z, Kobe B, Nizet V, Itzstein M von, Walker MJ, Paton AW, Paton JC, Torres VJ, Jennings MP. 2020. All major cholesterol-dependent cytolysins use glycans as cellular receptors. *Sci Adv* 6:eaaz4926.
46. Abrami L, Goot FG van der. 1999. Plasma membrane microdomains act as concentration platforms to facilitate intoxication by aerolysin. *J Cell Biology* 147:175–184.
47. Novotná D, Flegelová H, Janderová B. 2004. Different action of killer toxins K1 and K2 on the plasma membrane and the cell wall of *Saccharomyces cerevisiae*. *FEMS Yeast Res* 4:803–813.
48. Hutchins K, Bussey H. 1983. Cell wall receptor for yeast killer toxin: involvement of (1,6)-beta-D-glucan. *J Bacteriol* 154:161–169.
49. Lukša J, Podoliankaitė M, Vepškaitė I, Strazdaitė-Žiėlienė Ž, Urbonavičius J, Servienė E. 2015. Yeast β -1,6-glucan is a primary target for the *Saccharomyces cerevisiae* K2 toxin. *Eukaryot Cell* 14:406–414.