**Efflux-mediated resistance to fluconazole could be modulated by sterol homeostasis in *Saccharomyces cerevisiae***

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**Saccharomyces cerevisiae** has long been used as a model organism in the study of the ergosterol pathway and its inhibitors. The Pdr5 protein (Pdr5p), an ATP binding cassette transporter, plays an important role in active efflux of azole antifungals and therefore in azole sensitivity and resistance in *S. cerevisiae*. We have identified the Fluconazole Dominant Resistance-1 (*FDR-1*) mutant, which has a single dominant mutation conferring high-level resistance to fluconazole. *FDR-1* has been found to be an activated allele of the Pleiotropic Drug Resistance-1 (*PDR-1*) gene (termed *PDR1-100*) and to upregulate *PDR5* transcription. Resistance of *PDR1-100* to fluconazole decreased in the background of mutations known to affect sterol homeostasis. Hence, the resistance to fluconazole of *PDR1-100* was paradoxically decreased in an *erg3 PDR1-100* double mutant. The *erg3* mutants are resistant to azoles and accumulate 14-methyl-fecosterol instead of ergosterol in the presence of azoles. These results reinforce the emerging evidence in both *S. cerevisiae* and *Candida albicans* that sterols could serve as substrates for Pdr5p for transport across membranes.

**Introduction**

Fluconazole is a triazole antifungal commonly used to treat yeast infections.1 Fluconazole selectively inhibits cytochrome P-450-dependent C-14 lanosterol demethylase (P-450 14DM), causing a combination of ergosterol depletion and the accumulation of toxic 14-methyl sterols.1,2 Despite the progress made in defining the molecular mechanisms of sensitivity to azoles in yeast, the modulators of the antifungal activity of this drug are not fully understood.

*Saccharomyces cerevisiae*, a genetically tractable fungus closely related to *Candida albicans*, is an attractive experimental system for studying azole resistance.1,3,4 A well-characterized mechanism of azole resistance in *S. cerevisiae* is conferred by loss-of-function mutations in sterol Δ5,6 desaturase which is encoded by the *ERG3* gene.2,4 Instead of accumulating the toxic 14α-methyl-3,6-diol under azole treatment, *erg3* mutants accumulate the less toxic 14α-methylfocosterol.2 Another mechanism of resistance and hypersensitivity to azoles is mediated by the pleiotropic drug resistance-5 gene (*PDR5*), an ATP binding cassette (ABC) transporter, as a result of increased efflux of the drug.1,5 Finally, loss of function of the *CPR1* gene, which codes for the NADPH-dependent cytochrome P-450 oxidoreductase, and of *YMR034c*, which codes for a putative sterol transporter, results in azole hypersensitivity.3,4

In this study, we provide genetic evidence that Pdr5p-mediated resistance to fluconazole is altered in the background of mutations that affect ergosterol homeostasis and suggest a model in which Pdr5p functions in the maintenance of ergosterol homeostasis by extrusion or transport of sterol intermediates.

**Materials and methods**

**Strains**

Preparation of the yeast growth medium, synthetic complete medium (SC) and standard techniques for the manipulation of yeast have been described.6 All work was done in the Σ1278b genetic background. The Tn3::LEU2::lacZ fusion library was used as a disruption mutagen and one fluconazole-resistant mutant with a disruption of *ERG3* and three fluconazole-hypersensitive mutants with disruptions of the *PDR5*, *CPR1* and *YMR034c* genes, respectively, were isolated.3 The yeast strains used in this study are described in the Table. Fluconazole was a gift from Pfizer, Inc. (New York, NY, USA).

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Testing for fluconazole sensitivity

Two assays were used to test independently for sensitivity to fluconazole. In the first assay, the growth of each yeast strain streaked out to form single colonies was examined on SC medium plates containing various concentrations of fluconazole. In the second assay, yeast growth was examined by spreading approximately 10⁵ cells in late logarithmic growth phase on SC medium and placing on this a paper disc containing 500 μg of the drug. In both assays growth was aerobic at 30°C for 3 days.

Screen for dominant resistance to fluconazole

Approximately 10⁸ cells of the diploid strain L5803 were plated on selective SC plates containing leucine and fluconazole at 256 mg/L and incubated for 7 days at 30°C. Genetic analysis showed that 22 of the 30 resistant mutants had dominant mutations in a single gene, which we called the Fluconazole Dominant Resistance-1 gene (FDR-1).

Molecular biological and biochemical methods

Cloning of the FDR-1 gene. Genomic DNA from FDR-1 was partially digested with MboI. A genomic DNA library was constructed on the URA3-based vector PRS 316. Wild-type yeast (strain 10560-14C; Table) was transformed with the FDR-1 genomic DNA library and plated on SC plates containing uracil. The transformation plates were then replicated to SC plates containing uracil and fluconazole at 128 mg/L to select for fluconazole-resistant colonies among the transformants. Resistant colonies were purified and screened to determine which had a URA3 plasmid-dependent fluconazole resistance phenotype. The plasmid-dependent fluconazole resistance phenotype was evaluated by the identification of Ura⁺ non-resistant candidates by both 5-fluoro-orotic acid (5-FOA) medium selection and the passive loss of URA3 plasmid by streaking in yeast peptone dextrose (YPD) medium. The same wild-type strain was then retransformed with DNA from the candidate clones. Ura⁺ fluconazole-resistant transformants were deemed true positives, and DNA was sequenced from their corresponding plasmids.

The β-galactosidase assays were performed in triplicate on extracts of exponential-phase cultures in SC + fluconazole at 8 mg/L as described previously.⁶

Results and discussion

FDR-1 is an activated allele of PDR1 and it overexpresses Pdr5p

The FDR-1 allele was mapped to chromosome VII and was centromere linked. Linkage analysis with a URA3-marked pdr1 mutant (constructed by transforming yeast with a pdr1Δ plasmid pAB6.5)⁷ showed that FDR-1 was an allele of PDR1, which we called PDR1-100. Dominant point mutations of PDR1, a transcription factor, resulting in

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Sterol homeostasis and azole resistance

pleiotropic drug resistance through overexpression of PDR5 mRNA have been described. The diploid strain DC188 had an eight-fold increase in β-galactosidase levels in SC medium and a 16-fold increase at low concentrations (8 mg/L) of fluconazole compared with the diploid strain DC13, by quantitative β-galactosidase assay. Finally, the cloning of the FDR-I library revealed one transformant that was highly resistant to fluconazole. Sequencing of the 12 kb genomic clone in the corresponding plasmid revealed the PDR1 locus.

An unexpected genetic interaction was discovered between PDR1-100 and erg3, both of which were resistant to fluconazole as single mutants; the double mutant erg3 PDR1-100 was sensitive to fluconazole (Figure). In con-

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**Figure.** (a) PDR1-100 is less resistant in the erg3 background. Growth responses of haploids WT (10560-14C), erg3 (DK1-4C), PDR1-100 (FDR-I) and erg3 PDR1-100 (DC187-4A, double mutant) on SC + fluconazole disc (containing 500 µg). (b) The genetic interaction between PDR1-100 and erg3 and PDR1-100 and fluconazole-sensitive mutants. SC plate minimal inhibitory concentrations of fluconazole for wild type (10560-14C), erg3 (DK1-4C, resistant), pdr5 (DK13-5D, sensitive), cpr1 (HS62, sensitive), ymr034c (DK 300, sensitive) and PDR1-100 (FDR-I, resistant) and for the double mutants erg3 pdr5, erg3 PDR1-100, pdr5 PDR1-100 and cpr1 PDR1-100.
The erg3 pdr5 double mutant was as resistant as erg3 (Figure, b). In addition, the resistance of PDR1-100 was also decreased in the ymr034c and cpr1 backgrounds (Figure, b). Loss of function of CPR1 has been shown to influence ergosterol levels. In addition, YMR034c, which encodes a putative sterol transporter, could also affect ergosterol homeostasis.

Other than its role in detoxification of chemically unrelated compounds, the physiological role of Pdr5p has been elusive. There is emerging evidence that Pdr5p could catalyse the transport of sterols such as oestradiol or dexa-methasone across membranes. Furthermore, it has been reported that oestradiol and corticosterone are substrates of Cdr1p, which is the homologue of Pdr5p in C. albicans, and that fluconazole leads to the accumulation of these steroids. However, such steroid hormones are not natural components of steroid homeostasis in yeast. The decreased efficiency of the Pdr5p efflux pump in the background of mutations that affect ergosterol homeostasis, reported in this study and that recently published by Kaur and Bachhawat, provide additional genetic evidence supporting this concept. Future biochemical validation of this hypothesis could better explain the role of efflux pumps in lipid homeostasis. Alternatively, alterations of lipid fluidity in those mutants could affect the transport function of Pdr5p and its efficiency in active extrusion of the drug to varying degrees. There have been reports that reduction of both the Cdr1p-mediated resistance to fluconazole and the P-glycoprotein (the mammalian homologue of Pdr5p) -mediated multidrug resistance follows alterations of the membrane lipid fluidity.

Our work has potential therapeutic implications. If azoles compete with endogenous sterols for Pdr5p, strategies for reversing efflux-mediated resistance to these drugs by inhibiting other components of the ergosterol pathway (such as Erg3p), in addition to Erg11p, will be useful. Alternatively, if non-ergosterol sterols affect lipid fluidity and subsequently the activity of Pdr5p, this model will affect drug design. For example, alternative means ofazole delivery such as liposomes could theoretically overcome Pdr5p-mediated resistance. Since transporter-mediated resistance to azoles appears to be the most prevalent in vivo, further efforts to elucidate the modulators of such resistance could lead to new therapeutic strategies.

Acknowledgements

A portion of this work was performed at the Whitehead Institute for Biomedical Research in the laboratory of G. R. Fink in Cambridge, MA, when D. P. K. was a fellow in the Clinical Investigator Training Program in the Harvard–MIT Division of Health Sciences and Technology, which is supported by Pfizer, Inc., and a fellow in Infectious Diseases at Massachusetts General Hospital, Harvard Medical School in Boston, MA. D. P. K. thanks C. A. Styles, G. R. Fink and members of the Fink laboratory for helpful advice, and the Goffeau laboratory (Université Catholique de Louvain, Louvain-la-Neuve, Belgium) for kindly providing the plasmid pΔB6.5.

References

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Received 30 June 1999; returned 30 November 1999; revised 4 January 2000; accepted 1 March 2000