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Alanine scanning of transmembrane helix 11 of Cdr1p ABC antifungal efflux pump of *Candida albicans*: identification of amino acid residues critical for drug efflux

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***Objectives*: To investigate the role of transmembrane segment 11 (TMS11) of *Candida albicans* drug resistance protein (Cdr1p) in drug extrusion.**

***Methods*: We replaced each of the 21 putative residues of TMS11 with alanine by site-directed mutagenesis. The *Saccharomyces cerevisiae* AD1-8u$^-$ strain was used to overexpress the green fluorescent protein tagged wild-type and mutant variants of TMS11 of Cdr1p. The cells expressing mutant variants were functionally characterized.**

***Results*: Out of 21 residues of TMS11, substitution of seven residues, i.e. A1346G, A1347G, T1351A, T1355A, L1358A, F1360A and G1362A, affected differentially the substrate specificity of Cdr1p, while 14 mutants had no significant effect on Cdr1p function. TMS11 projection in an α-helical configuration revealed with few exceptions (A1346 and F1360), a distinct segregation of mutation-sensitive residues (A1347, T1351, T1355, L1358 and G1362) towards the more hydrophilic face. Interestingly, mutation-insensitive residues seem to cluster towards the hydrophobic side of the helix. Competition of rhodamine 6G efflux, in the presence of excess of various substrates in the cells expressing native Cdr1p, revealed for the first time the overlapping binding site between azoles (such as ketoconazole, miconazole and itraconazole) and rhodamine 6G. The ability of these azoles to compete with rhodamine 6G was completely lost in mutants F1360A and G1362A, while it was selectively lost in other variants of Cdr1p. We further confirmed that fungicidal synergism of calcineurin inhibitor FK520 with azoles is mediated by Cdr1p; wherein in addition to conserved T1351, substitution of T1355, L1358 and G1362 of TMS11 also resulted in abrogation of synergism.**

***Conclusions*: Our study for the first time provides an insight into the possible role of TMS11 of Cdr1p in drug efflux.**

Keywords: transmembrane segment 11, azoles, synergy, multidrug resistance, rhodamine 6G

Introduction

One of the most clinically significant mechanisms of azole resistance in the pathogenic yeast, *Candida albicans*, is overexpression of the multidrug transporter protein Cdr1p (*Candida* drug resistance), belonging to the ABC (ATP Binding Cassette) super-family of transporters.[1] Overexpression of Cdr1p in clinically resistant isolates of *C. albicans* has been shown to be involved in extrusion of therapeutic azoles.[1–3] Thus Cdr1p has not only acquired significant clinical importance, but is also considered an important target in the designing of strategies to combat antifungal resistance.

The predicted topology of Cdr1p exhibits characteristic features typical of an ABC transporter, namely, two highly hydrophobic transmembrane domains (TMDs) and two cytoplasmically localized nucleotide binding domains (NBDs). Each TMD comprises

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six transmembrane segments (TMS), which are envisaged to confer substrate specificity to Cdr1p. The range of substrates that can be exported by Cdr1p vary enormously and include structurally unrelated compounds such as azoles, lipids and steroids.[4–6] This promiscuity towards substrates is a characteristic feature of most ABC-type drug transporters and hence makes them all the more complex to understand.

In recent years, several structure–function studies have been performed on human MDR1/Pgp, a mammalian homologue of Cdr1p, and on the other ABC transporters to identify the domains and residues implicated in drug recognition, binding and efflux. Photoaffinity labelling and site-directed mutagenesis have identified TMS1, TMS5/6 and TMS11/12 as important helices involved in substrate recognition and transport.[7] Cross-linking studies with engineered cysteine residues demonstrated the physical interaction between TMS6 and TMS12 of Pgp in drug binding.[8–10]

In an effort to develop an understanding of the molecular details of drug binding and transport by Cdr1p, we have recently shown that amino acid residues of TMS6, TMS11, TMS12, NBD1 and NBD2 are important for surface localization, drug transport and ATP binding and hydrolysis.[11–16] While recent basic structural and functional analysis of Cdr1p has suggested that the drug-binding sites are scattered throughout the entire protein and that probably more than one residue of different helices is involved in drug binding and extrusion, there is insufficient information to predict where the most common antifungals, such as fluconazole, itraconazole and ketoconazole bind and are transported out. The importance of TMS11 in Cdr1p and in other homologous ABC transporters is reflected in the present study, in which we subjected all the putative 21 amino acid residues of this helix to alanine scanning. Our results revealed distinct clustering of mutation-sensitive residues, which are important for substrate binding and transport towards the more hydrophilic face, while mutation-insensitive residues appear to segregate towards the hydrophobic face of the helix. While competition assays using fluorescent substrate, rhodamine 6G, in the presence of various substrates revealed for the first time the overlapping binding site between azoles (such as ketoconazole, miconazole and itraconazole) and rhodamine 6G in native Cdr1p, the mutant variants displayed the differential competitive ability of azoles. Our results further demonstrate that the well reported synergism of calcineurin inhibitor FK520 is mediated by more than one amino acid residue of TMS11, and in addition to the well conserved residue T1351, other residues—namely T1355, L1358 and G1362—are also involved.

Materials and methods

Materials

Anti-GFP monoclonal antibody was purchased from BD Biosciences Clontech, Palo Alto, CA, USA. DNA-modifying enzymes were purchased from Roche Molecular Biochemicals, Germany. Protease inhibitors: PMSF, leupeptin, pepstatin A, aprotinin and drugs: miconazole (MIC), ketoconazole (KTC), itraconazole (ITC), cycloheximide (CYH), anisomycin (ANISO), rhodamine 6G (R6G) and other molecular grade chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA). Fluconazole (FLC) was kindly provided by Ranbaxy Laboratories, India. FK520 was a generous gift from Merck & Co., Inc., Rahway, USA, and tritylimidazole (tritylimz) and tricyclohexyltin chloride (hexyltin-Cl) were gifts from John Golin.

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 mg/L). The yeast strains were cultured in YEPD broth (Bio101, Vista, CA, USA) or SD-ura$^-$ (Bio101). For agar plates 2% (w/v) bacto agar (Difco, BD Biosciences, NJ, USA) was added to the medium.

Site-directed mutagenesis and development of transformants

Site-directed mutagenesis was performed by using the Quick-Change Mutagenesis kit from Stratagene (La Jolla, CA, USA). The mutations were introduced into plasmid pPSCDR1GFP[11] according to manufacturer's instructions, and the desired nucleotide sequence alteration was confirmed by DNA sequencing of the ORF. The mutated plasmid, after linearizing with *Xba*I, was used to transform AD1-8u$^-$ cells, as described previously.[11] Transformation of yeast cells was performed by the lithium-acetate method using routine laboratory protocols.[11]

Immunodetection of Cdr1p in plasma membrane

The plasma membranes (PM) were prepared from *Saccharomyces cerevisiae* cells, as described previously.[11] The western blot analysis was conducted using anti-Cdr1p polyclonal antibody (1:500 dilution), anti-GFP monoclonal antibody (1:1000 dilution) and anti-Pma1p polyclonal antibody (1:10 000 dilution), as described previously.[11] Proteins on immunoblots were visualized using the enhanced chemiluminescence assay system (ECL kit, Amersham Biosciences, Arlington Heights, IL, USA).

Confocal microscopy

The cells were grown to late log phase in SD-ura$^-$ medium, except for AD1-8u$^-$ where uridine (0.02%) was added to the SD-ura$^-$ medium. The cells were then washed and resuspended in an appropriate volume of 50 mM HEPES pH 7.0. The cells were placed on glass slides and then imaged under oil immersion objective at 100× magnification on a confocal microscope (Radiance 2100, AGR, 3Q/BLD; Bio-Rad, UK).

Drug susceptibility and other functional parameters

The susceptibilities of *S. cerevisiae* cells to different drugs were tested by microtitre plate assay and spot assay, as described previously.[17] The Cdr1p-associated ATPase activity of the purified PM was measured as oligomycin-sensitive release of inorganic phosphate, as described previously.[11] Glucose-mediated efflux of R6G was essentially performed as described elsewhere.[18] R6G was used at a final concentration of 10 μM and for competition between R6G and various substrates, a five-fold concentration (50 μM) of R6G was used for each substrate.

Results

In an effort to develop an understanding of the molecular details of drug binding and transport, we had earlier generated several mutant variants of Cdr1p.[11,12,14–16] We observed that several point mutations could drastically affect the substrate specificity of Cdr1p.[11,12,16] In order to investigate the role of putative TMS11 in drug binding and transport, all the 21 amino acid residues were subjected to alanine scanning by site-directed mutagenesis using mutagenic oligonucleotides depicted in Figure 1. To avoid the introduction of new side chains, the four existing alanines in TMS11 (A1346, A1347, A1350 and A1365) were replaced by

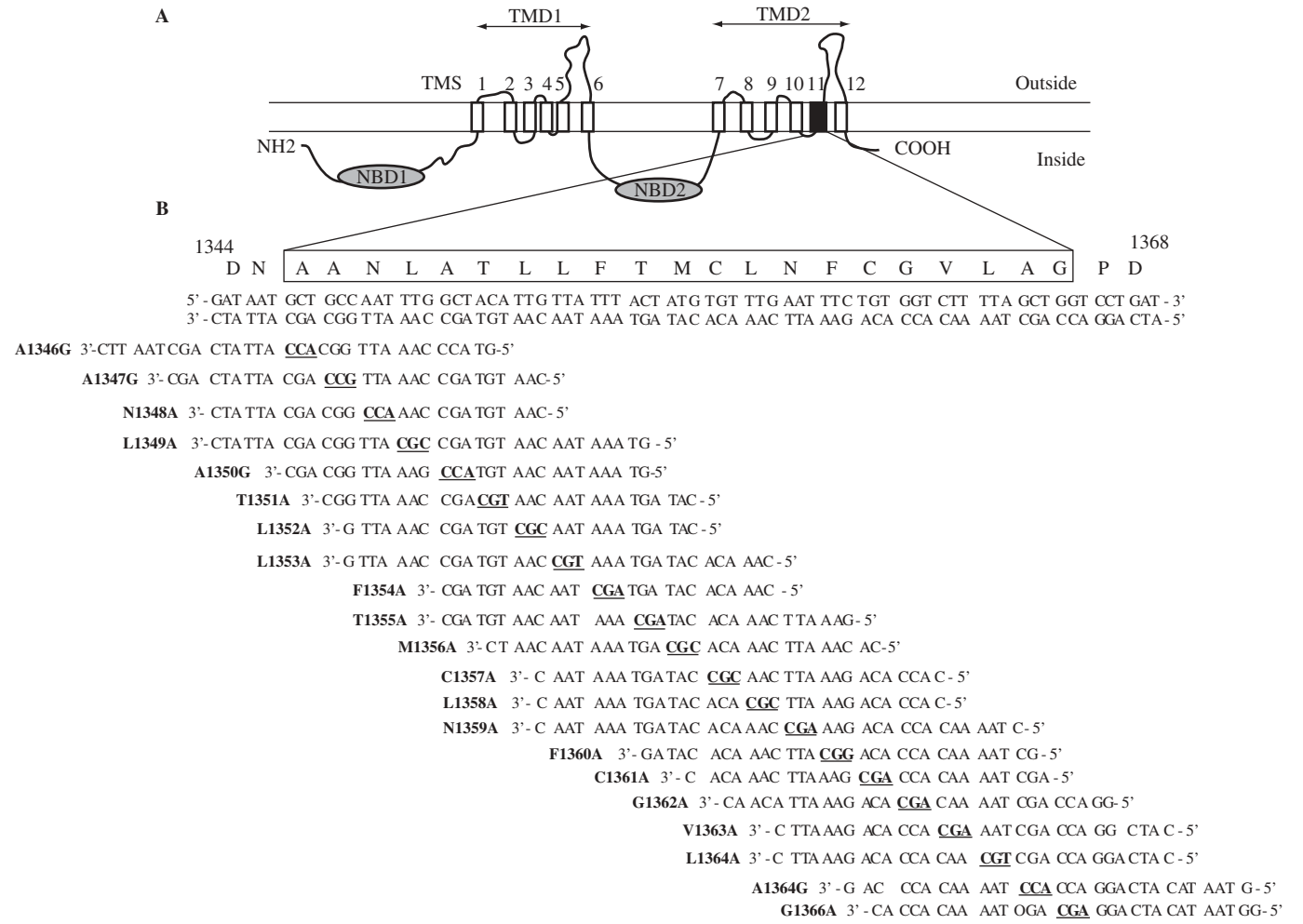

Figure 1. Site-directed mutagenesis of predicted TMS11 of *Candida albicans* Cdr1p. (A) The predicted topology of Cdr1p with two TMD and two NBD. The putative TMS11 is indicated in black. (B) The nucleotide and amino acid sequences (boxed) of TMS11 between positions 1344 and 1368 and the sequence of the oligonucleotides used for site-directed mutagenesis. The mutagenized codons are in bold and underlined. Transmembrane segments of the protein sequence of Cdr1p were determined with the program HMMTOP.[33–36]

glycines. For functional analysis of the newly generated TMS11 mutant variants, a heterologous hyperexpression system, where Cdr1p is stably overexpressed from a genomic *PDR5* locus in an *S. cerevisiae* mutant AD1-8u$^-$, was used.[19] The host AD1-8u$^-$ was derived from a *Pdr1-3* mutant strain with a gain of function mutation in the transcription factor Pdr1p, resulting in a constitutive hyperinduction of the *PDR5* promoter. 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.

Substitutions of TMS11 residues with alanine drastically affect substrate specificity

Confirmed positive mutant variants of TMS11 of Cdr1p were screened for their sensitivity to different substrates by two independent methods: by MIC_{80} determination and by spot assay. Based on spot assays, out of the 21 TMS11 mutants, only seven variants, namely A1346G, A1347G, T1351A, T1355A, L1358A, F1360A and G1362A led to a change in susceptibilities towards the tested substrates to varying degrees (Figure 2A). No significant change was observed in the other 14 mutants (data not shown). The substitutions A1347G, T1351A, F1360A and G1362A led to hypersensitivity to various substrates, while variant A1346G was the least sensitive (Figure 2A). Interestingly, substitutions T1355A and L1358A exhibited different sensitivity to all the tested substrates. The sensitivity of these mutant variants towards rhodamine 6G and fluconazole needs special mention. As depicted in Figure 2(A), T1355A and L1358A continued to display resistance to rhodamine 6G and fluconazole, which was comparable to native Cdr1p, thus suggesting that these substitutions do not affect the extrusion of both rhodamine 6G and fluconazole (discussed later). Recently, Golin *et al.*[20] have used imidazole and trialkyltin chloride derivates to determine the minimum prerequisite of Pdr5p-substrate interaction and transport. They demonstrated that it is the size of the substrate and not the hydrophobicity that is critical for efficient Pdr5p mediated transport. We tested the susceptibilities of Cdr1p mutant variants A1346G, A1347G, T1351A, T1355A, L1358A, F1360A and G1362A towards one such imidazole and trialkyltin chloride derivative, i.e. tritylimidazole and tricyclohexyltin chloride, respectively. Interestingly these two compounds had an opposite effect on all the seven TMS11 mutant

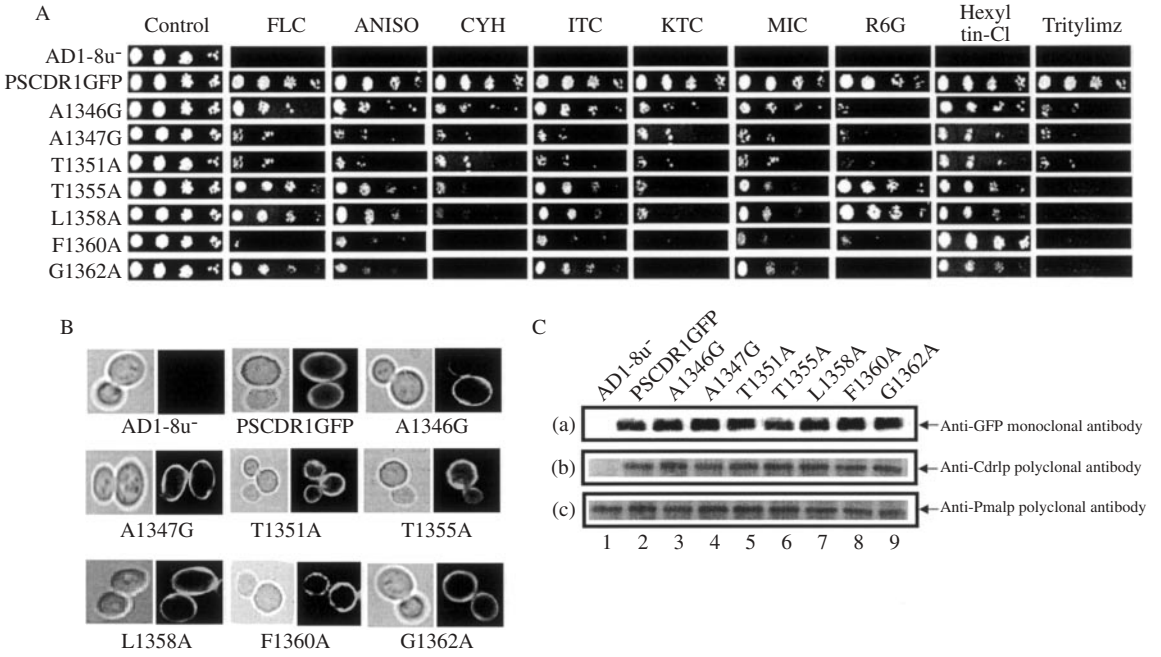

Figure 2. Substrate specificity, localization and expression of wild-type and mutant Cdr1GFP variants. (A) Spot assay: for this assay, a 5 μL sample of a five-fold serial dilution of each yeast strain (cells suspended in normal saline to an OD_{600} of 0.1) was spotted on YEPD plates in the absence (control) or in the presence of: FLC, 4 mg/L; ANISO, 0.8 mg/L; CYH, 0.08 mg/L; ITC, 0.1 mg/L; KTC, 0.125 mg/L; MIC, 0.09 mg/L; R6G, 6 mg/L; tricyclohexyltin (hexyltin-Cl), 4 mg/L; and tritylimidazole (tritylimz), 0.5 mg/L. Cell growth was monitored after 48 h of incubation of plates at 30°C. (B) Confocal images to show the localization of wild-type and mutant Cdr1GFP variants. (C) Expression of wild-type and mutant Cdr1GFP variants in *S. cerevisiae*: PM (25 μg) protein from AD1-8u⁻ (lane 1); PSCDR1GFP (lane 2); A1346G (lane 3); A1347G (lane 4); T1351A (lane 5); T1355A (lane 6); L1358A (lane 7); F1360A (lane 8); and G1362A (lane 9) were separated by 8% SDS-PAGE, electroblotted onto a nitrocellulose membrane and incubated with mouse monoclonal anti-GFP antibody (diluted 1:1000) (a), rabbit polyclonal anti-Cdr1p antibody (diluted 1:500) (b) and rabbit polyclonal anti-Pma1p antibody (diluted 1:10 000) (c). Proteins were immunodetected as described in the Materials and methods section. A colour version of this Figure is available with the online article.

variants. For example, none of these mutant variants showed significant change in susceptibility towards tricyclohexyltin chloride, while all displayed supersensitivity to tritylimidazole (Figure 2A). The drug sensitivities revealed by spot assay generally matched well with MIC_{80} assay (data not shown).

Mutant variants were properly expressed, surface localized and displayed unaltered ATPase activity.

In order to exclude the possibility that the observed hypersusceptibility of the mutant variants was due to poor expression or impaired surface localization, we compared the PM localization of wild-type and mutant Cdr1GFP variants by confocal microscopy and also checked the expression of Cdr1GFP by western blot analysis. The confocal images confirmed that there was no difference in cell surface localization of Cdr1GFP between the cells expressing wild-type and mutant variants (Figure 2B). Immunoblot analysis on PM from cells expressing wild-type and mutant Cdr1GFP variants confirmed that the expression level of the mutant variants was similar to the wild-type Cdr1GFP; as detected with anti-GFP monoclonal antibody (Figure 2C, panel a) and with anti-Cdr1p polyclonal antibody (Figure 2C, panel b). PM-ATPase was used as a marker (Figure 2C, panel c) to check the purity and quantity of protein in the PM fraction.

To rule out the possibility that the introduced mutations in TMS11 have, in any way, altered the catalytic cycle of Cdr1p, which might result in impaired efflux of substrates, we checked the hydrolysis of ATP. For this the purified PM proteins from wild-type as well as mutant Cdr1GFP variants of cell expression were analysed for their oligomycin-sensitive ATPase activity.[11] Cdr1GFP mutant variants exhibited ATPase activity comparable to wild-type protein (data not shown).

Rhodamine 6G efflux remained unaltered for mutant variants T1355A and L1358A

As mentioned above, of all the mutants tested, substitutions T1355A and L1358A did not affect sensitivity towards rhodamine 6G or fluconazole (Figure 2A). In other words, it means that the efflux of these substrates in mutants T1355A and L1358A remained unaffected as compared with other variants of TMS11. To further validate this possibility, we performed rhodamine 6G efflux. We demonstrated that as compared with AD1-8u⁻ host cells, native Cdr1GFP-expressing cells mediated energy-dependent (glucose induced) efflux of rhodamine 6G (Figure 3A, inset), which was reduced to 60–65% in variants A1346G, A1347G, T1351A, F1360A and G1362A (Figure 3A). On the other hand, mutants T1355A and L1358A, in agreement with the drug susceptibility data (Figure 2A), showed rhodamine 6G efflux comparable to native protein (Figure 3A).

Competition assays revealed a common binding site for rhodamine 6G and azoles

To examine the effect of TMS11 mutations more quantitatively, we compared rhodamine 6G efflux in the presence of various substrates. Competition assays with rhodamine 6G were performed in the presence of 5× excess of ketoconazole, itraconazole,

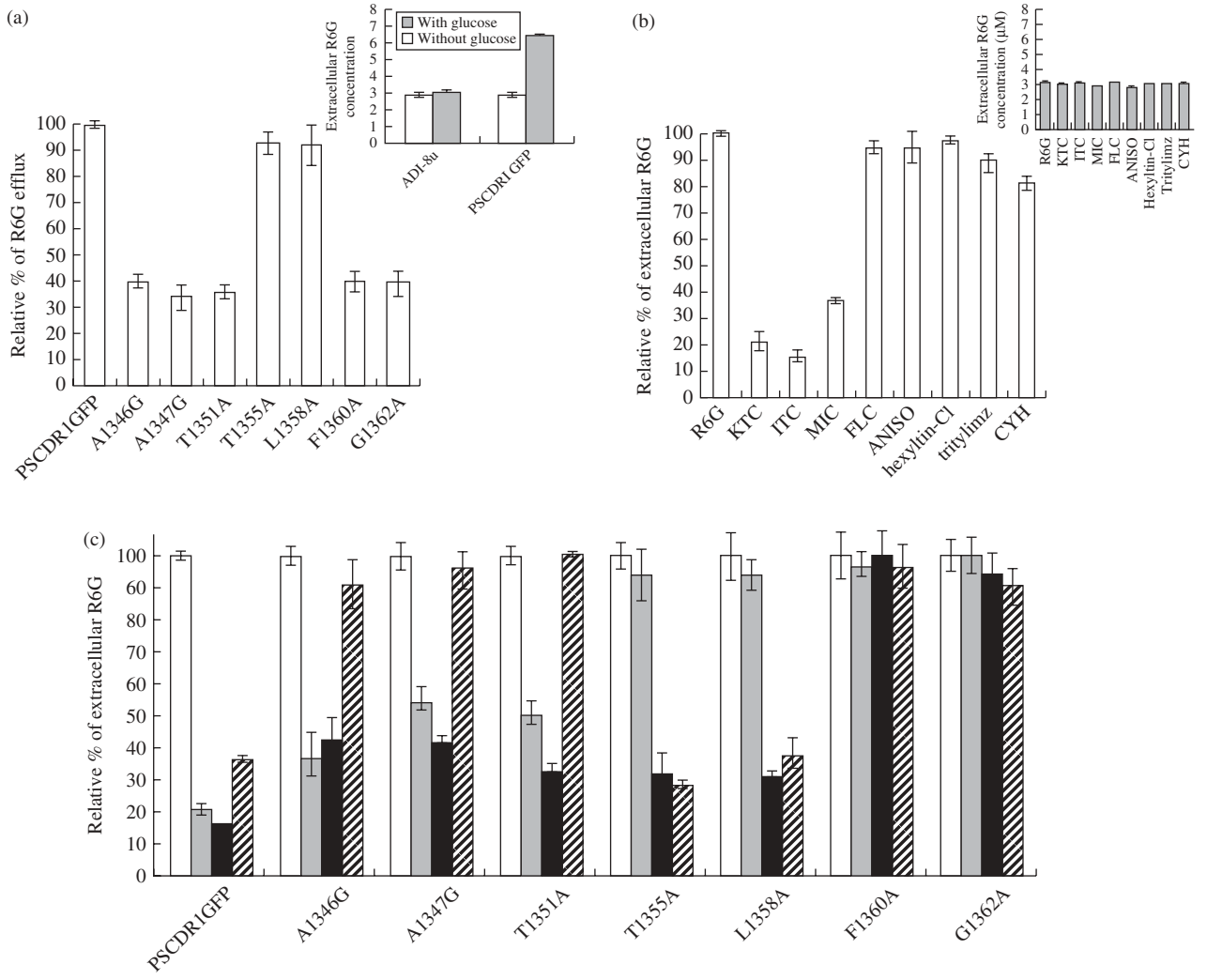

Figure 3. R6G efflux from the wild-type and mutant Cdr1GFP variant-expressing cells. (A) Efflux of R6G from wild-type and mutant Cdr1GFP-expressing strains. The assay was performed essentially as described in the Materials and methods section. The results are shown as relative change in % with R6G efflux from wild-type Cdr1p as 100%. The inset shows the extracellular concentration of R6G in the presence and absence of glucose in AD1-8u$^-$ and wild-type Cdr1p. (B and C) Relative % of R6G efflux with respect to R6G alone from the cells expressing wild-type Cdr1GFP (B) and AD1-8u$^-$ cells (B inset) in the presence of a five-fold molar excess of substrates (KTC, ITC, MIC, FLC, ANISO, hexyltin-Cl, tritylimz and CYH) and mutant variants (C) in the presence of a five-fold molar excess of substrates (KTC, grey bars; ITC, black bars; and MIC, striped bars). The efflux of R6G alone in (C) is shown by white bars. The results shown are the means of more than three independent experiments ±SD.

miconazole, fluconazole, anisomycin, tricyclohexyltin-chloride, tritylimidazole and cycloheximide. Of note, 5× concentration of competing substrates was sufficient to cause inhibition of R6G efflux in PSCDR1GFP cells (data not shown). Additionally, there was no change in the extracellular concentration of R6G if these substrates were added in excess to AD1-8u$^-$ cells (Figure 3B, inset). As shown in Figure 3(B), out of the eight substrates tested, only three substrates—namely ketoconazole, itraconazole and miconazole—could compete with rhodamine 6G efflux in the cells expressing wild-type Cdr1p, thus suggesting their common binding sites with rhodamine 6G. Of note, fluconazole could not compete with rhodamine 6G (Figure 3B). The mutant variants showed interesting patterns of rhodamine 6G efflux upon competition with these substrates (Figure 3C). For example, none of the substrates could compete with rhodamine 6G efflux in F1360A and G1362A variants. Ketoconazole could not compete with rhodamine 6G in T1355A and L1358A, while miconazole's competitiveness was totally abrogated in A1346G, A1347G and T1351A.

TMS11 mutant variants affect FK520 synergism with azoles

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.[2,3] Recently, it was observed that the protein phosphatase calcineurin allows survival of *C. albicans* during membrane stress exerted by azoles.[21] The calcineurin inhibitors cyclosporine A and tacrolimus (FK506) exhibit fungicidal synergism with azoles in *C. albicans*, *Candida glabrata*, *Candida krusei* and in *S. cerevisiae*.[22–24] We had earlier observed that mutating threonine 1351 to phenylalanine in TMS11 of Cdr1p abrogated synergism of

FK520 (a structural analogue of FK506) with various drugs, particularly azoles.[12] To check this possibility of synergistic behaviour of azoles in combination with FK520 in the newly constructed Cdr1GFP TMS11 mutant variants, we performed spot assays in the presence and absence of FK520 using non-toxic concentrations of FK520 (10 mg/L) and azoles (Figure 4). We observed that out of the seven mutant variants, which displayed hypersensitivity to drugs, only substitutions T1355A, L1358A and G1362A resulted in loss of synergism observed between FK520 and azoles (Figure 4). Of note, substitution of T1351 with alanine did not show abrogation of synergistic behaviour, which was seen when T1351 was replaced with phenylalanine.[12] The observed loss in synergism of FK520 with azoles for the mutants T1355A, L1358A and G1362A was further confirmed by MIC_{80} assay using ketoconazole, miconazole and fluconazole. Interestingly, the MIC_{80} of azoles for cells overexpressing wild-type PSCDR1GFP and variants A1346G, A1347G, T1351A and F1360A was reduced significantly in the presence of FK520 (10 mg/L), while, under similar conditions, the addition of FK520 did not change MIC_{80} for AD1-8u$^-$ cells (data not shown). Thus, it is apparent that FK520, at the non-toxic concentration used, requires Cdr1p for its synergistic effects. The addition of the FK520 also did not change the MIC_{80} of azoles in variants T1355A, L1358A and G1362A, suggesting the importance of these residues in the FK520-mediated synergy of azoles in Cdr1p-expressing cells (data not shown).

As discussed above, the substitution of T1351 by phenylalanine[12] instead of alanine (Figure 4) displayed abrogation of FK520 synergism. In the present study, we mutagenized the T1355 residue to phenylalanine and to a conservative substitution, serine. Interestingly, substitution T1355F, similar to T1351F variant, led to a severe increase in susceptibility to tested substrates (Figure 5A). Of note, conservative substitution of T1351 and T1355 by serine did not affect substrate susceptibility, surface localization and expression or FK520 synergism with azoles (Figure 5). However, interesting differences between the two threonine residues emerged when we examined the synergism of FK520. It is clear from Figure 5(D) that T1355-mediated synergism is lost only when it is replaced with alanine but not when substituted by phenylalanine, while these substitutions had an opposite effect at T1351. It should be mentioned that we could not show the synergistic effect of FK520 with fluconazole in variant T1355F as this mutant was severely hypersensitive to fluconazole and the minimum non-toxic concentration required for its growth led to loss of synergism even in the case of native Cdr1p.

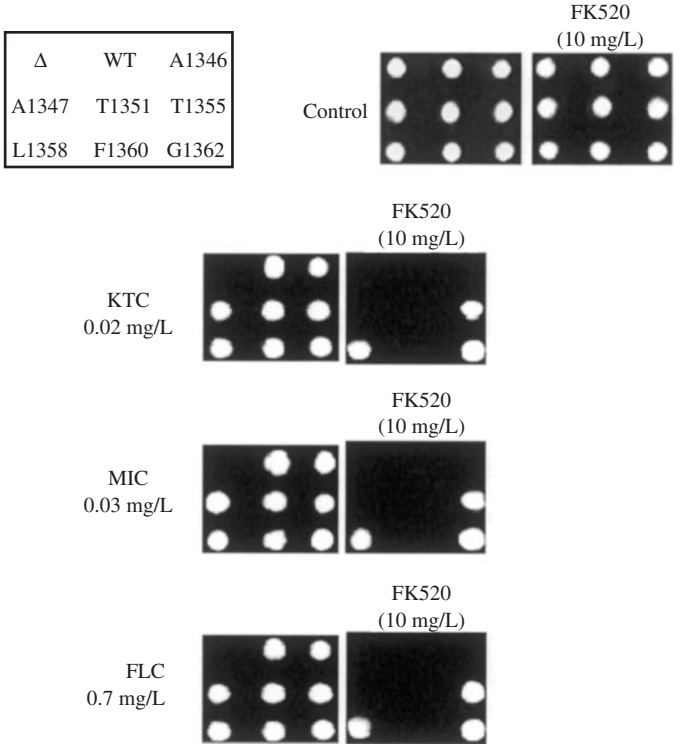

Figure 4. Synergy of FK520 with azoles in wild-type and mutant Cdr1GFP expressing cells. Spot assay in the presence and absence of FK520. The cells were suspended in normal saline to an OD_{600} of 0.1. A 5 μL sample of a 1:5 dilution of each strain was spotted onto YEPD agar plates in the presence of the indicated concentration of drugs and FK520 alone or in combination as indicated. Cell growth was monitored after 48 h of incubation of agar plates at 30°C. Δ, AD1-8u$^-$; WT, PSCDR1GFP.

Discussion

One of the key unresolved issues in the study of Cdr1p-mediated drug resistance is the mechanism by which it recognizes and transports structurally heterogeneous substrates. Identifying the specific segments and discrete amino acid residues implicated in the recognition and transport of a large number of structurally unrelated compounds that form the MDR spectrum thus becomes a prerequisite for the designing of effective antifungals capable of blocking Cdr1p function. Our previous work has already revealed that TMS6, TMS11 and TMS12 of Cdr1p harbour important amino acid residues, which affect drug susceptibilities.[11–13] Of note, evidence also points out that there is a conserved functional homology between Cdr1p, Pdr5p and human Pgp.[11,25] Based on our preliminary studies, as well as reports from other groups, it is apparent that TMS11 of Cdr1p and Pdr5p, another homologous ABC protein of *S. cerevisiae*, is critical not only for drug binding and transport, but also in displaying fungicidal synergism with calcineurin inhibitors FK520.[12,25,26] To further define the role of amino acid residues of TMS11 of Cdr1p in drug binding/transport, a detailed analysis of this transmembrane segment was undertaken in the present study, wherein all the putative 21 amino acid residues were subjected to alanine scanning.

Our results point out that of 21 residues of TMS11, substitution of only seven of them A1346G, A1347G, T1351A, T1355A, L1358A, F1360A and G1362A could affect drug susceptibilities of yeast cells. This indicated that substitutions in all the seven mutant Cdr1GFP variants could result in poor efflux of the tested substrates, which may reflect their impaired binding/transport by mutant proteins. A closer examination of the sequence alignment revealed the degree of conservation of mutation-sensitive residues in TMS11 of Cdr1p with other fungal transporters (Figure 6A). The helical wheel projection of TMS11 further revealed the clustering of most mutation-sensitive residues at a comparatively more polar face of the helix, except for A1346 and F1360 residues, which appeared to be towards the hydrophobic side of the helix. This topological sidedness of membrane spanning stretches is well known and is also reported in cases of mouse and human Pgp as well as Pdr5p.[25,27,28] On the basis of the helical wheel arrangement of TMS11 of Cdr1p, it appears that the hydrophobic side of the helix might interact with lipid matrix in the membrane bilayer and the more hydrophilic side may be involved in interaction between TMS11 and other TM helices of the protein, or alternatively may

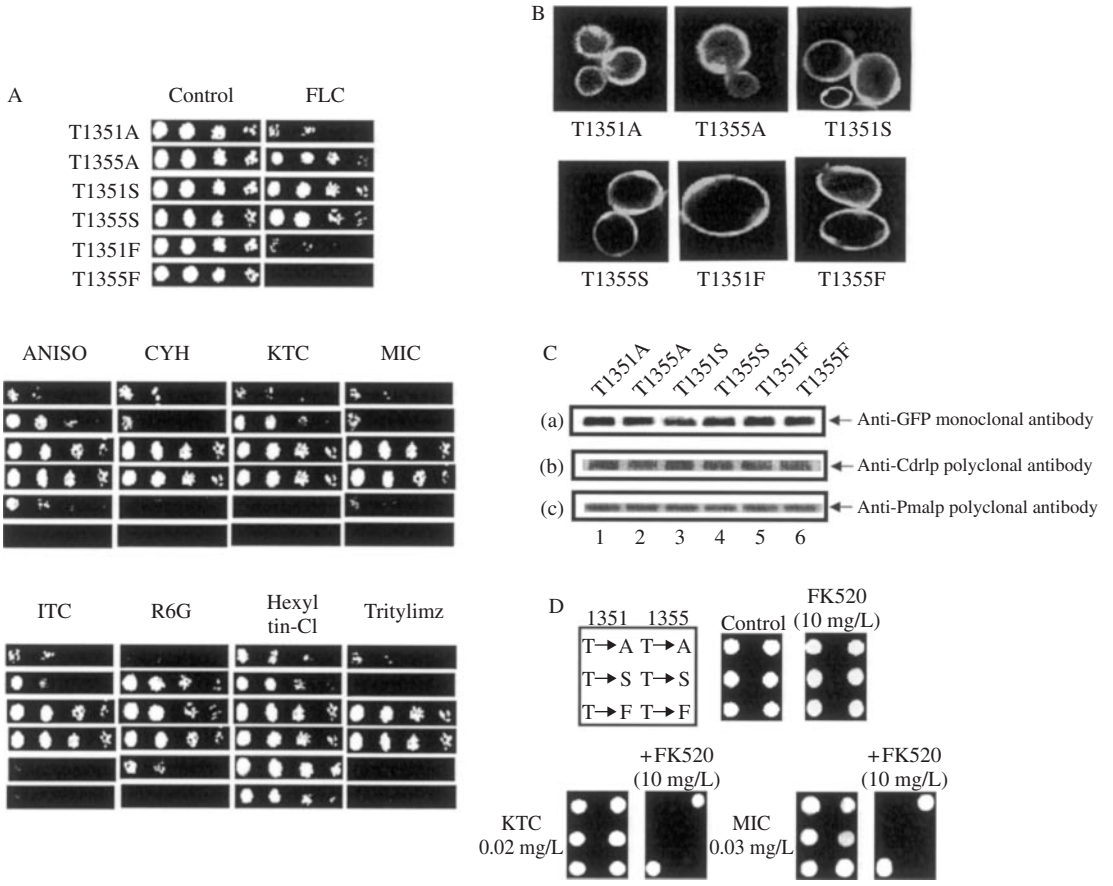

Figure 5. Substrate specificity, localization, expression and synergism with FK520 in mutant variants T1351A*/S/F and T1355A*/S/F. (A) Spot assay: for this assay, a 5 μL sample of a five-fold serial dilution of each yeast strain (cells suspended in normal saline to an OD_{600} of 0.1) was spotted on YEPD plates in the absence (control) or in the presence of: FLC, 4 mg/L; ANISO, 0.8 mg/L; CYH, 0.08 mg/L; ITC, 0.1 mg/L; KTC, 0.125 mg/L; MIC, 0.09 mg/L; R6G, 6 mg/L; hexyltin-Cl, 4 mg/L; and tritylimz, 0.5 mg/L. Cell growth was monitored after 48 h of incubation of plates at 30°C. (B) The plasma membrane localization of mutant variants T1351A/S/F and T1355A/S/F. (C) Expression of mutant variants T1351A/S/F and T1355A/S/F: PM (25 μg) protein from T1351A (lane 1), T1355A (lane 2), T1351S (lane 3), T1355S (lane 4), T1351F (lane 5) and T1355F (lane 6) were separated by 8% SDS-PAGE, electroblotted onto a nitrocellulose membrane and incubated with mouse monoclonal anti-GFP antibody (diluted 1:1000) (a), rabbit polyclonal anti-Cdr1p antibody (diluted 1:500) (b) and rabbit polyclonal anti-Pma1p antibody (diluted 1:10 000) (c). Proteins were immunodetected as described in the Materials and methods section. (D) Synergy of FK520 with KTC and MIC in mutant variants T1351A/S/F and T1355A/S/F. The cells were suspended in normal saline to an OD_{600} of 0.1. A 5 μL sample of a 1:5 dilution of each strain was spotted onto YEPD agar plates in the presence of the indicated concentration of drugs and FK520 alone or in combination as indicated. Cell growth was monitored after 48 h of incubation of agar plates at 30°C. (*) represents T1351A and T1355A (these mutants have been selected again for better comparison of all the substitutions of T1351 and T1355). A colour version of this Figure is available with the online article.

interact directly with substrate molecules. Of note, of the two sensitive residues A1346 and F1360, which appear to be localized on the hydrophobic side of the helix as compared with the rest of the five sensitive residues, substitution of F1360 by alanine (F1360A) showed the highest susceptibility to tested drugs. Notably, substitution F1360A yielded a protein product whose localization, expression and ATPase activity were indistinguishable from wild-type Cdr1p (Figure 2B and C). It is, however, possible that F1360 substitution by alanine either could result in local alteration in the protein–lipid matrix interaction, in such a way that there is a change in the drug binding domain(s), or alternatively, being hydrophobic, this residue might interact with the hydrophobic portion of the drugs.

The efflux of rhodamine 6G provided interesting clues about the substrate specificity of Cdr1p. Out of seven mutant variants, rhodamine 6G efflux was abrogated in all the mutants except in variants T1355A and L1358A (Figure 3A). The same residues also displayed unaltered resistance to rhodamine 6G (Figure 2A), thus confirming that substitutions T1355A and L1358A do not affect the efficiency of Cdr1p in effluxing rhodamine 6G. In the wild-type Cdr1p, various substrates in general could not compete with rhodamine 6G efflux, except for azoles ketoconazole, itraconazole and miconazole (Figure 3B). It would mean that among the various tested substrates, rhodamine 6G may have some commonality with regard to the binding and transport of these azoles. Of note, azoles could not compete with rhodamine 6G efflux in cases of Pdr5p.[25] This indicates that although Pdr5p is more closely related to Cdr1p (sequence similarity 73% and identity 56%), functional differences exist between the two proteins.

The results of competition assays with mutant variant proteins gave further insight into the specificity of Cdr1p-mediated transport. For example, efficient competitors, such as ketoconazole, itraconazole and miconazole of rhodamine 6G efflux became totally ineffective in the cells expressing F1360A and G1362A variant proteins. The inability of ketoconazole to compete with rhodamine 6G in T1355A and L1358A and of miconazole in cells expressing

A

			*	*			*			*			*		*		*							
C. albicans Cdr1	1346	**A**	**A**	N	L	A	**T**	L	**L**	F	**T**	M	C	**L**	N	**F**	C	**G**	V	L	A	G	1366
C. albicans Cdr2	1344	**A**	**A**	N	L	A	**T**	T	**L**	F	**T**	L	C	**L**	M	**F**	C	**G**	V	L	A	G	1364
C. albicans Cdr3	1326	**A**	**A**	N	W	I	S	L	**L**	L	**T**	I	S	M	I	**F**	C	**G**	V	I	A	T	1346
C. albicans Cdr4	1333	**A**	**A**	N	L	S	V	L	**L**	F	**T**	M	C	**L**	A	**F**	C	**G**	V	L	V	V	1353
C. glabrata Cdr1	1343	**A**	**A**	N	M	A	S	L	M	F	**T**	L	S	**L**	S	**F**	C	**G**	V	L	V	T	1363
C. dubliensis Cdr1	1346	**A**	**A**	N	L	A	**T**	L	**L**	F	**T**	M	C	**L**	N	**F**	C	**G**	V	L	A	G	1366
C. dubliensis Cdr2	1345	**A**	**A**	S	L	A	**T**	T	**L**	F	**T**	L	C	**L**	M	**F**	C	**G**	V	L	A	G	1365
S. cerevisiae Pdr5	1355	**A**	**A**	N	L	A	S	L	**L**	F	**T**	M	S	**L**	S	**F**	C	**G**	V	M	T	T	1375
S. cerevisiae Pdr10	1409	**A**	**A**	N	L	A	S	L	F	F	I	M	S	**L**	S	**F**	C	**G**	V	L	A	T	1429
S. cerevisiae Pdr15	1370	**A**	**A**	H	M	G	**T**	L	**L**	F	**T**	M	A	**L**	S	**F**	C	**G**	V	M	A	T	1390
S. cerevisiae Pdr12	1322	**A**	S	M	I	N	S	N	**L**	F	A	A	M	**L**	L	**F**	C	**G**	I	L	Q	P	1342
S. cerevisiae Snq2	1333	**A**	N	V	I	L	G	L	C	L	S	F	M	**L**	S	**F**	C	**G**	V	T	Q	P	1353

B

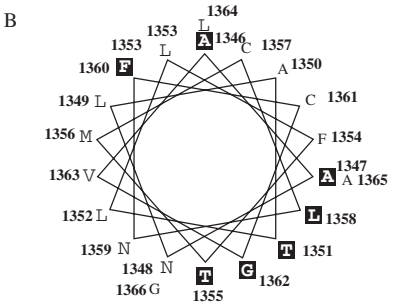

Figure 6. The sequence alignment and helical wheel projection of the predicted TMS11 of Cdr1p. (A) Sequence alignment of the predicted TMS11 of *C. albicans* Cdr1p, Cdr2p, Cdr3p and Cdr4p, *C. glabrata* Cdr1p, *Candida dubliensis* Cdr1p and Cdr2p and *S. cerevisiae* Pdr5p, Pdr10p, Pdr15p, Pdr12p and Snq2p. The amino acids are numbered according to their positions in the protein. Residues that affected the functionality of the Cdr1p after substitution are represented in bold and are shown by asterisks (*). (B) Helical wheel projection of the primary amino acid sequence was constructed, using 3.6 amino acids per turn of the helix by the EMBOSS PEPWHEEL program.[38] Mutations that affected the functionality of the Cdr1p are shown by white letters on a black background.

A1346G, A1347G and T1351A variants further suggests differential interactions of residues of TMS11 with various substrates. For example, it appears that while F1360 and G1362 may represent part of a substrate-binding pocket for all three azoles, T1351 and L1358 may only interact with ketoconazole, whereas A1346G, A1347G and T1351A may be specific to miconazole binding. Our results further confirmed close functional homology between human and mouse Pgp with Cdr1p.[27,28] In the mammalian homologues also, TMS11 play a major role in forming the binding sites of various substrates and are responsible for their subsequent efflux, although the residues involved are different between the two sets of proteins.[27,28] Recently, by using a variety of novel substrates of Pdr5p, Golin *et al*.[20] have reported that this ABC drug transporter from *S. cerevisiae* has at least three drug binding sites and suggested that some substrates might even interact at more than one binding site. The fact that the efflux of rhodamine 6G gets competed out by only three out of the eight substrates tested also supports the existence of multiple drug binding sites in Cdr1p.

In this context, it is also interesting to compare the effects of tricyclohexyltin chloride and tritylimidazole, two closely related drug substrates of Cdr1p, on the mutant variants generated by us. Of note, Golin *et al*.[20] have recently shown that both tricyclohexyltin chloride and tritylimidazole bind to the same site on Pdr5p, a homologous ABC transporter from *S. cerevisiae*. The ineffectiveness of tricyclohexyltin chloride to drastically alter the susceptibilities of most of the mutants is in sharp contrast to the severe effect that tritylimidazole has on drug susceptibilities of Cdr1p mutant variants. This could imply that the aromatic moieties of the latter are critical for its transport, while this might not be the case with tricyclohexyltin chloride. However, the possibility that the binding sites for the two drugs might be different in Cdr1p cannot be excluded from our present observations.

Our data revealed that not only the well established fungicidal synergism of calcineurin inhibitor FK520 in combination with azoles is mediated through T1351, but also shows for the first time that other residues of TMS11, such as T1355A, L1358A and G1362A are also involved. Interestingly, substitution of threonine by phenylalanine/alanine at position 1351 and 1355 had an opposite effect on FK520 synergism. Abrogation of synergism in T1351F and in T1355A suggests the importance of the length/size of the side chains of the residues at these positions in interacting with FK520. Of note, residues that are involved in the abrogation of synergism and thus in turn might form the FK520 binding site also seems to cluster towards the more polar, mutation-sensitive face of the helix (Figure 6A). The very fact that the other mutant variants of Cdr1p in TMS11 itself remained synergistically susceptible to FK520 indicates that only T1351F, T1355A, L1358A and G1362A specifically contribute to this synergy. Since antifungal agents of various structures, including azole derivatives, are substrates of Cdr1p, it is very likely that the immunosuppressants or their analogues might increase the intracellular level of drugs by competition, thus blocking the pump activity directly.[29,30] The mechanisms underlying the azole-FK520 synergy are not fully understood and opinions differ about its interaction with the efflux protein. Recently we, as well as others, have shown that Cdr1p and Cdr2p transporters can affect cell tolerance to FK520 and suggest a possible involvement of these transporters in the synergism between azoles and FK520 in *C. albicans*.[12,31,32] Earlier Egner *et al*.[25,26] have also observed loss of FK506 synergism in S1360F/T and T1364F/S/A variants of Pdr5p of *S. cerevisiae*. Of note, TMD prediction of a Pdr5p sequence by using a PHDsec algorithm, as was conducted for Cdr1p, depicted S1360 (equivalent to T1351 of Cdr1p) and T1364 (equivalent to T1355 of Cdr1p) as present in TMS11 (Figure 6A).[33–36]

While most of the studies point out that ABC transporters like Cdr1p, Cdr2p and Pdr5p affect the fungicidal synergism of calcineurin inhibitors, there is also a report that rules out any such interaction between calcineurin inhibitors, FK520 and efflux proteins.[37]

In conclusion, our alanine scanning results of TMS11 revealed that this domain is an important structural and functional determinant of drug transport by Cdr1p. The predicted topology of TMS11 that gives it an amphipathic character and conserves its mutation-sensitive amino acids in other fungal homologues proteins, strongly suggest that clustered residues on the hydrophilic face of TMS11 play a major role in drug binding/transport. What constitutes the substrate/drug binding pocket and how TMS11 interacts with other helices of Cdr1p are some of the issues that remain to be resolved. Such studies should lead to an understanding of how Cdr1p can transport a wide variety of substrates and may improve our approach in the design of new inhibitors/modulators of drug transporter for clinical applications.

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