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Identification of Nile Red as a fluorescent substrate of the *Candida albicans* ABC transporters Cdr1p and Cdr2p and the MFS transporter Mdr1p

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Abstract

Clinically relevant azole resistance in the fungal pathogen *Candida albicans* is most often associated with the increased expression of plasma membrane efflux pumps, specifically the ATP-binding cassette (ABC) transporters CaCdr1p and CaCdr2p and the major facilitator superfamily (MFS) transporter, CaMdr1p. Development of potent pump inhibitors that chemosensitize cells to azoles is a promising approach to overcome antifungal resistance. Here we identify Nile Red as a new fluorescent substrate for CaCdr1p, CaCdr2p and CaMdr1p. Nile Red was effluxed efficiently from *Saccharomyces cerevisiae* cells heterologously expressing these transporters. Enniatin selectively inhibited the efflux of Nile Red from *S. cerevisiae* cells expressing CaCdr1p or CaMdr1p but not from cells expressing CaCdr2p. This indicates that Nile Red can be used for the identification of inhibitors specific for particular transporters mediating antifungal resistance in pathogenic yeast.

Keywords

MDR; yeast; fluconazole; efflux pumps; fluorescent substrate; *Candida albicans*

Introduction

The resistance of microorganisms to antibiotics and synthetic chemotherapeutics is one of the biggest challenges for modern chemotherapy. Clinical resistance to commonly used antifungal drugs can also occur [1;2] and there are few alternative classes of antifungal drugs available. The azoles are a major class of drugs used to treat many fungal infections and although they have good pharmacokinetic properties, and are well tolerated, several fungi show innate or acquired azole resistance. A significant mechanism of azole resistance is the overexpression of membrane proteins that actively efflux drugs out of the cells (reviewed by Cannon et al [3]). Representatives of both the ATP-binding cassette (ABC) and major facilitator superfamily (MFS) classes of transporter are present in the opportunistic fungal pathogen, *C. albicans*, but clinically relevant azole resistance is most often associated with increased expression of

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mRNAs for the ABC genes *CaCDR1* and *CaCDR2* [4;5;6;7]. Furthermore, our recent demonstration using protein expression studies that CaCdr1p is the major contributor to azole resistance in many resistant isolates [8] has been confirmed by gene deletion experiments in *C. albicans* [9]. In some strains, however, the MFS transporter gene *MDR1* mediates resistance [10;11;12]. Deletion of either *Cdr1* [13] or *Mdr1* [14] from *C. albicans* isolates resulted in decreased fluconazole resistance of the respective mutant strains, and experimental overexpression of *CDR1* [15] or *MDR1* [16] in each case conferred resistance to fluconazole, confirming the role of these pump proteins in azole resistance. Efflux pumps are often also responsible for azole resistance in other pathogenic fungi such as CneMdr1p in *Cryptococcus neoformans*, CgCdr1p and CgPdh1p in *Candida glabrata* and CkAbc1p in *Candida krusei* (reviewed by Cannon et al [3]).

An important strategy for combating the phenomenon of multidrug resistance in pathogenic microorganisms is the use of chemical compounds (chemosensitizers) co-administrated with chemotherapeutics to restore drug susceptibility in multidrug resistant cells. Previous studies that identified yeast transporter inhibitors used agar diffusion assays, liquid growth assays, measurement of the effluxed substrate outside of the cell [17;18] or substrate accumulated within cells [19;20].

We have developed a flow cytometry based high-throughput screen (HTS) platform for the identification and characterization of substrates and inhibitors of human ABC transporters [21]. Here we present results obtained by applying a similar approach, using a heterologous expression system in the model yeast *Saccharomyces cerevisiae* [22;23;24], in order to identify a fluorescent substrate of fungal efflux pumps suitable for HTS discovery of fungal efflux pump inhibitors. This yeast expression system [22;23;24] achieves consistent and equivalent hyperexpression of individual alleles of efflux pump proteins such as *C. albicans* Cdr1p, Cdr2p and Mdr1p. The system is based on the integration of a cloning cassette, derived from plasmid pABC3 and containing the heterologous gene, into the genome of the host *S. cerevisiae* strain at the *PDR5* locus under the control of the constitutively active *PDR5* promoter. In the *S. cerevisiae* host strain seven endogenous efflux pump genes have been disrupted and the *pdr1-3* mutation facilitates the reproducible and equivalent overexpression of transporters of interest, allowing the identification of substrates and inhibitors of Cdr1p, Cdr2p or Mdr1p efflux activity.

Nile Red is a fluorescent dye, known as a probe of intracellular lipids and hydrophobic domains of proteins [25]. It is a vital, lipid-soluble, highly selective stain for yeast lipid particles [26]. Nile Red is strongly fluorescent, but only when it is present in a highly hydrophobic environment. The ability of Nile Red to sense its environment has made it a useful biological imaging tool. We tested this dye in our system to see if it is a substrate for Cdr1p, Cdr2p, or Mdr1p transporters and compared its efflux to that of Rhodamine 6G (R6G), previously reported as a specific substrate for Cdr1p and Cdr2p [8;27]. We have also tested the known Cdr1p inhibitor Enniatin [22] as a potential inhibitor of Nile Red efflux by these transporters.

Methods

Strains and culture conditions

All *S. cerevisiae* strains were based on AD1-8u⁻ [23;24] and contained the *CaCDR1*, *CaCDR2* or *CaMDR1* gene, integrated as a cassette from plasmid pABC3, at the *PDR5* locus as previously described [22]. These strains were denoted AD/CDR1, AD/CDR2, and AD/MDR1 respectively. A control strain, AD/pABC3, was constructed which contained the pABC3 cassette, but without a heterologous efflux pump gene. All fungal strains were grown in complete supplement medium without uracil (CSM-ura), which contained 2.67% (wt/vol) minimal synthetic defined (SD) base (Clontech Laboratories Inc., CA), 0.077% (wt/vol)–Ura

Drop Out (DO) Supplement (Clontech Laboratories Inc.), and 2% (wt/vol) D-glucose. Cells were incubated at 30°C with shaking (250 rpm) until the cultures reached an OD₅₄₀ of 0.25.

Nile Red and Rhodamine 6G accumulation assay

Accumulation of Nile Red (Invitrogen, Eugene, OR, USA) and R6G (Invitrogen) by *S. cerevisiae* cells was measured by flow cytometry with a Cyan™ flow cytometer (Dako Cytomation, Fort Collins, CO). Exponential phase yeast cells (5 µL; 2.5×10^6 cell/ml CSM-ura) were dispensed into the wells of 384-well microtiter plates (Greiner, Germany). Nile red (3.5 mM stock in dimethyl sulfoxide (DMSO)) or R6G (7.5 mM stock in DMSO) were prepared in diluted medium (containing one part of CSM-ura and two parts of water) containing 2% (w/v) glucose and 10 µl added to each microtiter well to give final concentrations of 7 µM Nile Red or 15 µM R6G. Enniatin (5 mM stock in DMSO; Alexis Biochemicals, San Diego, CA, USA) or DMSO control, was added to give a final concentration of 50 µM Enniatin. The total volume of the mixture in each well was 15 µL. After 20 minutes incubation at room temperature, the samples were excited with a 488 nm laser and PE-Texas Red and PE filters were used to detect Nile Red and R6G, respectively. The HyperCyt system [20;28] was used for aspiration of samples and delivery to a Cyan™ flow cytometer (Dako Cytomation, Fort Collins, CO) for analysis. Specialized software (IDLeQuery) was used to analyze the data file.

Characterization of Nile Red as a substrate

In order to determine optimal concentration of Nile Red as a substrate and to investigate Nile Red fluorescence stability in the assay over time, three-fold dilutions of Nile Red (final concentrations ranging from 7.6 nM to 50 µM, prepared in diluted medium as described above) were added to the control strain, AD/pABC3, and to strains overexpressing Cdr1p, Cdr2p, or Mdr1p. After 5, 15, 30, and 60 min incubation at room temperature the samples were excited with a 488 nm laser and a PE-Texas Red filter was used to determine median channel fluorescence (MCF) values.

Inhibition assay

In order to determine if Nile Red efflux from *S. cerevisiae* strains overexpressing Cdr1p, Cdr2p or Mdr1p could be inhibited by Enniatin, the compound was added to the yeast cells (final concentrations ranging from 137 nM to 100 µM). Nile red and 2% glucose were added to each strain, followed by 20 min incubation at room temperature. Cells were examined by flow cytometry, as described above, to determine pump activity.

Results

Nile Red is a substrate of Cdr1p, Cdr2p and Mdr1p

The ability of Cdr1p, Cdr2p and Mdr1p to efflux R6G or Nile Red was tested by measuring the fluorescence of AD/CDR1, AD/CDR2, and AD/MDR1 cells, relative to control AD/pABC3 cells, when incubated with the dyes under energized conditions (the assay medium contained glucose). Both dyes were taken up by the control AD/pABC3 cells which have negligible pump activities (Fig. 1, A and B). If a dye is the substrate of a heterologously expressed pump then *S. cerevisiae* cells expressing this pump will not accumulate the dye and have a low fluorescence. Significantly lower levels of R6G fluorescence were detected in AD/CDR1 and AD/CDR2 cells compared to the control strain AD/pABC3, indicating an active efflux of R6G from these strains (Fig. 1A). For AD/MDR1 cells, however, accumulation of R6G was equivalent to the control strain, suggesting no efflux activity by the Mdr1p transporter (Fig. 1A). In the presence of 50 µM Enniatin, the R6G fluorescence of AD/CDR1 cells (Fig. 1A) was ~5-fold higher than that without inhibitor; equivalent to the fluorescence of control AD/pABC3 cells whereas fluorescence of the AD/CDR2 cells remained low, and unaffected

by Enniatin. The transporters showed a different specificity towards the dye Nile Red (Fig. 1B). Nile Red was actively effluxed from AD/CDR1, AD/CDR2 and, in addition, by AD/MDR1 cells, which did not efflux R6G. Addition of Enniatin significantly inhibited Nile Red efflux from AD/CDR1 and AD/MDR1, but not from AD/CDR2 cells (Fig. 1B). In the presence of Enniatin, AD/pABC3 cells accumulated more Nile Red than in the absence of Enniatin. We also noted that Enniatin did not completely inhibit Nile Red efflux from AD/CDR1 and AD/MDR1 cells (Fig. 1B).

Characterization of Nile Red as a pump substrate

Criteria for good fluorescent substrates for HTS assays are: 1) the transporter maintains an equilibrium for the substrate and efficiently effluxes the substrate at a given concentration; 2) the fluorescence (median channel fluorescence units (MCF) range difference between control and pump-expressing cells) should be large enough to provide a good signal. Nile Red meets these criteria at concentrations ranging from 5 μ M to 17 μ M (Fig. 2). All three pump-expressing strains efficiently effluxed Nile Red at concentrations below 17 μ M (Fig. 2, enlarged portion of the graph on the right). Control cells (with no heterologous pump) accumulated the substrate in dose-dependent manner, reaching optimal fluorescence levels at 5 μ M and higher. We chose 7 μ M as the working concentration for Nile Red and tested the stability of the fluorescent signal over time. The Nile Red fluorescence of all strains tested showed minimal changes ($\leq 5\%$) over a period of 1 h, the time taken to complete the HTS assay (data not shown).

Confirmation that Enniatin inhibits Nile Red efflux by CaCdr1p, and CaMdr1p but not by CaCdr2p

The specificity of putative pump inhibitor Enniatin for Cdr1p and Mdr1p, was confirmed in dose-response assays. AD/CDR1, AD/CDR2 and AD/MDR1 cells were exposed to 7 μ M Nile Red and Enniatin at concentrations ranging from 100 nM to 100 μ M. The efflux of Nile Red was inhibited from AD/CDR1 and AD/MDR1 cells, but not from AD/CDR2 cells (Fig. 3). The IC₅₀ values for Cdr1p, Cdr2p, and Mdr1p were: 8.0 μ M, >100 μ M, and 9.26 μ M respectively. The 95% confidence intervals of the IC₅₀ values were 5.2 to 12.3 and 7.8 to 12.9 for CDR1p and MDR1p, respectively.

Discussion

The development of highly specific and potent fungal transporter inhibitors that chemosensitize resistant strains to existing antifungal drugs is a high priority. Several approaches have been developed to screen for modulators of drug resistance in yeast. Most methods used to identify efflux pump inhibitors are based on the ability of tested compounds to potentiate the action of the effluxed cytotoxic drug on the growth and viability of the tested cells [18;19], the ATPase activity of the pump [29], or the accumulation of radiolabeled substrate by cells [30]. Real-time detection of fluorescein diacetate accumulation in yeast cells was reported by Kolaczowski *et al.* as a method to identify inhibitors of *C. albicans* Cdr1p-mediated drug efflux [31]. Their approach was based on overexpression of the *CaCDR1* gene in a *S. cerevisiae* strain with a double *PDR5 SNQ2* knockout. However, the presence of other endogenous *S. cerevisiae* transporter genes, (for example *PDR10*, *PDR11*, *PDR12*, *PDR15*, *YOR1*, and *YCF1*) could represent a significant disadvantage of this strain compared to our multiply-deleted AD1-8u⁻ strain. Indeed, fluorescein is reported to be a substrate of PDR12 [32;33]. The use of fluorescein diacetate also introduces additional opportunities for experimental variation compared to inherently fluorescent substrates such as R6G or Nile Red, as the intracellular fluorescence resulting from fluorescein diacetate uptake depends on intracellular enzymatic cleavage to release the fluorescent product fluorescein [33].

Recently, we reported a flow cytometry-based HTS for identification of substrates and inhibitors of human ABC transporters [21]. We have adopted this assay for three fungal transporters from the pathogenic yeast *C. albicans*, (Cdr1p, Cdr2p and Mdr1p) each individually expressed in a *S. cerevisiae* host strain depleted of seven endogenous transporters. We have previously reported that overexpression of the *C. albicans* efflux pumps in *S. cerevisiae* resulted in decreased susceptibilities to fluconazole and a variety of structurally unrelated toxic compounds [22;34], demonstrating functionality and applicability of this expression system for the discovery of fungal drug efflux transporters. The ability of a fluorescent substrate to accumulate in the *S. cerevisiae* host strain and to be effluxed by the transporters for which it is a substrate, allows the identification of fluorescent dyes with different specificities to the different transporters. It also allows us to identify new inhibitors that will affect the efflux of the specific substrate by these transporters. This assay can be easily developed into a high throughput screening assay.

As part of a larger study, we have characterized the interaction of a library of fluorescent compounds as substrates for the human ABC transporters, ABCB1, ABCC1, and ABCG2. Here, our aim was to identify a fluorescent dye that is a substrate of all the major transporters responsible for *C. albicans* azole resistance: Cdr1p, Cdr2p and Mdr1p. R6G was previously reported as a specific substrate for Cdr1p and Cdr2p [24;35], but it is not a substrate of Mdr1p [22]. Our results confirm these reports; we demonstrated R6G efflux from AD/CDR1 and AD/CDR2 cells but not from AD/MDR1 cells. Nile Red, on the other hand, was a substrate for all three yeast transporters. It was actively effluxed from AD/CDR1, AD/CDR2 and AD/MDR1 cells, reducing their fluorescence levels from 299 ± 11.5 to 5 ± 5.0 , 7 ± 3.2 and 6 ± 5.5 MCF units, respectively. Nile Red exhibited stable fluorescence levels inside the cells, demonstrating its applicability as a fluorescent probe appropriate for whole cell assays. We tested a range of Nile Red concentrations and found that in our assay $7 \mu\text{M}$ Nile Red gave a good fluorescent signal and was efficiently effluxed from the pump-expressing strains. In preliminary experiments, we have also found that Nile Red is a substrate for three other fungal ABC transporters (*Candida glabrata* Cdr1p, *Candida krusei* Abc1 and *Cryptococcus neoformans* Mdr1p; Holmes *et al*, unpublished data) confirming the broad utility of Nile Red as a fluorescent probe for studies of fungal efflux pumps and their inhibitors.

In order to demonstrate the applicability of Nile Red to HTS discovery of pump inhibitors, we examined the effects of the known pump inhibitor, Enniatin, on the efflux of R6G or Nile Red. Enniatin was identified previously as an inhibitor of Cdr1p efflux activity, but not of Cdr2p [22]. In our assay, Enniatin inhibited R6G and Nile Red efflux by Cdr1p, but not by Cdr2p. Enniatin also inhibited Mdr1p-mediated efflux of Nile Red. The differences in substrate specificity of the Mdr1p transporter, may indicate that different binding sites are responsible for recognition of the different substrates. CaMdr1p also does not efflux certain other known substrates of the ABC transporters such as the azoles itraconazole and miconazole [22].

We found some inhibition by Enniatin of Nile Red efflux from the control strain AD/pABC3, but not of R6G efflux from this strain, which suggests the inhibition of some other transporters that are still present in the host strain at a much lower level of expression. Although the AD1-8u-strain is deleted in all major *S. cerevisiae* ABC transporters, a number of MFS transporters, such as Tpo1, a polyamine transporter [36] and for which Nile Red, but not R6G, could be a substrate, are still present and may be expressed at low levels. This background activity, however, did not prevent the identification of Nile Red as a substrate, and Enniatin as an inhibitor, of the heterologously expressed *C. albicans* transporters. To our knowledge, Nile Red is the first reported fluorescent substrate of *C. albicans* ABC transporters (Cdr1p, Cdr2p) and *C. albicans* MFS transporter Mdr1p. It can be used as a powerful tool for discovery of novel broad-spectrum inhibitors of Cdr1p, Cdr2p, and Mdr1p efflux pumps.

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Fig. 1. (A) R6G and (B) Nile Red content of *S. cerevisiae* control strain AD/pABC3 (vector only) and strains expressing *C. albicans* Cdr1p (AD/CDR1), Cdr2p (AD/CDR2), or Mdr1p (AD/MDR1) transporter genes. Yeast cells were incubated with 15 μ M R6G (A) or 7 μ M Nile Red (B) in DMSO (1%) for 20 min at 30°C. Enniatin (50 μ M final) was added to strains preloaded with substrate and incubated for 20 min at 30°C. MCF: median channel fluorescence units. Each bar represents the median $\pm$ SD (n=3).

Fig. 2.
Dose-dependent Nile Red accumulation in control and pump-expressing strains.
Pump-expressing strains can efficiently efflux the substrate and maintain equilibrium at concentrations below 17 μM , while the control strain continuously accumulates Nile Red.

Fig. 3. Dose-response analysis of Cdr1p, Cdr2p, and Mdr1p efflux activities to inhibition by enniatin (137 nM - 100 μM) using Nile Red as a substrate. Variable slope non-linear curve fit was applied for analysis.