Functional Interactions between Synthetic Alkyl Phospholipds and the ABC Transporters P-Glycoprotein, Ste-6, MRP, and Pgh-1[†]

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ABSTRACT: The ABC superfamily of transporters includes the mammalian P-glycoprotein family (Class I and Class II P-gps), the multidrug resistance-associated protein (MRP), the Pgh-1 product of *Plasmodium falciparum* gene *pfmdr1*, all of which are associated with cellular pleiotropic drug resistance phenomena. STE6, the yeast transporter for the farnesylated peptide pheromone **a**, is also a member of this family. Structural similarities in this family translate into functional homology as expression of mouse Mdr3S (P-gp), *P. falciparum* Pgh-1, and human MRP partially restore mating in a sterile yeast mutant lacking a functional *STE6* gene. The demonstration that Class II P-gps function as phosphatidylcholine (PC) translocators raise the possibility that other ABC transporters may also interact with physiological lipids. We report the identification of the synthetic lipid and PC analog ET-18-OCH_3 (edelfosine) as a substrate for not only Class II P-gp but also for Class I P-gps and surprisingly for the other ABC transporters MRP, Pgh-1, and STE6. Expression of these proteins in the yeast *Saccharomyces cerevisiae* JPY201 was found to confer cellular resistance to cytotoxic concentrations of this lipid by a factor of 4−20-fold in a growth inhibition assay. The noted activity of ABC transporters toward this synthetic lipid was specific as a mutant variant of Mdr3 (Mdr3F) with reduced activity could not convey cellular resistance to ET-18-OCH_3. ET-18-OCH_3 was also found capable of blocking **a**-peptide pheromone transport and STE6 complementation by these ABC proteins. The inhibitory effect of ET-18-OCH_3 on cell growth and **a**-factor transport could be abrogated by incubation with the lipid acceptor protein BSA or by enzymatic cleavage by microsomal alkylglycerol mono-oxygenase (MAMO). MAMO and BSA reversal of the ether lipid effect was only seen in the presence of a functional transporter. These results suggest that the group of cytotoxic synthetic PC analogs studied reveal possible structural and functional aspects common to the ABC transporters tested. Furthermore, the studies with BSA and MAMO suggest that the mechanism of transport of ET-18-OCH_3 by these ABC transporters may be related to the flippase mechanism of PC transport by Mdr2.

The emergence of multidrug resistance (MDR)[1] in cultured cells *in vitro* and in some tumors *in vivo* is caused by the overexpression of P-glycoprotein (P-gp), an integral membrane protein. Experiments *in vitro* indicate that P-gp overexpression causes cellular resistance to an intriguingly large number of structurally unrelated cytotoxic molecules that only share in common a high degree of hydrophobicity (*1*−*3*). These "MDR drugs" include bacterial antibiotics (anthracyclines), plant alkaloids (Vinca alkaloids), cytotoxic peptides (valinomycin, gramicidins), and a vast array of small lipid soluble cationic molecules. Transport studies in intact cells expressing P-gp or membrane vesicles derived from them, or with purified P-gp reconstituted in proteoliposomes have shown that P-gp-mediated drug resistance is caused by a direct ATP-dependent transport mechanism that ultimately results in decreased intracellular accumulation of the drugs (*3*, *4*).

Primary amino acid sequence analysis from cloned cDNAs (*mdr*), secondary structure predictions, and direct topology mapping experiments (*3*, *4*) have shown that P-gp is composed of two sequence homologous and symmetrical halves, each encoding six transmembrane (TM) domains and an ATP binding site of the Walker type (*5*). P-gp defines a small family of two highly similar proteins in humans and three proteins in the mouse, which have been functionally classified by their ability to convey drug resistance upon overexpression in transfected cells *in vitro*. Class I P-gps (human MDR1, mouse Mdr1/Mdr3) can convey MDR (*6*, *7*), while Class II P-gps (human MDR2 and mouse Mdr2) cannot do so in the same assay (*8*, *9*). Although the exact mechanism by which Class I P-gps can recognize and transport a large number of structurally different substrates remains debated, important clues have been obtained from the identification of the normal function and substrate of the liver-specific Class II P-gps. The study of mutant mice

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[1] Abbreviations: ABC, ATP binding cassette; BSA, bovine serum albumin; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine; ET-18-OH, 1-*O*-octadecyl-*sn*-glycero-3-phosphocholine; IC_{50}, 50% inhibitory concentration; MAMO, microsomal alkylglycerol mono-oxygenase; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; NB, nucleotide binding; PBS, phosphate-buffered saline; PC, dipalmitoylphosphatidylcholine; Pte·H_4, tetrahydropteridin; TLC, thin-layer chromatography; TM, trans-membrane.

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bearing a null allele at the *mdr2* locus identified a severe liver pathology associated with an absence of biliary lipids in these mice (*10*). Direct transport studies in yeast secretory vesicles expressing a recombinant mouse Mdr2 isoform showed that Mdr2 functions as a lipid flippase to translocate phosphatidylcholine (PC) from the inner leaflet to the outer leaflet of the membrane lipid bilayer (*12*). Similar results have been obtained from the study of fibroblasts expressing human MDR2 (*13*, *14*) and from experiments in membrane vesicles from the sinusoidal and canalicular domains of rat hepatocytes (*15*). Finally, data supporting a broad lipid transport activity for short chain phospholipids has recently been obtained for the Class I P-gp (*11*, *14*−*17*). Together, these results have suggested a mechanism by which drug molecules would be recognized by P-gp within the context of the lipid bilayer and would be translocated by a flippase mechanism to the outer leaflet of the membrane or to the cell exterior, to ultimately reduce the intracellular accumulation.

P-gp belongs to a large superfamily of structurally related proteins that together form the ABC (ATP binding cassette) family of membrane traffic ATPases (*18*, *19*) which spans both the prokaryotic and eukaryotic kingdoms. P-gp homologs have also been identified in lower and higher eukaryotes (60% overall homology). In this group are found the *pfmdr1* gene (Pgh-1 protein) of the malarial parasite *Plasmodium falciparum* (*20*) in which mutations have been associated with resistance to 4-amino quinoline drugs (*21*, *22*), and the yeast *STE6* gene, which is responsible for the transmembrane transport of the farnesylated dodecapeptide mating pheromone **a**-factor (*23*, *24*). In humans, the ABC family includes the MRP protein which was initially identified as responsible for adriamycin resistance in lung cancer cells (*25*) and subsequently shown to be capable of transporting glutathione adducts (*26*−*28*). Other members of this family include the ALDP and PMP70 heterodimer expressed in the membrane of peroxisomes (*29*, *30*), the sulfonylurea receptor expressed in pancreatic islets and involved in regulating a K^+ channel (*31*), and the *CFTR* gene in which mutations cause cystic fibrosis in humans (*32*).

Our laboratory has used the yeast *Saccharomyces cerevisiae* as an expression system to study the mechanism of action of the Class I (*33*−*36*) and Class II P-gps (*12*, *37*), and also of other ABC transporters (*22*, *38*). P-gps can be functionally expressed in *S. cerevisiae* and Class I P-gps can convey cellular resistance to antifungal drugs such as FK506, and carry out drug transport in secretory vesicles derived from these cells (*34*−*36*), while Class II P-gp-mediated PC transport can be demonstrated in the same system (*12*). Likewise, overexpression of wild type but not mutant variants of Pgh-1 in yeast causes resistance to 4-aminoquinoline drugs (*22*), while overexpression of MRP in a yeast mutant hypersensitive to lipophylic drugs and DNA-damaging agents causes cellular resistance to adriamycin (*38*), and this by a direct transport mechanism. Importantly, we showed that the structural homology between these different members of the ABC superfamily translates into functional homology: expression of either P-gp (*33*), Pgh-1 (*22*, *39*), or MRP (*38*) can complement a null allele at the *ste6* locus, restoring **a**-peptide pheromone transport and mating in an otherwise sterile *ste6* null mutant (*33*). These results suggest the possibility that evolutionary distant ABC transporters may recognize common structural determinants on their specific substrates and transport them by a similar mechanism, possibly related to the PC translocase activity of P-gps.

In the present study, we wished to test this hypothesis further and determine if the mode of action of P-gps, MRP, STE6, and Pgh-1 was related to the demonstrated lipid translocase activity of P-gps. We have expressed these various ABC transporters in yeast and have tested the ability of these proteins to interact with the cytotoxic analog of phosphocholine ET-18-OCH_3.

EXPERIMENTAL PROCEDURES

Materials. 1-*O*-Octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (ET-18-OCH_3) and 1-*O*-octadecyl-*sn*-glycero-3-phosphocholine (ET-18-OH) were obtained from Biomol (Plymouth Meeting, PA). All other chemicals and biochemicals were of high-quality reagent grade.

Yeast Strain, Plasmids, Transformation, and Culture Conditions. The *S. cerevisiae* strain JPY201 (*MAT***a**, *ste6*Δ*::his3 ura3*) (*24*) was used throughout this study. To express the various proteins in yeast, full-length cDNAs for the three wild type mouse *mdr* genes (*mdr1, mdr2, mdr3S*) or a mutant variant of *mdr3* with severely reduced activity (*mdr3F*), the *P. falciparum pfmdr1* gene (*21*), the *S. cerevisiae STE6* gene (*23*, *24*), and the human *MRP* gene (*25*) were introduced in the plasmid vector pVT101-U (pVT) (*40*). Plasmid transformation, growth conditions for transformed yeast cells, and monitoring of heterologous protein expression were carried out as we have described in detail elsewhere (*34*). Briefly, DNA transformation of JPY201 cells was performed by the lithium acetate method of Ito et al. (*41*), and stable transformants were selected on SD−ura plates. Routinely, five to ten independent transformants were picked, pooled, grown as a mass culture in SD−ura medium, followed by freezing at −80 °C in SD−ura medium supplemented with 30% glycerol. All subsequent cultures were always started from these stock samples and grown either in SD−ura or YCG medium (0.75% yeast nitrogen base without amino acids, 0.35% casein base, 2% glucose), as indicated in the text.

Liquid Growth Inhibition Assay. The cytotoxicity of synthetic lipids ET-18-OCH_3 and ET-18-OH for yeast transformants expressing either wild type or mutant P-gps, MRP, STE-6, or Pgh-1 was measured by a growth inhibition assay in liquid medium (*34*). JPY201 transformants grown in YCG medium to mid-logarithmic phase (OD_{600} = 1.5−2) were diluted in the same medium to exactly OD_{600} = 0.10. The cultures were diluted an additional 20-fold (~2×10^5 cells) in YCG medium and 50 μL aliquots were subsequently pipetted in 96-well microtiter plates. After addition of 50 μL aliquots of ET-18-OCH_3 or ET-18-OH containing YCG medium (2× amount of the final indicated concentrations), the plates were incubated at 30 °C and time dependent growth was determined by optical density (A_{600}) using a standard ELISA plate reader. In some experiments, bovine serum albumin (BSA; detergent-free, Boeringher Mannheim) was added to the medium, as an acceptor molecule for the cytotoxic lipids, for various length of time after initial exposure to the toxic lipid. Growth of the various transformants in cytotoxic lipids was calculated as relative growth compared to the same transformants grown in drug-free medium and was expressed as a percentage. The IC_{50} is the drug dose necessary to cause a 50% growth inhibition of individual populations of yeast transformants, and the level

of resistance is calculated by comparing the IC_{50} values of individual yeast populations to the IC_{50} of the control cells transformed with the pVT plasmid vector.

Quantitative Mating Assay. Quantitative mating was performed as described previously (*34*) with some minor modifications. Briefly, JPY201 transformants were grown at 30 °C in YCG medium to mid-logarithmic phase (OD_{600} = 1.5−2) and diluted with the same medium to OD_{600} = 0.6. ET-18-OCH_3 or ET-18-OH was added from 10 mg/mL stock solutions (in 100% ethanol) as indicated in the figure legends. After an additional growth period of 4 h at 30 °C, 0.75 mL aliquots were mixed with 0.25 mL of a mid-logarithmic phase culture (OD_{600} = 1.6) of the DC17 *MAT*α tester strain. One-half of each suspension was then concentrated onto a glass fiber filter (Whatman, GF/C filters), transferred to a YPD (1% yeast extract, 2% Bactopeptone, 2% glucose) plate, and further incubated for 3 h at 30 °C to allow mating. The cells were then removed from the filters by vigorous vortexing in MM medium (0.68% yeast nitrogen base without amino acids, 2% glucose). Aliquots of appropriate dilutions in MM medium were plated in parallel on SD−ura plates (number of total haploid JPY201 cells) and on MM plates (number of diploids formed after successful mating), and the ratio of the two numbers was used to define the mating frequency.

Analytical Methods. Microsomes were isolated from the livers of Sprague-Dawley rats following a standard protocol (*42*). Microsomal protein concentration was determined according to the procedure of Bradford (*43*) and adjusted to 20 mg/mL. Pte·H_4-dependent *O*-alkyl cleavage of ET-18-OH was performed according to a published method (*44*), with some minor modifications. The cleavage reaction was initiated by adding to the 750 μL aliquots of JPY201 yeast cultures 125 μL of the enzyme reaction buffer (YCG medium containing 5 mM Pte·H_4, 25 mM reduced glutathione, 25 mM $(NH_4)_2SO_4$, 250 mM Tris-HCl, pH 9, supplemented with 1000 units of catalase). After addition of microsomes (25 μL), the samples were further incubated in the shaker for 1 h at 30 °C, and then mixed with 100 μL of a mid-logarithmic phase culture (OD_{600} = 1.6) of the DC17 *MAT*α tester strain culture which was concentrated 2.5 times (OD_{600} = 4). Subsequently, the effect of *O*-alkyl cleavage on ET-18-OH inhibition of mating by the various transformants was tested as described above. For the control experiments on *O*-alkyl cleavage by the tetrahydropteridin-requiring (Pte·H_4) microsomal alkylglycerol mono-oxygenase (MAMO) shown in Figure 4, 25 μL of the reaction buffer diluted to 140 μL (to 130 μL in conditions C) with YCG medium was supplemented with 5 μL of microsome (diluted to 10 mg/mL) and 5 μL of the ET-18-OH or ET-18-OCH_3 stock solutions. After incubation under described conditions, 5 μL of a PC (dipalmitoylphosphatidylcholine) stock solution (10 mg/mL, dissolved in ethanol) was added to each samples to monitor efficiency of subsequent lipid extraction. Lipids were extracted with a mixture containing 150 μL of chloroform and 50 μL of methanol, followed by vigorous vortexing and quick centrifugation (30 s at 14000*g*). The organic phase (solvent) was collected, and equal amounts (∼one-fifth of the total volume) were loaded on a TLC plate. Lipids were separated under acidic solvent conditions (chloroform/methanol/acetic acid/ddH_2O = 25/15/4/2) and visualized by ninhydrin staining. To obtain PC micelles, 2 mg of dipalmitoylphosphatidylcholine was dried down under nitrogen followed by resuspension in 200 μL of PBS. Micelles were obtained by sonicating the sample in five 1 min intervals in a bath sonicator.

RESULTS

Expression of the Class I (Mdr1/Mdr3) and Class II P-gps (Mdr2) in Yeast Causes Cellular Resistance to the Synthetic Lipid ET-18-OCH_3. The goal of the present study was to determine the extent of functional similarity between the Class I (Mdr1/Mdr3) and Class II (Mdr2) P-gp isoforms, as well as between P-gps and other ABC transporters (STE6, MRP, Pgh-1). Since at the time we initiated these experiments, the only known physiological substrates for these transporters was either a lipid (PC for Mdr2, wide range of short-chain phospholipids for Mdr1/Mdr3) or a lipidated peptide (**a**-mating pheromone for STE6), we tested whether synthetic and cytotoxic phospholipid analog may reveal functional aspects of transport common to these ABC transporters. Among synthetic alkyl-lysophospholipids tested for toxicity and antitumor activity *in vitro* (tumor cell lines) and *in vivo* (transplantable tumors) (*45*, *46*), 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (ET-18-OCH_3, also known as edelfosine) shows very high cytotoxicity for mammalian cells (*47*). Since ET-18-OCH_3 is a close analog of lysophosphatidylcholine, a direct precursor of the Mdr2 substrate (PC), we speculated that ET-18-OCH_3 may also be recognized by Mdr2.

To test this hypothesis, we first determined if ET-18-OCH_3 was cytotoxic for *S. cerevisiae* JPY201 cells, and then tested whether overexpression of Mdr2 in these cells would alter their sensitivity to cytotoxic concentrations of this lipid analog (Figure 1). Using a growth inhibition assay in liquid medium (*34*), we observed that low micromolar concentrations of ET-18-OCH_3 (1−2 μg/mL) were sufficient to almost completely inhibit growth of JPY201 cells (data not shown) or JPY201 transformed with the control plasmid pVT (IC_{50} < 1 μg/mL) (Figure 1). In contrast, expression of Mdr2 in the same cells caused the appearance of cellular resistance to ET-18-OCH_3. In dose−response experiments, Mdr2 transformants showed an IC_{50} of 18 μg/mL (20-fold above JPY201 background), and significant growth was notable in ET-18-OCH_3 concentrations up to 25 μg/mL. In drug-free medium, pVT control and Mdr2 yeast transformants grew at the same rate over the 22 h observation period (data not shown). These results suggest that ET-18-OCH_3 may be a substrate for Mdr2.

We next tested the possibility that Class I P-gps (Mdr1/Mdr3) may also recognize ET-18-OCH_3 as a substrate. For this, we used the growth inhibition assay described above to establish in dose−response experiments the relative level of susceptibility/resistance to ET-18-OCH_3 of JPY201 transformants expressing similar amounts of Mdr1 or Mdr3 (*34*). Results in Figure 1 clearly show that expression of either Mdr1 or Mdr3 in JPY201 cells causes cellular resistance to ET-18-OCH_3, with IC_{50}s of 8 μg/mL (Mdr1; 8× resistance) and 9 μg/mL (Mdr3; 9× resistance), respectively. In these experiments we also included a JPY201 transformant stably expressing a mutant variant of Mdr3 (S939F; Mdr3F) to control for nonspecific effects of P-gp expression on cytotoxicity of ET-18-OCH_3. This mutant carries a serine to phenylalanine substitution at position 939 in transmembrane domain 11 which causes a severe reduction in transport

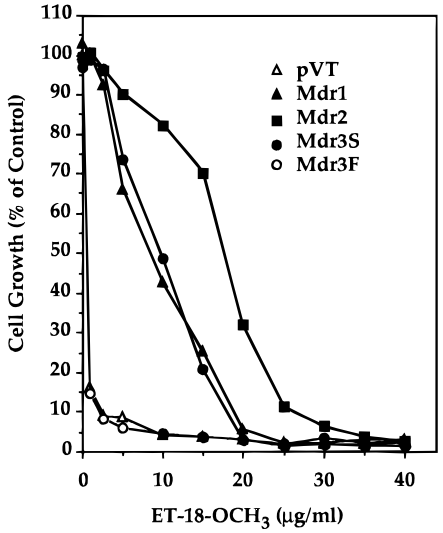


FIGURE 1: Growth inhibition properties of ET-18-OCH_3 for JPY201 cells expressing mouse Mdr1, Mdr2, or Mdr3 P-gp isoforms. The growth inhibitory properties of ET-18-OCH_3 for control yeast JPY201 cells (open triangles), and for JPY201 transformants expressing Mdr1- (filled triangles), Mdr2- (filled squares), Mdr3S- (filled circles), and an inactive Mdr3 mutant, Mdr3F (open circles) were determined in liquid medium as described under Materials and Methods. JPY201 transformants were diluted to an OD_{600} = 0.05 and 50 μL aliquots seeded in 96-well microtiter plates, followed by addition of 50 μL aliquots of YCG medium containing 2× concentrations of ET-18-OCH_3. Plates were incubated at 30 °C, and growth was determined by optical density (A_{600}) in a standard ELISA plate reader (*34*). Cell growth is expressed as a ratio (percentage) of growth measured in control conditions in the absence of ET-18-OCH_3 after an 18 h incubation period. Data represent the mean of three independent experiments. Standard errors on the mean were less than 5% (not shown for clarity).

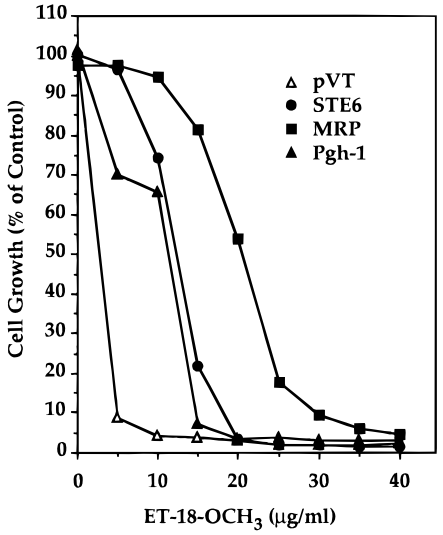


FIGURE 2: Growth inhibition properties of ET-18-OCH_3 for JPY201 cells expressing STE6, MRP, or Pgh-1 (*pfmdr-1*). The growth inhibitory properties of ET-18-OCH_3 for control pVT transformants (open triangles) and for transformants expressing STE6- (filled circles), MRP- (filled squares), or Pgh-1 (filled triangles) were determined as described in the legend to Figure 1. Data represent the mean of three independent experiments. Standard errors on the mean were less than 5% (not shown for clarity).

activity in cultured mammalian cells (*48*) and in yeast cells (*33*, *34*, *36*). Mdr3F expressing cells were found to be as sensitive to ET-18-OCH_3 as control pVT transformants (Figure 1), indicating that the observed effect of Class I and Class II P-gp expression on cellular resistance to this synthetic lipid was specific and required an intact transporter.

Expression of the ABC Transporters STE6, MRP, and Pgh-1 in Yeast Causes Cellular Resistance to the Synthetic Lipid ET-18-OCH_3. ET-18-OCH_3 is unique in that it is one of the first-reported substrates which seems to be recognized by both Class I and Class II P-gp isoforms (*49*). This observation prompted us to test the possibility that this unique property may also be shared by other ABC transporters such as STE6, MRP, and Pgh-1. We previously expressed the STE6, Pgh-1, and MRP proteins in yeast (*22*, *32*, *38*) and used the above described growth inhibition assay to monitor a possible effect of these proteins on cellular resistance of JPY201 cells to ET-18-OCH_3. As shown in Figure 2, expression of these three proteins caused significant levels of resistance to this lipid, with calculated IC_{50}s of 12 μg/mL (STE6), 13 μg/mL (Pgh-1), and 22 μg/mL (MRP). Resistance levels calculated for STE6 and Pgh-1 were similar to those measured for Class I P-gps (Mdr1/Mdr3), while the level of resistance of MRP-expressing cells was similar to that produced by Class II P-gp (Mdr2). Cytotoxicity experiments shown in Figures 1 and 2 therefore indicate that expression of phylogenetically distant ABC transporters in yeast causes cellular resistance to the same synthetic lipid molecule.

*Effect of ET-18-OCH_3 on Complementation of Mating by P-gp and Other ABC Transporters in a Null*Δ*ste6 Yeast Mutant.* To further explore the molecular basis of this interaction, we monitored a possible effect of ET-18-OCH_3 on the demonstrated capacity of P-gp, MRP, Pgh-1 to transport the lipidated peptide mating pheromone **a**-factor in yeast and restore mating in an otherwise sterile null mutant at *ste6* (Δste6). This was based on the proposal that ET-18-OCH_3 may interact with a site on ABC transporters that is important for transport of this lipidated peptide pheromone. This was done by testing the effect of ET-18-OCH_3 exposure on the formation of diploid cells after mating of haploid P-gp, MRP, and Pgh-1 expressing cells to a haploid tester stock of the opposite mating type in a quantitative mating assay (*34*). Therefore, sterile JPY201 cells (*MAT a*), carrying a Δste6 deletion (*33*) overexpressing either wild type (Mdr3S) or mutant P-gp (Mdr3F) or expressing MRP, Pgh-1, or control STE6 proteins were analyzed for their ability to mate with DC17 cells (*MAT*α) with or without a prior 3 h treatment of JPY201 transformants with ET-18-OCH_3. Results of these experiments are summarized in Table 1. A 3 h treatment of Mdr3S cells with a high concentration of ET-18-OCH_3 (125 μg/mL) resulted in a 2-fold reduction in viability of these cells (∼40 % reduction); however, this treatment caused a nearly 10-fold decrease in the capacity of Mdr3S cells to mate with the DC17 tester stock (90% reduction). Similar but more pronounced results were also obtained when a higher concentration of ET-18-OCH_3 was used (250 μg/mL) for the same incubation time (data not shown).

The effect of ET-18-OCH_3 on the ability of MRP, Pgh-1, and the control yeast STE6 protein to complement a Δste6 mutant was evaluated by the same experimental protocol (Table 1). Overall, we observed a similar inhibitory effect of ET-18-OCH_3 on mating complementation by these other ABC transporters. Treatment of the various transformants with 125 μg/mL ET-18-OCH_3 resulted in a 40−50% reduction in cell viability, but consistently resulted in near 10-

Table 1: Inhibitory Properties of ET-18-OCH_3 on Growth and Mating Activity of JPY201 Cells Expressing ABC Transporters

			conditions	
plasmid	assay	control	ET-18-OCH_3[a]	ET-18-OCH_3/BSA[b]
pVT	growth[c]	100 ± 6	36 ± 5	35 ± 4
	mating[d]	ND	ND	ND
Mdr3S	growth	100 ± 4	63 ± 12	84 ± 3
	mating	100 ± 3	11 ± 2	62 ± 3
Mdr3F	growth	100 ± 5	33 ± 5	29 ± 8
	mating	ND	ND	ND
Ste-6	growth	100 ± 6	50 ± 4	77 ± 4
	mating	100 ± 3	10 ± 4	66 ± 8
MRP	growth	100 ± 3	56 ± 4	88 ± 6
	mating	100 ± 4	11 ± 5	64 ± 6
Pgh-1	growth	100 ± 4	59 ± 11	79 ± 12
	mating	100 ± 5	9 ± 5	61 ± 6

[a] JPY201 transformants (~2.8×10^7 cells) expressing ABC transporters Mdr3S, mutant Mdr3F, STE6, MRP, and Pgh-1 were incubated with ET-18-OCH_3 (125 μg/mL) for 4 h at 30 °C. After 3 h of incubation in the presence of the drug, the samples were either incubated further or [b] treated with the lipid acceptor protein bovine serum albumin (BSA), as described under Material and Methods. [c] To determine the cell viability, 75 μL aliquots (diluted 10^4) were plated on SD−ura and incubated 48 h at 30 °C. [d] For mating experiments, JPY201 (Δ*ste6*) transformants were mixed with DC17 *MAT*α tester cells, and mating was monitored by formation of diploid colonies on selective medium. Half of each suspension was concentrated on glass fiber filters, transferred to a YPD plate and incubated for 3 h at 30 °C to allow mating. The cells were then recovered from the filters and plated on either SD−ura (number of surviving haploid cells) or on minimal agar (number of diploids formed). Mating efficiency was calculated as the ratio of haploid cells introduced in the assay divided by the number of diploids formed by successful mating; it is expressed as a percentage of the mating frequency observed for the same transformant under control conditions. Data represent means of eight determinations of two independent experiments.

fold reduction in their ability to mate. Overall, these results show that the cellular resistance to ET-18-OCH_3 caused by expression of P-gp and other ABC transporters in yeast is paralleled by an inhibitory activity of this compound toward complementation of mating (**a**-pheromone transport) by these ABC transporters.

A series of control experiments were carried to determine if the effect of ET-18-OCH_3 on yeast transformants expressing ABC transporters were specific. First, we introduced in the assay BSA, a lipid acceptor molecule which binds ET-18-OCH_3 in solution (*50*). Addition of BSA to the extracellular medium for 1 h after a 3 h exposure to ET-18-OCH_3 resulted in significantly increased survival of Mdr3S, MRP, Pgh-1, and STE6 transformants (Table 1) but also restored the mating capacity of these cells back to near-normal levels measured in untreated controls (Table 1). Secondly, the reversal of ET-18-OCH_3 toxicity caused by a 1 h treatment with BSA on induced cytotoxicity was only seen in cells expressing a functional ABC transporter (e.g., Mdr3S), but was not observed when a mutant Mdr3 variant with reduced activity (Mdr3F) was expressed in yeast cells. This indicates that the reversal effect of BSA on lipid toxicity requires a functional transporter and suggests that BSA may act as a lipid acceptor for ET-18-OCH_3 transported by Mdr3S and other ABC transporters. Thirdly, mating frequencies calculated in our mating assay were independent of the precise number of haploid **a**-cells introduced into the assay (and that may vary due to ET-18-OCH_3 cytotoxicity). Increasing the number of Mdr3S haploid transformants (Figure 3A, filled circles) while the number of haploid DC17 tester stock introduced in the assay was kept constant resulted in an identical overall mating frequency calculated as the ratio of diploid to haploid JPY201 cells present in the assay (Figure 3A, open circles). Fourthly, to exclude the possibility that a cytotoxic effect of ET-18-OCH_3 on the tester stock DC17 may influence the results of mating assays, DC17 cells were treated for 5 min with the equivalent concentration of ET-18-OCH_3 either 1) prior to the addition of Mdr3S-expressing cells and concentration on the nitrocellulose filter (Figure 3B, conditions 2 and 3), or DC17 cells were treated with ET-18-OCH_3, concentrated on the filter, followed by filtering of the Mdr3S transformants (Figure 3B, conditions 5 and 6). In both cases, the final mating frequency obtained with treated DC17 cells was similar to that obtained with control untreated cells (Figure 3B, conditions 1 and 4). Taken together, these results indicate that ET-18-OCH_3 can abrogate complementation of mating by the ABC transporters tested, suggesting that ET-18-OCH_3 and the **a**-factor may interact with a common transport site(s).

Effect of ET-18-OH on Complementation of Mating by P-gp and Other ABC Transporters in a Null Δste6 Mutant. To further test the specificity of the interaction between ET-18-OCH_3 and ABC transporters, we carried additional experiments with the closely related ether lipid analog 1-*O*-octadecyl-*sn*-glycero-3-phosphocholine (ET-18-OH). ET-18-OH differs from ET-18-OCH_3 only by a substitution of the methoxy for a hydroxyl group at the *sn-2* position of the glycerol backbone. However, unlike ET-18-OCH_3, the cytotoxicity of ET-18-OH can be completely abrogated (*44*) by incubation with the tetrahydropteridin (Pte·H_4) requiring microsomal enzyme alkylglycerol mono-oxygenase (MAMO; 1-*O*-alkyl cleavage) (*51*, *52*). Control experiments showed that the cytotoxicity of ET-18-OH for JPY201 cells was similar to that of ET-18-OCH_3 while expression of Mdr3S, STE6, MRP, as well as Pgh-1 in these cells also caused cellular resistance to this compound (Table 2 and data not shown). Therefore, sensitivity to MAMO treatment can be used to ascertain specificity of the detected ET-18-OH effects.

Since we had to adapt a published protocol (*44*) for the microsomal Pte·H_4-dependent 1-*O*-alkyl cleavage reaction to our assay system in yeast, we first verified the efficacy of the cleavage reaction in this system. For this, ET-18-OH was extracted from the enzyme reaction mixture at various time points and analyzed on TLC plates prior to (Figure 4, conditions 2 and 3) and after incubation at 30 °C (conditions 4−6; new conditions) or 37 °C (conditions 7 and 8) (Figure 4). Under our assay conditions, complete destruction of ET-18-OH was observed after 30 min at 30 °C, or after 10 min at 37 °C. As expected, ET-18-OCH_3 was not affected by the enzyme (conditions 9−12). These controls show that ET-18-OH but not ET-18-OCH_3 can be efficiently cleaved in our system by MAMO. To determine if MAMO could cleave ET-18-OH molecules present in membranes, pure PC micelles were incubated in the reaction buffer and mixed with ET-18-OH prior to addition of MAMO: incubation of ET-18-OH under these conditions (13−16) resulted in a time-dependent cleavage which was complete by 30 min, suggesting that ET-18-OH can be cleaved by MAMO both in aqueous solutions and in the context of a lipid bilayer.

The effect of ET-18-OH on complementation of mating by P-gp and other ABC transporters was tested as described in Table 1 for ET-18-OCH_3. Briefly, aliquots of Mdr3S,

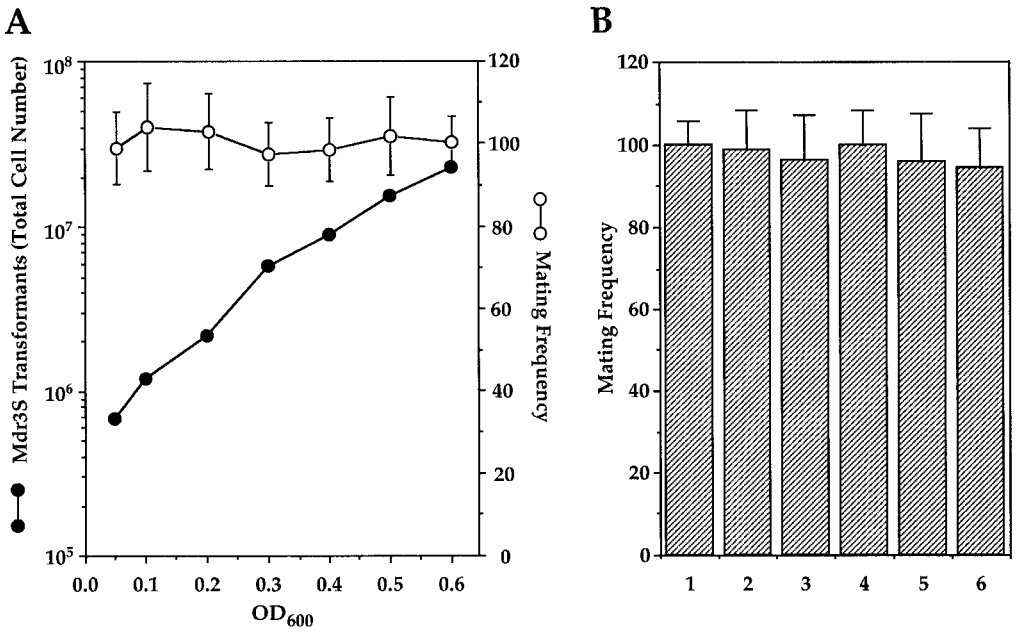


FIGURE 3: Effect of JPY201 and DC17 cell numbers on calculated mating frequencies. (A) Increasing numbers of Mdr3S JPY201 transformants (filled circles) were incubated with fixed numbers of DC17 cells, and mating frequencies were determined (percentage of control conditions, open circles) as described under Materials and Methods. (B) To determine if possible cytotoxic effects of ET-18-OCH_3 for DC17 cells may affect the calculated mating frequencies, DC17 cells were mixed for 5 min with ET-18-OCH_3 prior to addition of an aliquot of Mdr3S cells ($\sim 2.8 \times 10^7$ cells) and concentration on glass fiber filters (condition 1, no drug;, condition 2, +125 μg/mL ET-18-OCH_3; condition 3, +250 μg/mL ET-18-OCH_3). Alternatively, ET-18-OCH_3 treated DC17 cells were first concentrated on the filter prior to filtering the Mdr3S cells containing aliquot ($\sim 2.8 \times 10^7$ cells) on the same filter (condition 4, no drug; condition 5, +125 μg/mL ET-18-OCH_3; condition 6, +250 μg/mL ET-18-OCH_3). Cells were then recovered from the filters, and mating frequencies were calculated and are expressed as a percentage of those obtained under control conditions.

Table 2: Inhibitory properties of ET-18-OH on Growth and Mating Activity of JPY201 Cells Expressing ABC Transporters

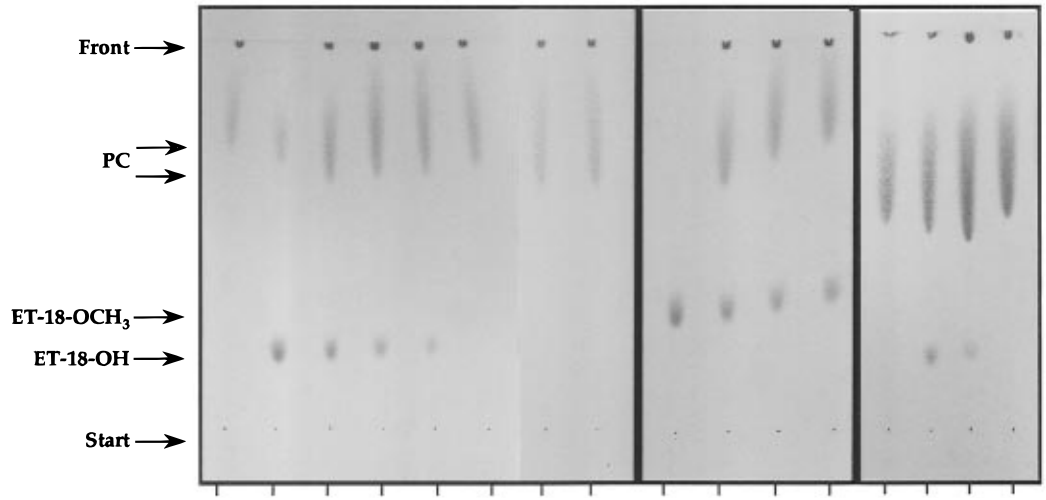
			conditions	
plasmid	assay	control	ET-18-OH[a]	ET-18-OH/MAMO[b]
pVT	growth[c]	100 ± 4	34 ± 4	36 ± 9
	mating[d]	ND	ND	ND
Mdr3S	growth	100 ± 2	58 ± 10	76 ± 12
	mating	100 ± 3	18 ± 7	91 ± 13
Mdr3F	growth	100 ± 3	28 ± 7	26 ± 5
	mating	ND	ND	ND
Ste-6	growth	100 ± 4	58 ± 12	80 ± 11
	mating	100 ± 4	9 ± 3	87 ± 8
MRP	growth	100 ± 6	62 ± 12	76 ± 11
	mating	100 ± 2	27 ± 10	90 ± 8
Pgh-1	growth	100 ± 7	54 ± 7	75 ± 8
	mating	100 ± 2	18 ± 9	78 ± 13

[a] JPY201 transformants ($\sim 2.8 \times 10^7$ cells) expressing ABC transporters Mdr3S, mutant Mdr3F, STE6, MRP, and Pgh-1 were incubated with ET-18-OH (125 μg/mL) for 4 h at 30 °C. After 3 h incubation in the presence of the drug, the samples were either incubated further or [b] treated with the enzyme MAMO, as described under Materials and Methods. [c] To determine the cell viability, 75 μL aliquots (diluted 10^4) were plated on SD−ura and incubated 48 h at 30 °C. [d] For mating experiments, JPY201 (Δ*ste6*) transformants were mixed with DC17 *MAT*α tester cells, and mating was monitored by formation of diploid colonies on selective medium. Mating frequencies were calculated as described in the legend of Table 1 and represent means of eight determinations of two independent experiments.

Mdr3F, MRP, Pgh-1, and STE6 transformants were first incubated for 3 h with ET-18-OH (125 μg/mL), and then groups were further incubated with either MAMO or in control conditions. As summarized in Table 2, ET-18-OH caused reduction in viability and vegetative growth of all yeast transformants expressing wild type ABC proteins (125 μg/mL, ∼40−50% reduction) similar to that seen for ET-18-OCH_3 (Table 1). ET-18-OH toxicity for yeast Mdr3S, MRP, Pgh-1, and STE6 transformants was abrogated by MAMO treatment, restoring viability to near-control levels seen in cells untreated with ET-18-OH. In contrast, the toxicity of ET-18-OH for Mdr3F expressing cells or pVT plasmid controls (65% inhibition at 125 μg/mL) was not altered by enzymatic treatment with MAMO. This suggests that accessibility of the cytotoxic lipid to MAMO treatment requires the presence of a functional transporter. ET-18-OH was also found to inhibit mating of the various ABC transporters (Table 2), in a fashion very similar to that seen for ET-18-OCH_3. For example, although exposure to 125 μg/mL ET-18-OH reduced viability of Mdr3S-expressing cells by about 40%, it reduced mating in these cells (formation of diploids) by approximately 90%. Destroying the lipid compound enzymatically with MAMO resulted in a ∼90% recovery of both, the vegetative growth as well as the mating capacity. A similar effect was seen for the other ABC transporters (Table 2). Again this suggests that ET-18-OH has to be exposed in the outer leaflet of the cell membrane to be accessible to MAMO, since treatment of control and Mdr3F cells with the enzyme did not affect the growth inhibition in these cells.

DISCUSSION

ABC transporters are a superfamily of integral membrane proteins that have retained a common architecture throughout evolution, consisting of twelve TM domains and two highly conserved nucleotide binding sites of the Walker type (*5*). ABC transporters have been shown to act on a wide range of substrate molecules, including lipids (Class I and Class II P-gps), lipophylic cations (Class I P-gps) and anions (MRP), peptides (TAP-1/TAP-2, STE6), ions (CFTR) and others (SUR, sulfonylurea receptor) (*3*, *4*). A puzzling aspect of this protein family is that although some ABC transporters



Condition	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16
Microsomes (μl)	5	---	5	5	5	5	5	5	---	5	5	5	5	5	5	5
PC micelles (μl)	---	---	---	---	---	---	---	---	---	---	---	---	10	10	10	10
ET-18-OH (μl)	---	5	5	5	5	5	5	5	---	---	---	---	---	5	5	5
ET-18-OCH3 (μl)	---	---	---	---	---	---	---	---	5	5	5	5	---	---	---	---
Incubation (min)	0	0	0	10	20	30	10	30	0	0	10	30	0	0	10	30
Temperature (°C)	0	0	0	30	30	30	37	37	0	0	37	37	0	0	30	30

FIGURE 4: Enzymatic cleavage of ET-18-OH and ET-18-OCH_3 by MAMO. Lanes 1−12: Reaction conditions and reagents were as listed below the figure, with a final reaction volume of 140 μL of YCG medium. The microsomes preparation stock was at 10 mg/mL. After incubation under the indicated conditions, an aliquot of phosphatidylcholine PC was added and total lipids were extracted with chloroform−methanol. Lipids were analyzed by thin-layer chromatography (TLC) followed by staining with ninhydrin and photography. Lanes 13−16: Conditions were similar to those used for 1−12 except that 10 μL of PC micelles were mixed with 5 μL of ET-18-OH (10 mg/mL) and incubated on ice for 5 min. Microsomes were then added and cleavage of the synthetic lipid monitored by TLC analysis of the chloroform−methanol extract, as described above.

appear to transport a single substrate (STE6, Class II P-gp), others (Class I P-gp, MRP, Pgh-1) seem to be less specific and can apparently act upon a large range of structurally related or unrelated molecules. The profiles of sequence conservation among ABC transporters (high in the NB sites and low in the membrane region), together with the direct analysis of their mechanism of transport (requires ATP hydrolysis) has suggested a model in which ABC proteins have evolved to develop the capacity to work on different types of substrates but have retained a common mechanistic aspect of transport. Although intuitively appealing, very little data supporting a functional parallel to the noted structural similarity among members of this family had been obtained until recently. Indeed, expression studies in the yeast *S. cerevisiae* have shown that P-gp (mouse Mdr3) can complement a *ste6* mutant and partially restore **a** mating pheromone transport and mating in this sterile mutant (*33*). Pgh-1 (*22*, *39*) and MRP (*38*) were recently shown to also complement STE6 in yeast. Results from cytotoxicity studies and inhibition of mating by the synthetic alkyl phospholipids reported here further strengthen the notion of functional conservation among ABC transporters.

To date, the mammalian P-gp family has been classified functionally into Class I (mouse Mdr1/Mdr3, human MDR1) and Class II proteins (mouse Mdr2, human MDR2), by virtue of their capacity to convey (Class I) or not (Class II) MDR. On the other hand, the class II isoforms have been shown by us (mouse Mdr2) and others (human Mdr2) to function as membrane flippases for the phospholipid, phosphatidylcholine; Furthermore, the Mdr2 activity is highly specific for PC since the aminophospholipids phosphatidylserine and phosphatidylethanolamine are not translocated by Mdr2 (*12*, *13*, *15*). The normal physiological role of the Class I isoforms has until recently remained elusive (*14*). In this study, we used cytotoxic lipid analogs to study the substrate specificity of Mdr2. Lipids such as ET-18-OCH_3 and ET-18-OH differ from PC only by a substitution of the *sn-2* position of the alkyl chain (methoxy or hydroxyl group) and by an ether linkage at the *sn-1* position of the second hexadodecyl chain. In contrast to PC, however, these molecules are extremely toxic to mammalian and yeast cells and thus, suitable for analysis by growth inhibition of intact cells expressing this group of proteins. Here, we show that the synthetic ether lipid and PC analogs ET-18-OCH_3 and ET-18-OH are substrates not only for Class II but also for Class I mouse P-gp isoforms (Figure 1). These findings and interpretations are in agreement with recent studies by Van Helvoort et al. (*14*, *15*) in polarized epithelial cells which suggest that Class I P-gp isoforms may be broad-specificity transporters for short-chain phospholipids.

In this study, we show that ET-18-OCH_3 and ET-18-OH are also substrates for other ABC transporters encoded by the mammalian MRP, the *Plasmodium* Pgh-1, and the yeast STE6 proteins. Indeed, overexpression of these proteins (and P-gps) in JPY201 cells causes the appearance of cellular resistance to toxic concentrations of these lipid analogs in growth inhibition assays (Figure 2). In addition, both synthetic lipids efficiently block transport of the farnesylated peptide **a** mating pheromone by these ABC transporters, and can block complementation of a Δste6 mutant, as measured by the formation of diploid cells in a mating assay (Tables 1 and 2). The observed effects of the synthetic lipids on cell cytotoxicity and mating appeared to be specific, as opposed to reflecting nonspecific effects of the lipids on membrane fluidity or cell fusion events or through inhibiting of farnesyl transferase activity. First, they could be blocked by either the lipid acceptor molecule BSA or by enzymatic cleavage of the lipid (ET-18-OH) by MAMO (Table 2).

Second, the BSA and MAMO protective effects required the presence of a functional transporter and were not seen in cells expressing either no variant or a mutant inactive variant of Mdr3 (Mdr3F). Therefore, one tempting interpretation of these results is that the PC analogs ET-18-OCH_3 and ET-18-OH define a common structural determinant that is important for substrate recognition by the ABC transporters tested above. This structural determinant would also be conserved in the peptide **a** mating pheromone, as ET-18-OCH_3 and ET-18-OH block mating by heterologous ABC transporters but also by the endogenous STE6 (Tables 1 and 2). The farnesyl moiety of the **a**-peptide pheromone may be the structure homologous to the synthetic lipids tested here, suggesting that the farnesyl lipid modification of the **a**-peptide may be not only important for membrane targeting of the pheromone but also for its recognition and transport by STE6. Although additional biochemical characterization of these interactions is required to validate this proposition, such an hypothesis is supported by independent studies that have shown (1) inhibition of azidopine photolabeling of P-gp by simultaneous incubation with prenylcysteine methyl esters and (2) stimulation of P-gp ATPase activity by prenylcysteine methyl esters (*53*).

Our results also suggest that ET-18-OCH_3 and ET-18-OH transport by Class I and Class II P-gps and by the other ABC transporters analyzed may be by a flippase mechanism to translocate the lipid analogs from the inner leaflet of the membrane to the outer leaflet. This is based on the following observations. The reversal effect on ET-18-OCH_3 and ET-18-OH cytotoxicity induced by either the lipid acceptor BSA or after enzymatic treatment with MAMO requires the presence of functional ABC transporters (Mdr3S, MRP, Pgh-1, and STE6) and is not seen in cells expressing a mutant inactive variant (pVT control and Mdr3F transformants). Therefore, accessibility of the ET-18-OCH_3 and ET-18-OH to either BSA or MAMO in the presence of active ABC transporters may reflect presence of the lipid at the cell surface where it would be accessible or direct transport into the medium. It is tempting to speculate by analogy with the demonstrated capacity of Mdr2 to translocate PC molecules from the inner to the outer leaflet of the lipid bilayer (*12*) that the Class I P-gps and ABC transporters tested may also transport ET-18-OCH_3 and ET-18-OH by a similar mechanism. The transporters would concentrate the substrate to the outer lipid layer of the membrane where it would become accessible for binding to BSA or to enzymatic cleavage by MAMO. This would not occur in control pVT and Mdr3F transformants. Exposure to BSA or MAMO would liberate transport sites on the transporters, therefore increasing the "off" rate of transport and restoring both cellular resistance to ET-18-OCH_3 and ET-18-OH and **a**-factor transport. Although highly speculative, this model is in good agreement with our previous studies showing that Mdr2-mediated translocation of NBD-labeled phosphatidylcholine (NBD-PC) in yeast secretory vesicles can be positively modulated by bile acids (*37*). Pre-loading these vesicles with a high enough concentration of the primary bile salt taurocholate caused a significant enhancement of Mdr2-mediated NBD-PC translocation across the bilayer. As the underlying mechanism, we demonstrated that taurocholate behaves as a potent lipid solubilizer and directly extracts NBD-PC out of the inner membrane and forms intravesicular NBD-PC/taurocholate aggregates. The constant removal of the reporter lipid from the membrane caused an indirect positive regulation of the Mdr2 transport system to translocate more PC molecules across the membrane bilayer.

In conclusion, these experiments have identified cytotoxic phospholipids as common substrates for several ABC transporters. Results from experiments using BSA as an acceptor molecule and MAMO treatment to inactivate the toxic lipid together suggest that the mechanism of lipid transport by these ABC transporters may be similar to the mechanism of PC transport by Mdr2 which involves translocation of the lipid from the inner to the outer phase of the membrane.

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