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Atomic modelling and systematic mutagenesis identify residues in multiple drug binding sites that are essential for drug resistance in the major *Candida* transporter Cdr1

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ABSTRACT

The ABC (ATP-Binding Cassette) transporter Cdr1 (Candida drug resistance 1) protein (Cdr1p) of *Candida albicans*, shows promiscuity towards the substrate it exports and plays a major role in antifungal resistance. It has two transmembrane domains (TMDs) comprising of six transmembrane helices (TMH) that envisage and confer the substrate specificity and two nucleotide binding domains (NBDs), interconnected by extracellular loops (ECLs) and intracellular loops (ICLs) Cdr1p. This study explores the diverse substrate specificity spectrum to get a deeper insight into the structural and functional features of Cdr1p. By screening with the variety of compounds towards an in-house TMH 252 mutant library of Cdr1p, we establish new substrates of Cdr1p. The localization of substrate-susceptible mutants in an ABCG5/G8 homology model highlights the common and specific binding pockets inside the membrane domain, where rhodamines and tetrazoliums mainly engage the N-moiety of Cdr1p, binding between TMH 2, 11 and surrounded by TMH 1, 5. Whereas, tin chlorides involve both N and C moieties located at the interface of TMH 2, 11, 1 and 5. Further, screening of the in house TMH mutant library of Cdr1p displays the TMH12 interaction with tetrazolium chloride, trimethyltin chloride and a Ca^{2+} ionophore, A23187. *In silico* localization reveals a binding site at the TMH 12, 9 and 10 interface, which is widely exposed to the lipid interface. Together, for the first time, our study shows the molecular localization of Cdr1p substrates-binding sites and demonstrates the participation of TMH12 in a peripheral drug binding site.

1. Introduction

Opportunistic fungal infections have increased over past few decades because of the increase in the number of immuno-compromised patients, particularly, undergoing advanced healthcare procedures [1]. Among the different fungal infections, *Candida* infection has steadily increased from past three decades [2]. Infections caused by *C. albicans* are commonly treated with either azoles or non-azole antifungal drugs [3, 4]. Widespread and prolonged use of azoles in recent years, have led to the increased tolerance to drugs leading to persistence of infections. The clinical azole resistant (AR) isolates not only show decreased susceptibility towards azoles but also show the collateral resistance towards several structurally unrelated drugs, thus displaying the multi-drug resistance (MDR).

The prominent mechanisms of drug resistance to azoles in *C. albicans* includes the alteration or an over-expression of the target enzyme P450 14α-lanosterol demethylase (P45014DM) involved in the ergosterol biosynthesis, change in the sterol composition of plasma membrane, and an overexpression of efflux pump proteins which belong to two superfamilies of membrane transporters that include ATP Binding Cassette (ABC[1]) and Major Facilitator Superfamily (MFS[2]). Although, there are large number of members of both the superfamilies in *Candida* genome, only two ABC[1] transporters Cdr1p and Cdr2p, and one MFS[2] transporter, Mdr1p, of *C. albicans* are shown to have the clinical relevance in the development of azole tolerance. These transporter proteins are over produced in AR isolates and as a result display the reduced accumulation

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[1] ABC, ATP-binding cassette.
[2] MFS, major facilitator superfamily.

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of therapeutic drugs due to their rapid extrusion that facilitates their survival. Considering the importance of major antifungal transporters, the focus of recent research has been to understand the structure and function of these proteins, together with the mechanism of drug extrusion and to design the inhibitors/modulators to block the pump protein activity so that the drug already in use could again sensitize the resistant yeast cells [5,6].

Over the years, Cdr1p has acquired significant clinical importance and thus considerable attention is focussed on understanding the structural and functional aspects of this protein. Cdr1p transports a huge array of structurally unrelated compounds such as azoles, lipids, and steroids [7,9]. This promiscuity towards substrates is the characteristic feature of most ABC[1] drug transporters and hence makes their functionality all the more complex to understand [8,9].

Cdr1p is expressed as a single polypeptide of 1501 amino acids, comprising of two Nucleotide Binding Domains (NBD[3]s) and two Trans Membrane Domains (TMDs[4]). Each TMD[4] comprises of 6 transmembrane helices (TMHs[5]) which imparts substrate promiscuity to the transporter. Cdr1p belongs to the ABC2 efflux family and is predicted to consist of two homologous halves, each comprising of one NBD[3] as an N-terminal hydrophilic domain, followed by a C-terminal hydrophobic TMD[4] [10]. The hydrophilic domain comprises of a conserved ABC[1] region, including the ATP-binding motifs known as the Walker A and Walker B [11] and another highly conserved motif, ABC[1] signature, preceding the Walker B motif. Notwithstanding the overall conservation of the domain architecture of the TMDs[4] of ABC[1] exporters, their primary sequences are extremely variable among related proteins. Thus, the extent of conservation is poor among the TMHs[5] of the fungal ABC[1] transporters as compared to the NBDs[3], which are highly conserved domains. It is the variability of the TMHs[5] that provides promiscuity to Cdr1p.

Most structure-function studies of ABC[1] transporters have been performed on human P-glycoprotein (P-gp[6]). Those studies have shown that nearly all the TMHs[5] are directly or indirectly involved in the drug transport [12]. Two distinct substrates-binding sites, H (Hoechst 33342 binding site) and R (Rhodamine 123 binding site), were pharmacologically defined for P-gp[6] [13]. Competition experiments subsequently suggested that P-gp[6] could contain at least seven different drug-binding sites [14]. The crystal structures of mouse, nematode and algae P-gp[6]s, together with biochemical data, indicate that these proteins contain a large internal binding cavity that can accommodate structurally unrelated compounds of different sizes and shapes [15,16,17,18]. Extensive structural and functional analysis suggests that Cdr1p probably contains at least three drug binding sites [7]. One site is probably responsible for the efflux of rhodamine 6G (R6G[7]) and azoles, such as ketoconazole (KTC[8]), miconazole (MCZ) and itraconazole (ITC), while a separate site(s) interacts with, and transports, fluconazole (FLC[9]) only. A third binding site may exist for the prazosin analogue iodoarylazidoprazosin [19]. Several important amino acid residues in *Saccharomyces cerevisiae* (*S. cerevisiae*) Pdr5p, a close homolog of Cdr1p, have also been identified as critical for drug binding and transport [20]. Several novel Pdr5p substrates indicate that this transporter, like Cdr1p, contains at least three drug-binding sites and that some substrates may interact with more than one site [21]. By performing the systematic mutagenesis of the primary sequences of both TMDs[4], recently the nature of the drug-binding pocket of Cdr1p has been examined in detail which provided the first insight into the drug binding pocket of the transporter. Together, the study suggests that the drug binding sites in Cdr1p is scattered throughout the protein and probably more than one residue of different helices are involved in binding and extrusion of the drugs [22]. Recently, the X-ray structure of the ABCG5/G8 heterodimer that belong to the same ABC2 porters family was released, revealing a specific 3-dimensional organization, distinct to that of P-gp[6]-like exporters. Since Cdr1p belong to ABC2 type porters family, likewise such data is fundamental in understanding the molecular mechanism by which exporters of the family translocate drugs out of the cells [10,23].

The structure-function studies on Cdr1p done so far by us and others have confirmed the participation of all the TMHs[5] in drug binding and transport, although the number of interacting residues varies among the different helices [22]. To better visualize the interactions with the drugs, we constructed a homology model of Cdr1p based on the structure of ABCG5/G8 and performed molecular docking. We highlight the role of each TMHs of Cdr1p in binding the known and new substrates. A most noteworthy exception has been TMH12 where none of its residues have been shown to interact with any of the tested substrates of Cdr1p. This study not only characterizes new substrates of Cdr1p but also demonstrates a range of new substrates which also interacts with TMH12 of the protein.

2. Experimental procedures

2.1. Materials

Media chemicals were obtained from Hi-Media (Mumbai, India). All the drugs and compounds were obtained from Sigma Chemical Co, (St. Louis, MO, USA).

2.2. Yeast strains and culture media

The yeast strains used in this study were grown in the yeast extract peptone dextrose medium at 30 °C as described in our previous publication [22]. The *S. cerevisiae* strain used as a heterologous host for the expression of Cdr1p was AD1-8u$^-$, provided by Richard D. Cannon, University of Otago, Dunedin, New Zealand. The host AD1-8u$^-$ having seven major ABC[1] transporters deleted, was suitably modified to clone GFP-tagged Cdr1p and its mutant variants. All the cloned mutants showed proper membrane localization and expression of the Cdr1p [24].

2.3. Transport assay

Rhodamine efflux was determined essentially as described previously [25]. Briefly, log phase cells (1×10^6 cells) were washed and suspended as a 2% suspension (*w*/*v*) in PBS and incubated for 2 h in the presence of 10 μM final concentration of rhodamine. After washing, the cells were suspended in PBS with 2% glucose. After 40 min, 1 ml aliquots from it was centrifuged, and absorbance of the supernatant was measured at 527 nm, 540 nm and 511 nm for rhodamine 6G (R6G[7]), rhodamine B (RB[10]) and rhodamine123 (R123[11]) respectively [25,26].

2.4. Statistical analysis

Data is in the form of means ± standard deviation (SD) from duplicate samples, taken of at least three independent experiments. Differences between the mean values were analyzed by Student's *t*-test, and the results were considered as significant when $p < 0.05$.

2.5. Drug susceptibility assay

The susceptibility of mutants to various drugs was tested by the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) and serial dilution assays as described previously [27,28]. For serial dilution assays, cells were suspended in normal saline to an optical density of 0.1 at 600 nm, followed by 5-fold serial dilutions.

[3] NBD, nucleotide binding domain.
[4] TMD, transmembrane domain.
[5] TMH, transmembrane helix.
[6] P-gp, P-glycoprotein.
[7] R6G, rhodamine 6G.
[8] KTC, ketoconazole.
[9] FLC, fluconazole.