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Rational Mutational Analysis of a Multidrug MFS Transporter CaMdr1p of Candida albicans by Employing a Membrane Environment Based Computational Approach

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Abstract

CaMdr1p is a multidrug MFS transporter of pathogenic Candida albicans. An over-expression of the gene encoding this transporter is linked to clinically encounteredazole resistance. In-depth knowledge of the structure and function of CaMdr1p is necessary for the development of effective inhibitors of this efflux transporter. Towards this goal, in this study, we have employed 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 \( R_{E_M} \)) were calculated from Multiple Sequence Alignment (MSA) by separately considering distinct physico-chemical properties of transmembrane (TM) and inter-TM regions. The residues of CaMdr1p with high \( R_{E_M} \) which were predicted to be significantly important were subjected to site-directed mutational analysis. Interestingly, heterologous host Saccharomyces cerevisiae, over-expressing these mutant variants of CaMdr1p wherein these high \( R_{E_M} \) 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, we could also predict the role of these functionally critical residues 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.


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Introduction

In yeasts, including the pathogenic Candida, an up-regulation of multidrug transporter genes belonging to either ATP Binding Cassette (ABC) or Major Facilitator Superfamily (MFS) is frequently observed in the cells exposed to the drugs leading to the phenomenon of multidrug resistance (MDR) [1]. Among the 28 putative ABC and 95 MFS transporter genes identified in the C. albicans genome, only ABC transporters CaCdr1p and CaCdr2p and MFS transporter CaMdr1p, are found to be the major determinants of azole resistance [2,3]. The reversal of the functionality of these multidrug efflux pump proteins represents an attractive strategy to combat azole resistance.

The major ABC transporters such as CaCdr1p, CaCdr2p bear similar topology and exist as two homologous halves. These, like any other member of the ABC superfamily have four distinct modules: two transmembrane domains (TMDs) each consisting of six transmembrane segments (TMSs) and two nucleotide binding domains (NBDs) located on the cytosolic side of the membrane. Though similar in topology and promiscuity towards substrate specificity, these ABC multidrug transporters of C. albicans display selectivity to the range of substrates they can export [4].

The transporters belonging to MFS, consists of membrane proteins from bacteria to higher eukaryotes and these are involved in symport, antiport or unport of various substrates [5,6]. One of the 17 families of MFS transporters uses the proton motive force to drive drug transport and has been identified in both prokaryotes and eukaryotes [7]. Crystal structures of MFS proteins such as lactose permease (LacY), glyceral-3-phosphate (GlpT), EmrD and oxalate: formate antiporter (OxI), suggest high structural resemblance among this family of proteins [8]. These consist of 12 TMS, arranged with a similar predicted topology, strongly supporting a common structural architecture or fold across all the MFS transporters [9–12]. The fungal MFS members particularly those involved in drug transport are poorly explored in terms of their structure and function [13]. The multidrug MFS transporter...
Author Summary

Membrane proteins belonging to the Major Facilitator Superfamily (MFS) transport molecules, including drugs, across the membrane and are known to be associated with drug resistance. CaMdr1p is one such MFS major multidrug efflux pump whose over-expression is linked to frequent, untowardly encounteredazole resistance in hospital isolates of C. albicans. Amino acid residues critical for a protein’s function are conserved across members of the protein family. However, the traditional measure of conservation is not a useful parameter in mapping a functionally important residue in membrane proteins e.g., hydrophobically conserved stretches form helical transmembrane regions of the protein and are responsible for membrane localization, which individually have limited effect on binding and transport. We have developed a method that uses information theory to score the conservation of a residue relative to its context within the membrane and hypothesize that these residues would be critical for the protein’s function. The relevance of predicted residues in the functioning of MFS is validated on CaMdr1p.

CaMdr1p belongs to DHA1 family which is widely distributed and includes both drug-specific and multidrug efflux pumps [14]. Random and site-directed mutational strategies have been extensively used to understand the structure and function of these MDR efflux proteins. For example, random mutational analysis of an ABC transporter, ScPdr5p of budding yeast identified several amino acid residues that alter its substrate specificity and sensitivity to various inhibitors [15,16]. Tutulan-Cunita et al. observed that several point mutations led to significant changes in drug specificity of ScPdr5p which are distributed throughout the length of the protein [17]. Site-directed mutagenesis followed by an elegant screen done by Golli’s group has revealed interactions between TM2 and the NBD which may help to define at least part of the translocation pathway for coupling ATP hydrolysis to drug transport mediated by ScPdr5p. Recently, Schmitt et al. have elucidated the role of H1068 in H-loop of ScPdr5p which couples ATP hydrolysis with drug transport [18].

Site-directed mutational analysis of multidrug ABC multidrug transporter CaCdr1p (a close homologue of ScPdr5p) has revealed insight into its drug binding and efflux properties. These studies have implicated some of the amino acid residues of TMS 5, 6, 11 and 12 as the components of the substrate binding pocket(s) of CaCdr1p [19,20]. Together, these studies suggest that the drug binding sites in CaCdr1p are scattered throughout the protein and probably more than one residue of different helices are involved in binding and extrusion of drugs. However, there is still insufficient information available to predict where and how exactly the most common antifungals such as azoles bind and how are they extruded by CaCdr1p.

Site-directed mutational strategies rely on conservation of residues in a Multiple Sequence Alignment (MSA). The conservation of a residue is calculated from the amino acid frequency distribution in the corresponding column of a MSA. However, the physicochemical conservation is not necessarily responsible for a protein’s structure and function but could reflect a more general function such as membrane localization. Thus conservation alone is not sufficient to distinguish between residues responsible for the protein function and membrane localization. Membrane proteins differ from soluble proteins because of their inter-TM hydrophobic and TM hydrophobic propensities, which have allowed the development of efficient membrane protein TM prediction methods [21] and of membrane protein specific substitution matrices [22].

The quantification of residue conservation has evolved over the last few years to the use of information theoretic measures [23]. Relative entropy is a distance measure commonly applied to multiple alignments by comparing the observed frequency distribution with a background distribution. In the present study, we have developed and employed a new method using information theory to rationalize mutation strategies and also applied it to a MFS multidrug transporter CaMdr1p [24]. Relative Entropy (RE) or the Kullback-Liebler divergence is an information theoretic measure of the difference between two probability distributions and has been increasingly applied in bioinformatics to identify functional residues [24,25]. The use of RE with background frequencies [26] can improve the prediction of a protein’s functional residues [27–32] as well as detect residues that determine the functional subtype of proteins [28]. Though the basic Kullback-Liebler equation has not changed, its intelligent application in our method calculates Relative Entropy (RE\textsubscript{MA}) relative to its context within the membrane. The RE\textsubscript{MA} scoring scheme has been improved by treating TM and inter-TM regions of MFS proteins separately which has drastically increased the credibility over the existing methods [23]. In this manuscript, we have compared traditional treatment of conservation, and standard RE, with our improved method. We validated our predictions by replacing the predicted highest RE\textsubscript{MA} positions of CaMdr1p with alanine by site-directed mutagenesis. We show that most of these residues when replaced with alanine showed decreased resistance to drugs which was corroborated by abrogated efflux of drugs. Additionally, we could further confirm the functional relevance of each of these high RE\textsubscript{MA} residues by predicting their location in deduced 3D model of CaMdr1p and their role in maintaining apparent inter-helical interactions. With this approach, our method enabled us to accurately predict MFS-wide function-specific residues, validated by using CaMdr1p.

Results

RE\textsubscript{MA} considers conservation as well as the background probability of each alignment position of a MSA.

A comprehensive non-redundant data set, sourced from all MFS sequences present in the 56.2 release, was generated. This data set was then aligned using a membrane-specific multiple alignment program, which stacked the helices appropriately. A highly conserved residue in a multiple alignment is predicted to have a functional significance. We calculated conservation values using the algorithm from Jalview. Residues shown to be conserved dominate the TM helices, and on closer evaluation are largely hydrophobic residues associated with membrane localization. The traditional relative entropy and our modified treatment of the method (RE\textsubscript{MA}) were calculated on the same alignment using scripts written in-house. Fig. 1 shows a representative section of the alignment along with the RE\textsubscript{MA}, RE and conservation scores; see supplementary Table S1 for the RE\textsubscript{MA}, RE and conservation scores for the entire MSA. The distribution curve generated on the basis of RE\textsubscript{MA} from the MSA is shown in Fig. 2A. Notably the dominant signal using a conventional conservation measure lies in the TM helices and traditional RE also issues high scores to these residues which are less frequent in nature. Our treatment using a RE\textsubscript{MA} dampens the membrane localization signals further increasing the signal from atypically occurring conserved residues (Figure S1). Since RE\textsubscript{MA} considers conservation as well as the background probability of a residue at a particular alignment position, it is an improved index of the functional significance of a residue. To emphasize this fact, thirty residues with highest values were shortlisted each from the conservation list, RE list and RE\textsubscript{MA} list. On comparison, it was found that there are thirteen residues
shortlisted as both conserved and with high RE while it was found that only nine out of these short-listed columns are both conserved and have high REM (Supplementary Table S2). The thirty alignment positions with high REM were further studied to assess their functional relevance. Expectedly, all residues predicted using REM are not present in every protein in the family. In CaMdr1p, 16 residues were identical with the most frequently occurring residue in the thirty highest scored alignment columns, and were mutated to alanine to directly validate the prediction (Fig. 2B).

Residues with high REM are part of the known motifs of Major Facilitator Superfamily

These sixteen out of the top thirty positions, wherein the residue in CaMdr1p matched with the most occurring residue across that alignment position in MSA were analyzed for further studies by site-directed mutagenesis. Interestingly, most of the sixteen residues with high REM turned out to be part of the well-known motifs of the MFS. These motifs are identified as Motif A (GxxLxxDxxxG), Motif B (bxxxRxxGxxgaa) which are conserved throughout the MFS, Motif C (gxxGxxxGxxG) only in 12 and 14-TMS family and Motif D2 exclusive to 12-TMS family [7]. Three out of the sixteen residues short-listed for CaMdr1p; E178, G183 and R184 are a part of Motif A; residues L211, R215 and G219 are a part of Motif B and G256 is a part of motif C. In addition to the known motifs mentioned above, two new motifs have been identified by our study. The residues in these stretches 27WxRxx27 and 28xSyxx28 have high REM scores. However, the known motif D2 does not appear to be highly conserved in our alignment and is thus not predicted to be family-wide function-specific.

Site-specific mutagenesis of residues with high REM shows that they are functionally critical

All the sixteen residues selected on the basis of high REM were mutated by employing site-directed mutagenesis and were replaced with alanine except G165, G183 and G256 which were replaced by leucine. For functional analysis of the mutant variants, a heterologous hyper-expression system, where GFP-tagged CaMdr1p (CaMDR1-GFP) was stably over-expressed from a genomic PDR3 locus in a S. cerevisiae mutant AD1-8u, was used [33]. The host AD1-8u developed by Goiffeau’s group, was derived from a Pdr1-3 mutant strain with a gain-of-function mutation in the transcription factor Pdr1p, resulting in constitutive hyper-induction of the PDR5 promoter [34]. A single-copy integration of each transformant at the PDR5 locus was confirmed by Southern hybridization (data not shown). Two positive clones of each mutant were selected to rule out clonal variations. These residues with high REM score showed increased drug susceptibility and abrogated efflux of substrates such as [3H]MTX and [3H]

![Figure 1. A portion of Multiple Alignment showing conservation and REM for each column.](image-url)
The multidrug MFS transporter CaMdr1p harbors a conserved antipporter ‘motif C’ within TMS 5. Our recent study has revealed that the conserved and critical residues of this motif and of TMS 5 are bunched together on the same face of its helical wheel...
projection and are critical in drug efflux [35]. However, the structure and function aspects of this major multidrug transporter remain poorly understood. To address some of these questions, in this study, we have rationalized conventional mutational strategy and applied computational approach to predict functionally critical residues of CaMdr1p.

The sequence set described in this manuscript represents a comprehensive non-redundant coverage of annotated MFS sequences from SWISSPROT. Many methods have been developed to improve the MSA of membrane protein families for accurate predictions of residues critical for structure and function [36]. Membrane proteins have fold signals which are easily mapped to the primary sequence as TM and inter-TM stretches. Considering the differences in physico-chemical properties of these two regions, membrane protein specific substitution matrices have been developed [22]. However, we argued that a conservation score on the basis of identity or physico-chemical similarity still remains inadequate as the background frequencies of their immediate environmental milieu are radically different with respect to hydrophilic and hydrophobic propensities. This is also apparent from the conservation scores of the MSA wherein a large proportion of the conserved columns correspond to hydrophobic TM regions. Notably, two CaMdr1p residues (F216 and L217) with high conservation but low \( R_{E_{MA}} \) were taken as controls, when replaced by alanine showed no change in the phenotype (data not shown). One of the most basic fold specific signals is the hydrophobic core in globular proteins, and the TM region in membrane proteins. Unlike globular proteins, the hydrophobic TM region is continuous in the membrane protein's primary structure, and indeed this still remains one of the preferred methods to identify membrane proteins, and map their TM regions. While it is intuitive that the synchronous stretch of hydrophobic residues is responsible for membrane localization, the application of a scoring method that can distinguish these residues from family-wide alignment columns associated with other functions has not yet been deployed. In essence, we require a method that can objectively separate the TM signals from other signals. To overcome these limitations, we improved existing method(s) of information theory wherein \( R_{E_{MA}} \) was calculated on the basis of MSA of MFS proteins, keeping in mind the differences in the environmental milieu. We thus treated TM and inter-TM regions by different background probabilities for calculation of \( R_{E_{MA}} \). These \( R_{E_{MA}} \) scores helped us to predict those sites which have amino acid distributions very different from the respective background distribution thereby statistically predicted to be functionally critical. Not all the residues predicted using \( R_{E_{MA}} \),

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**Figure 3. Drug susceptibility and transport assays of mutant variants of CaMDR1-GFP in *S. cerevisiae*.** Panel A: Drug resistance profile of wild type and mutant CaMDR1-GFP yeast strains by spot assay. For spot assay, cells were freshly streaked, grown overnight and then resuspended in normal saline to an \( A_{600} \) of 0.1 (1 \( \times 10^6 \) cells) and 5 \( \mu l \) of five-fold serial dilutions, namely 1 (1:5), 2 (1:25), 3 (1:125) and 4 (1:625), of each strain was spotted on to YEPD plates in the absence (control) and presence of the following drugs: FLU (0.20 \( \mu g / ml \)), CYH (0.20 \( \mu g / ml \)), 4-NQO (0.20 \( \mu g / ml \)) and MTX (65 \( \mu g / ml \)). Growth differences were recorded following incubation of the plates for 48 hrs at 30 \( ^\circ \)C. Growth was not affected by the presence of the solvents used for the drugs (data not shown). B: \([3H] \) MTX and \([3H] \) FLU accumulation in the different mutant variants of CaMdr1p-GFP. Controls AD1-8u and RPCaMDR1-GFP have also been included for comparison. The results are means \pm standard deviations for three independent experiments.

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Figure 4. Protein expression profiles of CaMdr1p-GFP and its mutant variants in S. cerevisiae. Panel A: Western Blot analysis of the PM fraction of mutant variants with anti-GFP antibody. B: Confocal and FACS analysis of all the mutant variants to check their expression and localization in comparison with AD1-8u (negative control) and RPCaMDR1-GFP (positive control) [46].

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are present in every protein in the family. In CaMdr1p, 16 residues were identical with the most frequently occurring residue in the thirty highest scored alignment columns, and were mutated to directly validate the prediction (Fig. 2B). Our results of drug susceptibility assays revealed that almost all of these matched residues with high RE_M when replaced with alanine displayed sensitivity to the tested drugs and showed abrogated drug transport (Fig. 3). Interestingly, when we mutated residues which had high conservation values but lower RE_M values (negative control), none showed alterations in drug susceptibilities and thus did not retain the functionally critical stringency as was evident from residues with higher RE_M. For example, analysis of a few conserved columns of the MSA, such as F216, L217 and L171 having RE_M values between 0.57-0.44 revealed that their replacement with alanine did not affect the function of CaMdr1p (data not shown). This strengthens the fact that our method takes into account the conservation along with the background frequency and thus lists out residues which affect the function. Also, to check the efficiency of the method, another negative control used was to mutate residues which are having low conservation and low RE_M values but lie in the vicinity of one of the 16 selected high RE_M residues. For example, when C225 which is closer to the critical G219 and D235, was mutated to C225A, the functioning of the protein was not affected (data not shown). Similarly, for critical G256, when residues A248, A253 and V254 which are within its vicinity were mutated as A248G, A253G and V254A, the mutant variants continued to behave as W1-CaMDR1-GFP [35].

To further elucidate the role of predicted residues in the functionality of CaMdr1p, a homology model based on the available crystal structures of lac permease, glycerol-3-phosphate, and oxalate: formate transporter was deduced [9-11]. The RE_M method predicts the relative importance of a residue purely from sequence analysis and is independent of the protein’s structure. However, the role a residue plays in the protein’s function is not readily apparent from its sequence. We exploited the protein’s 3D model as a guide to reason why a residue is functionally critical. The deduced 3D model suggested that similar to other MFS structures, the 12 TM helices of the CaMdr1p span the membrane in such a way that they form the channel pore particularly aligned by residues of TMS 2, 4, 5, 7, 8, 10 and 11. From the deduced homology model of CaMdr1p, a symmetric contact map was generated to highlight the inter-helical interactions of the protein (Fig. 5B). Based on the predictions from the distance map, we could show that many high RE_M residues are indeed a part of inter-helical interactions (Fig. 6B). It is apparent that more than one residue pair is predicted to be involved in maintaining the interactions between helices (Fig. 6A).
Our aim in developing this method was to identify residues with high specificity which would play a critical role across this entire MFS protein family. Although signals associated with antipporter motifs have been identified using this method, a finer granularity in function such as substrate specificity determining residues is not expected, as these signals would not be family-wide. Since the enlisted residues with high RE3 values which are functionally critical for CaMdr1p are expected to be family-wide function-specific and thus critical for the entire MFS protein data set, we validated their relevance from the earlier published work. It is known that Motif A of the MFS transporters span an eight residue long loop between TMS 2 and 3 and is suggested to be involved in maintaining a β-turn linking the adjacent TM helices [14]. In the present study, G183 and R184 in the loop between TMS 2 and TMS 3 of CaMdr1p were picked up as family-wide function-specific residues thus corroborating that these residues are a part of Motif A (GxLaDxGxGrkxG) which holds importance throughout the MFS transporters. The hypothesized rocking motion in MFS presumably requires conformational changes in the TMS and the β-turns. In this, the transporter inter-converts between Cβ (inward facing) and Cα (outward facing) states for translocation of substrates. In glycerol-3-phosphate of E. coli, it was seen that D88 was involved in inter-conversion between these Cβ and Cα states of the protein [10]. Interestingly, D88 corresponds to E178 of CaMdr1p which also lies in Motif A which upon mutation to alanine turns out to be critical for drug susceptibility and efflux (Table 1).

Motif B (LxxRxxqGxxg) of all MFS has a role in energy coupling which spans the N-terminal half of TMS 4 [7]. CaMdr1p contact map reveals that residues in Motif B interface with residues 162GxxG 169 on TMS 2. Motifs rich in glycine and proline residues promote formation of special backbone conformation including kinks in TMS, tight interactions between TMS and very flexible β-turns. In human VAChT, Motif B and the adjacent sequences contain a total of nine nontag signatures. A nontag allows two helical TMS to approach each other unusually closely because small side chains are located at the interface. R124 of PcoK of Pseudomonas putida which is equivalent to high RE3 R215 of CaMdr1p of C. albicans is shown to be critical for helix packing [37]. Interestingly, G111 of LacY of E.coli which also occupies a position in the same alignment column is also critical and earlier shown to be a residue at a kink.

Residues from Motif C (GxxGxxGxGxG) which is exclusive to 12-TMS family are also picked up by our calculations [7]. G130 of LacY of E.coli which is equivalent to high RE3 G226 of CaMdr1p is function-specific for LacY protein [36]. A stretch of conserved residues 296PeprG 300, previously unidentified, at the end of TMS 6 were also predicted with high RE3. We have mutated equivalent residues P296A, E297A and T298A of CaMdr1p that overlap with the consensus residues in the stretch and found that cells expressing these mutated variants displayed increased sensitivity to drugs. However, the functional significance of these residues is yet to be established.

There are a few exceptions which emerged from our method. For example, our method did not pick up any residue of Motif D2. This could be an artifact of the method used for alignments in earlier studies. In this study we have employed a membrane protein specific alignment method whereas earlier reports have used standard multiple alignments substitution matrices with smaller data sets. However, when we repeated the alignment using
Figure 6. Summary of inter-helical interactions via high REM residues. Panel A: The table summarizes predicted inter-helical interactions mediated via selected residues with high REM. More than one residue pair is predicted to be involved in maintaining the interactions between the helices. B: Pictorial representation of inter-helical interactions via these high REM residues. Figure shows that the residues involved in these interactions are majorly confined to the N-terminal half of the protein.

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MUSCLE [39] and with the standard substitution matrix (BLOSUM 62) [40] on the complete data set the motif still did not appear (data not shown). Motif D2 is assumed to have a structural significance as it holds a major kink within TMS 1 but mutations in this motif do not alter the backbone conformation. As an example of the possibly insignificant role of the motif, in human VAll, the mutation of L49G in this motif completely eliminates propensity for a kink or notch and abolishes activity while normally a glycine itself is expected to be present at this position and is supposed to be involved in maintaining a major kink in this motif [41].

Out of the 16 residues that were mutated, T160A, L211A, W273A and R274A did not lead to any phenotypic changes. It is known that for some of the positions in alignment, the most frequent amino acid does not match with the residue of CaMdr1p at that site. One reason for this could be that some of the

<table>
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<td>[9]</td>
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<td>TM5</td>
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<td>PcaK</td>
<td>Pseudomonas putida</td>
<td>R124 (1.9)</td>
<td>TM4</td>
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<td>[37]</td>
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<tr>
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<td>TM4</td>
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The residues with high REM positions and their predicted roles in the case of other MFS members are enlisted. The residues of CaMdr1p at the same position in the alignment are shown to be critical in this study.

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functionally important residues co-evolve i.e., those residues may mutate, with compensatory mutation occurring elsewhere in the protein to regain function [42]. T160 where the most frequent residue is a serine at that position may be one such case. Another reason may be that the alignment used in this study involved prediction of TMS with the possibility of errors in demarcating the edges of TM helices. Residues from columns lining the edges of the helices may be wrongly assigned to TM and inter-TM regions. This probably explains the lack of any effect of mutation on residues T160 of TMS 2 and L211 of TMS 4 which lie at the edge of the respective TMS. Other exceptions to our predictions are the mutation of W273 and R274 which though highly conserved and probably a part of the new motif but do not abrogate function upon mutation. Although a few tryptophans in an ABC transporter MRPl, have been shown to be involved in substrate binding and transport [43], generally, in a membrane protein tryptophan residues located on the surface of the molecule are mainly positioned to form hydrogen bonds with the lipid head groups while their hydrophobic rings are immersed in the lipid part of the bilayer [44]. We predict that W273 and R274 may be associated with membrane helix orientation and this function may not be perturbed by mutating them individually through alanine scanning. Alternatively, the tryptophan-arginine residues could be functionally critical in tandem and compensate each other for the loss of either one of them.

Of note, in our predictions, substrate specific residues with high RE20 are not picked up which predominantly occur in C-terminal of MFS proteins. It should be mentioned that since our alignment considers the entire MFS, residues responsible for substrate specificity would only be selectively conserved within a subfamily and would not have sufficiently strong signals to be visible in this present family-wide study. For this, the same method may be applied to a data set classified on the basis of substrate selectivity to identify residues critical to the functioning of that subfamily.

There are a number of conservation methods known but none has yet achieved both biological and statistical rigor. We have used RE31 to separate conserved residues from the background function of TM localization. The interpretations support the well-known fact that MFS has a conserved N-terminal half which has residues important for maintenance of a specific fold for this class of proteins while C-terminal half has a more specific role in substrate binding and recognition [7]. Taken together, our study provides an insight into the molecular details of MFS transporters in general and CaMdr1p in particular. Our method of scaled RE31 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.

Materials and Methods

Materials

Anti-GFP monoclonal antibody was purchased from BD Biosciences Clontech, Palo Alto, CA, USA. DNA modifying enzymes were purchased from NEB. The drugs cycloheximide (CYH), 4-Nitroquinoline oxide (4-NQO), Methotrexate (MTX) and Protease inhibitors (Phenylmethylsulfonyl fluoride, Leupeptin, Aprotinin, Pepstatin A, TPCK, TLCK) and other molecular grade chemicals were obtained from Sigma Chemicals Co. (St. Louis, MO, USA) Fluconazole (FLU) was generously provided by Ranbaxy Laboratories, India. [3H] Fluconazole was custom prepared and [3H] Methotrexate (MTX) was purchased from Amersham Biosciences, United Kingdom.

Media and strains

Plasmids were maintained in Escherichia coli DH5α. E.coli was cultured in Luria-Bertani medium (Difco, BD Biosciences, NJ, USA) to which ampicillin was added (100 μg/ml). The S. cerevisiae strain used was AD1-8u+ (MATa pdr1-3 his1 ura3 Δpir1::hisG Δmq2::hisG Δpdr5::hisG Δpdr10::hisG Δpdr11::hisG Δψf1::hisG Δpdr3::hisG Δpdr15::hisG) provided by Richard D. Cannon, University of Otago, Dunedin, New Zealand [33], [34]. The yeast strains used in this study are listed in the Supplementary Table S3. The yeast strains were cultured in YEPD broth (Bio101, Vista, CA, USA) or in SD-ura- dropout media (0.67% yeast nitrogen base, 0.2% dropout mix, and 2% glucose; Difco). For agar plates, 2.5% (w/v) Bacto agar (Difco) was added to the medium.

Methods

Multiple sequence alignment. 561 MFS sequences having 12 TMS as predicted by TMHMM [21] were extracted from SWISSPROT release 56.2. Redundancy in the sequences is reduced to 90% on the basis of identity using BLASTclust by setting the identity threshold S to 90 and keeping all other parameters to default values (http://genomes.ucsc.edu/manuals/blast/blastclust.html). The resulting 342 sequences were then aligned by PRALINETM using TMHMM as the method for predicting TM lengths and keeping all other parameters at their default values [36,43] (See Supplementary Dataset S1).

Calculation of conservation score, RE and REM. Conservation of an amino acid in an alignment column was calculated using Jalview using available scoring schemes which are restricted to soluble proteins, was modified to accommodate different background probabilities for TM and inter-TM regions. RE is calculated as the deviation of the amino acid distribution P(a)/ from a background distribution f(a). 

\[
REM = \sum_{a=1}^{20} P(a) \log \left( \frac{P(a)}{f(a)} \right)
\]

where \(P(a)\) is the probability of the occurrence of amino acid \(a\) in column \(i\) of the MSA \(f(a)\) is the background probability of amino acid \(a\) and is classically estimated from SWISSPROT as the probability of occurrence of an amino acid in a large data set of proteins.

In our method REM, \(f(a)\) is the background probability of amino acid \(a\) and is classically estimated as the probability of occurrence of an amino acid in a large data set of proteins. \(f(a) = f(a)_{TM}\) or \(f(a)_{IM}\) where \(f(a)_{TM}\) and \(f(a)_{IM}\) is the background probability calculated separately for TM and inter-TM regions of the alignment. The background probability is thus replaced with this environment specific background frequency. A classical description of entropy also does not take into consideration the absence of data points which are gaps in the case of a multiple alignment column. We used a scaling factor which is equal to number of amino acid positions excluding gaps in column \(i\) divided by total number of sequences. The scores were then divided by this scaling factor to give scaled REM score.

Site-directed mutagenesis of CaMdr1p. Site-directed mutagenesis was performed by using the Quick-Change Mutagenesis kit (Stratagene, La Jolla, CA, USA) as described previously [46]. The mutations were introduced into the plasmid pRPCaMDR1-GFP according to the manufacturer’s instructions, and the desired nucleotide sequence alterations were confirmed by DNA sequencing of the ORF. The primers used for the purpose are listed in Supplementary Table S4. The mutated plasmid, after linearising...
with \( \Delta \text{val} \), was used to transform AD1-Ru\(^{+} \) cells for uracil prototrophy by lithium acetate transformation protocol [46]. Integration was confirmed by Southern Blot analysis (data not shown).

Preparation of the plasma membranes and immuno-detection of CaMdr1p and its mutant variants. The plasma membranes (PM) were prepared from S. cerevisiae cells, as described previously [46]. The PM protein concentration was determined by bicinchoninic acid assay using bovine serum albumin as the standard. For Western Blot analysis the immunoblot was incubated with anti-GFP monoclonal antibody 1:5000, JL-8 BD Biosciences as described previously. Immunoreactivity of GFP antibody was detected using goat anti-mouse horseradish peroxidase-labelled antibody (1:5000) and was visualized using the enhanced chemiluminescence assay system (ECL kit, Amersham Biosciences, Arlington Heights, IL, USA) [46].

Drug susceptibility. The susceptibilities of yeast cells, harboring wild type CaMdr1-GFP and its mutant variants, were tested to different drugs by spot assay. For spot assay, 3 µl samples of five-fold serial dilutions of yeast culture each with cells suspended in normal saline to an OD of 0.1 \( \times 10^{6} \) cells at \( \lambda_{600} \) were spotted onto YEFP plates in the absence control or in the presence of the drugs [53]. Growth differences were recorded following incubation of the plates for 48 hrs at 30°C.

Drug transport of mutant variants. The accumulation of \(^3\text{H}\) MTX (specific activity, 8.60 Ci/mmol and \(^3\text{H}\) FLL (specific activity, 19 Ci/mmol) was determined by protocol described previously [55]. Cells from mid-log phase were centrifuged at 5000 xg for 3 min and resuspended in fresh YEFP medium as 3 x 10\(^5\) cell suspension. 100 µl of cell suspension was incubated in shaking water bath at 150 rpm at 30°C and \(^3\text{H}\) MTX was added to a final concentration of 1.50 x 10\(^{-3}\) M. The cells were incubated with \(^3\text{H}\) MTX 35 µM or \(^3\text{H}\) FLL 100 nM for 30 min. Filtered rapidly and washed twice with 1x PBS, pH 7.4 on Millipore manifold filter assembly using 0.45 µm nitrocellulose filter discs Millipore, USA. The filter discs were dried and put in cocktail-O and the radioactivity was measured in a liquid scintillation counter (Packard, Beckman, USA). The model was expressed relative to the wild type CaMdr1p-GFP.

Molecular modeling of CaMdr1p. Structures of known MFS proteins [PDB id - 1wp6 [9], 1wpv [10], 1ucz [11]] were retrieved from Protein Data Bank www.rcsb.org and aligned using MODELLER9v5 [47]. MFS sequences containing CaMdr1p aligned earlier with PRALINE/TM were aligned to this structure alignment after removing the sequences which corresponded to known structures. These were aligned using the profile-profile alignment option of ClustalW. The Profile alignment was then manually refined based on helix packing information of known MFS structures. Manual refinement was restricted to incorporating gaps in the loop regions without disturbing either the structural alignment or PRALINE sequence alignment. The homology model for the target sequence CaMdr1p was generated using MODELLER9v5 [17] using 1wp6, 1wpv and 1ucz as template sequences. The initial 90 residues from N-terminal of CaMdr1p did not align with any of the templates, and were occluded from further study. The model was evaluated and validated by PROCHECK.

Generating the contact map. The contact map displays the distances between C\( \beta \) of one residue and C\( \beta \) of every other residue within 8 Å distance (C\( \beta \) is considered in case of glycine). Using the homology model a symmetric contact map for CaMdr1p was generated using a PERL program written in-house.

Supporting Information

Dataset S1 The PRALINE/TM alignment of 342 MFS sequences as described in Materials and Methods. Found at: doi:10.1371/journal.pcbi.1000624.s001 (0.77 MB DOC)

Table S1 RE\(_{50}\), RE and conservation scores for all the positions of the MSA of 342 MFS sequences. Found at: doi:10.1371/journal.pcbi.1000624.s002 (2.01 MB DOC)

Table S2 Comparison of RE\(_{50}\) and conservation scores. Found at: doi:10.1371/journal.pcbi.1000624.s003 (0.26 MB DOC)

Table S3 List of yeast strains used in this study. Found at: doi:10.1371/journal.pcbi.1000624.s004 (0.04 MB DOC)

Table S4 List of oligonucleotides used for site-directed mutagenesis. Found at: doi:10.1371/journal.pcbi.1000624.s005 (0.05 MB DOC)

Figure S1 Comparative plot of conservation red, RE (green) and RE\(_{50}\) (blue) across the entire alignment. The conservation scores are scaled for comparison with RE and REM. Positions of the highest scoring alignment columns by each method are shown above the graph, along with the results of mutation of matching residues. Out of these top scoring positions by three different calculations, the mutated positions showing resistant phenotype are marked in red triangles, those showing sensitive on all drugs are marked in green triangles while those which were not mutated are marked by empty triangles. Locations of the transmembrane regions are marked by black bars on the x-axis. Found at: doi:10.1371/journal.pcbi.1000624.s006 (0.03 MB PDF)

Acknowledgments

We thank R. D. Cannon for providing us with the plasmid and the strains used in this study. We further thank Rainbaxx Laboratories Limited, India for providing us with Flu-crev. We are thankful to K. Narajan and Devapriya Choudhury for their valuable comments on our manuscript.

Author Contributions

Conceived and designed the experiments: KK AMR PRP. Performed the experiments: KK MR AR. Analyzed the data: KK MR RP AMR RP. Contributed reagents/materials/analysis tools: AMR RP. Wrote the paper: KK AMR RP.

References


Multidrug Resistance in Yeast *Candida*

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The opportunistic human pathogens *Candida albicans* and other non-*albicans* species have acquired considerable significance in the recent past due to the enhanced susceptibility of immunocompromised patients. These pathogenic species of *Candida* derive their importance not only from the severity of their infections but also from their ability to develop resistance against antifungals. Widespread and prolonged use of azoles has led to the rapid development of the phenomenon of multidrug resistance (MDR), which poses a major hurdle in antifungal therapy. Various mechanisms that contribute to the development of MDR have been implicated in *Candida* as well as in other human fungal pathogens, and some of these include overexpression of or mutations in the target enzyme of azoles, lanosterol 14α-demethylase, and transcriptional activation of genes encoding drug efflux pump proteins belonging to ATP-binding cassette (ABC) as well as to major facilitator superfamilies (MFS) of transporters. The ABC transporters, *CDR1*, *CDR2*, and an MFS pump *CaMDR1*, play a key role in azole resistance as deduced from their high level of expression found in several azole-resistant clinical isolates.

**KEY WORDS:** Multidrug resistance, ABC transporter, MFS transporters, Azoles, Efflux pumps, *Candida*, Ergosterol. © 2005 Elsevier Inc.

I. Introduction

Due to a sudden spurt in immunocompromised patients, fungal infections have become more common. Among various human fungal pathogens, *Candida albicans* accounts for the majority of systemic infections. But
infections caused by non-\textit{albicans} species, such as \textit{C. glabrata}, \textit{C. parapsilosis}, \textit{C. tropicalis}, and \textit{C. krusei}, are also common, particularly in neutropenic patients and neonates (Coleman \textit{et al.}, 1998; Ghannoum and Rice, 1999; Vanden Bossche and Koymans, 1998; Vanden Bossche \textit{et al.}, 1994a; White \textit{et al.}, 1998). Recently, incidences of \textit{C. albicans} cells acquiring resistance to antifungals like azoles have increased considerably, which has posed serious problems in successful chemotherapy (Hitchcock, 1993; Prasad \textit{et al.}, 2000; Scholer and Polak, 1984; Sternberg, 1994; Vanden Bossche, 1995). Unfortunately, the incidence of antifungal resistance is not restricted to \textit{C. albicans} alone since the non-\textit{albicans} species, such as \textit{C. glabrata}, \textit{C. parapsilosis}, \textit{C. tropicalis}, and \textit{C. krusei}, also display this phenomenon (Bennett \textit{et al.}, 2004; Coleman \textit{et al.}, 1998; Vanden Bossche and Koymans, 1998). Both \textit{C. albicans} as well as non-\textit{albicans} species have evolved a variety of mechanisms to combat antifungal resistance (Table I). This review focuses on the molecular mechanisms of antifungal resistance with a special emphasis on major drug efflux pump proteins of \textit{Candida}.

\section{II. Resistance to Antifungals}

\subsection{A. Antifungals and Their Targets}

The antifungals, which are commonly used to treat \textit{Candida} infections and their mode of actions, are listed in Table II. Most of the antifungals in use or at various stages of development belong to five major classes based on their mode of action: (1) azoles, which represent the most common class in use, inhibit ergosterol synthesis by blocking 14\textsubscript{z}-lanosterol demethylase (\textit{ERG11}), (2) allylamines block the synthesis of ergosterol (\textit{ERG1}) and result in the accumulation of toxic squalene, (3) polyenes, on the other hand, directly bind to membrane ergosterol resulting in pore formation, which makes the cell membrane leaky and results in cell death, (4) candins constitute a class of antifungals that inhibits fungal cell wall synthesis by inhibiting the synthesis of the major structural polymer \(\beta\)-1,3-glucan, and (5) pyrimidines, like flucytosine, inhibit nucleic acid synthesis by inhibiting thymidylate synthase.

\subsection{B. Mechanisms of Resistance to Antifungals}

Resistance to antifungals can be visualized as a gradually evolving process wherein different mechanisms may appear during the course of chemotherapy. Studies so far suggest that antifungal resistance in \textit{Candida}
is a multifactorial phenomenon (Fig. 1) (Balkis et al., 2002; Morschhauser, 2002). The main mechanisms of antifungal resistance include alterations in the ergosterol biosynthetic pathway by an overexpression of the ERG11 gene, which encodes the drug target enzyme 14α-demethylase or by an alteration in target enzymes (point mutations), which leads to reduced affinity to fluconazole. Reduced intracellular accumulation of drugs is another prominent mechanism of resistance in Candida cells wherein genes encoding drug extrusion pumps belonging to ABC (the ATP-binding cassette) and MFS (major facilitator) superfamilies of proteins are over-expressed. It has been well documented that clinical azole-resistant isolates of C. albicans display transcriptional activation of genes encoding ABC (Cdr1p, Cdr2p) or MFS (CaMdrlp) proteins. Invariably, resistant Candida cells, which show enhanced expression of efflux pumps encoding genes, also show a simultaneous increase in the efflux of drugs. Rapid efflux of incoming drug prevents cells from accumulating lethal concentration of azoles and enable them to survive (Prasad et al., 1995; Sanglard et al., 1995; White 1997a). Interestingly, drug inactivation, which is a very common mechanism in bacteria, has not been observed in Candida cells. Recent gene profiling results already suggest even more complexities, which may affect and control the phenomena of antifungal resistance (De Backer et al., 2001; Kaur et al., 2004; Krishnamurthy et al., 2004; Rogers and Barker, 2002, 2003). Some of the most common mechanisms of azole resistance found in Candida cells are discussed next.

1. Lipids in Drug Resistance

a. 14α-Lanosterol Demethylase (ERG11)  Azoles resistance in C. albicans occurs primarily through ERG11, which encodes 14α-lanosterol demethylase (CYP51, also known as P45014DM) involved in sterol biosynthesis. Azoles inhibit this step in the ergosterol biosynthesis in fungi by binding to and inhibiting P45014DM (Vanden Bossche et al., 1989; Wilkinson et al., 1972, 1974). The inhibition of P45014DM leads to high levels of 14-methylated sterols, which causes disruption of membrane structures. The alteration of the target protein P45014DM by point mutation and an overexpression of its gene (ERG11) encoding the target protein are the most predominant resistant mechanisms adopted by fungal cells (Ghannoum and Rice, 1999; Marichal, 1999; White 1997b).

i. Alterations in Erg11p  Since Erg11p is the target of azole derivatives, it can be expected that amino acid substitution could affect the affinity of the drug. Indeed, many studies have documented point mutations in the P45014DM (ERG11) gene, which resulted in changes in the affinity of the azoles to its target protein leading to resistance (Favre et al., 1999; Kakeya et al., 2000; Kelly et al., 1999; Lamb et al., 1997; Loffler et al., 1997; Marichal
<table>
<thead>
<tr>
<th>Fungi</th>
<th>Antifungals</th>
<th>Target gene</th>
<th>Target</th>
<th>Mechanism of resistance</th>
<th>References</th>
</tr>
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<tr>
<td><em>Candida albicans</em></td>
<td>Azoles</td>
<td><em>ERG11</em></td>
<td>Cytochrome P450</td>
<td>Point mutation in the target enzyme</td>
<td>Lamb et al., 1997; Sanglard et al., 1995</td>
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<td></td>
<td></td>
<td></td>
<td>14α-demethylase</td>
<td>alters the affinity of the enzyme to azoles</td>
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<td>Overexpression of target gene</td>
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<td></td>
<td><em>CaCDR1</em></td>
<td>ABC transporters</td>
<td>Overexpression of ABC and MFS family of multidrug transporters</td>
<td>Prasad et al., 1995; Sanglard et al., 1995; White, 1997a</td>
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<td></td>
<td><em>CaCDR2</em></td>
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<td></td>
<td><em>CaMDR1</em></td>
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<td></td>
<td><em>ERG3</em></td>
<td>Δ^5,6^-Desaturase</td>
<td>Alteration of the sterol</td>
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<td>Nonazoles</td>
<td>5-Flucytosine</td>
<td>Thymidylate synthase</td>
<td>Not reported</td>
<td></td>
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<td></td>
<td>Amphotericin B</td>
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<td>Increased membrane fluidity</td>
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<td>Nystatin</td>
<td>Membrane ergosterol</td>
<td>Alterations in membrane sterols</td>
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<td><em>ERG11</em></td>
<td>Cytochrome P450</td>
<td>Overexpression of target gene</td>
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<td>14α-demethylase</td>
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<td><em>CdCDR1</em></td>
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<td>Overexpression of ABC and MFS family of multidrug transporters</td>
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<td><em>CdMDR1</em></td>
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<td>Defects in the sterol</td>
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<td><em>ABC1</em></td>
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<td>Katiyar and Edlind, 2001; Orozco et al., 1998</td>
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<td><em>ABC2</em></td>
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<td>Cytochrome P450</td>
<td>Reduced susceptibility of target enzyme to inhibition</td>
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<td><strong>Mitochondrial loss</strong></td>
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<td><strong>Decreased membrane ergosterol content</strong></td>
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TABLE II
Structure of Representative Antifungals of Each Class along with Its Target and Mode of Action in a Yeast Cell

<table>
<thead>
<tr>
<th>Class</th>
<th>Drug and structure</th>
<th>Site of action</th>
<th>Target</th>
<th>Mode of action</th>
<th>Resistant organisms</th>
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<tbody>
<tr>
<td>Polyenes</td>
<td>Amphotericin B</td>
<td>Membrane</td>
<td>Membrane ergosterol</td>
<td>Binds to sterols, mainly ergosterol, in cell membrane resulting in change in permeability of the membrane and cell death</td>
<td>Fusarium spp., Aspergillus nidulans, Candida spp., Trichosporon spp.</td>
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<td>Nystatin</td>
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<td>Pyrimidines</td>
<td>5-Flucytosine</td>
<td>Nucleic acid function and synthesis</td>
<td>Cytosine permease, cytosine deaminase, uracil:phosphoribosyltransferase</td>
<td>It enters cells via cytosine permease and is deaminated to active form 5-FU by cytosine deaminase, 5-FU is then converted to FUMP by uracil:phosphoribosyltransferase. FUMP can be converted into FUTP or FdUMP, which inhibits protein synthesis or DNA synthesis, respectively</td>
<td>Candida spp., Cryptococcus neoformans, Aspergillus</td>
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<td>Azoles</td>
<td>Ketoconazole</td>
<td>Fluconazole</td>
<td>Itraconazole</td>
<td>Miconazole</td>
<td>Voriconazole</td>
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<td><img src="image" alt="Ketoconazole Structure" /></td>
<td>Ergosterol</td>
<td><img src="image" alt="Itraconazole Structure" /></td>
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<td><img src="image" alt="Voriconazole Structure" /></td>
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<td>Inhibition of cytochrome P450 14α-demethylase; accumulation of lanosterol leading to perturbation of fungal cell membrane</td>
<td>Cytochrome P450 14α-demethylase</td>
<td>Inhibition of cytochrome P450 14α-demethylase</td>
<td>Inhibition of cytochrome P450 14α-demethylase</td>
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(continued)
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<th>Site of action</th>
<th>Target</th>
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<td>β-1,3-Glucan synthase</td>
<td>Inhibition of cell-wall glucan synthesis leading to susceptibility of fungal cell to osmotic lysis</td>
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<td>Allylamines</td>
<td>Terbinafine</td>
<td>Ergosterol synthesis</td>
<td>Squalene epoxidase</td>
<td>Inhibition of squalene epoxidase; fungicidal effect may be due to accumulation of toxic squalene rather than deficiency of ergosterol</td>
<td>Saccharomyces cerevisiae, Ustilago maydis, Candida glabrata</td>
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FIG. 1 Predominant drug resistance mechanisms of Candida. (A) Reduced drug uptake (import) due to compositional changes in the cell wall or plasma membrane. (B) Rapid efflux of drugs mediated by the ABC or MFS transporters. (C) Overexpression of the genes encoding drug target ERG11 and/or of efflux pumps, e.g., CDR1, CDR2, and CaMDRI. (D) Mutation in drug target Erg11p (P45014DM) leads to reduction or loss in drug binding. (E) Activation of alternate ergosterol pathways such as $\Delta^{5,6}$-desaturase.

et al., 1999; Perea et al., 2001; Sanglard et al., 1998a; White et al., 2002; Xiao et al., 2004). White's group who analyzed a series of C. albicans strains isolated earlier by Redding et al. (1994) from a single HIV patient over a period of 2 years and identified a single amino acid substitution, viz. R467K in Erg11p. Since this mutation is close to amino acid cysteine, which participates in the coordination of the iron atom in the heme cofactor of enzyme, it has been proposed that the mutation causes structural changes associated with the heme. It has been further reported that R467K alone can confer azole resistance by reducing the affinity of the enzyme for fluconazole (Lamb et al., 2000). Based on the architecture of the active site of the enzyme, Lamb et al. (1997) introduced a point mutation T315A in Erg11p and observed that the mutant variant protein had higher MIC values for fluconazole and ketoconazole. The purified mutated protein exhibited reduced enzyme activity and affinity for azoles, thus providing an example of a single amino acid change in the target protein leading to azole resistance. There are several
reports wherein in response to azoles, many point mutations in Erg11p have been identified (Marichal et al., 1999). The known point mutations in C. albicans have been compiled in a graphic representation by Marichal et al. (1999) to show the frequency and position of each substitution. It is observed that four mutations, D116E, K128T, E266D, and G464S, occurred with highest frequency, whereas G464S was the only substitution exclusively seen in azole-resistant isolates. The exact placement of these mutations in a three-dimensional model of the protein shows that these mutations are not randomly distributed but rather clustered in three hot spot regions between amino acid residues 105–165, 266–287, and 405–488 (Marichal et al., 1999).

ii. Up-regulation of ERG11 Resistance to fluconazole in many clinical isolates has often been associated with the transcriptional activation of ERG11 (the gene-encoding target protein P45014DM) (Harry et al., 2002). However, it has been difficult to correlate the up-regulation of the gene with the observed fluconazole resistance mainly due to the simultaneous occurrence of mutations in ERG11 or to the overexpression of the efflux pumps encoding genes. Gene amplification is one of the common mechanisms of resistance in eukaryotic cells (Stark and Wahl, 1984; Van der Bleik et al., 1988). However, overexpression of ERG11 in C. albicans has not been linked to gene amplification (Vanden Bossche et al., 1992, 1994b; Marichal et al., 1997). In a clinical isolate of C. glabrata, an increased level of P45014DM was shown to be associated with the amplification of the ERG11 gene (Vanden Bossche et al., 1992, 1994b; Marichal et al., 1997). The amplification of the ERG11 gene in this isolate was linked to chromosomal duplication, which in turn resulted in high levels of the P45014DM protein (Marichal et al., 1997). That gene conversion or mitotic recombination could also play a role in fluconazole resistance in C. albicans was apparent from a study done by White's group in which additional genetic variations in a clinical isolate of C. albicans with R467K substitution have been reported (White, 1997c). It was shown that all allelic differences present in sensitive isolates of C. albicans were eliminated in the resistant isolates from the ERG11 by gene conversion or mitotic recombination (White, 1997c). The resulting strain had an R467K mutation in both copies of ERG11 and was more resistant to azoles as compared to a strain with single allelic substitution.

b. \( \Delta^{5,6} \)-Desaturase (ERG3) Another enzyme of the ergosterol biosynthesis pathway, \( \Delta^{5,6} \)-desaturase (ERG3), has been shown to contribute toazole’s resistance. A defect in ERG3 leads to the accumulation of 14\( \alpha \)-methylfecosterol instead of 14\( \alpha \)-methylergosta-8,24(28)-dien-3\( \beta \).6\( \alpha \)-diol. Accumulation of sufficient amounts of 14\( \alpha \)-methylfecosterol compensates for ergosterol in the membranes and thus contributes toazole resistance in C. albicans (Ghannoum and Rice, 1999; Vanden Bossche and Koymans, 1998; White et al., 1998). The lethality of the S. cerevisiae disruptant of ERG11 can be
suppressed by Δ5,6-desaturase (Kelly et al., 1997a). The decrease in ergosterol content due to a defect in Δ5,6-desaturase in fluconazole-resistant clinical isolates of C. albicans also results in cross-resistance to amphotericin B (Kelly et al., 1997a).

c. Δ22-Desaturase (ERG5) Another cytochrome P450, Δ22-desaturase (CYP61 and also ERG5) has been purified from an ERG11 (P45014DM)-disrupted strain of C. glabrata (Lamb et al., 1999). The purified enzyme showed desaturase activity in a reconstituted system. Δ22-Desaturase and its homologues have also been identified in C. albicans and Schizosaccharomyces pombe. The spectral analyses obtained with azole antifungal compounds, viz. ketoconazole, fluconazole, and itraconazole in reconstituted Δ22-desaturase, suggest that these drugs directly interact with the cytochrome heme (Lamb et al., 1999).

d. Membrane Lipid Composition In addition to membrane ergosterol, which mainly provides rigidity, stability, and resistance to physical stresses, there are other membrane lipid components, which also affect drug susceptibilities of Candida cells (Loffler et al., 2000; Mukhopadhyay et al., 2002). It is well documented that clinical as well as adopted azole-resistant isolates of C. albicans exhibit altered membrane phospholipids as well as sterol composition (Loffler et al., 2000; Mukhopadhyay et al., 2002). Additionally, recent reports suggest that the interactions between membrane ergosterol and sphingolipid are important determinants of drug susceptibilities of C. albicans cells (Mukhopadhyay et al., 2002). In a recent study, close interactions between ergosterol and sphingolipid, which appeared to be disrupted in erg mutants, were found to be critical for drug sensitivity of C. albicans cells. It is observed that Candida cells, when grown in the presence of fumonisin B1 (specific inhibitor of sphingolipid synthesis), had a lower sphingolipid content and, similar to erg mutant cells, became hypersensitive to drugs (Mukhopadhyay et al., 2004).

The existence of discrete membrane microdomains, known as lipid rafts, within a lipid bilayer, predominantly composed of sphingolipid and sterol, is well documented in several systems. Interestingly, the acquisition of the MDR phenotype is also accompanied by the up-regulation of lipids and proteins that constitute lipid rafts (Lavie and Liscovitch, 2001; Lavie et al., 1998). Luker et al. (2000) and others (Demeule et al., 2000) recently observed that human P-gp (ABC transporter) is predominantly localized in cholesterol-enriched membrane domains and depletion of cholesterol impairs human Pgp-mediated drug transport. Liscovitch and Lavie (2000) hypothesize that raft-dependent cholesterol efflux pathways may play a role in delivering drugs from various intracellular membranes to the plasma membrane, from which drugs can be extruded from the cells by drug efflux.
pumps. The existence of ergosterol and sphingolipid-rich microdomains (membrane raft) in \textit{C. albicans} has recently been established (Martin and Konopka, 2004). Whether drug efflux proteins of \textit{Candida} are also localized within these domains remains to be established.

The alteration in the physical state of plasma membrane lipid is another factor that seems to affect drug susceptibility of yeast cells. By employing isogenic \textit{erg2}, \textit{erg3}, \textit{erg4}, and \textit{erg6} mutants of \textit{S. cerevisiae} strains, it was observed that due to defective ergosterol biosynthesis and accumulation of various intermediates therein, these mutants possessed high membrane fluidity. These mutants became sensitive to several tested drugs and elicited enhanced level of passive diffusion. This suggested that passive diffusion of drugs could contribute to hypersensitivity of \textit{erg} mutants. However, when a membrane fluidizer benzyl alcohol enhanced membrane fluidity, it was observed that the increment in fluidity alone did not affect the susceptibility of the tested drugs in \textit{S. cerevisiae} cells. Thus, it appears that the change in membrane fluidity and increased diffusion therein alone are not sufficient to result in the observed higher susceptibility of \textit{erg} mutants. In conclusion, the hypersensitivity of \textit{erg} mutants of \textit{S. cerevisiae} could be attributed to membrane permeability changes, which may involve changes in passive diffusion across the membrane or in active transport of these drugs. In support of the former, Van Den Hazel \textit{et al.} (1999) have shown that \textit{S. cerevisiae} cells lacking \textit{PDR16} and \textit{PDR17} (encoding homologues of Sec14p) result in altered phospholipid and sterol composition and render cells hypersensitive to many drugs due to their increased passive diffusion. In support of the later possibility, ABC transporter Pdr5p of \textit{S. cerevisiae} has been shown to function less efficiently in \textit{erg6}-deleted cells (Emter \textit{et al.}, 2002; Kaur and Bachhawat, 1999). Taken together, it is becoming apparent that the membrane lipid composition, with changes in membrane fluidity and interactions between membrane ergosterol and sphingolipid, particularly affects drug susceptibilities of \textit{Candida} cells.

2. Drug Efflux and Import

\textbf{a. Efflux of Drugs} The permeability constraints imposed by the pathogen to drugs (mainly by way of increased efflux) represent one of the important molecular mechanisms of antifungal resistance. Several azole-resistant clinical isolates of \textit{C. albicans} as well as of other fungal pathogens like \textit{Aspergillus fumigatus} and \textit{Cryptococcus neoformans} display transcriptional activation of efflux pump-encoding genes and often show reduced intracellular accumulation of drugs (Nascimento \textit{et al.}, 2003; Posteraro \textit{et al.}, 2003; Semighini \textit{et al.}, 2002; Slaven \textit{et al.}, 2002; Thornewell \textit{et al.}, 1997; Tobin \textit{et al.}, 1997). The azole-resistant isolates mainly overexpress genes encoding multidrug efflux transporters proteins (MET) belonging to two superfamilies: the ABC transporters and MFS (Fig. 2).
i. **ABC Efflux Proteins** Cdr1p was the first ABC transporter identified as a drug efflux pump of *C. albicans* (Prasad *et al.*, 1995). The gene encoding *CDR1* was cloned by complementation of the *S. cerevisiae pdr5* mutant exhibiting hypersensitivity to cycloheximide and other drugs. Cdr1p is a close homologue of the ABC transporter human MDR1/P-gp. To date, Cdr1p and Cdr2p, which are very close homologues, represent two major drug extrusion pumps of *C. albicans*. These pump proteins not only efflux azoles and its derivatives but also extrude a variety of structurally unrelated compounds. Despite a high level of structural similarities, Cdr1p and Cdr2p display major functional differences particularly with regard to substrate specificity (Table III). Other homologues of *CDR1* and *CDR2*, namely *CDR3* and *CDR4* (75% similarity), have also been identified, but neither an overexpression nor a deletion of *CDR3* or *CDR4* genes affects drug susceptibilities of *C. albicans* (Balan *et al.*, 1997; Franz *et al.*, 1998; Sanglard *et al.*, 1998b) (Table IV). Cannon’s group has recently found that there is allelic variation in a number of genes involved in fungal drug resistance (Cannon *et al.*, 2004). There are two *CDR1* alleles designated A and B, for example, in *C. albicans* ATCC 10261. Sequencing of both alleles revealed 37 synonymous single nucleotide polymorphisms (SNPs) and 6 nonsynonymous SNPs. When hyperexpressed in *S. cerevisiae*, allele A conferred slightly less resistance to fluconazole (FLU) (MIC 200 μg/ml) than allele B (MIC 300 μg/ml) (Cannon *et al.*, 2004). Considering the widely observed polymorphism of human P-gp/MDR1 (Brinkmann and Eichelbaum, 2001; Cascorbi *et al.*, 2001; Hoffmeyer *et al.*, 2000; Woodahl and Ho, 2004) the characterization of an allelic variation of drug extrusion proteins of *Candida* represents an interesting possibility, which may also contribute to azole resistance. This definitely needs to be examined to assess if allelic variations in MDR genes of *Candida* have any clinical relevance. The reported glucose-induced phosphorylation of CgCdr1p and Pdh1p, drug efflux proteins of *C. glabrata*, suggests that posttranslational modification of efflux proteins could represent yet another novel mechanism of drug resistance (Wada *et al.*, 2002).

**Structure and Function of ABC Proteins.** The molecular mechanisms that govern the function of Cdr1p or Cdr2p as efflux pumps for azoles are not well known, and information is needed to (1) understand how the protein can bind a structurally diverse range of compounds including different azoles, (2) define drug-substrate binding, and (3) determine how ATP binding and hydrolysis are linked to drug transport (Fig. 3). The analysis of the molecular mechanism of azole transport by efflux pump proteins is expected to provide the basis for rational designing of a modulator/inhibitor for the proteins.

Similar to mammalian homologues, ABC transporters of *Candida* and of other yeasts like *S. cerevisiae* possess specific domains for membrane association, ATP binding, and hydrolysis. A typical Cdr1p, like most of the fungal ABC drug transporter, is composed of two homologous halves, each made
up of a hydrophilic, cytoplasmic nucleotide-binding domain (NBD) and a transmembrane domain (TMD) represented by six transmembrane segments (TMS) (Fig. 2). Candida Dbase predicts several ABC transporters, which show NBD conserved sequences upon multiple alignments (http://genolist.pasteur.fr/CandidaDB). Although many transporters are predicted to have an (NBD-TMD)_2 topology, there are also some putative ORFs that have only (NBD-TMD)_1, and thus appear to be half proteins. Among all the putative ABC proteins, only CDR1 and CDR2 are experimentally implicated in azole resistance. The structural and functional analysis of human P-gp/MDR1 and its other homologues in mouse has demonstrated the importance of NBDs and TMDs in drug extrusion (Loo and Clarke, 1993, 1994a,b, 1995a,b). In comparison, studies pertaining to the identification of the molecular determinants of yeast ABC drug transporters have only been recently initiated (Egner et al., 1998, 2000).

In an effort to develop an understanding of the molecular details of drug binding and efflux, in a recent study Cdr1p was overexpressed as a GFP-tagged fusion protein in a heterologous hyperexpression system (Shukla et al., 2003) and was characterized for drugs and nucleotide binding (Shukla et al., 2003). Iodoarylazidoprazosin (IAAP, a photoaffinity analogue of the P-gp substrate prazosine) and azidopine (a dihydropyridine photoaffinity analogue of the P-gp modulator verapamil) were shown specifically to bind with Cdr1p-GFP. Interestingly, IAAP binding with Cdr1p-GFP was competed out by nystatin, while azidopine binding could be competed out only by miconazole, thus demonstrating the possibility of different drug-binding sites for the two analogues (Shukla et al., 2003). For detailed structural and functional analysis, point mutations were also introduced in Cdr1p. Several point mutations yielded interesting phenotypes (Table V and Fig. 4). The studies conducted so far with Cdr1p suggest that in spite of topological differences with human P-gp, there is a conserved functional homology between the two multidrug transporters (Shukla et al., 2003). Gauthier et al. (2003) have recently shown that membranes prepared form Cdr1p and Cdr2p expressing cells are able to bind the photoaffinity analogue of rhodamine 123.

FIG. 2 ABC and MFS drug transporters of Candida. Putative topology of multidrug transporters. (A) Diagrammatic representation of an ABC transporter. The structural organization is characterized by two homologous halves (NBD-TMD)_2, each comprising a hydrophilic nucleotide-binding domain (NBD) followed by a hydrophobic region transmembrane domain (TMD) containing six transmembrane segments (TMS). (B) Diagrammatic representation of an MFS transporter. The MFS transporter depicted here has two structural units each of six transmembrane spanning α-helical segments, linked by cytoplasmic loops. (C) The NBDs of the ABC transporters hydrolyze ATP thus facilitating the transport of drugs out of the cell. ABC protein functions as a pump. (D) MFS protein utilizes a proton gradient to expel the drug.


<table>
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<tr>
<th>Class of transporter</th>
<th>Efflux pump</th>
<th>Substrates</th>
<th>References</th>
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<td>Fluconazole, itraconazole, ketoconazole, cycloheximide, amorolfine, fluphenazine, rhodamine 6G, cerulenin, brefeldin, miconazole, chloramphenicol, sulfomethuron methyl, rhodamine 123, β-estradiol, 4-nitrosoquinoline, O-phenanthroline, erythromycin, oligomycin, nystatin, dinitrophenol, corticosterone, FK-520, anisomycin, azidopine, IAAP</td>
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<td>Gene</td>
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<sup>a</sup>Subfamily based on sequence similarity with human ABC transporters ALDP, MRP/CFTR, MDR, and yeast (PDR, YEF3).

<sup>b</sup>Number of amino acid residues.

<sup>c</sup>NBD, nucleotide-binding domains; TMS, transmembrane segment.
FIG. 3 Sequence of probable steps involved in the expulsion of fluconazole or any other drug from yeast cell. (1) Interaction of drug with drug-binding site (DBS) present in TMS. (2) Drug binding may stimulate ATP hydrolysis in the nucleotide-binding domain (NBD). (3) ATP hydrolysis in NBDs initiates conformational changes in TMDs. (4) The conformational changes result in release and efflux of drugs. None of these steps (1-4) has been proven experimentally for azole efflux. These steps are only predictions based upon observations from human MDR1/MRPs (Pajeva et al., 2004).

([125I]iodo-ary lazido-rhodamine123, IAARh123), and that both N-terminal and C-terminal halves of Cdr2p contribute to rhodamine binding.

NBDs in ATP Binding and Hydrolysis. An important characteristic feature of ABC drug transporters is that they use the energy of nucleotide (ATP) hydrolysis to transport drugs across the membrane, against the concentration gradient. The conserved NBDs located at the cytoplasmic periphery are the hub of such an activity. In an attempt to understand the molecular basis of ATP hydrolysis of Cdr1p, recently the active N-terminal nucleotide-binding domain (NBD1) of Cdr1p was purified and characterized. It is demonstrated that this purified domain elicits a cation-dependent general ribonucleotide triphosphatase activity (Jha et al., 2003a). Jha et al. (2003a) found an evolutionary divergence in this domain wherein a conserved variation exists within the catalytically crucial Walker A motif of NBD1. The
Cdr1p Walker A motif contains an atypical but conserved cysteine-193, which appears to be a common feature of fungal ABC transporters. It was found to be critical for ATP hydrolysis. Reports from nonfungal transporters suggest that the Walker A motifs of NBDs have a well-conserved lysine residue at an equivalent position, which is indispensable for ATP hydrolysis (Azzaria et al., 1989). In a recent in vivo study, the relative contribution of both the N- and C-terminal NBDs in ATP binding, hydrolysis, and transporter activity of native Cdr1p (full protein) was examined wherein an atypical Cys-193 of Walker A of NBD1 (C193K) and a conserved Lys-901 (K901C) of Walker A of NBD2 were replaced (Jha et al., 2004). The drug resistance profile of Cdr1p mutant variant cells harboring C193K or K901C provided interesting insight into the functioning of the two NBDs. The cells expressing K901C were hypersensitive to drugs as compared to the C193K variant or to native Cdr1p. This clearly establishes that the two NBDs respond asymmetrically to the substitution of conserved residues of their respective Walker A motifs. The divergence in functioning of two NBDs was further evident when the efflux ability of these mutant proteins was compared. An in-depth analysis of the catalytic cycle, namely nucleotide binding, hydrolysis, and substrate binding/efflux, is required to determine the reasons behind the dichotomy in functioning of the NBDs. This study, however, demonstrated that a diverse N-terminal NBD (GxxGxC/GCS/T) of Cdr1p is functional where uncommon C193 is critical. Considering that all other fungal ABC transporters, including the well-studied Pdr5p of S. cerevisiae, have uncommon cysteine in Walker A of NBD1 (with the exception of Ste6p of S. cerevisiae), it is expected that this residue will have an indispensable role in the catalytic cycle (Jha et al., 2003b).

ii. MFS Efflux Proteins The MFS was originally defined as a superfamily of permeases characterized by two structural units of six TMS-α-helical segments, linked by a cytoplasmic loop. Out of several MFS proteins listed in the Candida database (http://genolist.pasteur.fr/CandidaDB), CaMDR1, its alleles, and FLU1 are the only drug transporters. CaMDR1 was initially identified as a gene, which conferred resistance to the tubulin-binding agent benomyl and the tetrahydrofolate reductase inhibitor methotrexate (Ben-Yaacov et al., 1994; Fling et al., 1991). CaMDR1 expression in S. cerevisiae confers resistance to several unrelated drugs and its overexpression has been linked to azole resistance in C. albicans. The expression of CaMDR1 in C. albicans cells is enhanced by benomyl, methotrexate, and several other unrelated drugs, and is found to be more pronounced in some of the azole-resistant clinical isolates (Becker et al., 1995; Gupta et al., 1998). Morschhäuser and his group employed a proteomic approach to understand the molecular basis of drug resistance in C. albicans. By comparing the protein expression pattern of matched pairs of fluconazole-resistant
<table>
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and susceptible clinical isolates, they identified several proteins whose expression was up-regulated specifically when only the \textit{CaMDR1} gene was overexpressed. These proteins, mostly belonging to the putative aldo-keto reductase family, were not up-regulated in a fluconazole-resistant strain that overexpressed only \textit{CDR1/CDR2} and not \textit{CaMDR1}. This implied that the expression of efflux pump-encoding genes is controlled by a different regulatory network (Kusch \textit{et al.}, 2004).

Recently \textit{FLU1}, another gene encoding the MFS protein of \textit{C. albicans}, was cloned by a complementing strain of \textit{S. cerevisiae}, which was hypersensitive to fluconazole. However, \textit{FLU1} is not involved in the development of fluconazole resistance in clinical isolates of \textit{C. albicans}. Interestingly, studies revealed that the preferred substrate of Flu1p is mycophenolic acid rather than fluconazole. Although more than two dozen putative MFS genes have been identified in the \textit{Candida} genome, except for CaMdrlp none of the other proteins of this superfamily of MFS is reported to have any direct role in clinical fluconazole resistance.

In contrast to the ABC drug transporter, the structure–function relationship of MFS has not been generalized in detail due to the diversity in their nucleotide and amino acid sequences. There are, however, some reports that suggest that the N-terminal halves of different major facilitator families share greater similarities than their C-terminal halves, which suggests that C-terminal regions are involved in substrate recognition and N-terminal regions are involved in proton translocation (Paulsen \textit{et al.}, 1996; Saier and Reizer, 1991).

\textbf{b. Import of Drugs} The hydrophobic nature of drugs permits their easy import by passive diffusion. However, the contribution of drug import to the overall scenario of MDR is not well established since technically it has not been possible to separate efflux of drugs from their import. Nonetheless, there are a few studies particularly with mammalian cells in which passive diffusion of drugs through a lipid bilayer has been shown to be an important determinant of MDR (Ferte, 2000). The variations in membrane fluidity are expected to affect passive diffusion of drugs and, in turn, their sensitivity.

The enhanced fluidity has been linked to enhanced diffusion of drugs (Mukhopadhyay \textit{et al.}, 2002). There are factors, other than membrane

\footnote{Data taken from Shukla \textit{et al.} (2003).}
\footnote{Location of each mutation is marked in the two-dimensional representation of Cdr1p in Fig. 5.}
\footnote{Aniso, anisomycin; Cyh, cycloheximide; Flu, fluconazole; Mic, miconazole; Nys, nystatin.}
\footnote{Phenotype was checked in the presence of Flu, Cyh, Aniso, Mic, and Nys.}
fluidity, that can also influence passive diffusion of drugs across the membrane bilayer and thus can affect drug susceptibilities (Van Den Hazel et al., 1999). The import of drugs and its impact on drug resistance need to be analyzed more carefully. It is expected that with better experimental designs, the contribution of import of drugs in MDR can be established.

3. Other Mechanisms of Drug Resistance

In addition to the mechanisms of drug resistance commonly found in Candida (discussed above), other mechanisms may also be important contributors to antifungal resistance.

a. Mitochondrial Respiration  The petite mutants (which lack mitochondrial DNA) of S. cerevisiae display resistance to fluconazole due to an uncoupling of oxidative phosphorylation. Interestingly, petite mutants that also have an erg3 mutation were found to be sensitive to fluconazole. It has been suggested by Kontoyiannis and his group that an erg3 mutant lacking mitochondria could accumulate toxic steroid intermediates that arrest growth (Kontoyiannis, 2000). A relationship between mitochondrial function and azole resistance is also observed in pathogenic C. glabrata wherein blockage of respiration by inhibitors or by mutation leads to decreased susceptibility to azoles (Kaur et al., 2004). The resistance to azoles accompanied with the loss of mitochondria was also linked to the up-regulation of CgCDR1 and CgCDR2, genes encoding ABC pumps of C. glabrata (Brun et al., 2003, 2004). Petite mutants of C. albicans are known to exist but so far a relationship between mitochondrial function and azole resistance has not been demonstrated (Aoki et al., 1990; Arie et al., 1998; Roth-Ben et al., 1998). However, given the similarities between Candida species, such as association is expected. Genome-wide expression profile analysis revealed coordinately regulated genes associated with azole resistance in clinical isolates of C. albicans. Many genes that are involved in the oxidative stress response are also regulated, which may contribute to azole resistance (Rogers and Barker, 2002, 2003). Taken together, mitochondrial respiratory status

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**FIG. 4** Two-dimensional depiction of Cdr1p topology that displays the site of point mutations introduced in this protein. The NH$_2$ and COOH mark the N-terminal and C-terminals, respectively. The conserved Walker and signature regions are indicated in a gray background. The two transmembrane domains and individual transmembrane segments are marked. Numbers indicate the beginning and end of transmembrane segments. The mutated residues are indicated at respective positions. The mutations in TMS11 are depicted in an enlarged helix (Shukla et al., 2003).
affecting azole resistance could represent another mechanism of drug resistance in yeasts.

b. Calcium Homeostasis  Azoles are fungistatic rather than fungicidal to Candida cells and this tolerance to azoles contributes to the development of resistance encountered in clinical isolates from immunocompromised patients (St-Gremain et al., 2001; White et al., 1998, 2002). Recently, it was observed that the protein phosphatase calcineurin allows survival of C. albicans during membrane stress exerted by azoles (Cruz et al., 2002). The calcineurin inhibitors cyclosporine A (CsA) and tacrolimus (FK506) exhibit fungicidal synergism with azoles in C. albicans, C. glabrata, C. krusei, and S. cerevisiae (Edlind et al., 2002; Kaur et al., 2004; Onyewu et al., 2003). Recently, Shukla et al. (2004) observed that the T1351F mutant variant of Cdr1p exhibited abrogated synergism of FK520 (a structural analogue of FK506) with fluconazole, ketoconazole, and cycloheximide. The fact that the other mutant variants of Cdr1p, which have been substituted in different domains spanning the entire protein molecule, remained synergistically susceptible to FK520 suggests that T1351 of the predicted TMS11 specifically contributes to this synergism. Since antifungal agents of varied structures including azoles derivatives are substrates of Cdr1p, it is very likely that the immunosuppressants or their analogues might increase intracellular levels of drugs by competition and thus by blocking pump activity directly (Maesaki et al., 1998; Marchetti et al., 2001, 2003). The inhibition of fluconazole efflux by FK520 suggests the possibility of a direct interaction of FK520 with Cdr1p. That FK520 synergism is mediated at least in part by its interaction with Cdr1p is supported by a set of observations: (1) T1351 replacement abrogates synergism, (2) FK520 is ineffective in cells not expressing Cdr1p, (3) FK520 shows synergism with other substrates of Cdr1p, and (4) FK520 competes with fluconazole efflux. Recently Raymond’s group has also shown that Cdr1p can affect cell tolerance to FK520 and suggested a possible involvement of these transporters in the synergism between azoles and FK520 in C. albicans (Gauthier et al., 2003). However, Marchetti et al. (2003) in a simultaneously appearing report suggested that the fungicidal synergism of fluconazole with cyclosporine may not be dependent on the MDR transporters of C. albicans. Since azoles are actively exported by ABC transporters such as Cdr1p and Cdr2p, a direct inhibitory effect of the immunosuppressants on these transporters is likely. Egner et al. (1998, 2000) also observed that the replacement of S1360 (equivalent to T1351 of Cdr1p) of Pdr5p to phenylalanine resulted in the loss of synergism of FK506 with fluconazole. Further, if serine was changed to alanine, it resulted in hypersensitivity to FK506. Their observations suggested a direct role of S1360 in mediating the synergy of fluconazole activity with FK506 in Pdr5p. The sequence alignment of TMD11 of various ABC transporters of C. albicans and their
comparison with Pdr5p reveals that instead of a serine residue, which is present in Pdr5p, the major drug transporters of *C. albicans* (Cdr1p and Cdr2p) instead possess threonine. When TMD11 of Cdr1p was looked at in a helical wheel projection, an amphipathic structure with a hydrophilic and a hydrophobic side was revealed, where T1351 lies near the boundary of the two faces of the putative helix. The importance of T1351 in drug as well as in inhibitor susceptibility is well evident from its placement in a helical wheel projection wherein probably the hydrophilic face of the helix plays a crucial role in the recognition/transport of Cdr1p substrates. Thus it is likely that the hydrophobic face of TMD11 could interact with the lipid layer while the hydrophilic face may interact with other TM helices or could have direct contact with the substrate molecule. The interactivity of the other residues of TMD11 positioned at the hydrophilic face remains to be examined.

c. *An Alkane Inducible Cytochrome P450 (CaALK8)* The modification of drugs to their nontoxic forms mediated by cytochrome P450 represents an important mechanism by which a cell could confer resistance to different drugs. The role of cytochrome P450 as the detoxifying enzymes in prokar­yotes as well as in eukaryotes is well established (Graham-Lorence and Peterson, 1996; Neber and Gonzalez, 1987). Although, in yeast, the existence of two different classes of cytochrome P450, viz. P45014DM and P450alk (alkane-inducible), has been shown, neither has been linked to xenobiotics metabolism (Kappeli, 1986; Vanden Bossche and Koymans, 1998). Recently, an alkane-inducible cytochrome P450 gene of *C. albicans* has been identified and it has been shown that it is involved in multidrug resistance (Panwar et al., 2001). This gene, designated *CaALK8*, shows sequence homology to a family of alkane-inducible cytochrome P450 genes involved in hydrocarbon assimilation. Interestingly, when *CaALK8* is expressed in *S. cerevisiae* or in *C. albicans*, it confers resistance to fluconazole, cycloheximide, *o*-phenanthroline and nitrosoquinoline oxide, miconazole, and itraconazole. Eight members of P450alk genes have already been identified in *C. maltosa* and *C. tropicalis* and the availability of sequences in the *Candida* database (http://alces.med.umn.edu/Candida.html) suggests the existence of a multigene family of ALK genes in *C. albicans* (Ohkuma et al., 1995; Seghezzi et al., 1992). The involvement of all *CaALK* genes in conferring multidrug resistance and the mechanism by which they render the drug nontoxic are two important aspects that require attention. An interesting possibility could be that the incoming drug is modified by *CaALK8* like the alkanes. However, so far the metabolic conversion of drugs has not been shown to be part of the drug resistance mechanism in *Candida*. Kelly et al. (1997b) observed that CYP61 (*Δ*²²-desaturase), which is involved in 22-desaturation in ergosterol biosynthesis in *S. cerevisiae*, can metabolize xenobiotics.
III. Concluding Remarks

The involvement of various mechanisms of antifungal resistance suggests that it is a multifactorial phenomenon. Among different mechanisms, drug efflux represents one of the predominant determinants of antifungal susceptibility. Given the promiscuity of efflux pumps with regard to substrate specificity, it is necessary to determine if the new antifungals under development are not potential substrates of efflux pump proteins, otherwise the resistance to new compounds could eventually develop. Alternatively, inhibitors or modulators of efflux pump proteins could be developed and exploited to block pump activity and thus increase efficacy. There are certain efflux protein inhibitors that increase the susceptibility of Candida cells to azoles (Chamberland et al., 1999; Schuetzer-Muehlbauer et al., 2003a). Recently Shukla et al. (2004) observed that the drug disulfiram, used for treatment of alcoholism, reverses Cdr1p-mediated drug resistance in C. albicans by inhibiting both ATP and substrate binding to the transporter. They speculate that this drug may be useful as a modulator of Cdr1p in antifungal therapy to overcome drug resistance in certain strains. Notwithstanding the need for further research in this area, the possibility of an inhibitor or a modulator of efflux pump protein is very promising. It is worth mentioning a recent report in which Niimi et al. (2004), by using a D-octapeptide combinatorial library, identified a Pdr5p ATPase inhibitor that chemosensitized clinical isolates of C. albicans probably by inhibiting ATPase activity of Cdr1p and Cdr2p.

References

Multidrug resistance in *Candida*.


MULTIDRUG RESISTANCE IN CANDIDA


