The impact of amino acid changes in the substrate binding site of fungal sterol 14α-demethylase on protein structure and function using *S. cerevisiae* as an expression model

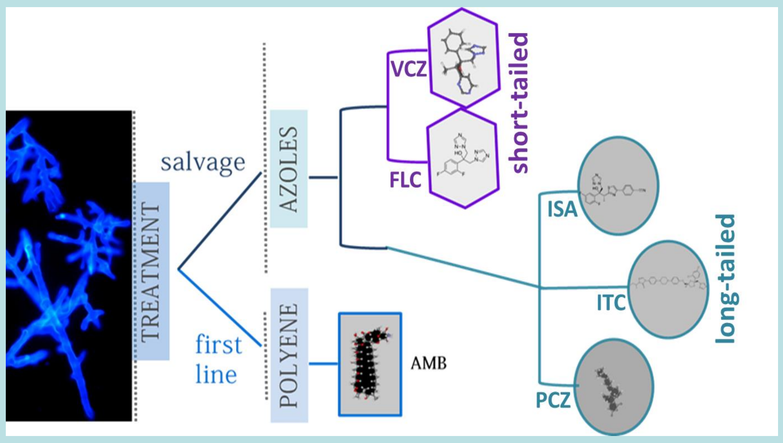
M Lackner[1*], S Toepfer[1], C Mueller[2], MV Keniya[3], BC Monk[3]

[1]*Division of Hygiene and Medical Microbiology, Medical University of Innsbruck, Innsbruck , Austria*
[2]*Department of Pharmacy, Ludwig-Maximilians-University Munich, Munich, Germany*
[3]*Sir John Walsh Research Institute and the Department of Oral Sciences, Faculty fo Dentistry, University of Otago, Dunedin , New Zealand*

***Michaela.Lackner@i-med.ac.at**

Background

Why the short-tailed azoles voriconazole (VCZ) and fluconazole (FLC) lack activity against their target lanosterol 14α-demethylase (LDM) of mucormycetes while the long-tailed azoles itraconazole, posaconazole (PCZ) show *in vitro* and *in vivo* activity is not fully understood.

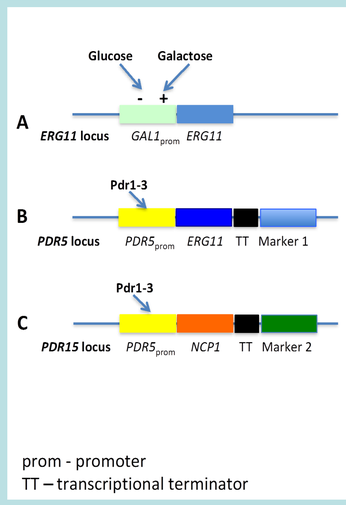


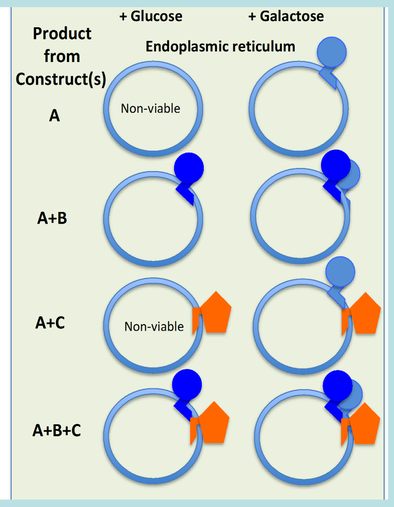
Alignment of LDM primary sequences identified pan-Mucorales conservation of a phenylalanine 129 substitution in the LDM F5 isoforms of 6 mucormycete species. A high resolution X-ray crystal structure of *Saccharomyces cerevisiae* LDM in complex with VCZ was used to generate a homology model of *Rhizopus arrhizus* LDM F5. Structural and functional knowledge of *S. cerevisiae* LDM suggested that the F129 residue in LDM F5 is responsible for intrinsic resistance of Mucorales to short-tailed azoles, with a V to A amino acid (aa) substitution in helix I potentially playing a role (1).

HYPOTHESIS: Innate resistance to short-tailed azoles in at least six Mucorales species appears to be mediated by the LDM substitutions Y129F (*S. cerevisiae* LDM Y140) in the loop between helices B and C of LDM F5 and V291A (*S. cerevisiae* LDM V311) in helix I.

Material & Methods

S. cerevisiae host strain AD2Δ (2) is deleted of 7 ABC transporters, the *PDR3* transcriptional regulator and contains the gain-of-function *pdr1-3* mutation. This enables constitutive expression of the gene encoding LDM (*ERG11*) from the *PDR5* locus and comparable expression of a cognate NADPH-cytochrome P450 reductase gene (*NCP1*) from the *PDR15* locus when its promoter is replaced with the *PDR5* promoter. In some instances the native *ERG11* promoter was replaced with the *GAL1* promoter i.e. native LDM protein is expressed during growth on galactose, but is not expressed when grown on glucose. This allows phenotypic analysis of the expressed heterologous gene(s) without the impact of the native LDM.

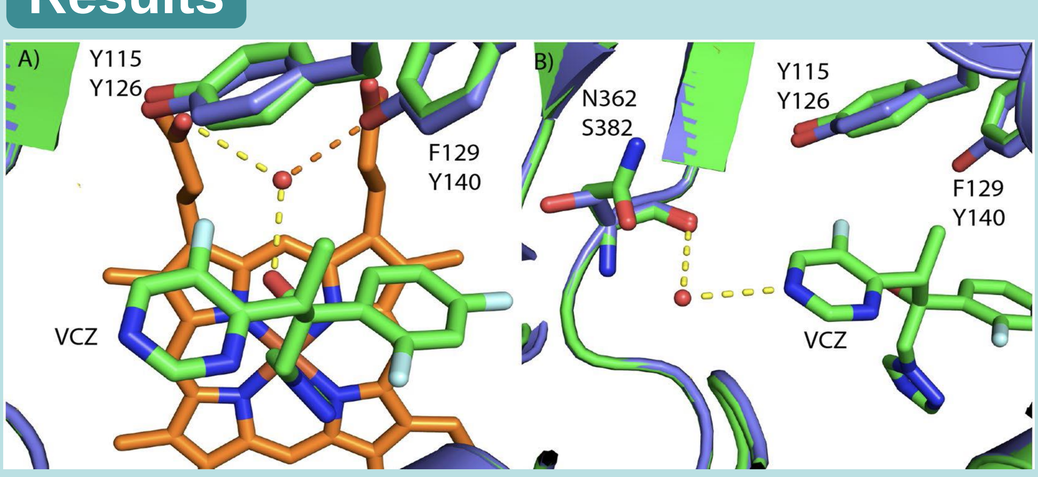




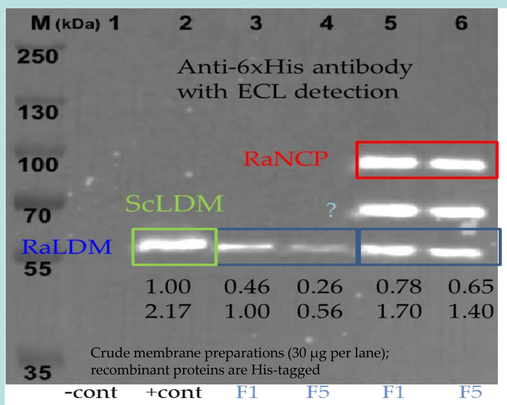
APPROACH:

- Expression of *R. arrhizus* LDM F1 and LDM F5 (at *PDR5* locus) +/- coexpression of cognate NCP1 (at *PDR15* locus) using *S. cerevisiae*
- Revert substitution(s) (i.e. F129Y and/or A291V) in LDM F5 + NCP1

Results

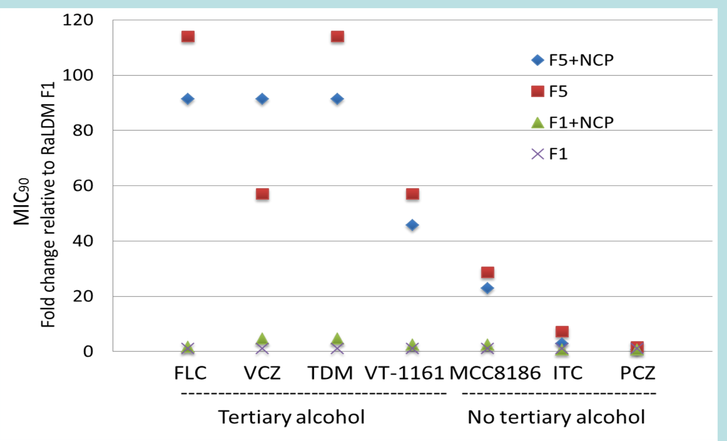


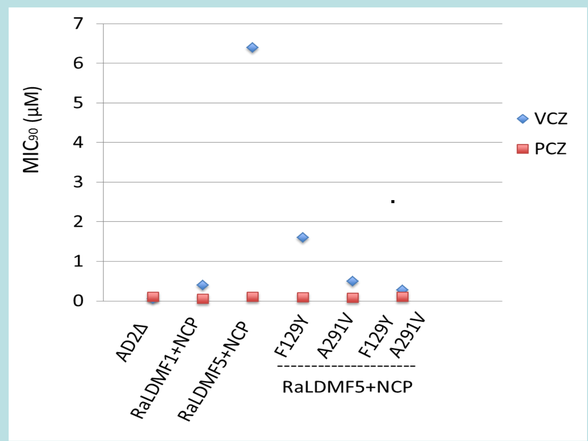
Substitution of *R. arrhizus* LDM F5 F129 for *S. cerevisiae* LDM Y140 blocks interaction of VCZ with a **water-mediated hydrogen bond network**. Substitution of *R. arrhizus* LDM F5 N362 for *S. cerevisiae* LDM S382 does not modify existing polar interactions with VCZ.



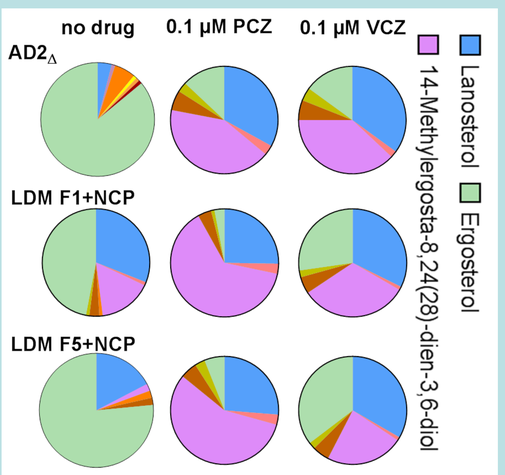
Recombinant hexa Histidine-tagged mucormycete *R. arrhizus* LDM F1 and F5 are **functionally expressed** in *S. cerevisiae* in modest amounts. Coexpression with the cognate NCP1 reduces susceptibility to all azole drugs tested by increasing the amounts of LDM F1 and F5 expressed. *R. arrhizus* is intrinsically resistant to VCZ but susceptible to PCZ. **The MICs show the LDM F5 isoform determines VCZ resistance and susceptibility to PCZ.**

The *R. arrhizus* LDM F1 isoform is susceptible to VCZ and the azoles with a tertiary alcohol-containing linker, but the *R. arrhizus* LDM F5 isoform confers resistance to those drugs. *R. arrhizus* LDM F1 and F5 isoforms are both susceptible to ITC and PCZ. Susceptibility of *R. arrhizus* LDM F5 to VT-1161 resembles VCZ, but susceptibility to MCC8186 is closer to PCZ. MCC8186 is a congener of VT-1161 that contains a oxolane linker like PCZ. The water-mediated hydrogen bond network involving the azole tertiary alcohol is therefore only partially responsible for resistance to FLC and VCZ. **Reversion of *R. arrhizus* LDM F5 F129Y (*S. cerevisiae* LDM Y140) plus A291V (*S. cerevisiae* LDM V311) in LDM F5 is required to confer full susceptibility VCZ.**





drug [µM]	strain	main sterols [%] lanosterol	14-methylergosta-8,24(28)-dien-3,6-diol	ergosterol
no drug	AD2Δ	4	0	86
	LDM F1+NCP	31	16	47
	LDM F5+NCP	17	2	75
0.1 PCZ	AD2Δ	33	42	13
	LDM F1+NCP	25	63	3
	LDM F5+NCP	26	56	6
0.1 VCZ	AD2Δ	35	38	15
	LDM F1+NCP	32	32	27
	LDM F5+NCP	33	23	35



The sterol composition of the *S. cerevisiae* AD2Δ host strain was 86% ergosterol (end-product), 4% lanosterol (substrate), 10% intermediate products, and 0% cytotoxic 14-methylergosta-8,24(28)-dien-3,6diol (14MEDD). As AD2Δ, LDM F1+NCP and LDM F5+NCP are susceptible to PCZ, composition changes detected included increased lanosterol content (to 25-33%), increased 14MEDD (to 42-63%) and reduced ergosterol (3-13%). While AD2Δ, LDM F1+NCP are susceptible to VCZ, LDM F5+NCP is resistant. This was mirrored by 10% more 14MEDD and an 8-20% lower ergosterol content in AD2Δ and LDM F1+NCP compared with LDM F5+NCP.

KEY FINDING:
Innate resistance to short-tailed azoles is mediated by the substitutions Y129F in the B-C loop and V291A in helix I of LDM F5.

Conclusion

By using an *S. cerevisiae* platform to heterologously express recombinant *R. arrhizus* LDM F1 and F5 +/- their cognate NADPH-cytochrome P450 reductase, we have shown that the F5 isoform is responsible for the intrinsic resistance to FLC and VCZ. These properties are reflected in the lipid composition of the recombinant yeast strains on drug exposure. Reversion of key aa residues in LDM F5 homologous to residues found in the F1 isoform demonstrate that the VCZ resistance is due to aa substitutions F129 in the B-C loop and A291 in helix I. Alignment of LDM primary sequences suggests that this explanation applies to a wide range of mucormycetes.

Funding

This research was supported by a Catalyst Fund Seeding grant from the Royal Society of New Zealand, the Health Research Council of New Zealand and the FWF der Wissenschaftsfonds.

REFERENCES: (1) Intrinsic short-tailed azole resistance in mucormycetes is due to an evolutionary conserved aminoacid substitution of the lanosterol 14α-demethylase. Caramalho R, Tyndall JDA, Monk BC, Larentis T, Lass-Flörl C, Lackner M. Sci Rep. 2017 Nov 21;7(1):15898. doi: 10.1038/s41598-017-16123-9. (2) Structural insights into binding of the antifungal drug fluconazole to *Saccharomyces cerevisiae* Lanosterol 14α-demethylase. Sagatova AA, Keniya MV, Wilson RK, Monk BC, Tyndall JDA. Antimicrob Agents Chemother. 2015 Aug;59(8):4982-9. doi: 10.1128/AAC.00925-15. (3) Antifungal drug testing by combining minimal inhibitory concentration testing with target identification by gas chromatography-mass spectrometry. Müller C, Binder U, Bracher F, Giera M. Nat Protoc. 2017 May;12(5):947-963. doi: 10.1038/nprot.2017.005