Summary
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From the relative simplicity of bacterial cells, fungi and protozoa to the complexity of human cancer cells, resistance has become problematic. Stated in its simplest terms, drug resistance decreases the chance of providing successful treatment against a plethora of diseases. A major mechanism of resistance in *Candida albicans* as well as in cancer cells is the membrane protein-catalyzed extrusion of drugs from the cell. One of the most clinically significant mechanisms of azole resistance in pathogenic yeast *Candida albicans* is the overexpression of multidrug transporter protein Cdr1p (*Candida Drug Resistance*), belonging to the ABC (ATP Binding Cassette) Superfamily of transporter (Calderone, 2002; Prasad et al., 2002; Prasad et al., 1996). In view of the functional importance of Cdr1p in azole resistance and its ability to transport wide variety of substrates, there is a need to characterize the protein in terms of its substrate interaction and transport. The molecular mechanisms which govern Cdr1p functions are not well known and information is needed (1) to understand how the protein can bind to structurally diverse range of compounds (2) to define the drug-Substrate binding and (3) to determine how ATP binding and hydrolysis are linked to the drug transport.

Three different strategies were employed to know the structure-function relationship of Cdr1p. To check the localization of Cdr1p, GFP was tagged at the C-terminal end of *CDR1* and integrated at the *PDR5* locus in the *S. cerevisiae* mutant AD1-8u- in which the mutation in the transcription factors *pdr1-3* causes the hyperinduction of *PDR5* promoter and thus overexpression of Cdr1p.

1. Functional analysis of drug extrusion pump Cdr1p of *C. albicans* by employing site directed mutagenesis
• Considering the importance of putative transmembrane domain 11 (TMD11) in case of Pgp as well as in PDR5 in substrate recognition and transport, in this study, we have investigated the role of all hydrophilic residues as well as some hydrophobic residues within TMD11 in determining the substrate specificity of Cdr1p.

• Localization and expression of mutant Cdr1p variants remained unaltered: All mutant Cdr1p variants were localized at the plasma membrane and showed similar expression levels.

• Resistance profile: Mutations N1348A, N1359A, T1351S, T1355S, L1353A and C1361A had no significant effect on drug resistance where as T1355F, T1351F and F1360A drastically affected the resistance profile to all the tested drugs, clearly demonstrating the severity of substitution in the indicated respective order: T1355> F1360> T1351> Wild type.

• ATPase activity remains unaltered: Cdr1p mutant variants exhibited the oligomycin sensitive ATPase activity comparable to that of wild type. However, we were unable to detect the drug-stimulated ATPase activity that would have been a correct measure of the ATPase activity contributed by the active protein. Unlike mammalian MDR proteins, we as well as others are so far unable to demonstrate the drug stimulated ATPase activity of any of the yeast ABC drug transporters.

• Binding with azidopine (a photoaffinity analogue of dihydropyridine): both the wild type as well as mutant Cdr1GFP variants showed similar binding with azidopine that could be competed out by miconazole (100μM) (Shukla et al 2003). But at the same time, change in binding with other substrates of Cdr1p in mutant Cdr1GFP variants cannot be ruled out (Shukla et al 2003).

• Transport of rhodamine 6G and (³H) Fluconazole: Results from rhodamine 6G efflux assay and (³H) Fluconazole accumulation assay demonstrated distinct sensitivity levels of Cdr1GFP variants, which is T1355F > F1360A > T1351F > wild type.
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• The data also indicate that despite the highly conserved alpha helical wheel projection of TMD11, Cdr1p and Pdr5p display major difference with respect to the interaction of T1355 in case of Cdr1p and its equivalent T1364 in case of Pdr5p with FK520 in combination with azoles.

• In conclusion: our results suggest i) all the polar residues are not involved in determining the substrate specificity, ii) Only non-conservative substitution (Phe) of Thr resulted in enhanced susceptibility to drugs whereas more conservative substitution (Ser) had no effect iii) Elimination of the aromatic side chain of Phe1360 decreased the resistance to several fold for all the tested drugs. Taken together our results strongly implicate the TMD11 in the mechanism of Cdr1p drug recognition and efflux and indicate a close functional homology between Cdr1p and Pgp in which the TMD11 has also been shown to influence substrate specificity.

2. Domain analysis: Functional relevance of cytoplasmic region comprising NBD1 of Cdr1p

• Confocal images of Cdr1p having hydrophilic domains comprised of two NBD1 rather than NBD1 and NBD2 revealed poor cell surface localization of the variant Cdr1p.

• Resistance profile: Cells expressing both type of variant Cdr1p were hypersensitive to the all the tested drugs as compared to wild type Cdr1p.

• Improved localization of variant Cdr1p: The variant Cdr1GFP showed improved surface localization with increasing concentrations of cycloheximide. With other drugs also, like miconazole and anisomycin improved surface localization was observed but the effect was more pronounced in the case of cycloheximide.

• Rhodamine6G efflux and azidopine binding: Variant Cdr1GFP cells grown in the presence of cycloheximide did not transport substrates like rhodamine6G but retained the ability to bind the [3H]-azidopine.
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- In conclusion, these results suggest the non-equivalence of the two hydrophilic regions comprising of NBD1 and NBD2.

3. Functional analysis of drug extrusion pump, Cdr1p of *C. albicans* by using chimeric approach

- Various chimeras were constructed between *CDR1* and *CDR3* to know the functional domain of *CDR1* responsible for drug resistance.

- Resistance profile of wild type Cdr1p and various chimeras: All the chimeras except Cdr1(1-1436)/Cdr3(1420-1501)GFP showed hypersensitivity to all the tested drugs while Cdr1GFP and Cdr1(1-1436)/Cdr3(1420-1501)GFP showed almost similar drug resistance profile.

- Surface localization of the wild type Cdr1GFP and Cdr1/Cdr3GFP chimeras: The confocal images showed comparable cell surface localization of Cdr1GFP and Cdr1(1-1436)/CDR3(1420-1501)GFP; whereas rest of the chimeras showed poor cell surface localization.

- Western blot analysis of plasma membrane protein showed the comparable cell surface localization of Cdr1GFP and Cdr1(1-1436)/Cdr3(1420-1501)GFP where as no signal was detected in rest of the chimeras and in case of crude membranes (CM) Cdr3N/Cdr1CGFP and Cdr1(1-1173)/Cdr3(1154-1501)GFP was poorly expressed.

- ATPase activity of Cdr1(1-1436)/Cdr3(1420-1501)GFP: Since all the chimeras only Cdr1(1-1436)/Cdr3(1420-1501)GFP conferred drug resistance whereas rest of them were almost as sensitive as AD1-8u-, further functional analysis was performed with Cdr1(1-1436)/Cdr3(1420-1501)GFP. Both Cdr1GFP and Cdr1(1-1436)/Cdr3(1420-1501)GFP elicited comparable oligomycin sensitive ATPase activity.

- [*3H*]-fluconazole and rhodamine-6G transport: Cdr1GFP as well as Cdr1(1-1436)/Cdr3(1420-1501)GFP showed almost comparable level of fluconazole accumulation and rhodamine-6G efflux activity as compared to AD1-8u-.
In conclusion, our result suggest that replacement of either the N- or C-terminal half of \textit{CDR1} with the corresponding region of \textit{CDR3} resulted in non-functional chimeras as this replacement led to the trafficking problem, however exchanging only TM12 resulted in functional chimera as the residues predicted to participate in substrate interaction is highly conserved between \textit{CDR1} and \textit{CDR3} (Pawagi et al 1994) thus demonstrating the functional exchangeability of TM12 of \textit{CDR1} with \textit{CDR3}. 