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From the relative simplicity of bacterial cells, fungi and protozoa to the complexity of human cancer cells, MDR has become problematic and decreases the chance of providing successful treatment against a plethora of diseases. *Candida albicans* is an opportunistic diploid fungus that causes infection in immunocompromised and debilitated patients [Odds, 1988]. Wide spread and prolonged usage of azoles in recent years has led to the rapid development of the phenomenon of azole resistance which poses a major threat to antifungal therapy [Calderone, 2002; White *et al.*, 1998]. MDR is a multifactorial phenomenon where a combination of mechanisms could contribute to drug resistance [White, 1997; Franz *et al.*, 1998; Lopez-Ribot *et al.*, 1998]. One of the most clinically significant mechanisms of azole resistance in pathogenic yeast *Candida albicans* is the over-expression of the drug extrusion pump encoding genes belonging to either ABC Superfamily, e.g. *CDR1* and *CDR2* or MFS e.g. *CaMDR1* [Fling *et al.*, 1991; Ben-Yaacov *et al.*, 1994; Prasad *et al.*, 1995; Sanglard *et al.*, 1997]. The ABC and MFS superfamilies are designated by Transport commission (TC) system as TC 3.A.1 and TC 2.A.1, respectively [Busch and Saier, 2002]. Whereas the ABC transporters bind ATP and require ATP hydrolysis for transport activity, the MFS mediated transport is driven by the proton-motive force. Members of both classes are found in all three kingdoms of life and are apparently involved in transport of solutes across the plasma membrane or across intracellular membranes. Subfamilies have been defined on the basis of structural and functional criteria [Pao *et al.*, 1998; Saier, 1999] but the physiological substrates are known only for a few transporters. Both ABC and MFS transporters are encoded by large gene families that have been characterized extensively [Decottignies and Goffeau, 1997; Goffeau *et al.*, 1997; Nelissen *et al.*, 1997; Bauer *et al.*, 1999; Wolfger *et al.*, 2001; Sa-Correia and Tenreiro, 2002]. Members of both classes can have broad and overlapping substrate specificities. Owing to the fact that all eukaryotic genomes encode several gene families capable of encoding MDR functions, among which the ABC and MFS transporters are the largest, the number of candidate MDR genes in both these superfamilies means that study of the drug resistance properties of an organism cannot be effectively carried out without taking a wider perspective. In comparison to ABC transporters Cdr1p and Cdr2p which are well-studied, the MFS transporter CaMdr1p is poorly explored.
[Shukla et al., 2003; Saini et al., 2005; Gupta et al., 1998; Pasrija et al., 2007]. In-depth knowledge of structure and function of CaMdr1p is necessary for an effective designing of modulators or inhibitors of this efflux transporter.

1. Random mutational analyses of the multidrug MFS transporter CaMdr1p

- A random mutational analysis of MFS transporter CaMdr1p using the traditional measure of conservation as the criteria for selection of the residues was followed. The conserved residues of CaMdr1p were mutated to alanine to elucidate their role in the functioning of CaMdr1p.


- Localization and expression of mutant CaMdr1p variants remained unaltered: All mutant CaMdr1p variants were localized at the plasma membrane and showed similar expression levels.

- The Class I mutant variants showed an increase in accumulation of $[^3H]$MTX and $[^3H]$FLU while Class II and III mutant variants had levels like that of the wild-type.

- Cysteine-scanning was employed so as to assess the structure-function relationship of this protein. Individual cysteine mutations to alanine did not lead to any effect on the drug susceptibilities of these mutant variants. A cysless-CaMdr1p was created which opens up as plethora of options to explore the mechanistic details of this MFS antiporter. It was sensitive to drugs as compared to the wild-type but still exhibited a considerable resistance towards these drugs.

- Localization of these individual cysteine mutant variants as well as the cysless-CaMdr1p was not affected.
An analysis of the unique N-terminal of CaMdr1p revealed by site-directed mutagenesis followed by construction of deletion mutants predicts its role in surface localization of this protein.

Of note, this random site-directed mutational study reveals the structural and functional details of this transporter but a better approach was required for identifying the critical residues for specific function of CaMdr1p. Moreover, efficacy of site-directed mutagenesis was comparatively low. Taken together, a more rationalized approach was needed to be developed which involved the usage of bioinformatics.

2. Membrane environment based rational computational approach for identification of family-wide-function specific residues of CaMdr1p

A rational mutational analysis was done which uses a membrane environment based computational approach to predict the functionally critical residues of CaMdr1p. For this, Information theoretic scores which are variants of Relative Entropy (Modified Relative Entropy REM) were calculated from Multiple Sequence Alignment (MSA) of 361 MFS sequences, by separately considering distinct physico-chemical properties of transmembrane (TM) and inter-TM regions.

The residues of CaMdr1p with high REM which were predicted to be significantly important were subjected to site-directed mutational analysis. Interestingly, heterologous host S. cerevisiae, over-expressing these mutant variants of CaMdr1p wherein these high REM residues were replaced by either alanine or leucine, demonstrated increased susceptibility to tested drugs.

The hypersensitivity to drugs was supported by abrogated substrate efflux mediated by mutant variant proteins and was not attributed to their poor expression or surface localization.

Additionally, by employing a distance plot from a 3D deduced model of CaMdr1p, the role of these functionally critical residues could be predicted in maintaining apparent inter-helical interactions to provide the desired fold for the proper functioning of CaMdr1p.
Residues predicted to be critical for function across the family were also found to be vital from other previously published studies, implying its wider application to other membrane protein families.

This study provides an insight into the molecular details of MFS transporters in general and CaMdr1p in particular. This method of scaled \( R_{EM} \) calculations improves its performance over other information theoretic methods.

Additionally, this study also provides a method for rational mutational analysis not only for MFS proteins but can be applied to any class of membrane proteins and thus makes it possible to predict and locate family-wide functionally relevant residues.

3. Identification of drug-proton antiporter function specific residues of CaMdr1p by employing information theoretic measures

This study employs information theoretic measures to present a structure and functional analysis of this multidrug-proton antiporter Mdr1p of *Candida albicans* by predicting residues important for drug-proton antiporter function.

Since CaMdr1p belongs to drug-proton antiporter (DHA1) family of MFS transporters, this DHA1 family (antiporters) was contrasted with Sugar Porter family (symporters). Cumulative Relative Entropy (CRE) calculated for these two sets of alignments enabled us to selectively identify conserved residues of not only CaMdr1p but for the entire DHA1 family.

Based on CRE, the highest scoring thirty positions were selected and predicted to impart functional specificity to CaMdr1p as well as to other drug-proton antiporters. Nineteen positions wherein the CaMdr1p residue matched with the most frequent amino acid at a particular alignment position of DHA1 members were subjected to site-directed mutagenesis and were replaced with either alanine or leucine.

All these residues, except one, displayed either complete or selective sensitivity to the tested drugs. The enhanced susceptibility of these mutant variants was corroborated with the simultaneously abrogated efflux of substrates. Taken together, based on scaled CRE between two MFS sub-families, this study could accurately predict the functionally relevant residues of CaMdr1p.
An extrapolation of these predictions to the entire DHA1 family as validated from previously published data shows that the equipositional residues in other members of the DHA1 family are also functionally critical.

This analysis effectively shows that few residues in or around the pore differ on the basis of the mechanism of an MFS transporter and are thus specific for a particular family. Such residues are important for classifying a sequence to be a MFS-MDR transporter as validated from CaMdr1p.

In conclusion, this study shows that by using an information theoretic measure, it is possible to rationally conduct structure and functional study of a major MFS antiporter CaMdr1p. The function-specific residues identified therein are not only critical for CaMdr1p but are valid for the entire DHA1 family.