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Review

Amino Acid Residues Affecting Drug Pump Function in *Candida albicans*
－*C. albicans* Drug Pump Function－

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Abstract

Membrane-located drug transporters are important components in the multidrug resistance of microbial cells and human tissues. In fungi, clinically important resistance to antifungal drugs most often results from the over-expression of efflux pump proteins in the plasma membrane of the resistant cell. This review describes studies of the ATP binding cassette (ABC) family of membrane efflux pumps in the opportunistic human pathogen *Candida albicans* and, in particular, examines how changes in the polypeptide sequence can affect pump function. The identification of amino acid residues affecting pump function can provide new insights into efflux pump mechanisms and the relationship between structure and function. Such information will be important for the design of pump inhibitors which could supplement existing antifungal drugs.

Key words: *Candida albicans*, antifungal efflux, allelic variation, single nucleotide polymorphisms (SNPs)

The problem of antifungal resistance

The development of resistance by microbes to drug treatments is a major health issue, and particularly so for antifungal therapy where few classes of drugs are available. In the 1990s, the widespread use of prolonged therapy with the fungistatic drug fluconazole (FLC) to prevent recurrent candidiasis or cryptococcal meningitis[1] led to an increased frequency of oropharyngeal candidiasis (OPC) treatment failure due to FLC-resistant *C. albicans* strains[2, 3]. This became a major problem in the treatment of AIDS patients[4]. The incidence of OPC in HIV-infected individuals peaked in 1997[5] and has since declined following the introduction of HAART (highly active anti-retroviral therapy; reviewed by Kaplan *et al.*[6]). Despite the use of HAART, *Candida* infections still cause severe complications during the advanced stages of AIDS[7]. There is also increasing evidence for significant development of drug-resistant HIV in the HAART era[8, 9] which may result in the re-emergence of AIDS-related infections such as candidiasis.

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Resistance mechanisms

A variety of resistance mechanisms contribute to FLC resistance[10－13], but the most clinically significant resistance mechanism is considered to be energy-dependent drug efflux from *C. albicans* cells[14]. This reduces the intracellular concentration of FLC, thus preventing inhibition of the drug target, Erg11p (cytochrome P450 lanosterol demethylase). *C. albicans* possesses genes with homology to two classes of drug efflux pumps: the ATP binding cassette (ABC) family, and the major facilitator superfamily (MFS) of membrane transporters. Of all potential drug pumps in *C. albicans*, expression of mRNAs encoding the ABC transporters CaCdr1p and CaCdr2p most often correlates with FLC resistance in *C. albicans* clinical isolates[15－20].

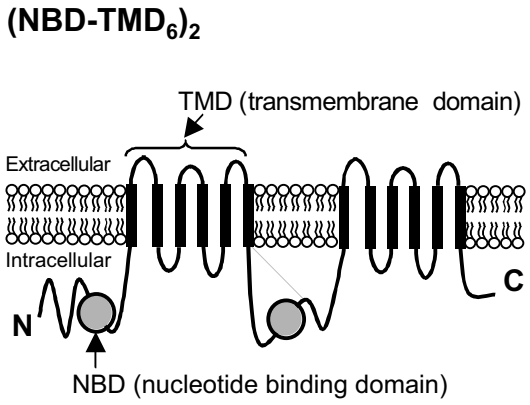

Fig. 1. Diagrammatic representation of a member of the Pdr5p family of fungal ABC transporters within the plasma membrane.

An examination of the database strain (SC5314) sequence has identified 28 putative ABC transporters[21]. Six (*CDR1*, *CDR2*, *CDR99*, *CDR3*, *CDR4*, *SNQ1*) have the hallmark dual nucleotide binding domains (NBD) each followed by transmembrane domains (TMD) consisting of six transmembrane spans[11] (Fig. 1). Four of these genes (*CDR1-4*) have been functionally analysed, but only *CDR1* and *CDR2* have been demonstrated to be involved in efflux of azole drugs. Disruption of *CDR1* makes *C. albicans* hypersusceptible to azoles[22] and we have demonstrated that controlled over-expression of Cdr1p in a *C. albicans CDR1*-null mutant conferred resistance to FLC and other xenobiotics[23]. Although there is strong evidence for the involvement of Cdr1p in FLC resistance, RT-PCR evidence suggests that Cdr2p may also play a significant role[20]. Furthermore, we recently found that elevated expression of both Cdr1p and Cdr2p polypeptides in cell plasma membrane fractions also accompanied decreased susceptibility to azoles in sequential *Candida albicans* clinical isolates that developed FLC resistance (Holmes *et al.* unpublished data). Therefore, an understanding of the mechanism of action of these two transporters could help develop new approaches to reducing antifungal treatment failure. Although many studies (reviewed by Ernst *et al.*[24]) have focussed on the mechanisms involved in the development and regulation of elevated pump expression, primary sequence changes within an ORF also affect the expression and function of proteins. This brief review will describe how changes in single residues within ABC transporters can influence functions such as substrate recognition and inhibitor susceptibilities.

ABC transporters Cdr1p and Cdr2p: functional studies

ABC transporter proteins are located in the plasma membrane, or in organelle membranes, of organisms as diverse as *Escherichia coli* and humans. They are ATP-dependent translocators of a wide variety of small molecules, including many xenobiotics, and typically comprise alternating pairs of cytoplasmic NBDs and membrane-embedded TMDs that contain six transmembrane spans[25]. ABC transporters are important in many human genetic disorders[25-27]. In cancer patients, multidrug resistance (MDR) of neoplastic tissues can be a major obstacle in cancer chemotherapy, and the predominant cause of MDR is the over-expression and drug transport activity of P-glycoprotein (P-gp)[28]. The orientation of the four domains in ABC transporters varies in different organisms, depending on the functions performed by the pump[29, 30]. All NBD regions contain conserved motifs: Walker A and Walker B (also found in other nucleotide-binding proteins) and the family-defining C-loop or ABC signature motif (LSGGQ). *Saccharomyces cerevisiae* Pdr5p is the archetype of the fungal pleiotropic drug resistance (PDR) family of drug transporters; the largest grouping (10 members) among the ~30 ABC transporters in this yeast[31]. *C. albicans* Cdr1p and Cdr2p show approximately 70% homology with Pdr5p.

In order to study the function of individual fungal transporters, researchers, including the authors, have used heterologous expression in the genetically tractable model yeast *S. cerevisiae*[32-35]. The host *S. cerevisiae* strain AD1-8u$^-$ has been developed to contain a mutant transcriptional regulator Pdr1-3p which leads to constitutive expression of genes integrated at the *PDR5* locus. This gives high-level expression of functional heterologous proteins in the plasma membrane of *S. cerevisiae*[33, 36]. Seven of the endogenous efflux pump genes have been disrupted in this strain, so that the pump function measured is dominated by the introduced heterologous gene. We have demonstrated that we can use this system to clone and functionally express the individual alleles of *C. albicans* CaCdr1p[33] and other ABC and MFS transporters[37]. An analysis of plasma membrane fractions of *S. cerevisiae* cells hyper-expressing Pdr5p or individual alleles of Cdr1p or Cdr2p (from laboratory strain ATCC 10261) revealed that these proteins were expressed at levels equivalent to 25-29% of membrane protein (Fig. 2).

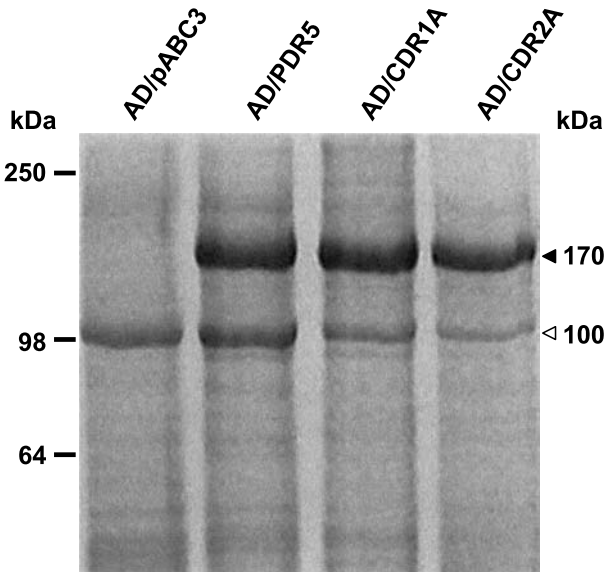

Fig. 2. SDS-PAGE analysis of plasma membrane fractions showing hyper-expression of efflux pump membrane proteins from *Saccharomyces cerevisiae* (Pdr5p) and *C. albicans* (Cdr1p and Cdr2p) in *S. cerevisiae* AD1-8u⁻. Gel was stained with Coomassie blue. Filled arrow: hyper-expressed pump proteins; open arrow: Pma1p; AD/pABC3: empty transformation cassette control.

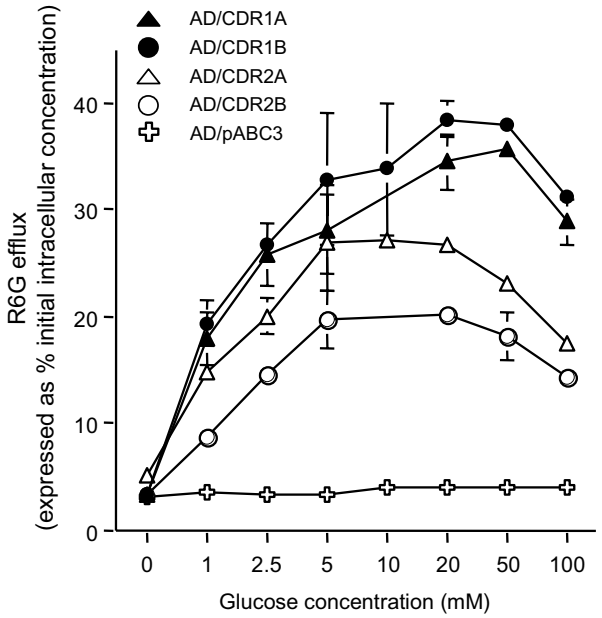

Fig. 3. Differential response to glucose of R6G efflux from *S. cerevisiae* strains expressing individual alleles of CaCdr1p or CaCdr2p from *C. albicans* ATCC 10261. Cells were pre-loaded with R6G under starvation conditions as described by Nakamura *et al.* (2001)[33]. Efflux of R6G was measured in filtered supernatants 10 min after the addition of glucose. Results shown are the means of triplicate samples in a representative experiment.

Heterologous expression of the efflux pump proteins allows direct functional comparison of pumps and identification of factors that affect function. A number of assays (both whole cell and *in vitro*) have been developed to assess the function of individual pump proteins. The simplest approach is to determine the inhibitory concentrations of antifungals on whole cells, using a liquid media-based minimal inhibitory concentration (MIC) microdilution assay such as that used in clinical laboratories[38] or a solid media drug diffusion[33] or drug dilution[39] resistance assay. A more direct measure of pump function is the quantification of substrate efflux using a radiolabelled substrate or a naturally fluorescent substrate such as rhodamine 6G (R6G)[33]. Fig. 3 shows the glucose-dependent R6G efflux from *S. cerevisiae* strains expressing either CaCdr1p or CaCdr2p, that had been preloaded with R6G under glucose-deprived conditions. Strains expressing either ATCC 10261 CaCdr1p allele achieved greater efflux than strains expressing either CaCdr2p allele, but this required higher glucose concentrations. Under the same conditions, no R6G efflux by the empty transformation cassette control strain AD/pABC3 was detected. There are also a number of assays to measure the function of the pump in purified membrane preparations (reviewed by[24]) such as the measurement of vandate- and oligomycin-sensitive ATPase activity[40].

Identifying amino acid residues important for efflux pump function

Many studies have highlighted the importance of single residues in substrate recognition and inhibitor susceptibilities of pump proteins[24]. Human ABC transporters have been subjected to directed mutational analysis, and mapping of natural mutations, in order to elucidate structure/function relationships (reviewed by Frelet and Klein[41]). More than 50 single nucleotide polymorphisms (SNPs) have been reported for human P-gp, many of which affect the function of the protein[27, 42]. In fungal ABC transporters, a commonly conserved lysine in the Walker A motif is replaced by cysteine and in *C. albicans* Cdr1p NBD1 C193 was shown to be essential for ATPase activity[43, 44]. The *C. albicans* NBD2 has the conventional lysine residue at a similar position, which is also critical for function[45]. Site-directed mutation has shown that F774 in TM segment 6 of Cdr1p affects the protein's trafficking and localisation[46]. A T1351F mutation in the TM segment 11 of Cdr1p[35] and

mutations in the TM-10 of *S. cerevisiae* Pdr5p[47] affect substrate specificity. Alanine scanning mutagenesis has identified single amino acid residues in Cdr1p TM-11 affecting function[34] and cysteine-scanning mutagenesis of human P-gp identified important residues for substrate binding in the TM-12 region of this pump protein[48]. Comparison of the primary structures of different ABC transporters can also provide important clues about the function and regulation of the fungal transporters. Although the fungal and human NBDs probably evolved by gene duplication from half sized transporters, fungal ABC transporters contain NBDs that appear less homologous and functionally less similar than their human counterparts. For example the Walker A, Walker B and signature motifs of the P-gp ATP binding site are highly conserved between its two NBDs. In contrast, the fungal signature motifs differ markedly between NBDs[43, 49]. Examination of these differences has provided further insight into structure/function relationships. Mutational analysis of the two NBDs of *C. albicans* Cdr1p revealed that they influence Cdr1p function differently[45]. In the *C. glabrata* Cdr1p NBD1, a Walker A motif

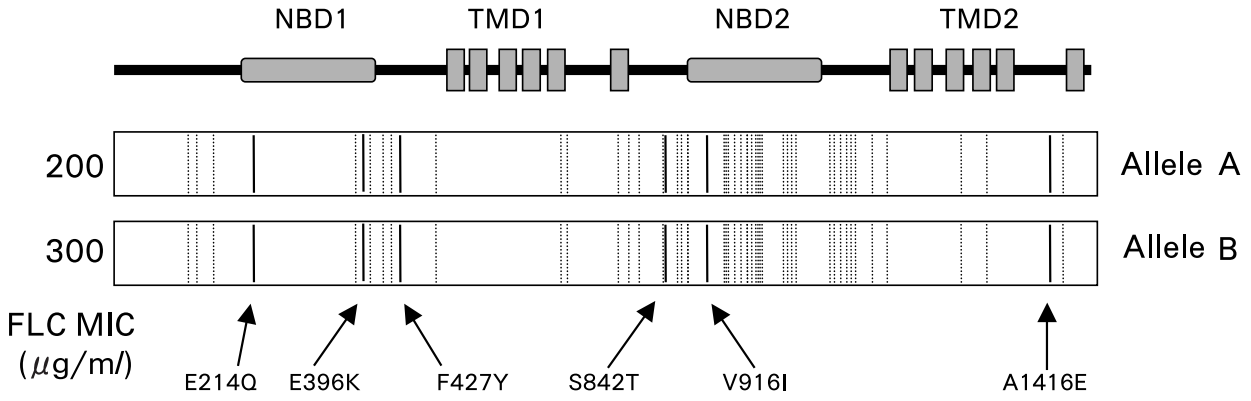

Fig. 4. Diagrammatic representation of the NBD and TMD regions of Cdr1p and the A and B alleles of the *C. albicans* strain ATCC 10261 *CDR1* gene. SNPs are indicated by broken lines (S-SNPs) or solid lines (NS-SNPs). The A allele amino acid residue is given first for each NS-SNP identified. The MICs of *S. cerevisiae* strains expressing the individual allele proteins are given.

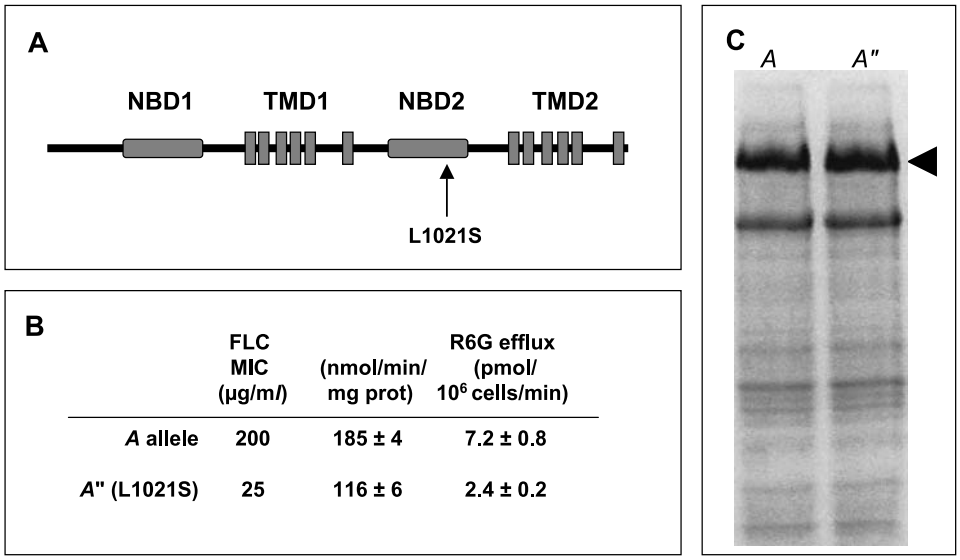

Fig. 5. Generation of a mutant allele of *C. albicans CDR1* with reduced function. The L1021S mutation was generated by recombinant PCR site-directed mutagenesis of the *C. albicans* ATCC 10261 *CDR1* A allele. The mutated allele was expressed in *S. cervisiae* AD1-8u$^-$. A. Diagram showing the NBD and TMD regions of Cdr1p indicating the position of the introduced mutation. B. Table showing reduced function of the mutated allele as determined by microdilution MIC assays, ATPase activities of purified membrane fractions and glucose-dependent R6G efflux from whole cells preloaded with R6G under starvation conditions. C. Coomassie blue stained SDS-PAGE separation of purified plasma membranes from *S. cerevisiae* strains expressing the parental or mutant allele. Arrow indicates heterologous Cdr1p.

C189A mutation diminished enzyme activity by 30-40% without significantly increasing susceptibility to FLC or the fluorescent substrate R6G, whereas the comparable mutation in the Walker A motif of NBD2 (K899A) eliminated ATPase activity and dramatically increased susceptibility to xenobiotic pump substrates (Tanabe *et al.* unpublished data).

Mapping of naturally occurring non-synonymous (NS)-SNPs that affect function has been applied widely to the identification of key amino acid residues in human ABC transporters[41]. We recently discovered that there is considerable heterozygosity in the *CDR2* gene of *C. albicans* isolates (up to 20 NS-SNPs) that results in functional variation between the two alleles (Holmes *et al.* unpublished data). The *CDR1* gene of *C. albicans* laboratory strain ATCC 10261 showed less extensive heterozygosity, but also contains six NS-SNPs that result in functional differences between the allele proteins, as determined by separately cloning and hyper-expressing each allele (denoted A or B) in *S. cerevisiae* AD1-8u$^-$ (Fig. 4). In order to investigate the effect of introducing additional NS-SNPs, *CDR1* was amplified under low fidelity conditions. Following cloning and transformation of *S. cerevisiae* AD1-8u$^-$ with the mutated DNA fragment, a strain with reduced fluconazole MIC (30 μg/m*l*) was isolated. Sequencing showed that this strain contained the A allele with 3 additional non-synonymous SNPs. Each individual mutation was inserted into the A allele by site-directed mutagenesis using recombinant PCR. Mutated Cdr1ps were expressed to a similar extent as the parental Cdr1pA (Fig. 5). Only mutation L1021S (obtained in strain A") showed reduced function, as demonstrated by a decreased FLC MIC, reduced glucose-dependent R6G efflux and lower ATPase activity, relative to the wild-type A allele. The L1021S mutation is immediately before the Walker B motif (LLFLDE) of NBD2 confirming that the NBD2 of *C. albicans* Cdr1p is critical for function, as previously reported[45].

In conclusion, recent studies have made valuable progress in the determination of structure/function relationships for the primary sequences of the Cdr pump proteins of *C. albicans*. Such studies are important as reference points, linking structural analysis to function. Combined use of data from mutational analysis and crystallography will provide the best opportunities to define the mode of action of lead inhibitors and for mapping the essential features of substrate and inhibitor binding sites.

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