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Substitution of threonine-1351 in the multidrug transporter Cdr1p of *Candida albicans* results in hypersusceptibility to antifungal agents and threonine-1351 is essential for synergic effects of calcineurin inhibitor FK520

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***Objectives*: Functional characterization of a mutant *Candida albicans* drug resistance protein (Cdr1p) by overexpression in *Saccharomyces cerevisiae*.**

***Methods*: We overexpressed green fluorescent protein-tagged Cdr1p in *S. cerevisiae* AD1-8u$^-$ host and introduced a point mutation to substitute T1351 with F in Cdr1p. The cells expressing T1351F mutant Cdr1p were analysed for their functional activity using minimum inhibitory concentration, spot assay, and fluconazole efflux. The binding activity of photoaffinity analogues 8-azidoATP, iodoarylazidoprazosin and azidopine to the mutant T1351F Cdr1p was also characterized.**

***Results*: The T1351F mutant Cdr1p-expressing cells were susceptible to anisomycin, cycloheximide, fluconazole, miconazole and nystatin. The mutant protein was expressed to the same level as that of native Cdr1p in *S. cerevisiae* cells and was properly localized to the cell surface. There was also no difference between the mutant variant and the native protein's ability to bind a photoaffinity analogue of ATP, 8-azidoATP, or the radiolabelled photoaffinity agents iodoarylazidoprazosin and azidopine. However, the substitution of T1351 resulted in considerable reduction in its ability to export fluorescent substrate rhodamine 6G. The synergy between calcineurin inhibitors FK520 and azoles was abrogated in cells expressing the T1351F mutant variant of Cdr1p.**

***Conclusions*: The results from this study suggest that the T1351 in the predicted transmembrane domain (TMD) 11 of Cdr1p is not only important for drug-substrate transport but also has a role in governing synergy of FK520.**

Keywords: *C. albicans*, azoles, synergy, multidrug resistance

Introduction

Candida albicans is a fungal pathogen which is responsible for opportunistic infections in immunocompromised patients. Several drugs are used to treat candidiasis and most target the ergosterol biosynthetic pathway or its final product, ergosterol, a cell membrane component that is unique to fungi. The most commonly used drugs in both the treatment and prevention of candidiasis are azoles.[1–3] Azoles are fungistatic rather than fungicidal to *Candida* cells. As a result, this tolerance to azoles contributes to the development of the resistance sometimes encountered in clinical isolates from immunocompromised patients.[2–5]

A number of mechanisms contribute to the development of azole resistance in *C. albicans*.[6–10] One of these is the energy-dependent drug efflux which results from an overexpression of genes encoding drug efflux pump proteins belonging to the ATP-binding cassette (ABC) as well as the major facilitators (MFS) superfamilies of transporters.[7,11–13] Among ABC transporters, *CDR1* and *CDR2* are major efflux pumps, whose overexpression plays a key role in azole resistance in *C. albicans*. Thus, invariably, transcript levels of *CDR1* and *CDR2* genes are elevated in azole-resistant clinical isolates recovered from patients receiving long-term antifungal therapy.[9,10] In many cases, the up-regulation of *CDR1* results in decreased intracellular

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levels of fluconazole thus corroborating its direct involvement in drug efflux.[14,15]

The molecular mechanisms which govern the function of Cdr1p or Cdr2p as efflux pumps for azoles are not well known and information is needed (1) to understand how the protein can bind a structurally diverse range of compounds including different azoles, (2) to define the drug–substrate binding, and (3) to determine how ATP binding and hydrolysis are linked to drug transport. In an effort to develop an understanding of the molecular details of drug binding and transport, recently, we overexpressed Cdr1p as a green fluorescent protein (GFP)-tagged fusion protein in a heterologous hyper-expression system of *Saccharomyces cerevisiae*. Based on conserved residues among various drug transporters, we had generated several mutant variants of Cdr1p.[16] We observed that several point mutations resulted in mutant variants of Cdr1p, which were susceptible to different drugs.[16] In this study, we chose a mutant T1351F (where a threonine 1351 of TM11 was replaced by phenylalanine), for functional characterization as it showed susceptibility to antifungal agents. Of note, T1351 is a conserved residue of TM11 of many drug transporters wherein either a threonine or a serine is present and has been implicated in drug susceptibility.[17,18] We show that the mutant T1351F Cdr1p was properly expressed and localized to the cell surface, exhibited similar binding of the ATP and photoaffinity analogues of drugs and had no difference in ATPase activity. However, the cells expressing the mutant protein were hypersusceptible to all the tested drugs. Additionally, the known synergy between azoles and calcineurin inhibitors was abrogated in mutant variant T1351F.[19–22] Our results for the first time demonstrate a direct involvement of Cdr1p in synergy between immunosuppressant analogue FK520 and azoles.

Materials and methods

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, miconazole, ketoconazole, nystatin, cycloheximide, anisomycin, rhodamine 6G and other molecular grade chemicals were obtained from Sigma Chemical Co (St. Louis, MO, USA). The radiolabelled [^{125}I]IAAP (Iodoarylazidoprazosin) (2200 Ci/mmol) was from Perkin–Elmer Life Sciences (Boston, MA, USA), [α-^{32}P]8-azidoATP (15–20 Ci/mmol) from Affinity Labeling Technologies, Inc. (Lexington, KY, USA), [^{3}H]fluconazole (20 Ci/mmol) and [^{3}H]azidopine (60 Ci/mmol) from Amersham Biosciences (Arlington Heights, IL, USA). The [α-^{32}P]8-azidoATP showed no detectable contaminating [α-^{32}P]8-azidoADP by thin-layer chromatography with 0.8 M LiCl as the solvent. Fluconazole was kindly provided by Ranbaxy Laboratories, India. FK520 was a generous gift from Merck & Co., Inc., Rahway, USA.

Bacterial and yeast strains and growth media

Plasmids were maintained in *Escherichia coli* XL-1 blue. *Escherichia coli* was cultured in Luria-Bertani medium (Difco, BD Biosciences, NJ, USA) to which ampicillin was added (100 mg/L). The *S. cerevisiae* strain used was AD1-8u^{-} (Mat a, *pdr1-3, his1, ura3, Δyor1::hisG, Δsnq 2::hisG, Δpdr5::hisG, Δpdr10::hisG, Δpdr11::hisG, Δycf1::hisG, Δpdr3::hisG, Δpdr15::hisG*) (provided by Professor Richard D. Cannon, University of Otago, Dunedin, New Zealand). PSCDR1-GFP and T1351F were AD1-8u^{-} derivatives expressing Cdr1p-GFP and its mutant protein (mutant Cdr1p-GFP), respectively. The yeast strains were cultured in Yeast Extract Peptone Dextrose (YEPD) broth (Bio101, Vista CA, USA). For agar plates, 2% (w/v) Bacto agar (Difco, BD Biosciences, NJ, USA) was added to the medium.

Site-specific mutagenesis and development of transformants

Site-directed mutagenesis was carried out by use of the QuickChange Mutagenesis system from Stratagene (La Jolla, CA, USA). The mutation was introduced into plasmid pPSCDR1-GFP,[16] according to manufacturer's instructions and the desired nucleotide sequence alteration was confirmed by DNA sequencing of the ORF. The mutated pPSCDR1-GFP after linearizing with *Xba*I was used to transform AD1-8u^{-} cells as described previously.[16]

Preparation of purified plasma membranes of S. cerevisiae cells

Crude membranes were prepared from *S. cerevisiae* cells grown in YEPD to late exponential phase. The protease inhibitor cocktail (1 mM PMSF, 1 mg/L leupeptin, pepstatin A and aprotinin) was added to the culture and the cells were harvested. The cells were broken with glass beads by vortexing the cells four times for 30 s followed by a 30 s interval on ice. The homogenization medium contained 50 mM Tris pH 7.5 and 2.5 mM EDTA and the protease inhibitor cocktail. The lysate was centrifuged at 1000 **g** to remove unbroken cells and the crude membranes were pelleted by ultracentrifugation of low speed supernatant at 100 000 **g** for 1 h. Finally, the crude membranes were suspended in suspension buffer (10 mM Tris pH 7.5, 0.5 mM EDTA and 10% glycerol). The crude membrane suspension was applied to a discontinuous gradient made of an equal volume of 53.5% (w/v) sucrose and 43.5% (w/v) sucrose. After centrifugation for 5 h at 100 000 **g** in a Beckman SW 28 rotor, the purified plasma membranes were recovered at 43.5/53.5 interface as described by Monk *et al.*[23]

Immunodetection of Cdr1p in plasma membrane

The western blot analysis was done with anti-Cdr1p polyclonal antibody (1:500 dilution) or anti-GFP monoclonal antibody (1:1000 dilution), anti-Pma1p polyclonal antibody (1:10 000 dilution) as described previously.[16]

Drug susceptibility and other functional parameters of S. cerevisiae

The susceptibilities of *S. cerevisiae* cells to different drugs were determined by microdilution and spot assays as described earlier.[24] The Cdr1p-associated ATPase activity of the purified plasma membrane was measured as oligomycin-sensitive release of inorganic phosphate as described previously.[16] Efflux of rhodamine 6G and the accumulation of [^{3}H]fluconazole were determined essentially by protocols as described previously.[24,25]

Photoaffinity labelling with [^{125}I]IAAP, [^{3}H]azidopine, [α-^{32}P]8-azidoATP

The plasma membrane (15 μg) protein was photoaffinity labelled with 3–6 nM [^{125}I]IAAP (2200 Ci/mmol) or 0.5 μM [^{3}H]azidopine (60 Ci/mmol) or with 10 μM [α-^{32}P]8-azidoATP (10 μCi/nmol) and competed with drugs as described previously.[16]

Results

For functional characterization of Cdr1p, we have previously used a hyper-expression system, where Cdr1p was stably overexpressed from a genomic *PDR5* locus in a *S. cerevisiae* mutant AD1-8u$^-$.[16] The 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.[11] We tagged the GFP gene at the C-terminal end of *CDR1*, which was overexpressed as a fusion protein in PSCDR1-GFP strain and demonstrated that GFP tagging and overexpression did not impair functional activity of the protein.[16] To dissect molecular details of drug binding and transport mediated by Cdr1p, site-directed mutagenesis was employed to generate several mutant variants.[16] The following study pertains to one such mutant variant of Cdr1p where threonine 1351 of TMD11 is substituted by phenylalanine (T1351F).

Drug resistance profile of T1351F mutant Cdr1p-GFP

The mutation T1351F was introduced in the overexpressing Cdr1p-GFP plasmid (pPSCDR1-GFP).[16] The mutated plasmid was integrated in *S. cerevisiae* (AD1-8u$^-$) as described previously.[16] Southern hybridization confirmed that the gene was inserted as a single copy at the genomic *PDR5* locus (data not shown). Two positive clones of the mutant were selected to rule out clonal variations. Confirmed positive mutants were screened for their sensitivity to drugs by two independent methods, by MIC_{80} determination and by spot assay. As depicted in Figure 1(a and b), the T1351F mutant cells became susceptible to anisomycin, cycloheximide, fluconazole, miconazole and nystatin compared to native Cdr1p-GFP. In the following experiments, we analysed some functional parameters to ascertain the phenotype of T1351F.

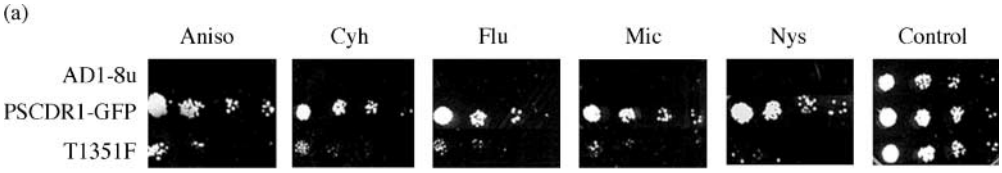
T1351F was properly expressed and localized at the cell surface

To explore if the observed changes in drug susceptibilities of T1351F mutant Cdr1p-GFP were due to alterations in protein functions rather than due to altered expression levels, we did a fluorescence activated cell sorting (FACS) analysis of the live cells which showed that the fluorescence intensity in T1351F cells was comparable to the wild-type PSCDR1-GFP (Figure 2a). The confocal images confirmed that the wild-type and mutant T1351F Cdr1p-GFP was properly localized at the cell surface (Figure 2b). The western blot of the mutant as well as the wild-type Cdr1p-GFP plasma membrane proteins with anti-GFP monoclonal antibody (upper panel Figure 2c) and with anti-Cdr1p polyclonal antibody (middle panel Figure 2c) showed that cells with T1351F mutant variant of Cdr1p-GFP expressed protein to the same level as that of wild-type. These data indicate that the observed hypersusceptibility of T1351F towards various drugs was not due to a difference in the expression level or the localization of mutant variant protein. Of note, the plasma membrane (PM-ATPase) (lower panel Figure 2c) was used as a marker for the purity and quantity of the protein in the plasma membrane fractions.

ATPase activity, nucleotide and substrate binding of mutant T1351F variant protein remain unchanged

We used photoaffinity analogues, 8-azidoATP (analogue of ATP), IAAP [a photoaffinity analogue of P-glycoprotein (P-gp) substrate, prazosin] and azidopine (a photoaffinity analogue of dihydropyridine),[16] to explore if the observed difference in drug sensitivity was not due to any change in nucleotide or drug binding. We used a purified plasma membrane fraction from the native and mutant variant expressing cells for photoaffinity analogue binding and observed that there was no significant

(a)



(b)

	MIC_{80} (mg/L)				
	Aniso	Cyh	Flu	Mic	Nys
AD1-8u$^-$	0.12	0.015	1	0.015	0.03
PSCDR1-GFP	16	1	64	4	2
T1351F	0.25	0.062	4	0.06	0.06

Figure 1. Drug sensitivity of Cdr1p-GFP and T1351F expressing cells. (a) AD1-8u$^-$, PSCDR1-GFP (expressing wild-type Cdr1p) or T1351F (mutant Cdr1p-GFP) cells were grown overnight on YEPD plates at 30°C. The cells were then resuspended in normal saline to an A_{600} of 0.1. Five microlitres of five-fold serial dilutions of each strain was spotted onto YEPD plates in the absence (Control) or in the presence of the following antifungal agents: anisomycin (Aniso) (5 mg/L), cycloheximide (Cyh) (0.5 mg/L), fluconazole (Flu) (10 mg/L), miconazole (Mic) (1 mg/L) and nystatin (Nys) (1 mg/L). Growth differences were recorded following incubation of the plates for 48 h at 30°C. Growth was not affected by the presence of the solvents used for the drugs (data not shown). (b) The drug resistance profile of AD1-8u$^-$, PSCDR1-GFP and T1351F cells. The MIC_{80} (mg/L) values of anisomycin (Aniso), cycloheximide (Cyh), fluconazole (Flu), miconazole (Mic) and nystatin (Nys) for AD1-8u$^-$, PSCDR1-GFP and T1351F cells, were determined as described in Materials and methods. The results are typical of one determination, which was confirmed by three independent experiments.

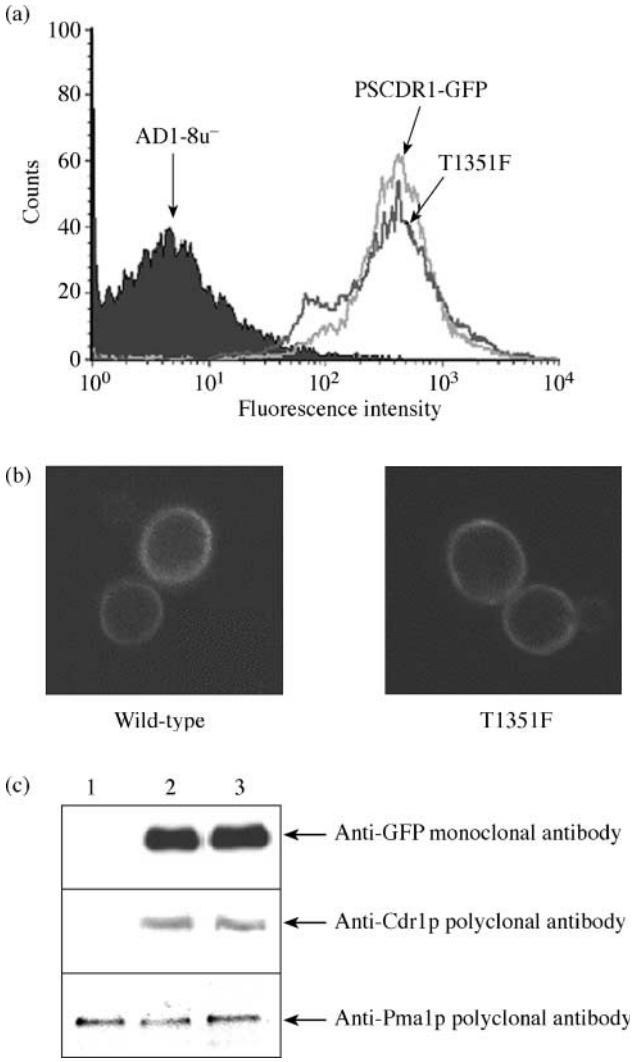


Figure 2. T1351F mutant Cdr1p-GFP is localized to the cell surface and expressed to the same level as wild-type protein. (a) Flow cytometry of *S. cerevisiae* AD1-8u$^-$, PSCDR1-GFP and T1351F Cdr1p-GFP cells: AD1-8u$^-$ (control), PSCDR1-GFP (Cdr1p-GFP) and T1351F cells were grown in YEPD medium to mid log phase and used for FACS analysis in a FACSort flow cytometer (Becton–Dickinson Immunocytometry Systems). Analysis was carried out with CellQuest software. The histogram derived from the CellQuest program depicts fluorescence intensity for AD1-8u$^-$, PSCDR-GFP and T1351F cells, respectively. (b) Confocal images of *S. cerevisiae* cells expressing GFP-tagged wild-type and T1351F mutant Cdr1p-GFP. Cells were grown in YEPD medium to late log phase. The cells were washed and resuspended in an appropriate volume of 50 mM HEPES pH 7.0. The cells were viewed directly, on a glass slide with a drop of antifade reagent to prevent photobleaching, with 100 × oil emulsion objective on a Bio-Rad confocal microscope (MRC 1024). (c) Expression of T1351F mutant Cdr1p-GFP in *S. cerevisiae*: The plasma membrane (20 μg) protein from AD1-8u$^-$ (lane 1), PSCDR1-GFP (lane 2) and T1351F (lane 3) were separated on 8% SDS–polyacrylamide gel, electroblotted on to nitrocellulose, and incubated with mouse monoclonal anti-GFP antibody (1:1000) (upper panel), rabbit polyclonal anti-Cdr1p antibody (1:500 dilution) (middle panel) and rabbit polyclonal anti-Pma1p antibody (1:10 000 dilution) (lower panel). Proteins were immunodetected as described in Materials and methods.

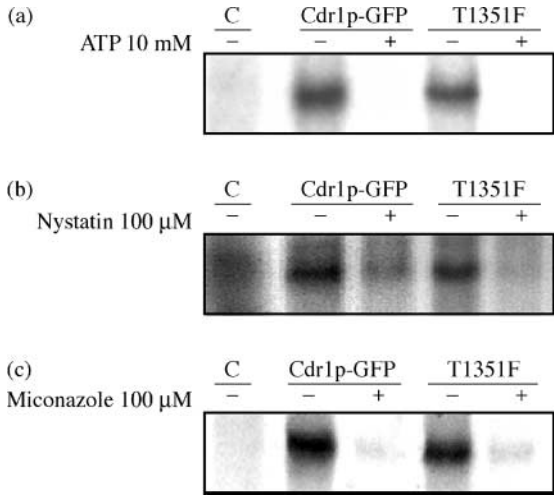


Figure 3. ATP and substrate analogue binding characteristics of T1351F mutant Cdr1p-GFP. (a) [α-^{32}P]8-azidoATP-labelling of AD 1–8u$^-$ (control), wild-type (Cdr1p-GFP) and T1351F mutant Cdr1p-GFP. The plasma membrane proteins (15 μg/50 μL) were photoaffinity labelled with 10 μM [α-^{32}P]8-azidoATP (10 μCi/nmol) in the absence (–) and presence of (+) of 10 mM ATP as described in Materials and methods. (b) [^{125}I]IAAP labelling of AD 1–8u$^-$ (control), wild-type (Cdr1p-GFP) and T1351F mutant Cdr1p-GFP. The plasma membrane proteins (15 μg) were labelled with 3–6 nM of [^{125}I]IAAP (2200 Ci/mmol) as described in Materials and methods; 100 μM of nystatin (+) was added to compete with IAAP binding. (c) [^{3}H]azidopine labelling of AD 1–8u$^-$ (control), wild-type (Cdr1p-GFP) and T1351F mutant Cdr1p-GFP. The plasma membrane protein (30 μg) was labelled with [^{3}H]azidopine, as described in Materials and methods, in the presence and absence of 100 μM miconazole as (+) indicated.

difference between the specific binding of [α-^{32}P]8-azidoATP to the wild-type and T1351F protein (Figure 3a). It was found that both the wild-type as well as the mutant Cdr1p-GFPs of T1351F showed similar binding of [^{125}I]IAAP which could be competed out with 100 μM nystatin (Figure 3b). That there was no difference in drug binding was further confirmed by examining the binding of [^{3}H]azidopine. It was observed that the [^{3}H]azidopine bound similarly to wild-type as well as mutant Cdr1p-GFP protein (Figure 3c) and could be competed out by miconazole (100 μM).

We as well as others have shown that Cdr1p elicits NBDs-dependent oligomycin-sensitive ATPase activity.[11,16] We assayed ATPase activity in the purified plasma membrane fraction from mutant T1351F and compared it with the wild-type Cdr1p-GFP-expressing cells. The oligomycin-sensitive ATPase activity of T1351F mutant (43.4 nmol Pi/min/mg) was found to be comparable to that of native Cdr1p-GFP (41.4 nmol Pi/min/mg). We did not get a drug-stimulated ATPase activity with wild-type as well as the mutant T1351F Cdr1p-GFP which would have been a correct measure of the ATPase activity contributed by the active protein. Of note, unlike mammalian homologues of yeast ABC drug transporters, drug-stimulated ATPase activity in any drug transporter of yeast has not been possible to demonstrate.[11,26]

T1351F Cdr1p-GFP shows reduced efflux of rhodamine 6G

We used fluorescent rhodamine 6G, a known substrate of Cdr1p to compare efflux ability of T1351F with native protein. It was observed that T1351F mutant variant protein showed reduced efflux of rhodamine 6G efflux (0.71 nmol/mL) compared with

the wild-type Cdr1p-GFP-expressing cells (2.53 nmol/mL). Of note the level of efflux by mutant variant T1351F was comparable to that of control AD1-8u$^-$ cells (0.53 nmol/mL), which did not express Cdr1p.

FK520 shows synergy with drugs and inhibits Cdr1p-GFP-mediated drug resistance

In view of the known fungicidal synergic effect of the immunosuppressive agent tacrolimus (FK506) with azoles in *C. albicans*,[27] we checked the drug resistance profile of the Cdr1p-GFP-expressing cells in presence and absence of FK520 (ascomycin), a structural analogue of FK506, to determine the interactions between this class of compounds and azoles. In a MIC assay, FK520 showed a MIC of 128 mg/L for PSCDR1-GFP cells, whereas it was 16 mg/L for the control cells (data not shown). In order to examine the reported synergy between immunosuppressant and antifungal agents, the AD1-8u$^-$ and PSCDR1-GFP cells were grown in the presence or absence of non-toxic concentrations of FK520 (10 mg/L) in combination with fluconazole in a spot assay (Figure 4). It was observed that both the cells could grow well in the presence of non-toxic concentration of FK520. As expected, PSCDR1-GFP cells were resistant to fluconazole and ketoconazole compared to host AD1-8u$^-$ cells, which did not express Cdr1p. However, the resistance to both the azoles displayed by PSCDR1-GFP cells was abrogated when FK520 was also included in the growth medium. Furthermore, this synergic effect of FK520 on Cdr1p-GFP-expressing cells was not limited to azoles since it also showed inhibition of growth with cycloheximide (Figure 4c) and anisomycin (data not shown).

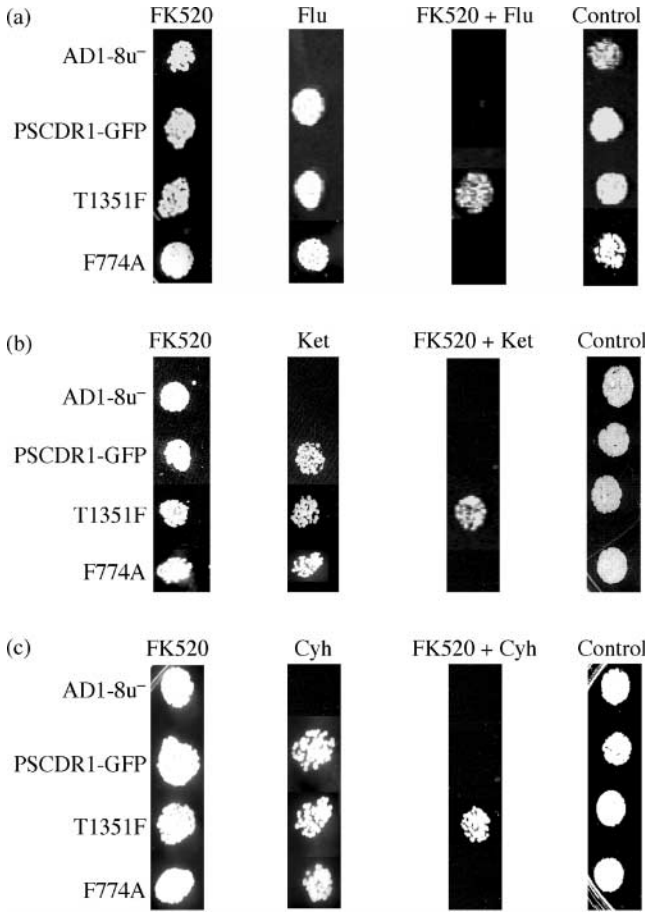


Figure 4. Synergy of FK520 with drugs in Cdr1p-GFP and its mutant variant T1351F expressing cells. AD1-8u$^-$, PSCDR1-GFP and T1351F cells were grown overnight on YEPD plates at 30°C. The cells were then resuspended in saline to an A_{600} of 0.1. Five microlitres of a 1:5 dilution of each strain was spotted onto YEPD plates in the absence (growth control) or in the presence of the following drugs: (a) FK520 (10 mg/L), (b) fluconazole (2 mg/L), (c) ketoconazole (100 μg/L) and (d) cycloheximide (20 μg/L) alone or in combination as indicated. Growth differences were recorded following incubation of the plates for 48 h at 30°C.

The synergic effect of FK520 was further confirmed by MIC determinations. Interestingly, the MIC of fluconazole for overexpressing PSCDR1-GFP cells was 64 mg/L, which was reduced to 16 mg/L in the presence of FK520 (10 mg/L) (Figure 5a). However, under similar conditions, the addition of FK520 did not change MIC value (1 mg/L) of fluconazole for AD1-8u$^-$ cells, which were neither expressing *CDR1* nor expressing any major efflux proteins of *S. cerevisiae* (Figure 5a). It is apparent from the data that FK520 at the non-toxic concentration used requires Cdr1p for its synergic effects. The addition of the FK520 also did not change the MIC value (4 mg/L) of fluconazole for T1351F mutant Cdr1p-expressing cells suggesting the importance of T1351 in the FK506 mediated synergy of fluconazole activity in Cdr1p-expressing cells (Figure 5a).

FK520 competes with fluconazole efflux

To further confirm the direct interaction of FK520 with Cdr1p and role of T1351 in this interaction, we checked if the accumulation of fluconazole could be competed out with immunosuppressant. For this, AD1-8u$^-$, PSCDR1-GFP and T1351F cells were incubated with or without FK520 (10 μM) with 100 nM of [^{3}H]fluconazole (20 Ci/mmol) and after 45 min of incubation, samples were removed for measurement of drug accumulation.[24] As shown in Figure 5(b), whereas AD1-8u$^-$ cells in the absence of FK520 accumulated 0.20 pmol of radiolabelled drug, PSCDR1-GFP cells showed 50% less accumulation of it (0.10 pmol). This is consistent with our model in which the efflux activity of PSCDR1-GFP cells expressing Cdr1p prevents accumulation of fluconazole as in control cells. Interestingly, whereas the presence of molar excess of FK520 did not show any significant effect on fluconazole accumulation in AD1-8u$^-$ cells, its presence increased the level of accumulation of the drug in Cdr1p-GFP expressing cells. These results demonstrate that FK520 can effectively compete with fluconazole and thus could directly interact with Cdr1p. This was further verified by the observation that the presence of a molar excess of FK520 did not have any effect on the residual fluconazole efflux ability of the mutant T1351F Cdr1p expressing cells (Figure 5b). These results conclusively prove that T1351 plays an important role in the FK520-mediated synergy of fluconazole.

T1351F cells are not susceptible to inhibition by FK520

In the following experiments, a direct interaction of Cdr1p with FK520 was again validated in the cells using the spot assay. Since the mutant T1351F cells were susceptible to fluconazole, we used a non-toxic concentration of it (2 mg/L) which allowed the mutant T1351F cells to grow in the presence of the drug. Under these conditions, one could see a synergy between

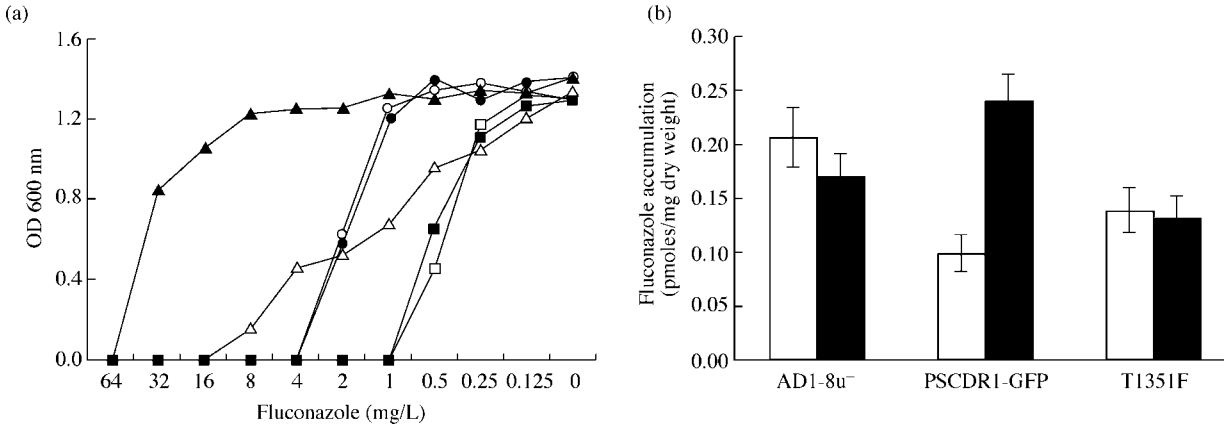


Figure. 5. FK506 inhibitory effect is Cdr1p specific: (a) Drug susceptibility of AD1-8u^{-}, PSCDR1-GFP and T1351F cells for fluconazole, in the presence and absence of FK520 (10 mg/L), was determined by microtitre assay.[24] The graph represents OD_{600} nm plotted against indicated concentration of fluconazole (filled squares, AD1-8u^{-}; open squares, AD1-8u^{-} + FK520; filled triangles, PSCDR1-GFP; open triangles, PSCDR1-GFP+FK520; filled circles, T1351F; open circles, T1351F+FK520). (b) Effect of FK520 on fluconazole accumulation in wild-type and mutant T1351F Cdr1p-GFP-expressing cells. The accumulation of fluconazole in AD1-8u^{-}, PSCDR1-GFP and T1351F cells was determined, in the presence (filled) and absence (open) of FK520 (10 μM), as described in Materials and methods. The values are given as the means±S.D. of three independent experiments.

FK520 and antifungal agents. Cdr1p-GFP expressing cells could not grow in non-toxic fluconazole concentration if FK520 was also present in the medium (Figure 4). However, the presence of the analogue FK520 prevented the drug-induced inhibition of growth of cells expressing mutant protein (Figure 4). Thus, the synergic ability of FK520 with drugs was severely reduced in the mutant T1351F cells (Figure 4a and b). To ensure that the reduced inhibitor susceptibility was because of T1351F mutation only and was not a generalized phenomenon, we checked the inhibitor susceptibility in another mutant, which has a mutation of F774A in the predicted TMD6 of Cdr1p.[16] Interestingly, F774A mutant behaved like wild-type Cdr1p-GFP-expressing cells and was susceptible to FK520 inhibition (Figure 4). Of note, several other mutant variants of Cdr1p further confirmed that the synergic effect of FK520 is mediated via T1351 of Cdr1p (data not shown).

Discussion

To understand the molecular mechanism of drug transport mediated by Cdr1p, we have recently generated several mutants of this major efflux protein.[16] In this study, we describe a mutant variant of Cdr1p where threonine 1351 of predicted TMD11 is replaced by phenylalanine (T1351F). We show that the T1351F mutant variant of Cdr1p becomes susceptible to anisomycin, cycloheximide, fluconazole, miconazole and nystatin even though the mutant protein was properly expressed, localized to the cell surface and displayed normal ATPase activity. The ability to export substrates like fluorescent dye rhodamine 6G was, however, significantly reduced in cells expressing the T1351 mutant variant protein. Thus it is very likely that the reduced efflux ability may contribute to observed enhanced drug sensitivity of T1351F cells. Of note, the hypersusceptible variant T1351F showed normal binding of the two photoaffinity analogues of drug substrates and ATP. In this context, it is difficult to point out from the available data, the reason behind reduced efflux and hypersusceptibility of T1351F cells. In view of the similar binding of photoaffinity analogues with T1351F protein variant, it is likely that the release of drugs from the binding site may be affected. A slower release of drug by T1351F protein would result in higher accumulation and hypersusceptibility phenotype. In a related study, Loo & Clarke[28] observed that the reduced resistance in a mutated version of human P-gp (a close homologue of Cdr1p), was accompanied by high affinity of the drug analogue for the protein. The relative reduction in drug resistance of the mutant protein was linked to the impairment of release of the drug during the transport cycle.[28]

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.[2–5] Recently, it was observed that the protein phosphatase calcineurin allows survival of *C. albicans* during membrane stress exerted by azoles.[29] The calcineurin inhibitors ciclosporin A (CsA) and tacrolimus (FK506) exhibit fungicidal synergy with azoles in *C. albicans*, *C. glabrata*, *C. krusei* and in *S. cervisiae*.[22,27] In this study, we observed that a T1351F mutant variant of Cdr1p exhibited abrogated synergy of FK520 (a structural analogue of FK506) with fluconazole, ketoconazole and cycloheximide. The fact that the other variants of Cdr1ps, which have substitution mutations in different domains spanning the entire protein molecule, remained synergically susceptible to FK520 suggests that T1351 of predicted TMD11 specifically contributes to this synergy. Since antifungal agents of varied structures including azole 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 blocking the pump activity directly.[19,21,30] The inhibition of fluconazole efflux by FK520 suggests such a possibility of a direct interaction of FK520 with Cdr1p (Figure 5b). That FK520 synergy is mediated at least in part by its interaction with Cdr1p is suggested from the set of observations: 1) T1351 replacement abrogates synergy, 2) FK520 is ineffective in cells not expressing Cdr1p, 3) FK520 show synergy with other substrates of Cdr1p, and 4) FK520 competes with fluconazole efflux. Recently, Raymond's group has also shown that Cdr1p can

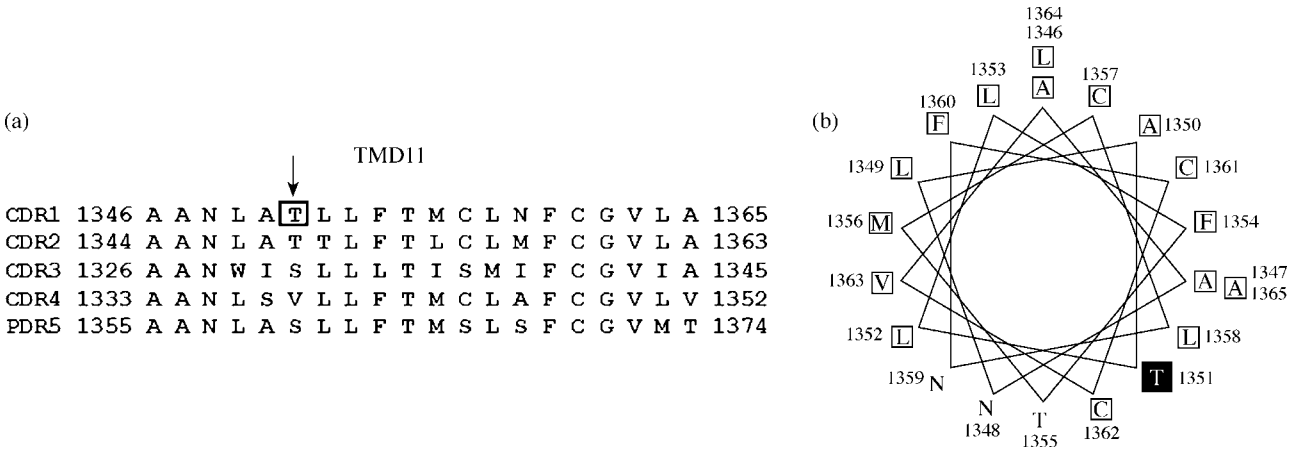


Figure. 6. The sequence alignment and helical wheel projection of the predicted TMD11 of Cdr1p with Cdr2p, Cdr3p, Cdr4p and Pdr5p. (a) Sequence alignment of predicted TMD11 of Cdr1p. The amino acids are numbered according to their positions in the proteins. The position of the mutated residue in Cdr1p with respect to other proteins is marked with an arrow and the residue mutated in Cdr1p is boxed. (b) Transmembrane domains of the amino acid sequence of Cdr1p were determined with the program HMMTOP.[38,39] The 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.[40] The hydrophobic amino acids are shown with open squares. The amino acid T1351, mutated to F, is shown by a white letter on a black background.

affect cell tolerance to FK520 and suggested a possible involvement of the transporter in the synergy between azoles and FK520 in *C. albicans*.[31] However, Marchetti *et al.*[32] in a report appearing simultaneously suggested that the fungicidal synergy of fluconazole with ciclosporin may not be dependent on the MDR transporters of *C. albicans*. It should be pointed out that Gauthier *et al.*[31] recently observed that the Cdr1p-expressing cells were susceptible to FK520 (50 and 70 mg/L) whereas PSCDR1-GFP cells in this study showed resistance at similar concentrations. This variation could be due to difference in the level of Cdr1p activity. The Cdr1p-expressing TY310 cells in their study showed only moderate resistance to fluconazole (25 mg/L),[31] compared to our observations (64 mg/L). Additionally, different host backgrounds could also contribute to such differences. Since azoles are actively exported by ABC transporters such as Cdr1p and Cdr2p,[14,15,33] a direct inhibitory effect of the immunosuppressants on these transporters cannot be ruled out. In fact, Egner and colleagues[17,18] have shown that the replacement of S1360 of Pdr5p (homologue of Cdr1p) to phenylalanine resulted in the loss of the synergy of FK506 with fluconazole. Further if serine was changed to alanine, it resulted in the hypersensitivity to FK506. Their observations suggested a direct role for S1360 in mediating the synergy of fluconazole activity with FK506 in Pdr5p. Of note, S1360 of Pdr5p is equivalent to T1351 of Cdr1p in its position and location. It should be pointed out that T1351 of Cdr1p[34] was earlier predicted to be in cytoplasmic loop 5 based on predictions described by Eisenberg *et al.*[35] We made a TMD prediction of Cdr1p as was done earlier for Pdr5p sequences by PHDsec algorithm and found that both T1351 of Cdr1p and S1360 of Pdr5p are present in TMD11.[36,37] The sequence alignment of TMD11 of various ABC transporters of *C. albicans* and their comparison with Pdr5p reveals that instead of the serine residue which is present in Pdr5p, the major drug transporters of *C. albicans* (Cdr1p and Cdr2p) possess threonine (Figure 6a). When TMD11 of Cdr1p was looked at in a helical wheel projection, it revealed an amphipathic structure with a hydrophilic and a hydrophobic side where T1351 lies near the boundary of the two faces of the putative helix (Figure 6b). The importance of T1351 in the drug as well as inhibitor susceptibility observed in this study is evident from its placement in 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 whereas the hydrophilic face may interact with other transmembrane 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.

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