Discussion
4 DISCUSSION

Cdr1p was first cloned and identified by us as a protein capable of effluxing a variety of unrelated cytotoxic drugs and subsequently demonstrated to be a transporter involved in azole resistance of *C. albicans* (Krishnamurthy et al. 1998c; Prasad et al. 1995). Further studies from our lab as well as from other groups established that the efflux of drugs by Cdr1p represents one of the major determinants of azole resistance in clinical isolates of *C. albicans* (Krishnamurthy et al. 1998c; Sanglard et al. 1999). Functional analysis of Cdr1p further revealed that in addition to drug extrusion activity, the protein is also capable of effluxing human steroid hormones and can translocate membrane phospholipids between the two monolayers of PM of *C. albicans* cells (Krishnamurthy et al. 1998c; Dogra et al. 1999). The promiscuity of Cdr1p towards substrates and its ability to mediate several functions suggest that the protein might contain mutually exclusive substrate binding sites that allow the efflux of variety of unrelated compounds with striking physical and chemical diversity. The molecular basis for the broad range of this transport capacity is largely unknown for Cdr1p as well as for other ABC drug transporters of yeast. Delineating the architecture of the drug binding sites of Cdr1p for the substrates will be invaluable not only to understand how drugs interact with this protein, but also to design more useful and specific inhibitors of Cdr1p. In this study, we have attempted to examine the structure and function relationship of Cdr1p.

4.1 Cdr1p is fully active as GFP-tagged protein

For detailed functional analysis, Cdr1p tagged with GFP, was overexpressed from a genomic *PDR5* locus in a *S. cerevisiae* mutant AD 1-8u derived from a *pdr1-3* mutant strain with a gain of function mutation in gene encoding transcription factor *PDR1*, resulting in a
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constitutive hyper-induction of the *PDR5* promoter (Nakamura *et al*. 2002). Thus Cdr1p-GFP expression driven by hyper induced *PDR5* promoter provided sufficient level of expression, confirmed by confocal microscopy, flow cytometry analysis and immunoblotting, for functional analysis. That GFP tagged Cdr1p was functional like native Cdr1p was established by assaying the MICs of *S. cerevisiae* host cells to various drugs. The fluorescence imparted by the GFP tagged Cdr1p when visualized under confocal microscope, confirmed its proper localization to PM.

### 4.2 Cdr1p harbors multiple drug binding sites

Cdr1p, Pdr5p, human P-gp and some of the MRPs function as drug extrusion pumps. Cdr1p structurally differs from the human full-length ABC transporters since it possesses inverted domain organization (NBD-TMD)$_2$ (Prasad *et al*. 1995) as compared to human P-gp (TMD-NBD)$_2$ (Gottesman *et al*. 1995). However, the human ABCG2 (mitoxantrone resistance-associated protein, MXR, breast cancer resistance protein, BCRP, or placenta ABC-protein, ABCP), the half transporter has domain organization similar to Cdr1p (Litman *et al*. 2001) and the functional unit of this transporter is a dimer (NBD-TMD)$_2$. Notwithstanding these domain based differences and low level of sequence identity outside conserved stretches, binding characteristics of Cdr1p have many commonalties with human P-gp. The binding of IAAP as well as azidopine to Cdr1p is one such case of similarity. Both analogues bind to human and murine drug transporting P-gps and Cdr1p (this study) wherein it has been exploited to map drug binding sites (Greenberger *et al*. 1991). The fact that IAAP binding to Cdr1p is competed out by nystatin while miconazole did not have any effect and that azidopine binding is competed out only by miconazole (Figure 2 c & 2 f) suggest that IAAP
and azidopine share two different binding sites in Cdr1p. We had earlier observed that the deletion of a 79 amino acid stretch from the C-terminal, which encompasses the TMS 12 of this transporter leads to selective impairment of drug resistance (Krishnamurthy et al. 1998a). The expression of ΔCdr1p led to decreased resistance to nystatin while resistance to miconazole was retained. Recently by using variety of novel substrates of Pdr5p, Golin et al (Golin et al. 2003) have reported that this ABC drug transporter from S. cerevisiae has at least three drug binding sites and that some substrates might interact even at more than one site. Taken together, our results suggest that Cdr1p harbors different drug binding sites, which recognize structurally dissimilar drugs. Of note, selective sensitivity to nystatin and miconazole revealed upon mutational analysis further reaffirmed that Cdr1p has multiple drug binding sites. While variant F774A retained the resistance to miconazole as compared to native Cdr1p, its sensitivity towards nystatin enhanced considerably (Figure 4 a and 4 b). This duality of Cdr1p towards these two drugs can be explained if one considers different binding sites. Interestingly, similar conclusion can be drawn from IAAP and azidopine binding. While the former gets competed out by nystatin, the latter is affected by miconazole. Taken together, this would suggest nystatin and IAAP share common binding site(s) while azidopine and miconazole bind to another site. The concept of multiple drug binding sites is further illustrated by the fact that the binding of IAAP and azidopine are not competed out by drugs like cycloheximide, anisomycin and fluconazole, which may bind to yet unidentified site(s).

Notwithstanding topological differences, the photoaffinity analogues, IAAP and azidopine could specifically label Cdr1p as well as human P-gp. The extent of binding of these photoaffinity analogues
to Cdr1p revealed even further similarity between the two ABC drug transporters. For example both IAAP and azidopine showed enhanced binding to Cdr1p-GFP variant of SS5G (F774A). Similar observations were reported by Chen et al. (Chen et al. 1997) where they observed that the deletion of F335 led to enhance IAAP binding to P-gp. In another study, Loo et al. (Loo and Clarke 1993) observed that the replacement of F335 by A of human P-gp, also led to enhanced binding of azidopine. Taken together, these results suggest F335 in TM6 of mammalian P-gp and its equivalent F774 in TM6 of Cdr1p behaves similarly and plays an important role in determining substrate specificity.

4.3 T1351 is important for susceptibility of Cdr1p to FK520

FK506, an immunosuppressant agent is not toxic to Pdr5p expressing *S. cerevisiae* cells, however, its presence reverses the resistance to azoles and other substrates conferred by the transporter (Egner et al. 1998). The modulation effect of FK506 has been attributed to a TMS 10 residue (S1360) of Pdr5p since its substitution with phenylalanine resulted in loss of Pdr5p susceptibility to inhibition by FK506 (Egner et al. 1998). In this study, Cdr1p expressed in *S. cerevisiae* also shows identical phenotype where resistance of wild type cells to ketoconazole and fluconazole could be reversed in the presence of FK 520 (Figure 8 a and 8 b). The secondary structure prediction of Cdr1p using a method of Eisenberg et al (Eisenberg et al. 1984) algorithm places T1351 in CL5, which is equivalent to S1360 of TMS 10 of Pdr5p. The mutation T1351F (SS15) in Cdr1p resulted in global sensitivity to all the tested drugs including ketoconazole and fluconazole. Of note the residual growth of SS15G (T1351F) observed in presence of ketoconazole and fluconazole was not completely inhibited in the presence of FK520. Thus implying that
the effectiveness of the immunosuppressant derivative is dependent on T1351.

4.4 ΔF774 in SS6G leads to poor localization of mutated Cdr1p

In order to analyze some of the interesting mutant variants of Cdr1p, F774A, ΔF774 and T1351F mutations were introduced in Cdr1p-GFP expression system driven by hyper induced PDR5 promoter (discussed above) for detailed analysis. It was observed that in SS6G (ΔF774) strain, where F774 of predicted TMS 6 was deleted, showed very low expression of mutant Cdr1p-GFP in the PM fraction when analyzed by western blot analysis. While mutant Cdr1p-GFP in SS6G (ΔF774) was poorly expressed in PM isolated from these cells, western blot did not show any steady state expression defect in SS5G (F774A) and SS15G (T1351F) membranes. Confocal microscopy and FACS analysis also confirmed poor surface expression of SS6G (ΔF774) mutant protein.

4.5 Cell surface expression of mutant Cdr1p (ΔF774) protein could be rescued by growth in the presence of cycloheximide

Our finding that the localization of the SS6G (ΔF774) mutant protein variant to PM could be improved by growing the cells in presence of cycloheximide (a specific substrate of Cdr1p), suggest that ΔF774 significantly interferes with the folding of the Cdr1p during biogenesis. Of note the mutant protein of ΔF774 which could be directed to PM in presence of cycloheximide was not only found to be at the cell surface but also became functionally active as was determined by its ability to efflux rhodamine 6G. These results suggest that the deletion mutation ΔF774 in TMS 6 inhibits the transporter from folding in to an active conformation, but the protein
can be made to fold into an active conformation if synthesized in the presence of cycloheximide.

Earlier misprocessing of other ABC transporters has also been observed. For example, the deletion of phenylalanine 508 in NBD1 of CFTR causes its improper localization on the cell surface (Cheng et al. 1990). Deletion of equivalent phenylalanine residue in NBD1 of Yor1p of *S. cerevisiae* also led to variant protein, which was retained in endoplasmic reticulum (ER) (Katzmann et al. 1999). Interestingly, a number of low molecular weight molecules have been shown to rescue the processing defect of mutant CFTR protein (Brown and Welch 1996). In addition, even lower temperature, presence of glycerol, trimethylamine N-oxide, or deuterated water has been shown to rescue ΔF508 CFTR mutant misprocessing phenotype (Denning et al. 1992; Sato et al. 1996). Similarly, TMS 6 mutant of human P-gp expressing misfolded protein was rescued when grown in the presence of substrates like cyclosporin A, capsaicin, vinblastine, or verapamil (Loo and Clarke 1997). In such rescuing instances an early on drug substrate and TMDs interaction is observed which stabilizes the folding intermediates in a native conformation thus escaping cell quality control mechanism. The exact mechanism by which cycloheximide acting as a chemical chaperone alters proper folding of ΔF774 mutant Cdr1p is not clear at present. Given the clinical importance of this protein, such residues, which affect the localization, could be exploited in rationally designing antifungals, which would prevent superfolding of nascent Cdr1p.

In conclusion, our study demonstrates that in spite of the topological differences in structure, there is a conserved functional homology between Cdr1p and human P-gp. This is evident amply
when one considers the fact that IAAP and azidopine specifically bind to both drug transporters and the amino acid residue (phenyalanine) critical for drug-substrate recognition is also appear to be same at equivalent positions in these transporters. In addition, similar to P-gp, TMS 6 in Cdr1p also appears to be a major contributor to drug-substrate binding sites. We show functional overexpression of GFP tagged Cdr1p wherein photoaffinity analogues binding can be exploited to get information on drug-substrate binding mechanisms using site directed mutagenesis approach. Considering the clinical importance of MDR in cancer patients and fungal infections, structure and functional study of Cdr1p should provide additional information about the mechanism of action of these yeast and mammalian drug transporters.