Results
3 Results

The *Candida* drug resistance protein (Cdr1p), a member of the ATP-binding cassette (ABC) super family, causes multidrug resistance by an active efflux mechanism, which keeps the intracellular level of antimycotic compounds such as azoles below a cell-killing threshold. Similar to other well-characterized multidrug ABC transporters, Cdr1p (~170kDa) comprises of two NBDs and 12 TMS. NBDs are the hub of ATP hydrolysis activity and are considered to be critical for ABC protein mediated drug efflux. In an attempt to functionally characterize the Cdr1p in terms of drug and nucleotide binding and the importance of domains in drug interactions, in this study we have overexpressed Cdr1p as a GFP tagged protein. The functional characterization of wild type Cdr1p-GFP and of the mutant variants generated by site directed mutagenesis are the subject of the following experiments.

3.1 Expression of Cdr1p in hypersensitive *S. cerevisiae* host

Earlier, A. Goffeau’s group constructed a *S. cerevisiae* strain (AD1234568) deleted in seven major ABC drug transporters (*pdr5Δ, snq2Δ, pdr10Δ, pdr11Δ, pdr15Δ, yor1Δ, ycf1Δ*) (Pleiotropic drug resistance 5, 10, 11, 15, 4-Nitro-quinoline oxide resistance, Yeast oligomycin resistance 1 and Yeast cadmium factor 1) (Decottignies et al. 1998). As a result, this disrupted AD1234568 strain turned hypersensitive to several tested drugs. The deleted mutant strain because of minimum interference by its own ABC transporters, has already proven to be an ideal host to express *C. albicans* ABC drug transporter *CDR1* gene which is used in this study (discussed below). This strain AD1234568 was transformed by a plasmid construct (pS12-35) which carried *CDR1* gene with its native promoter in a centromeric vector (pYEURA3) (Prasad et al. 1995) as described in
Results

'Materials and Methods'. The positive transformants were selected by their ability to grow in SD-URA media. Southern hybridization was done to confirm the presence of CDR1 by using a specific probe for it (data not shown). The transformants harboring CDR1 were designated as AD-CDR1. AD1234568 was also transformed with vector (pYEURA3) alone and such transformants designated as S6-20 cells.

In order to check the expression of CDR1 encoded protein (Cdr1p), purified PM were prepared from both AD-CDR1 and S6-20 cells. Cdr1p was detected in these proteins by Western analysis using anti-Cdr1p antibody. A protein band of approximately 170 kDa, corresponding to Cdr1p was observed only in AD-CDR1 cells which was distinctly absent in PM protein isolated from S6-20 cells (data not shown).

3.2 Expression of Cdr1p makes S. cerevisiae hyper-resistant to cytotoxic drugs

In a spot assay, it was observed that the cells expressing Cdr1p (AD-CDR1) grew in presence of fluconazole (2 µg/ml), cycloheximide (20 ng/ml), miconazole (250 ng/ml), nystatin (500 ng/ml) while S6-20 showed reduced or no growth (Figure 16). The demonstration of a hyper-resistant phenotype by the cells expressing Cdr1p indicated that the protein, which was expressed in S. cerevisiae, was functionally active and imparted drug resistance to the cells.
Figure 16: Drug resistance of *S. cerevisiae* cells expressing Cdr1p: Drug resistance was determined by the spot assay essentially as described in 'Materials and methods'. Briefly, S6-20 or AD-CDR1 cells were grown overnight on SD-URA plates at 30°C. The cells were then resuspended in normal saline to an A$_{600}$ of 0.1. Five microlitres of fivefold serial dilutions of each strain was spotted onto SD-URA plates in the absence (growth control) or in presence of the following drugs; (1) anisomycin (1 mg/ml), (2) cycloheximide (20 ng/ml), (3) fluconazole (2 mg/ml), (4) miconazole (250 ng/ml), (5) nystatin (500 ng/ml), (6) growth control. Growth differences were recorded following incubation of the plates for 48 hrs at 30°C.
3.3 Characterization of ATPase activity and transport functions of Cdr1p

3.3.1 ATPase activity

Functional Cdr1p should be able to hydrolyze ATP and this prompted us to check the ATPase activity of Cdr1p in the PM isolated from AD-CDR1 cells. Yeast PM also have PM-ATPase, which utilizes the energy derived from ATP hydrolysis for H+ transport. In order to minimize interference of PM-ATPase, we exploited the selectivity of oligomycin, which has been shown to be a specific inhibitor of ATPase of Pdr5p and Cdr1p (Decottignies et al. 1994; Wada et al. 2002; Nakamura et al. 2002). The difference in the ATPase activity, in the presence and absence of 20 μM oligomycin, for S6-20 and AD-CDR1 cells are shown in Figure 17 a. The purified PM of AD-CDR1 showed oligomycin sensitive ATPase activity, which was almost 3.5-folds higher than the activity of the PM protein isolated from the control S6-20 cells (Figure 17 a).

3.3.2 [³H]-fluconazole and rhodamine-6G transport

The enhanced efflux of drugs mediated by Cdr1p could be the main reason for AD-CDR1 cells to grow in the presence of drugs (Figure 16). This possibility was examined by assaying accumulation of radiolabeled fluconazole and by monitoring the efflux of fluorescent rhodamine 6G. Both these compounds are good substrates of Cdr1p (Maesaki et al. 1999; Smriti et al. 1999; Krishnamurthy et al. 1998a). The amount of [³H]-fluconazole (0.7 TBq/mmole) accumulated after 60 min, was checked by measuring the radioactivity retained within the cells. Expectedly because of increased efflux activity due to Cdr1p, AD-CDR1 cells showed 60% reduction in fluconazole accumulation as compared to S6-20 cells (Figure 17 b). The low intracellular
Figure 17: (a) Oligomycin-sensitive Cdr1p ATPase activity: PM protein (10 μg) isolated from S. cerevisiae S6-20 or AD-CDR1 cells were used for ATPase assay which was carried out at 30°C for 10 min as described in ‘Materials and methods’. The oligomycin sensitive activity was determined as the difference in the ATPase activity in the presence or absence of 20 μM oligomycin. The results are the means ± standard deviations of three independent experiments.

(b) [³H]-Fluconazole accumulation in S. cerevisiae cells: S6-20 and AD-CDR1 cells from mid log phase were used for fluconazole accumulation as described in ‘Materials and methods’. The accumulation is expressed as pmoles/mg dry weight. The values are mean of three independent experiments ± standard deviation.
accumulation of drug in AD-CDR1 cells implies rapid efflux of fluconazole mediated by Cdr1p, unlike the S6-20 cells, which lacked this transporter.

We compared the ability of AD-CDR1 cells to efflux another substrate rhodamine 6G with S6-20 (Maesaki et al. 1999). In order to enable cells to equilibrate rhodamine 6G in the absence of energy source, 10 μM of fluorescent rhodamine 6G was incubated with Cdr1p expressing and control cells in the absence of glucose for 2 h. Efflux was initiated by the addition of 2% glucose. An aliquot was taken after 45 minutes and the absorbance of the supernatant was measured at A527. As shown in Figure 18 a, the supernatant of AD-CDR1 cells showed a 1.51 nmoles rhodamine 6G/ml while S6-20 showed 0.246 nmoles rhodamine 6G/ml suggesting that AD-CDR1 cells effluxed seven folds more of rhodamine 6G as compared to the control cells.

3.4 Cdr1p translocates membrane phospholipid
Cdr1p translocates aminophospholipids from the inner monolayer of the PM to the outer monolayer (Dogra et al. 1999). In order to confirm the Cdr1p associated phospholipid translocation, fluorescamine, a fluorescent dye, which specifically labels exposed amino phospholipids (Diaz and Schroit 1996; Dogra et al. 1999), was used for labeling of AD-CDR1 and S6-20 cells. The percentage of fluorescamine labeled phosphatidylethanolamine (Ptd-Etn) was determined by isolating the membrane lipids as described in ‘Materials and Methods’. It was observed (Figure 18 b) that AD-CDR1 had 23% labeled Ptd-Etn whereas S6-20 cells had only 12.8% exposed Ptd-Etn. This showed that the expression of Cdr1p was linked to Ptd-Etn translocation, which was another evidence for the functional activity of Cdr1p, expressed in the S. cerevisiae cells.
Figure 18: (a) Rhodamine-6G efflux from *S. cerevisiae* cells: Approximately $10^7$ cells from an overnight culture of S6-20 and AD-CDR1 cells were inoculated in 250 ml of YEPD and grown for 5h at 30°C. The cells were pelleted and washed three times with phosphate buffered saline without glucose and used for Rhodamine-6G efflux assay as described in 'Materials and methods'. The values are mean of four independent experiments ± standard deviation.

(b) Percentage labeling of PE with fluorescamine in the PM of *S. cerevisiae* cells: S6-20 or AD-CDR1 cells were labeled with fluorescamine as described in 'Materials and methods'. The results presented here are the mean of three independent experiments ± standard deviation.
Results

Taken together by employing various functional parameters, we could show that the Cdr1p remained fully functional when expressed in a plasmid based expression in a heterologous hypersensitive *S. cerevisiae* AD1234568 host.

### 3.5 Overexpressed Cdr1p-GFP is fully functional

In order to confirm the plasma membrane localization of Cdr1p and for biochemical characterization, we used an expression system developed by Nakamura *et al* which was a generous gift from R. Cannon, New Zealand (Nakamura *et al.* 2002), where Cdr1p was stably overexpressed from a genomic *PDR5* locus in an *S. cerevisiae* mutant AD1-8u-. 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. The high level expression of Cdr1p was obtained by integration of the *CDR1* ORF to the *PDR5* promoter in AD1-8u- cells and the resulting strain was designated as AD1002 (Nakamura *et al.* 2002). We tagged green fluorescent protein (GFP) gene at the C-terminal end of *CDR1*, which was overexpressed as a fusion protein in PSCDR1-GFP strain (Figure 19). A single copy integration of GFP tagged *CDR1* was ensured by Southern hybridization of restricted genomic DNA by *CDR1* specific probe as described earlier (Nakamura *et al.* 2002) (data not shown).

The presence of higher amounts of Cdr1p and Cdr1p-GFP in the purified PM of AD1002 and PSCDR1-GFP cells could be detected on SDS-PAGE gel when stained by Coomassie brilliant blue G250 dye (Figure 20 a), which was further, confirmed by western blot analysis of the PM protein using Cdr1p polyclonal antibody. Figure 20 b confirms that the Cdr1p and Cdr1p-GFP were expressed in PM to the
Gain of function *Pdr1* & *Pdr3* mutation

Overexpressing PDR5 promoter

| Overexpressing PDR5 promoter | CDR1 ORF | GFP ORF |

Overexpression of *CDR1-GFP*

**Figure 19:** Cartoon showing overexpression cassette of Cdr1p-GFP: A gain of function mutation in the transcription factor *PDR1* and *PDR3*, resulted in a constitutive hyperinduction of the *PDR5* promoter. *CDR1* with a GFP tag at the C-terminal end was integrated under this promoter, which resulted in the overexpression of Cdr1p-GFP.
Figure 20 (a) and (b): Expression of Cdr1p and Cdr1p-GFP in *S. cerevisiae* AD1-8u: PM (20 μg) protein from AD1-8u-, AD1002 and PSCDR1-GFP were separated on 8% SDS-PAGE gel (a) stained by colloidal coomassie blue G-250 (b) electroblotted on to nitrocellulose, and incubated with rabbit polyclonal anti-Cdr1p antibodies (1:500 dilution). Antibodies were detected with HRP labeled anti-rabbit immunoglobulin using ECL kit.
same levels. There was an expected ~30 kDa difference in the observed molecular weight of Cdr1p-GFP compared to Cdr1p because of the GFP tag attached at the C-terminal end of Cdr1p. The intrinsic fluorescence of GFP imparted to the fusion protein also enabled us to detect the wild type and mutants of Cdr1p in live cells. Cdr1p-GFP expression at the cell periphery also confirmed that the GFP tagging of Cdr1p did not interfere with its localization (Figure 21). The expression of Cdr1p-GFP was further confirmed by FACS analysis where Cdr1p-GFP variant cells showed enhanced fluorescence intensity (Figure 22 a and 22 b).

We ensured that GFP tagging and over expression did not impair functional activity by analyzing drug susceptibilities of cells harboring CDR1 or the GFP-tagged variant. It was found that the cells expressing Cdr1p or Cdr1p-GFP cells imparted similar level of resistance to the tested drugs (Figure 23 a). The purified PM from PSCDR1-GFP containing Cdr1p-GFP also showed oligomycin-sensitive ATPase activity which was almost 3-fold higher, and comparable to the activity of overexpressed Cdr1p, than the activity of the PM protein isolated from the AD 1-8u' control cells (Figure 23 b). Taken together, it was thus established that overexpressing Cdr1p and its GFP tagged variant is properly localized and functional. In the following experiments these proteins were further examined for drug and nucleotide binding properties.

3.6 Photoaffinity analogues of ATP and drug-substrates bind to Cdr1p

Cdr1p utilizes ATP as an energy source by catalyzing its hydrolysis and coupling the energy released to the efflux of the drugs. Therefore, alternatively the binding of ATP to this protein can also be
Figure 21: Confocal microscopy of Cdr1p-GFP expressing cells: AD1-8u⁻ and PSCDR1-GFP cells were grown in SD-URA media to late log phase. The cells were washed and resuspended in appropriate volume of 0.5 mM HEPES pH 7.0. The cells were directly viewed, on a glass slide with a drop of antifade reagent to prevent photobleaching, with 100X oil immersion objective on a Biorad confocal microscope (MRC 1024).
Figure 22: (a) and (b) Flow Cytometry of cells expressing Cdr1p-GFP: AD1-8u- and PSCDR1-GFP cells were grown to mid log phase and used for FACS analysis as detailed in ‘Materials and methods’. Analysis was performed with CellQuest software. (a) The number of cells were plotted on a histogram with the green fluorescence plotted on a log scale and the mean fluorescence intensity was calculated. (b) The mean fluorescence intensity plotted on a histogram showing GFP fluorescence in AD1-8u- (control) and PSCDR1-GFP cells.
Figure 23: (a) The drug resistance profile of AD1-8u-, PSCDR1-GFP and AD1002 cells: The MIC$_{80}$ (μg/ml) of AD1-8u-, PSCDR1-GFP (Cdr1p-GFP) and AD1002 (wild type Cdr1p) cells for the indicated drugs, anisomycin (Aniso), cycloheximide (Cyh), fluconazole (Flu), miconazole (Mic) and nystatin (Nys), was determined as described in 'Materials and methods'. The results are typical of one determination, which was confirmed by three independent experiments.

(b) Oligomycin-sensitive Cdr1p ATPase activity in purified plasma membranes from AD1-8u-, PSCDR1-GFP (Cdr1p-GFP) and AD1002 (wild type Cdr1p) cells: ATPase assay were carried out using the PM protein (100 μg/ml) of AD1-8u-, AD1002, PSCDR1-GFP cells at 30°C for 10 min as described in 'Materials and methods'. The oligomycin sensitive activity was determined as the difference in the ATPase activity in the presence or absence of 20 μM oligomycin. The values are given as the means ± SD of three independent experiments.
Results

checked as a means of determining the functionality of this protein. The photoaffinity analogue, \( [\alpha^{32P}] \) 8-azidoATP has been used routinely for studying the interactions of nucleotide with the human P-gp, MRP1 and with other ABC transporters proteins (Booth et al. 2000; Dey et al. 1998; Sauna and Ambudkar 2001). The binding of \( [\alpha^{32P}] \) 8-azidoATP to Cdr1p was examined to see the interactions of ATP with the protein as detailed in 'Materials and Methods'. Figure 24 shows that the incubation of purified PM protein (15 μg) from AD1-8u’, AD1002 and PSCDR1-GFP with 10 μM \( [\alpha^{32P}] \) 8-azidoATP (10 μCi/nmole) followed by ultraviolet irradiation led to covalent incorporation of this analogue (Figure 24 lane 3). The labeling was competed out by 1000-fold excess of ATP (10 mM) confirming that the observed binding was specific (Figure 24 lane 4). The binding of \( [\alpha^{32P}] \) 8-azidoATP to Cdr1p-GFP variant protein was similar to native Cdr1p, which was also competed out by excess ATP (Figure 24 lane 5 and 6). The control PM from AD1-8u’ cells did not show any band at the corresponding position (Figure 24 lane 1 and 2). A Western blot of the gel confirmed that the observed bands, which cross-linked with 8-azidoATP were Cdr1p specific (data not shown). The binding of 8-azido-ATP analogue confirmed that the Cdr1p or its variant Cdr1p-GFP overexpressed in this heterologous system is functionally active in terms of its ability to bind ATP.

In order to monitor drug binding sites on human P-gp radiolabeled photoactive analogs of prazosin and dihydropyridine have been successfully used (Greenberger 1993; Bruggemann et al. 1989). Such studies have revealed two major areas, one on each homologous halves of the protein, as primary sites of drug interaction (Bruggemann et al. 1989). In this study, we used IAAP for analyzing interactions with Cdr1p. For this 15 μg of purified PM protein from
Figure 24: Binding of [α-32P] 8-azidoATP to Cdr1p and Cdr1p-GFP:
The PM (15 μg) protein were photoaffinity labeled with 10 μM [α-32P] 8-
azidoATP (10 μCi/nmole) under subdued light as described in 'Materials and methods'. Ice cold ATP (10 mM) was added to displace excess non-covalently bound [α-32P] wherever indicated. Lane 1: AD1-8u- PM, lane 2: AD1-8u- PM + 10 mM ATP, lane 3: AD1002 PM, lane 4: AD1002 PM + 10 mM ATP, lane 5: PSCDR1-GFP PM, Lane 6: PSCDR1-GFP PM + 10 mM ATP.
Results

AD1-8u', AD1002 and PSCDR1-GFP was incubated with 3-6 nM $^{125}$I-labeled IAAP at room temperature for 5 minutes followed by UV cross linking as detailed in 'Materials and Methods'. Figure 25 (lane 2) shows that while IAAP specifically labeled Cdr1p, there was no binding of it to the PM prepared from control AD1-8u' cells (Figure 25 lane 1). Cdr1p-GFP variant PM showed similar binding of IAAP as native Cdr1p (Figure 25, lane 3). In order to test whether the IAAP binding was specific, we carried out the binding with Cdr1p-GFP in the presence of molar excess of cycloheximide, nystatin, anisomycin and miconazole. Interestingly, IAAP was competed out by nystatin (100 μM), a known substrate of Cdr1p (Figure 26, lane 4) while the binding remained unaffected by the presence of the other drugs (Figure 26 lane 3, 5, 6). The competition of IAAP labeling by nystatin was concentration-dependent with a K_i of 5.9 μM (Figure 27).

Azidopine, a dihydropyridine photoaffinity analog, has been shown to bind specifically to mammalian drug transporting P-gps (Safa 1988; Kajiji et al. 1993; Loo and Clarke 1993; Loo and Clarke 1994c) and is a valuable tool used in determining the substrate binding sites on these multidrug transporters. We explored this possibility by examining the ability of azidopine to bind to Cdr1p. The PM protein (30 μg) from AD1-8u', AD1002 and PSCDR1-GFP was incubated with 0.5 μM $[^3]$H-azidopine for 5 minutes at room temperature and then photo-crosslinked. The incorporation of radioactivity in protein bands corresponding to Cdr1p and Cdr1p-GFP showed similar intensity implying that $[^3]$H-azidopine could bind to both proteins (Figure 28). The specificity of binding was assessed by the addition of 100 μM each of cycloheximide, nystatin, anisomycin or miconazole. Interestingly, $[^3]$H-azidopine binding to Cdr1p-GFP was not inhibited by nystatin as was the case with IAAP binding, but was...
Figure 25: $[^{125}\text{I}]-\text{IAAP}$ binding to Cdr1p and Cdr1p-GFP: Binding of $[^{125}\text{I}]-\text{IAAP}$ to Cdr1p and Cdr1p-GFP: The PM (15 μg) protein from AD1-8u (Lane 1), AD1002 (lane 2), and PSCDR1-GFP (lane 3) were incubated with 3-6nM $[^{125}\text{I}]-\text{IAAP}$ (2200 Ci/mmol) for 5 min under subdued light and were processed as described in 'Materials and methods'.
Figure 26: Effect of Cdr1p substrates on the binding of IAAP to Cdr1p-GFP: The PM (15 μg) protein from AD1-8u (control) (lane 1) or PSCDR1-GFP were incubated with the 100 μM of different drug substrates as indicated (lane 2: no drug, lane 3: cycloheximide, lane 4: nystatin, lane 5: anisomycin, lane 6: miconazole) for 10 min at 37°C in 50 mM Tris-HCl, pH 7.5. The samples were brought to room temperature and 3-6 nM [125I]-IAAP (2200 Ci/mmol) was added and incubated for an additional 5 min under subdued light. The samples were then illuminated with a UV lamp and were processed as described in ‘Materials and methods’.
Figure 27: Determination of $K_i$ of nystatin for IAAP binding to Cdr1p-GFP: The PM (15 µg) protein of PSCDR1-GFP were incubated with the increasing concentration (0.25-50 µM) of nystatin for 10 min at 37°C in 50 mM Tris-HCl, pH 7.5. The samples were brought to room temperature and 3-6 nM $[^{125}]$-IAAP (2200 Ci/mmol) was added and incubated for an additional 5 min under subdued light. The samples were processed and the radioactivity incorporated into the Cdr1p band was quantified as described in 'Materials and methods'. The data were fitted using the software GRAPHPAD PRISM 2.0 for the PowerPC Macintosh and are representative of three independent experiments.
Figure 28: Photoaffinity labeling of Cdr1p and Cdr1p-GFP with [³H]-azidopine: The PM (30 µg) protein from AD1-8u⁻ (lane 1), AD1002 (lane 2), PSCDR1-GFP (lane 3) were incubated with 0.5 µM [³H]-azidopine (60 Ci/mmol) for 5 min under subdued light. The samples were processed and analyzed as described in ‘Materials and methods’.
Results

instead found to be competed out by miconazole. (Figure 29). This inhibition of $[^3]H$-azidopine binding to Cdr1p-GFP by miconazole was concentration-dependent ($K_i = 8.7 \mu M$ see Figure 30).

3.7 Construction and expression of mutant variants of Cdr1p

A multiple sequence alignment of human P-gp, the ABC drug transporter of S. cerevisiae, Pdr5p and of C. albicans Cdr1p, Cdr2p, Cdr3p and Cdr4p, highlights the conservation of certain amino acid residues within TMS and NBDs which may have a role in the functioning of these transporters (Figure 31). Considering the functional importance of amino acid residues determined for other ABC drug transporters particularly of human P-gp and Pdr5p of S. cerevisiae, in this study we selected the equivalent of most homologous or conserved residues of Cdr1p for point mutations (Figure 32) as indicated. This was achieved by either replacing a conserved residue by another amino acid such as in SS1, SS2, SS3, SS4, SS5, SS7, SS8, SS9, SS10, SS11, SS12, SS13, SS14, SS15, SS16, SS17, SS18 or by deleting the residue altogether as in SS6. In total eighteen mutations (SS1-SS18) were introduced in the different predicted domains spanning across entire Cdr1p length, which are highlighted in, Figure 32.

Initially, all the mutations were introduced in CDR1 gene driven by its native promoter cloned in non-integrative pYEURA3 plasmid (pS12-35) (11) The hypersensitive AD 1234568 cells were then transformed with the mutated plasmids and the positive clones were selected by their ability to grow on SD-URA plates. The positive transformants were further confirmed by Southern hybridization (Figure 33). At least two positive clones of every mutant were selected for initial screening to rule out clonal variations.
Figure 29: Effect of Cdr1p substrates on the binding of [³H]-azidopine to Cdr1p-GFP: The PM (30 µg) protein of AD1-8u (control) (lane 1) or PSCDR1-GFP were incubated with the 100µM drug substrates as indicated (lane 2: no drug, lane 3: cycloheximide, lane 4: nystatin, lane 5: anisomycin, lane 6: miconazole) for 10 min at 37°C in 50 mM Tris-HCl, pH 7.5. The samples were brought to room temperature and 0.5 µM [³H]-azidopine (60 Ci/mmole) was added and incubated for an additional 5 min and the samples were processed as described in 'Materials and methods'.
Figure 30: Determination of $K_i$ of miconazole for $[^3H]$-azidopine binding to Cdr1p-GFP: The PM (30 μg) from PSCDR1-GFP were incubated with the increasing concentration (0.25-100 μM) of miconazole for 10 min at 37°C. The samples were brought to room temperature and 0.5 μM $[^3H]$-azidopine (60 Ci/mmole) was added and incubated for an additional 5 min. The samples were processed and quantitated as described in ‘Materials and methods’. The data were fitted using the software GRAPHPAD PRISM 2.0 for the PowerPC macintosh and are representative of three independent experiments.
**Figure 31: Alignment of protein sequences of various domains:** The alignment of the highly conserved regions NBDs, TMS 6, extracellular loop 6 and cytoplasmic loop 6 of human Mdr1, Cdr1, Cdr2, Cdr3, Cdr4 and Pdr5 proteins. The amino acids are numbered according to their positions in the proteins. The position of mutated residues in Cdr1p with respect to other proteins is marked with arrows and the residues mutated in Cdr1p are boxed.
Figure 33: Southern blot analysis of mutant Cdr1p expressing AD1234568 cells: The plasmid DNA from SS1-SS18 (lane 2-lane 20) cells was isolated and checked for the presence of CDR1 and its mutant variants as described in ‘Materials and methods’. Plasmid DNA from AD-CDR1 (lane 1) and S6-20 (lane 2) were used as positive and negative control respectively.
3.8 Drug resistance profile of Mutant Cdr1ps

Confirmed positive mutants denoted SS1-SS18 were screened for their sensitivity to drugs by determining their MIC₈₀ values (Figure 35) and also by spot test (Figure 34) analysis. The mutants were classified into three groups according to their sensitivity to the tested drugs. Substitutions like D232K (SS10) and T1351F (SS15) and deletion of phenylalanine, ΔF774, (SS6), resulted in a general drug hypersensitive phenotype towards all the tested drugs while strains with mutations K309E (SS1), R308S (SS2), G305A (SS3), G305V (SS4) F774A (SS5), G296D (SS11), G995S (SS12) and C1418Y (SS16) were selectively sensitive to one or more drugs when compared to the wild type. Mutants like V773A (SS7), V773I (SS8), D232E (SS9), G998S (SS13), G1000C (SS14), T1449I (SS17) and V1456I (SS18) retained hyper resistant phenotype comparable to wild type Cdr1p expressing cells (AD-CDR1).

In order to ascertain if the introduced mutation in Cdr1p and the observed altered drug susceptibilities therein are not due to poor expression or localization of the protein, we analyzed its expression in PM protein by immunoblotting. It was observed that all the mutants except ΔF774 (SS6) (discussed below) expressed the mutant variants of Cdr1ps to the same level (data not shown).

As shown in Figure 34 and 35, except for nystatin, the replacement of phenylalanine by alanine (F774A) in SS5 resulted only in marginal alterations in drug susceptibilities (Figure 35) when compared with MIC₈₀ values of wild type. The Cdr1p harboring ΔF774 variant in SS6 and T1351F in SS15 rendered hypersensitivity to all the tested drugs.
### Figure 34: Drug resistance profile of wild type and mutant CDR1 strains determined by the spot assay:

S6-20 or AD-CDR1 cells or the Cdr1p mutants (SS1-SS18) created by site directed mutagenesis were grown overnight on SD-URA 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 on to SD-URA plates in the absence (control) and presence of the following drugs, anisomycin (Aniso) (1 mg/ml), cycloheximide (Cyclo) (20 ng/ml), fluconazole (Flu) (2 mg/ml), miconazole (Mic) (250 ng/ml), nystatin (Nys) (500 ng/ml). Growth differences were recorded following incubation of the plates for 48 hrs at 30°C. Growth was not affected by the presence of the solvents used for the drugs.
3.9 Site directed mutagenesis in the overexpressed Cdr1p

In order to understand the molecular basis of observed phenotypes of these three variant mutant Cdr1ps expressing cells SS5 (F774A), SS6 (ΔF774) and SS15 (T1351F), they were analyzed further in greater detail. For this, same mutations were introduced in the overexpressing Cdr1p-GFP plasmids (pPSCDR1-GFP). The mutated plasmids were integrated in *S. cerevisiae* (AD1-8u) as described above. Southern hybridization was done using CDR1 specific probe to ensure that the gene was inserted as a single copy at the genomic PDR5 locus (data not shown). The new overexpressing strains harboring mutant Cdr1p-GFP were designated as SS5G (F774A), SS6G (ΔF774) and SS15G (T1351F).

3.10 Functional analysis of SS5G (F774A), SS6G (ΔF774) and SS15G (T1351F)

3.10.1 Drug susceptibility of SS5G (F774A), SS6G (ΔF774) and SS15G (T1351F)

The cells overexpressing GFP tagged Cdr1p variants were checked for their sensitivity to drugs. As shown in Figure 36, the SS6G (ΔF774) and SS15G (T1351F) were hypersensitive to the drugs while SS5G (F774A) showed marginal sensitivity to the tested drugs. These results were essentially similar to what we observed with cells expressing variant of Cdr1ps driven by its native promoter (Figure 34 and 35).

3.10.2 ATPase activity of Mutant Cdr1p-GFPs

The purified PM protein from SS5G (F774A), SS6G (ΔF774) and SS15G (T1351F) were analyzed for their oligomycin sensitive ATPase activity. Of note the oligomycin sensitive ATPase activity was higher in
**Drug susceptibility of wild type and mutant CDR1 strains determined by Microtitre assay**

<table>
<thead>
<tr>
<th></th>
<th>Aniso</th>
<th>Cyh</th>
<th>Flu</th>
<th>Mic</th>
<th>Nys</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS1 (K309E)</td>
<td>8</td>
<td>0.15</td>
<td>16</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>SS2 (R308S)</td>
<td>16</td>
<td>0.075</td>
<td>16</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>SS3 (G305A)</td>
<td>16</td>
<td>0.15</td>
<td>32</td>
<td>4</td>
<td>0.25</td>
</tr>
<tr>
<td>SS4 (G305V)</td>
<td>8</td>
<td>0.15</td>
<td>32</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>SS5 (F774A)</td>
<td>16</td>
<td>0.037</td>
<td>8</td>
<td>4</td>
<td>0.12</td>
</tr>
<tr>
<td>SS6 (ΔF774)</td>
<td>4</td>
<td>0.018</td>
<td>4</td>
<td>0.5</td>
<td>0.03</td>
</tr>
<tr>
<td>SS7 (V773A)</td>
<td>16</td>
<td>0.15</td>
<td>32</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>SS8 (V773I)</td>
<td>16</td>
<td>0.15</td>
<td>32</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>SS9 (D232E)</td>
<td>16</td>
<td>0.15</td>
<td>64</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>SS10 (D232K)</td>
<td>2</td>
<td>0.018</td>
<td>4</td>
<td>1</td>
<td>0.03</td>
</tr>
<tr>
<td>SS11 (G296D)</td>
<td>8</td>
<td>0.037</td>
<td>8</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>SS12 (G995S)</td>
<td>8</td>
<td>0.037</td>
<td>8</td>
<td>8</td>
<td>0.5</td>
</tr>
<tr>
<td>SS13 (G998S)</td>
<td>32</td>
<td>0.075</td>
<td>64</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td>SS14 (G1000C)</td>
<td>16</td>
<td>0.075</td>
<td>128</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>SS15 (T1351F)</td>
<td>0.5</td>
<td>0.008</td>
<td>8</td>
<td>0.12</td>
<td>0.03</td>
</tr>
<tr>
<td>SS16 (C1418Y)</td>
<td>32</td>
<td>0.15</td>
<td>64</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SS17 (T1449I)</td>
<td>8</td>
<td>0.15</td>
<td>64</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>SS18 (V1456I)</td>
<td>16</td>
<td>0.075</td>
<td>64</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>S6-20</td>
<td>1</td>
<td>0.004</td>
<td>2</td>
<td>0.25</td>
<td>0.015</td>
</tr>
<tr>
<td>AD-CDR1</td>
<td>32</td>
<td>0.3</td>
<td>64</td>
<td>8</td>
<td>1</td>
</tr>
</tbody>
</table>

**Figure 35: Drug resistance profile of wild type and mutant CDR1 strains determined by MIC assay:** The MIC
so (~g/ml) of S6-20 or AD-CDR1 cells or the CDR1 mutants (SS1-SS18) for the drugs indicated was determined as described in 'Materials and methods'. The results are typical of one determination of three independent experiments.
### Drug susceptibility of AD1-8u-, PSCDR1-GFP, SS5G, SS6G and SS15G cells

<table>
<thead>
<tr>
<th></th>
<th>Aniso</th>
<th>Cyh</th>
<th>Flu</th>
<th>Mic</th>
<th>Nys</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD1-8u-</td>
<td>0.12</td>
<td>0.015</td>
<td>1</td>
<td>0.015</td>
<td>0.03</td>
</tr>
<tr>
<td>PSCDR1-GFP</td>
<td>16</td>
<td>1</td>
<td>64</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>SS5G</td>
<td>8</td>
<td>0.5</td>
<td>16</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>SS6G</td>
<td>0.12</td>
<td>0.015</td>
<td>2</td>
<td>0.015</td>
<td>0.03</td>
</tr>
<tr>
<td>SS15G</td>
<td>0.25</td>
<td>0.062</td>
<td>4</td>
<td>0.06</td>
<td>0.06</td>
</tr>
</tbody>
</table>

**Figure 36: Drug susceptibility of AD1-8u-, PSCDR1-GFP, SS5G, SS6G and SS15G cells:** The MIC$_{80}$ (µg/ml) of AD1-8u-, PSCDR1-GFP, SS5G, SS6G and SS15G cells to the drugs indicated was determined as described in 'Materials and methods'. The results are typical of one determination which was confirmed by three independent experiments.
all the PM protein from cells expressing either native or mutant Cdr1p-GFP as compared to control AD1-8u- cells (Figure 37). However, the oligomycin sensitive ATPase activities of SS5G (F774A) and SS15G (T1351F) were comparable to that of native Cdr1p-GFP, the specific activity of SS6G (ΔF774) was ~50% lower (Figure 37). Thus SS6G (ΔF774) cells though unable to grow in presence of tested drugs (Figure 36) exhibited lower ATPase activity consistent with decreased expression of the mutant protein at the plasma membrane (Figure 39). It is important to mention that we were unable to detect drug-stimulated ATPase activity although that would have been a correct measure of the ATPase activity contributed by the active protein. In fact we, as well as others, have so far been unable to demonstrate drug-stimulated ATPase activity of any of the yeast ABC drug transporters (Nakamura et al. 2002; Decottignies et al. 1994).

3.10.3 Mutant Cdr1p-GFPs elicit reduced efflux of rhodamine6G

We checked the rhodamine 6G efflux and found that all the three mutants overexpressing GFP tagged Cdr1p variants exhibited reduced efflux as compared to cells expressing native protein. (Figure 38).

3.11 Expression of Mutant Cdr1p-GFPs

In order to ensure that the observed changes in drug susceptibilities and efflux activities of SS5G (F774A), SS6G (ΔF774) and SS15G (T1351F) were due to alterations in protein functions rather than due to altered expression levels, the steady state levels of Cdr1p-GFP variants were compared to those of the native protein by western analysis. The western blot of these three overexpressing mutant Cdr1p-GFP PM proteins with anti-Cdr1p polyclonal antibody showed that SS5G (F774A) and SS15G (T1351F) expressed mutant
Figure 37: Oligomycin sensitive ATPase activity in the PM of SS5G, SS6G and SS15G: The ATPase activity was determined for the wild type Cdr1p-GFP as well as mut-Cdr1p-GFPs from SS5G, SS6G and SS15G in the PM protein from the respective cells as described in legend to Figure 17 a. The results are the means ± standard deviations of three independent experiments.
Figure 38: Rhodamine-6G efflux in SS5G, SS6G and SS15G cells: Rhodamine-6G efflux for SS5G, SS6G and SS15 cells was determined as described in 'Materials and methods'. The results are the means ± standard deviations of three independent experiments.
Cdr1p-GFPs to the same level as that of wild type (Figure 39 lower panel). Hence the observed functional defects observed in SS5G (F774A) and SS15G (T1351F) expressing mutant variants of Cdr1p were not due to an effect on the level of the synthesis or trafficking of these proteins. As revealed by immunoblotting with a monoclonal antibody against GFP that mutant Cdr1p-GFP of SS6G (ΔF774) was poorly expressed at the cell surface (Figure 39 upper panel lane 4). Of note this faint band of mutant Cdr1p-GFP from SS6G, was picked up consistently when we used monoclonal antibody against GFP. The confocal microscopy and FACS data, however, also confirmed that there was considerably reduced cell surface expression of mutant Cdr1p-GFP in SS6G (ΔF774) cells (Figure 41 and 42). Thus ΔF774 Cdr1p-GFP mutant variant protein was consistently detected at a reduced steady state level relative to native Cdr1p-GFP, suggesting that this mutation harbors major defect which affects its surface localization (discussed below).

3.12 Characterization of ATP and substrate binding sites of mutant Cdr1p-GFP variants

In order to explore if the observed differences in drug susceptibilities accompanied with increased drug sensitivity and efflux activities are not due to any aberration in nucleotide or drug binding, these mutants were analyzed for their ATP and drug binding abilities by using [α-32P] 8-azidoATP, [125I]-IAAP and [3H]-azidopine.

3.12.1 [α-32P] 8-azidoATP binding

The radiolabeled analogue of ATP, [α-32P] 8-azidoATP was used to assess its binding to mutant Cdr1p-GFPs using the purified PM from SS5G (F774A), SS15G (T1351F) and SS6G (ΔF774). It is clear from Figure 6 b that there was no significant difference in the ATP
Figure 39: Expression of Cdr1p-GFP and mut-Cdr1p-GFP in *S. cerevisiae*: PM (20 μg) protein from AD1-8u- (lane 1), PSCDR1-GFP (lane 2), SS5G (lane 3), SS6G (lane 4) and SS15G (lane 5) were separated on 8% polyacrylamide gel, electroblotted on to nitrocellulose, and incubated with mouse monoclonal anti-GFP antibody (1:1000) (upper panel) and rabbit polyclonal anti-Cdr1p antibodies (1:500 dilution) (lower panel). Antibodies were detected with HRP labeled anti-rabbit immunoglobulin using ECL kit.
Results

binding to the PM of wild type, SS5G (F774A) and SS15G (T1351F). There was very little binding of [α-32P] 8-azidoATP to PM of SS6G (ΔF774), which correlated well with its poor expression in these cells. The binding of [α-32P] 8-azidoATP was, however, specific in all the mutants carrying Cdr1p variants as was evident from its competition by ATP (10 mM) (Figure 40 a).

3.12.2 [125I]-IAAP and [3H]-azidopine binding

It was found that although mutant Cdr1p-GFPs of both SS5G (F774A) and SS15G (T1351F) could bind [125I]-IAAP, the former variant showed 2.3-fold greater binding than the native Cdr1p-GFP (Figure 40 b). The [125I]-IAAP binding was considerably lower in SS6G (ΔF774) cells. Further like in the case of native Cdr1p, [125I]-IAAP binding was competed out by nystatin (100 μM) for the mutants also.

The drug-binding domain of mutant Cdr1ps was further probed by examining the binding of [3H]-azidopine. It was observed that the although [3H]-azidopine binds to mutant Cdr1p-GFPs from SS5G (F774A) and SS15G (T1351F), its binding to mutant Cdr1p-GFP from SS5G (F774A) was 1.8-fold higher as compared to wild type Cdr1p-GFP (Figure 40 c). On the other hand, the mutant Cdr1p-GFP from SS6G (ΔF774) showed very faint specific band, which corresponded with very little protein at the cell surface of this mutant protein. Irrespective of the extent of [3H]-azidopine binding among mutant variants of Cdr1p-GFP, it could be competed out by miconazole (100 μM).
Figure 40: (a) $[\alpha^{-32P}]$ 8-azidoATP labeling and competition of Cdr1p-GFP and mut-Cdr1p-GFP from SS5G, SS6G and SS15G: PM (15 μg) protein were photoaffinity labeled with 10 μM $[\alpha^{-32P}]$ 8-azidoATP (10 μCi/nmole) and competed as described in 'Materials and methods'. Ice cold ATP (10 mM) was added to displace excess non-covalently bound $[\alpha^{-32P}]$ wherever indicated.

(b) $[^{125}I]$-IAAP labeling and competition of Cdr1p-GFP and mut-Cdr1p-GFP from SS5G, SS6G and SS15G: The PM (15 μg) protein were labeled with 3-6 nM of $[^{125}I]$-IAAP (2200 Ci/mmol) and competed as described in 'Materials and methods'. 100 μM of nystatin, wherever indicated was added to compete IAAP binding.

(c) $[^3H]$-azidopine labeling and competition of Cdr1p-GFP and mut-Cdr1p-GFP from SS5G, SS6G and SS15G: The PM (30 μg) were labeled with $[^3H]$-azidopine (60 Ci/mmole) as described, and competed with 100 μM miconazole wherever indicated, in 'Materials and methods'.
3.13 The deletion of F774 leads to poor cell surface localization of the mutant Cdr1p-GFP in SS6G

The confocal images showed that while the expression of Cdr1p-GFP in wild type and mutant Cdr1p-GFPs in SS5G (F774A) and SS15G (T1351F) at the cell surface, (Figure 41) was comparable; it was very minimal in SS6G (ΔF774) cells. Although, some of the fluorescent protein was visible in the cytoplasm of SS6G (ΔF774), very small patches of fluorescence were seen on the cell surface, which showed that probably the mutant Cdr1p-GFP in SS6G (ΔF774) was not properly localized on the cell surface. These results were corroborated by western analysis where PM of SS6G (ΔF774) cells also showed significantly reduced expression of mutant Cdr1p-GFP (Figure 39). FACS analysis of the live cells showed fluorescence in SS5G (F774A) and SS15G (T1351F) that was comparable to the wild type PSCDR1-GFP. SS6G (ΔF774) cells, however, showed lower fluorescence intensity but it was still higher than that of the control cells (AD1-8u-) (Figure 42). The mean fluorescence intensity (arbitrary units) for wild type PSCDR1-GFP, SS5G (F774A), SS6G (ΔF774) SS15G (T1351F) and AD1-8u- were 500, 482, 160, 496 and 11, respectively. Taken together these results suggest that there was only a marginal expression of mutant Cdr1p-GFP in PM of SS6G (ΔF774) which was probably sufficient to show some functional activity (Figure 36-42) albeit with lower efficiency but was not sufficient enough to confer drug resistance (Figure 34 and 35)

3.14 Mutant ΔF774 Cdr1p-GFP can be brought to the cell surface by growing cells in the presence of Cdr1p substrate

We explored various possibilities to check whether the localization of mutant Cdr1p-GFP in SS6G (ΔF774) cells could be improved. For this, we added different drugs, which are substrates of
Expression of GFP-tagged wild-type and mutant Cdr1p-GFP variants

Figure 41: Confocal pictures of *S. cerevisiae* SS5G, SS6G and SS15G cells: Cells were grown in SD-URA media to late log phase. The cells were washed and resuspended in appropriate volume of 0.5 mM HEPES (pH 7.0). The cells were directly viewed, on a glass slide with a drop of antifade reagent to prevent photobleaching, with 100X oil immersion objective on a Biorad confocal microscope (MRC 1024).
Figure 42: Flow Cytometry of cells expressing Cdr1p-GFP and mut-Cdr1p-GFP: AD1-8u-L, PSCDR1-GFP, SS5G, SS6G and SS15G cells were grown to mid log phase and used for FACS analysis as detailed in ‘Materials and methods’. Analysis was performed with CellQuest software. The number of cells were plotted on a histogram with the green fluorescence plotted on a log scale and the mean fluorescence intensity was calculated.
Figure 43: (a)-(h) Confocal pictures of AD1-8u- and SS6G cells expressing mut-Cdr1p-GFP grown in presence of increasing concentrations of cycloheximide as described: (a) AD1-8u- (b) PSCDR1-GFP expressing native Cdr1p + 50ng/ml cycloheximide (c) SS6G expressing mut-Cdr1p-GFP grown without drug or (d) SS6G + 10 ng/ml or (e) SS6G + 20 ng/ml or (f) SS6G + 30 ng/ml or (g) SS6G + 40 ng/ml or (h) SS6G + 50 ng/ml of cycloheximide.
Cdr1p like cycloheximide, miconazole, anisomycin and nystatin (at concentrations less than respective MICs), just after the lag phase of the SS6G (ΔF774) cells (4-5 h). The cells were then grown for 12 more hours. Thereafter cells were checked by confocal microscopy for the localization of the protein. It was observed that the mutant Cdr1p-GFP showed improved surface localization with increasing concentrations of cycloheximide. The surface expression of mutant Cdr1p-GFP appeared almost like native Cdr1p-GFP at 40 ng/ml of cycloheximide (Figure 43 a-h). FACS analysis also confirmed that the SS6G (ΔF774) expressing mutant Cdr1p-GFP mutant grown in presence of 40 ng/ml of cycloheximide showed almost comparable expression to wild type (Figure 44). Interestingly, PM protein isolated from the SS6G (ΔF774) cells which were grown in increasing concentrations of cycloheximide when subjected to SDS-PAGE and western analysis (probed with monoclonal anti-GFP antibody), also showed a concentration-dependent increase in the amount of Cdr1p-GFP in the PM protein which was maximum in 40 and 50 ng/ml (Figure 45 a, and 45 b lanes 6 and 7). Despite growth inhibition due to the presence of cycloheximide, SS6G (ΔF774) cells expressing mutant variant could distinctly show improvement in surface localization while the presence of drug had no effect in cells expressing native Cdr1p-GFP (Figure 43 b, 43 h and 45). The observed rescuing effect was not specific to cycloheximide alone. Other substrates of Cdr1p like anisomycin and nystatin could also rescue the ΔF774 protein to the plasma membrane (data not shown).

3.15 Mutant Cdr1p-GFP from SS6G (ΔF774) cells grown in the presence of cycloheximide is functional

In order to test if improved localization led to resumption of transport functions; the efflux of rhodamine 6G was measured in the
Figure 44: Flow cytometry of SS6G (ΔF774) cells grown in presence of cycloheximide: The control (AD1-8u-), SS6G, PSCDR1-GFP grown in presence or absence of cycloheximide 40ng/ml wherever indicated were analyzed by flow cytometry as described in ‘Materials and methods’.
Figure 45: Expression of mutant Cdr1p-GFP from SS6G (AF774) cells grown in presence of cycloheximide: The PM protein from AD1-8 (lane 1), SS6G (lane 2) SS6G (grown in the presence of increasing concentrations of cycloheximide) as 10 ng/ml (lane 3), 20 ng/ml (lane 4), 30 ng/ml (lane 5), 40 ng/ml (lane 6) 50 ng/ml (lane 7) and PSCDR1-GFP (grown in presence of 50ng/ml) (lane 8) were separated on 8% polyacrylamide gel and (a) stained with colloidal coomassie G250 or (b) electroblotted on to nitrocellulose, and incubated with rabbit polyclonal anti-Cdr1p antibodies (1:500 dilution). Antibodies were detected with HRP labeled anti-rabbit immunoglobulin using ECL kit.
cells grown in presence of cycloheximide. The control (AD1-8u'), PSCDR1-GFP and SS6G (ΔF774) cells were grown in presence or absence of cycloheximide 40 ng/ml for 12-14 h and rhodamine 6G efflux was studied as described in ‘Materials and Methods’. Figure 46 shows that the SS6G (ΔF774) cells grown in presence of cycloheximide showed considerable increase in rhodamine 6G efflux when compared to the same cells grown in the absence of drug. The level of efflux was comparable to native PSCDR1-GFP cells grown at the same concentration of cycloheximide. There was some reduction in efflux of rhodamine 6G by CDR1-GFP cells when grown in presence of cycloheximide. The decrease in efflux by the cells expressing native Cdr1p could be due to the fact that cycloheximide, which is also a substrate for the Cdr1p transporter probably, competes with rhodamine 6G.

3.16 T1351 and FK520 sensitivity

FK506 and FK520 (a FK 506 derivative) are potent immunosuppressive antimicrobial agents, which modulate P-gp mediated MDR in mammalian cells. Egner et al (Egner et al. 1998) showed that the FK 506 inhibits the transport of drugs and steroids mediated by Pdr5p and reversesazole resistance in S. cerevisiae. S1360 of TMS 10 of Pdr5p is the residue found to be critical for FK506 sensitivity. In this study we observed that Cdr1p-GFP expressing S. cerevisiae cells which are resistant to ketoconazole and fluconazole turned sensitive in presence of FK520 (Figure 8 a and 8 b). Interestingly, the sensitivity to azoles in presence of FK520 was only partly restored in SS15G (T1351F) cells expressing mutant variant protein (Figure 47).
Figure 46: Rhodamine 6G efflux from SS6G (ΔF774) cells grown in presence of cycloheximide: The Rh-6G efflux by AD1-8u', SS6G, SS6G or PSCDR1-GFP (both grown in the presence of 40 ng/ml cycloheximide) and PSCDR1-GFP cells was determined as described in 'Materials and methods'. The results are the means ± standard deviations of three independent experiments.
Figure 47: Cdr1p-GFP activity is inhibited by FK520 and mut-Cdr1p-GFP from SS15G is not sensitive to this inhibition: AD1-8u\textsuperscript{-}, PSCDR1-GFP and SS15G cells were grown overnight on YEPD plates at 30°C. The five fold serial dilution of a 0.1 A\textsubscript{600} cells of each strain was spotted onto YEPD plates in the absence (growth control) or in presence of the following drugs, FK520 (40 µg/ml), (a) fluconazole (2 µg/ml), (b) ketoconazole (100 ng/ml) alone or in combination as indicated.