The Membrane Lipid Environment Modulates Drug Interactions with the P-Glycoprotein Multidrug Transporter†

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ABSTRACT: The P-glycoprotein multidrug transporter functions as an ATP-driven efflux pump for a large number of structurally unrelated hydrophobic compounds. Substrates are believed to gain access to the transporter after partitioning into the membrane, rather than from the extracellular aqueous phase. The binding of drug substrates to P-glycoprotein may thus be modulated by the properties of the lipid bilayer. The interactions with P-glycoprotein of two drugs (vinblastine and daunorubicin) and a chemosensitizer (verapamil) were characterized by quenching of purified fluorescently labeled protein in the presence of various phospholipids. Biphasic quench curves were observed for vinblastine and verapamil, suggesting that more than one molecule of these compounds may bind to the transporter simultaneously. All three drugs bound to P-glycoprotein with substantially higher affinity in egg phosphatidylcholine (PC), compared to brain phosphatidylserine (PS) and egg phosphatidylethanolamine (PE). The nature of the lipid acyl chains also modulated binding, with affinity decreasing in the order egg PC > dimyristoyl-PC (DMPC) > dipalmitoyl-PC (DPPC). Following reconstitution of the transporter into DMPC, all three compounds bound to P-glycoprotein with 2−4-fold higher affinity in gel phase lipid relative to liquid-crystalline phase lipid. The P-glycoprotein ATPase stimulation/inhibition profiles for the drugs were also altered in different lipids, in a manner consistent with the observed changes in binding affinity. The ability of the drugs to partition into bilayers of phosphatidylcholines was determined. All of the drugs partitioned much better into egg PC relative to DMPC and DPPC. The binding affinity increased (i.e., the value of K_d decreased) as the drug−lipid partition coefficient increased, supporting the proposal that the effective concentration of the drug substrate in the membrane is important for interaction with the transporter. These results provide support for the vacuum cleaner model of P-glycoprotein action.

Overexpression of the P-glycoprotein multidrug transporter (Pgp[1]) is one of the major causes of multidrug resistance (MDR) in human cancers. Pgp, which is a member of the ABC superfamily of membrane proteins, is an ATP-driven efflux pump for a diverse range of chemotherapeutic drugs, natural products, and hydrophobic peptides (for recent reviews, see refs *1*−*4*). From the primary sequence of Pgp and hydropathy plot analysis, the protein is predicted to be made up of two homologous halves, each comprising six putative hydrophobic transmembrane segments and a nucleotide binding (NB) domain on the cytoplasmic side of the membrane. This structural model has been confirmed by biochemical mapping studies (*5*, *6*). The Class I and II Pgp gene products are multidrug transporters, whereas the Class III Pgp appears to function as a lipid flippase, exporting phosphatidylcholine (PC) into the bile (*7*, *8*).

Pgp is an unusual ATP-driven transporter, in that it has a low affinity for ATP and exhibits a high level of constitutive or basal ATPase activity in the apparent absence of substrates. The basal ATPase activity of purified Pgp is further stimulated or inhibited by certain transport substrates, chemosensitizers, and peptides (for example, see refs *9*−*12*). The fact that drugs affect the ATPase activity indicates that there is some type of communication, or coupling, between the drug-binding sites, which are believed to reside within the membrane-spanning regions of the transporter, and the NB domains. Fluorescence spectroscopic studies of purified Pgp recently demonstrated the existence of such conformational communication between the two domains (*13*). The lipid environment appears to alter this coupling. The pattern of stimulation or inhibition of Pgp ATPase by four drugs was strongly modulated by the lipid environment in which the protein was reconstituted, whereas their inhibition of azidopine photoaffinity labeling remained unchanged (*14*). The interaction of Pgp with drugs also appears to be dependent on the lipid environment. The yeast steroid ergosterol was shown to inhibit photolabeling of Pgp by azidopine, as well as inactivate its drug transport function (*15*), and azidopine labeling was differentially modulated by various sterols (*16*).

The mechanism by which Pgp transports such a wide variety of hydrophobic compounds remains unclear. The so-

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; MDR, multidrug resistance; MIANS, 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid; NB, nucleotide binding; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Pgp, P-glycoprotein; PS, phosphatidylserine.

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called "vacuum cleaner" model proposes that drugs gain access to the substrate binding and transport site(s) of Pgp directly from the lipid bilayer, rather than from the aqueous solution (*17*). Substantial evidence has accumulated, indicating that this is indeed the case (summarized in ref *4*). Pgp appears to export drugs directly from the membrane (*18*), likely via a substrate-binding site (or sites) accessible from the cytoplasmic leaflet of the bilayer (*19*, *20*). A similar mechanism was recently demonstrated for the bacterial multidrug transporter lactococcal LmrP (*21*). It has also been suggested that the class I and II Pgps may be drug flippases, by analogy with the Class III gene product (*17*, *22*).

Given the fact that most Pgp substrates are hydrophobic and probably partition into the bilayer before they interact with the transporter, we might expect that the nature of the host lipid bilayer would play an important role in modulating drug binding to Pgp. This issue has been difficult to approach, since until recently there was a lack of quantitative methods available for measuring equilibrium drug binding to Pgp. Most studies of drug binding have employed photoaffinity labeling, a low efficiency, nonequilibrium technique, and its inhibition by competing compounds. The development in our laboratory of a fluorescence quenching technique for assessing equilibrium binding of both ATP and drugs to purified Pgp (*13*, *23*, *24*) has opened up the possibility of directly assessing the effects of the lipid environment on Pgp function.

The objectives of the present study were to determine how the properties of the host lipid bilayer modulate the interaction of transport substrates with Pgp, using fluorescence quenching to quantitate equilibrium drug binding. These experiments were carried out with purified Pgp and various phospholipids, both natural lipid mixtures and defined chain synthetic species. Parameters that were varied include the nature of the lipid headgroup, the acyl chain length, and the phase state of the bilayer. The biochemical and biophysical nature of the surrounding lipid was found to have large effects on the interaction of Pgp with drugs and chemosensitizers. For a series of PC lipids, the affinity of binding of drugs to Pgp was highly correlated with their ability to partition into the lipid bilayer, suggesting that substrates enter the bilayer before they interact with the transporter.

MATERIALS AND METHODS

Materials. Dimyristoyl-L-α-phosphatidylcholine (DMPC), dipalmitoyl-L-α-phosphatidylcholine (DPPC), egg PC, egg phosphatidylethanolamine (PE), and brain phosphatidylserine (PS) were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesterol, vinblastine, daunorubicin, verapamil, and 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane-sulfonate (CHAPS) were purchased from Sigma Chemical Co. (St. Louis, MO). 2-(4′-Maleimidylanilino)naphthalene-6-sulfonic acid (MIANS) was supplied by Molecular Probes (Eugene, OR).

MDR Cell Lines and Pgp Purification. Pgp was isolated from the plasma membrane of the highly drug-resistant cell line CH^RB30, using the detergent CHAPS, to give a highly enriched S_2 fraction, as described previously (*13*). Removal of contaminating glycoproteins by affinity chromatography on concanavalin A-Sepharose gave highly purified (90−95%) Pgp (*13*, *25*, *26*). Pgp preparations were kept on ice and used the same day.

Protein Assay. A microplate adaptation of the method of Bradford (*27*) was used to determine the protein content of CH^RB30 plasma membrane. For purified Pgp and reconstituted proteoliposomes, protein was quantitated by the method of Peterson (*28*). In both assays, bovine serum albumin (crystallized and lyophilized, fraction V, Sigma) was used as a standard.

Fluorescent Labeling of Pgp and Reconstitution into Proteoliposomes. Highly purified Pgp was labeled on two Cys residues in the NB domains using the fluorophore MIANS, as previously described (*13*, *26*). Reconstitution of MIANS-Pgp or the S_2 fraction into bilayer vesicles composed of various phospholipids was carried out using the gel filtration procedure reported previously (*25*, *26*). The final lipid/protein ratios of the reconstituted preparations were in the range 10−20:1 (w/w).

Determination of Drug-Binding Affinity by Fluorescence Quenching. To compare the binding affinities of verapamil, vinblastine, and daunorubicin to Pgp in various lipids (egg PC, egg PE, brain PS, DMPC, and DPPC), we carried out fluorescence quenching experiments as described previously (*13*). Quenching data were collected following addition of 5 μL aliquots of each drug from stock solutions prepared in 10% DMSO. Fluorescence intensities were corrected for dilution, vesicle scattering, and the inner filter effect, as described previously (*13*, *29*). Absorbance measurements for inner filter effect corrections were made throughout the titration following addition of each aliquot of drug to the Pgp-lipid sample. Background scatter corrections were made by titrating identical lipid samples with 5 μL aliquots of 10% DMSO at the appropriate temperature, so that background samples differed from the experimental Pgp-lipid samples by the absence of drug. Quenching data for monophasic binding of daunorubicin were fit by nonlinear regression (SigmaPlot, SPSS Inc., Chicago, IL) to an equation describing interaction of the drug with a single binding site of K_{d1}, as previously described (*13*, *23*).

$$100\Delta F/F_o = \frac{(100\Delta F_{max}/F_o)[S]}{K_{d1} + [S]}$$

In the case of the biphasic binding curves obtained for vinblastine and verapamil, data points from each section of the curve were fit independently to this equation, yielding two values for K_d, one of high affinity (K_{d1}) and one of low affinity (K_{d2}). Fitting of the biphasic curves to a two-component equation for binding of drug to two independent sites

$$100\Delta F/F_o = \frac{(100\Delta F_{max1}/F_o)[S]}{K_{d1} + [S]} + \frac{(100\Delta F_{max2}/F_o)[S]}{K_{d2} + [S]}$$

led to similar values for K_{d1} and K_{d2} but poorer fits to the experimental data, suggesting that the two drug-binding sites may not be occupied by drug independently of each other.

To compare the binding affinities of various drugs to Pgp in gel versus liquid-crystalline phase DMPC, we performed the fluorescence experiments in a similar manner using reconstituted DMPC vesicles containing purified MIANS-labeled Pgp as previously described (*26*).

Measurement of Drug−Lipid Partition Coefficients. Drug−lipid partition coefficients were determined using a modifica-

tion of the method described by Rogers and Davis (*30*). Lipid solutions were prepared in chloroform/methanol (4:1 v/v) and stored at −20 °C. Aliquots (10 mg) of the desired phospholipid were dispensed into glass tubes, dried under a stream of N_2, and pumped in a vacuum desiccator for at least 3 h to remove all traces of organic solvent. Duplicate samples of dried lipid were resuspended in 1 mL of buffer containing 100 μg/mL of the drug to be analyzed. The samples were allowed to equilibrate at 22 °C for approximately 24 h with occasional mixing using a vortex mixer. Following equilibration, the lipid/drug mixtures were transferred into 1.5 mL microcentrifuge tubes and the lipid vesicles were sedimented at 36000*g* for 40 min at 22 °C. The supernatant was removed, and the absorbance was measured at the absorption maximum of the drug being tested (i.e., verapamil 278 nm; vinblastine 270 nm; daunorubicin 484 nm). The concentration of drug remaining in the supernatant following centrifugation was calculated by comparing the absorbance of the aqueous supernatant with a standard curve generated using known aqueous concentrations of drug. Control samples consisting of lipid and buffer with no drug were treated in an identical manner. Following centrifugation, the absorbance of the control supernatants was determined and subtracted from that of the drug-containing samples.The partition coefficient, P_{lip}, of the drugs in each lipid was then calculated using the following equation:

$$P_{lip} = \frac{(C_T - C_W)W_1}{C_W W_2}$$

where C_T is the initial aqueous concentration of drug, C_W is the final aqueous concentration of drug in the supernatant, W_1 is the weight of aqueous phase, and W_2 is the weight of lipid.

Pgp ATPase Stimulation and Inhibition Profiles. The S_2 fraction of Pgp was reconstituted into proteoliposomes of egg PC, DMPC, and DPPC as reported earlier (*25*, *26*). A colorimetric assay was used to measure Pgp ATPase activity using an ATP concentration of 1 mM and an assay time of 20 min, as described previously (*31*, *32*). Drugs were added to Pgp as DMSO solutions 5 min before initiation of the ATPase assay. The final DMSO concentration did not exceed 1% (v/v), which had no effect on Pgp ATPase activity.

RESULTS

Determination of the Affinity of Drug Binding to Reconstituted MIANS−Pgp using Fluorescence Quenching. Binding of drugs, chemosensitizers, and hydrophobic peptides to Pgp can be quantitated using concentration-dependent quenching of purified Pgp labeled on Cys 428 and Cys 1071 with the fluorescent probe MIANS (*13*, *23*, *24*, *33*). The labeled Cys residues are located close to the bound nucleotide within the catalytic sites of Pgp (*34*), and binding of drugs to Pgp (presumably within the membrane-bound regions) results in quenching of MIANS fluorescence as a result of a conformational change in the protein. MIANS-labeled Pgp can bind both unmodified ATP and fluorescent TNP-ATP/ADP with unchanged affinity, but is unable to carry out ATP hydrolysis (*13*, *34*). In addition, ATP and drug can bind simultaneously, and prior binding of ATP does not alter the affinity of drug binding (*13*).

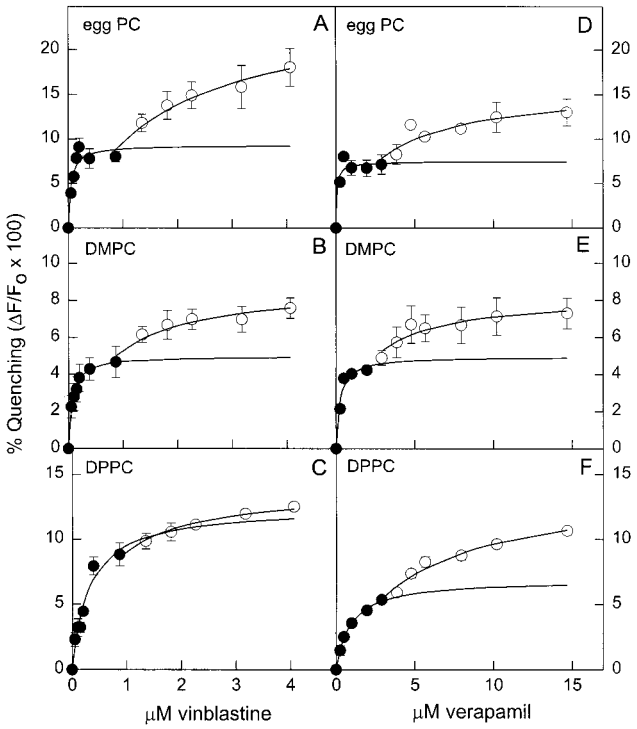

FIGURE 1: Fluorescence quenching curves for binding of vinblastine (A, B, C) and verapamil (D, E, F) to purified MIANS-labeled Pgp at 22 °C in the presence of 0.5 mg/mL egg PC (A, D), DMPC (B, E), and DPPC (C, F). The data points (shown by the symbols) represent the mean ± range ($n = 2$). Where not visible, error bars are included within the symbols. The biphasic binding curves are made up of data points from a high-affinity (●) and a low-affinity (○) region. The continuous lines represent the computer-generated fit of the data points in the high- and low-affinity regions to noninteracting sites of affinity K_{d1} and K_{d2}, respectively. Only the upper part of the fitted curve is shown for the low-affinity region of the plots.

We previously reported that, in the absence of phospholipids, drug-induced quenching was smaller and less reproducible (*13*), suggesting that a lipid environment is important either for refolding of detergent-solubilized Pgp into a more native conformation or as a reservoir into which drugs partition before interaction with the protein. Previous experiments in our laboratory used soybean phospholipids (asolectin) as the host lipid. In the present work, we investigated the effects of the lipid environment on the interaction with Pgp of the transported drug substrates vinblastine and daunorubicin, and the chemosensitizer verapamil (which is also transported). MIANS−Pgp was titrated with increasing concentrations of drug in the presence of various lipids differing in chemical structure, headgroup charge, acyl chain length, and lipid phase state. Quenching of the relatively low-affinity substrate daunorubicin ($K_d = 15-45$ μM) resulted in smooth hyperbolic quenching curves for all of the lipid systems tested. However, vinblastine and verapamil, which bind to Pgp with higher affinity ($K_d = 0.048-0.81$ and $0.063-1.31$ μM, respectively), showed biphasic quench curves in most lipids (see Figure 1), suggesting the existence of two binding sites with different affinities. Two-phase binding curves have recently been noted for the interaction of several ligands with Pgp in permeabilized intestinal cells (*35*), and other evidence supports the existence of two sites to which some drugs can bind simultaneously (*36*−*39*). Fluorescence quenching data were fit by nonlinear regression

Table 1: Effect of Lipid Headgroup on the Binding Affinity for Interaction of Drugs with Purified Pgp at 22 °C

	K_{d1}[a] (μM)			K_{d2}[a] (μM)		
lipid system	vinblastine	verapamil	daunorubicin	vinblastine	verapamil	daunorubicin
egg PC	0.048 ± 0.022	0.063 ± 0.06	18.4 ± 3.8	1.60 ± 0.25	3.06 ± 0.89	na
brain PS	0.19 ± 0.02	1.31 ± 0.31	36.9 ± 3.0	1.18 ± 0.10	4.18 ± 0.52	na
egg PE	0.81 ± 0.60	0.81 ± 0.10	31.6 ± 3.4	1.17 ± 0.23	2.97 ± 0.28	na

[a] Binding of drugs to purified MIANS-labeled Pgp was determined by fluorescence quenching at 22 °C as described previously (*13*, *23*), in the presence of 0.5 mg/mL of the appropriate phospholipid. The quenching data were fit to equations describing binding to either a single type of site with K_{d1} or, when appropriate, two separate sites with K_{d1} (high affinity) and K_{d2} (low affinity) (see Materials and Methods). The errors given show the goodness of fit of the data by nonlinear regression to the binding equation.

Table 2: Binding Affinity and Partition Coefficient for Interaction of Drugs with Purified Pgp in the Presence of Various PC Species at 22 °C

	vinblastine			verapamil			daunorubicin		
lipid	K_{d1}[a] (μM)	K_{d2} (μM)	P_{lip}[b]	K_{d1}[a] (μM)	K_{d2} (μM)	P_{lip}[b]	K_{d1}[a] (μM)	K_{d2} (μM)	P_{lip}[b]
egg PC	0.048 ± 0.022	1.60 ± 0.25	267	0.063 ± 0.06	3.06 ± 0.89	507	18.4 ± 3.8	na	425
DMPC	0.072 ± 0.008	0.67 ± 0.12	219	0.24 ± 0.09	1.67 ± 0.34	165	28.4 ± 3.6	na	338
DPPC	0.31 ± 0.09	0.54 ± 0.04	90.4	0.92 ± 0.08	4.66 ± 0.57	26.3	30.4 ± 3.9	na	131

[a] Binding of drugs to purified MIANS-labeled Pgp was determined by fluorescence quenching at 22 °C as described previously (*13*, *23*), in the presence of 0.5 mg/mL of the appropriate phospholipid. The quenching data (shown for vinblastine and verapamil in Figure 1) were fit to equations describing binding to either a single type of site with K_{d1} or, when appropriate, two separate sites with K_{d1} (high affinity) and K_{d2} (low affinity) (see Materials and Methods). The errors given show the goodness of fit of the data by nonlinear regression to the binding equation. The same batch of Pgp was used to generate each column of data. [b] Drug−lipid partition coefficients were determined at 22 °C for each drug−lipid combination as described in Materials and Methods.

to an equation for the interaction of the drug with either a single binding site of affinity K_{d1} or two binding sites, one with high affinity (K_{d1}) and the other with lower affinity (K_{d2}).

Effect of the Nature of the Lipid Headgroup on Drug Binding to Pgp. The binding affinity of Pgp for the three drugs was investigated in three highly fluid natural lipid mixtures with different headgroups, egg PC, brain PS, and egg PE. The first two lipids are bilayer-preferring under all conditions, and we have previously reported that egg PE (which can exist in the hexagonal H_{II} phase) also forms bilayers at temperatures below 40 °C (*40*). As shown in Table 1, changes in the lipid headgroup gave rise to pronounced changes in drug-binding affinity. All three drugs bound to Pgp with the highest affinity in egg PC. The largest changes were noted for vinblastine and verapamil, where K_{d1} was 17- and 13-fold lower, respectively, in egg PC than in egg PE. Daunorubicin showed a 2-fold reduction of K_{d1} in egg PC compared to brain PS. The low-affinity binding component showed relatively smaller changes in different lipids for vinblastine and verapamil (Table 1). The three lipids used are highly fluid and are expected to have similar acyl chain fluidity; the ratio of saturated to unsaturated fatty acids, S/U, is 0.80 for egg PC and egg PE, and 0.95 for brain PS (information provided by Avanti Polar Lipids). Thus, these results indicate that the nature of the lipid headgroup has a large modulatory effect on the affinity of binding of drugs to Pgp. Of the three lipids, egg PC was previously noted to stimulate the largest increase in the ATPase activity of detergent-solubilized Pgp (*11*), and was superior in restoring ATPase activity following inactivation by detergent delipidation (*32*).

Effect of the Nature of the Lipid Acyl Chains on Drug Binding to Pgp. Drug-binding affinity was also determined using a series of lipids with the same headgroup but different acyl chain composition: egg PC (largely 16C saturated, and 18 mono- and di-unsaturated chains), DMPC (14C saturated chains), and DPPC (16C saturated chains). The gel to liquid-crystalline phase transition temperatures for egg PC, DMPC, and DPPC under the experimental conditions are <0, 25, and 43 °C, respectively. Figure 1 shows the fluorescence quenching curves for vinblastine and verapamil in the series of three PCs. For all three drugs, binding affinity was highest (i.e., the value of K_{d1} was lowest) in egg PC, and binding affinity was lowest in DPPC, with DMPC displaying intermediate values (Table 2). The values of K_{d1} in egg PC relative to DPPC were over 6-fold lower for vinblastine and 15-fold lower for verapamil. Daunorubicin-binding affinity showed a smaller effect, with a 1.6-fold decrease in egg PC relative to DPPC. Thus the affinity of drug binding to Pgp is also modulated by the acyl chain composition of the lipid environment.

Effect of Lipid Phase State on Drug Binding to Pgp. To investigate the effect of the lipid phase state on drug-binding affinity, we reconstituted purified MIANS−Pgp into proteoliposomes of the defined phospholipid DMPC, which undergoes the gel to liquid-crystalline phase transition at ~25 °C in this system (*25*). We previously showed that the ability of Pgp to bind and hydrolyze ATP varies, depending on whether the DMPC bilayer is in the fluid liquid-crystalline phase or the solid gel phase (*26*). All three drugs bound to Pgp with higher affinity when the protein was present in gel phase DMPC (at 20 °C) relative to liquid-crystalline DMPC (at 30 °C) (Table 3). The K_{d1} values for vinblastine were 2.5-fold lower when the lipid was in the gel state, whereas the K_{d1} values for binding of verapamil and daunorubicin were 4- and 3-fold lower, respectively, in gel phase DMPC. The K_{d2} values for binding of vinblastine and verapamil were similarly reduced when the bilayer was in the gel state. The change in temperature from 20 to 30 °C by itself has little effect on the values of K_d for Pgp in egg PC (data not shown), which does not undergo a melting transition in this temperature range, suggesting that the differences in K_d observed for DMPC arise from changes in bilayer phase state.

Effect of Cholesterol on Drug Binding to Pgp. Previous reports indicated that yeast sterols and cholesterol modulated

Table 3: Binding Affinity for Interaction of Drugs with Pgp Reconstituted into Proteoliposomes of DMPC at Temperatures above (30 °C) and below (20 °C) the Gel to Liquid-Crystalline Phase Transition

	vinblastine		verapamil		daunorubicin	
temp (°C)	K_{d1}[a] (μM)	K_{d2}[a] (μM)	K_{d1}[a] (μM)	K_{d2}[a] (μM)	K_{d1}[a] (μM)	K_{d2}[a] (μM)
20	0.085 ± 0.015	0.68 ± 0.29	0.16 ± 0.02	1.26 ± 0.11	14.9 ± 3.8	na
30	0.21 ± 0.02	2.04 ± 0.35	0.63 ± 0.04	4.00 ± 0.20	45.2 ± 4.3	na

[a] Binding of drugs to purified MIANS-labeled Pgp was determined by fluorescence quenching as described previously (*13*, *23*), in the presence of 0.5 mg/mL DMPC, at either 20 °C (below the DMPC phase transition) or 30 °C (above the DMPC phase transition). The quenching data were fit to equations describing binding to either a single type of site with K_{d1} or, when appropriate, two separate sites with K_{d1} (high affinity) and K_{d2} (low affinity) (see Materials and Methods). The errors given show the goodness of fit of the data by nonlinear regression to the binding equation. The same batch of Pgp was used to generate each column of data.

the photoaffinity labeling of Pgp by azidopine (*15*, *16*). To determine the effect of cholesterol on drug binding, we reconstituted MIANS−Pgp into bilayers containing various amounts of cholesterol, and K_{d1} for drug binding was estimated. Addition of up to 30% (w/w) cholesterol to DMPC bilayers did not significantly affect the binding affinity of either verapamil or daunorubicin. However, when DMPC bilayers contained increasing amounts of cholesterol, the affinity of vinblastine binding decreased 10-fold at 20% (w/w) sterol (data not shown). The presence of cholesterol therefore appears to have effects on the binding of only certain drugs.

Effect of Lipid Environment on the Drug−ATPase Modulation Profile of Pgp. The pattern of stimulation or inhibition of Pgp by drugs and chemosensitizers may reflect the characteristics of their binding to the protein. To investigate this, we determined the concentration dependence of ATPase stimulation or inhibition for the three drugs in the presence of various lipids for which the K_d for drug binding had been determined. As previously reported (*9*−*11*, *41*), verapamil stimulated Pgp ATPase activity in a bimodal fashion (Figure 2B), whereas vinblastine and daunorubicin produced no stimulation and inhibited Pgp ATPase at the higher concentrations tested (Figure 2A,C).

Addition of verapamil to Pgp resulted in stimulation of Pgp ATPase activity up to a concentration of ~10 μM and inhibition at higher concentrations (Figure 2B). The extent of activation and inhibition varied with the lipid environment. Egg PC produced the highest stimulation of ATPase activity, about 2.5-fold, with DMPC showing a slightly lower level of stimulation. DPPC gave the smallest increase in ATPase activity of less than 50%. Thus the level of ATPase stimulation mirrors the binding affinity at the high-affinity site (K_{d1}); the higher the binding affinity, the higher the level of stimulation. In contrast, the verapamil concentration required to induce half-maximal stimulation was similar for all three lipids, despite the 15-fold difference in K_{d1} values.

There was essentially no difference in the ATPase profile for inhibition by vinblastine in egg PC, DMPC, and DPPC, despite the fact that the value of K_{d1} varied by over 6-fold (Table 2). These results suggest that the inhibitory activity of vinblastine on the ATPase activity may not reflect binding of drug to the high-affinity site. Instead, it may be related to binding to the low-affinity site, since K_{d2} for vinblastine did not change much in the three phospholipids (Table 2). It has been suggested by Gottesman et al. (*42*) that inhibition of Pgp ATPase activity by drugs may be related to binding to a lower-affinity inhibitory site. Inhibition of ATPase activity

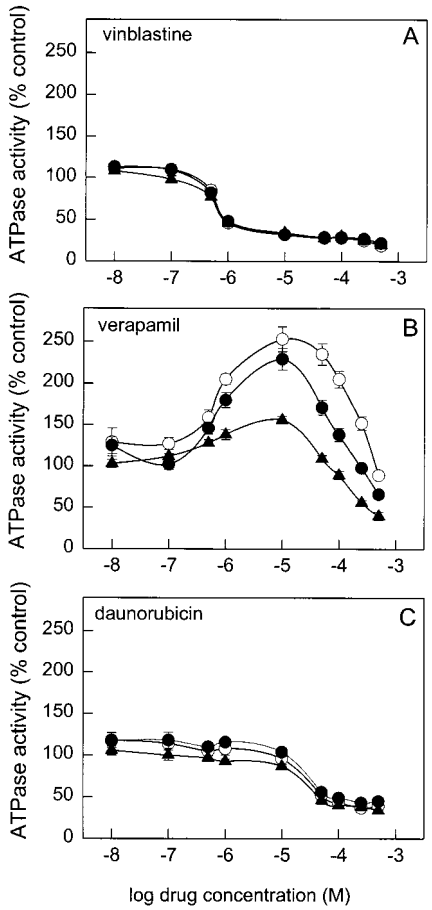

FIGURE 2: ATPase stimulation profiles of Pgp reconstituted into proteoliposomes of egg PC (○), DMPC (●), and DPPC (▲) for vinblastine (A), verapamil (B), and daunorubicin (C) at 22 °C. The data points (shown by the symbols) represent the mean ± SEM ($n = 3$); where not visible, error bars are included within the symbols. Data are expressed as a percentage of the control ATPase activity measured in the absence of drug.

by verapamil may be related to occupancy of the lower-affinity site (K_{d2}). Daunorubicin also inhibited Pgp ATPase activity, and the pattern of inhibition again did not change in the three different lipids. Since this drug appeared to bind to Pgp with only a single affinity which was not greatly affected by a change in lipids (Tables 1−3), these results suggest that it may interact with only the inhibitory site; that is, K_{d1} for daunorubicin is equivalent to K_{d2} for vinblastine and verapamil.

The ATPase stimulation and inhibition patterns of Pgp were also determined in DMPC at temperatures above (30 °C) and below (20 °C) the gel to liquid-crystalline phase transition, to compare with the measured K_{d1} values. As

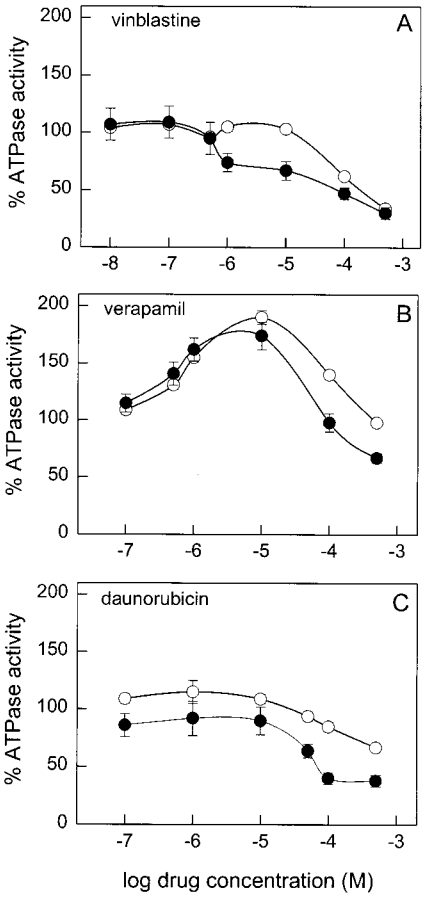

FIGURE 3: ATPase stimulation profiles of Pgp reconstituted into proteoliposomes of DMPC for vinblastine (A), verapamil (B), and daunorubicin (C), above (30 °C, ○) and below (20 °C, ●) the gel to liquid-crystalline phase transition temperature of DMPC (25 °C). The data points (shown by the symbols) represent the mean ± SEM (n = 3); where not visible, error bars are included within the symbols. Data are expressed as a percentage of the control ATPase activity measured in the absence of drug.

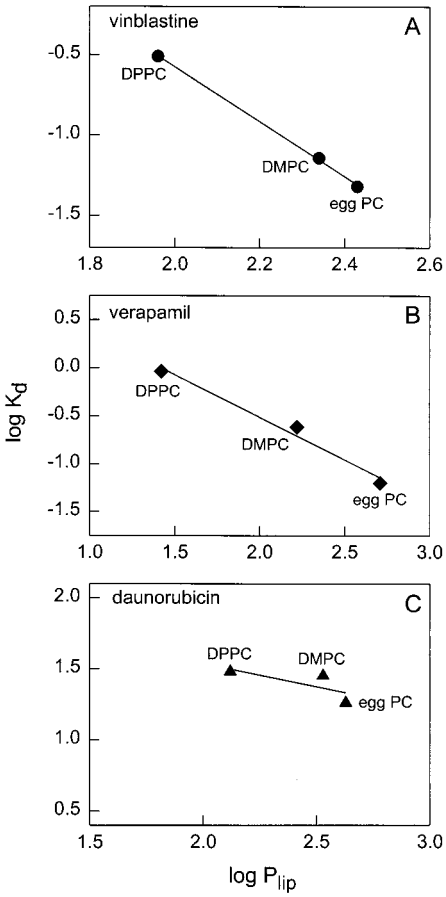

FIGURE 4: Correlation between the Pgp-binding affinity (K_d) and the lipid−water partition coefficient (P_{lip}) for (A) vinblastine, (B) verapamil, and (C) daunorubicin, in liposomes of egg PC, DMPC, and DPPC. The solid line represents the best fit of the data to a straight line by linear regression: (A) r = 0.9996, slope = −1.70, (B) r = 0.9906, slope = −0.89, (C) r = 0.742, slope = −0.33.

shown in Figure 3, only modest changes in the ATPase profile were noted for the three drugs. The maximal level of ATPase stimulation observed for verapamil was similar above and below the phase transition and was reached at a similar drug concentration. However, for all three drugs, there was less inhibition of Pgp ATPase activity at high concentrations in liquid-crystalline phase lipid, compared to gel phase lipid. Assuming that ATPase inhibition is associated with drug binding to the lower-affinity inhibitory site (K_{d2} for vinblastine and verapamil, K_{d1} for daunorubicin), these results are consistent with the weaker binding of drug to these sites in liquid-crystalline phase lipid (Table 3).

Correlation of Drug-Binding Affinity with the Lipid Partition Coefficient. The observed differences in drug-binding affinity shown in Figure 1 and Table 2 are for lipid species with the same polar headgroup (phosphocholine), which differ only in acyl chain composition, at the same temperature. The origin of the large differences in drug-binding affinity therefore cannot be attributed to altered electrostatic interactions, etc. We wondered whether the observed modulation of binding affinity by the acyl chain composition reflected the ability of the drugs to partition into bilayers of the different PCs. Since Pgp is proposed to extract its substrates from the lipid bilayer, rather than the aqueous phase, a higher lipid-water partition coefficient for a particular drug would have the effect of increasing the effective substrate concentration in the lipid phase, thereby increasing the apparent binding affinity. There is currently little or no information available on the ability of various Pgp substrates to partition into lipid bilayers. We therefore measured the partition coefficients, P_{lip}, for distribution of the three drugs between bilayers of various PC species (egg PC, DMPC, and DPPC) and water. Relatively large differences were noted between the ability of a given drug to partition into bilayers of these lipids (Table 2). All three drugs showed the highest partitioning into egg PC, less partitioning into DMPC, and substantially lower partitioning into DPPC. The partition coefficient for verapamil was most sensitive to the lipid bilayer composition, with the value of P_{lip} varying almost 20-fold from egg PC to DPPC. Vinblastine and daunorubicin showed 3- and 3.2-fold differences in P_{lip}, respectively, between egg PC and DPPC. When these P_{lip} values were compared to the K_{d1} values for the three drugs in the same lipids (Figure 4), it was observed that the binding affinity was highly correlated with the partition coefficient; that is, as the partition coefficient increased, K_{d1} decreased (binding affinity increased). These data suggest that the effective concentration of the drug substrate within the lipid bilayer is important for interaction with the transporter, and provides support for the vacuum cleaner hypothesis of Pgp action.

DISCUSSION

The present study is the first to examine the effects of the properties of the host lipid matrix on the affinity of equilibrium binding of drugs to purified Pgp. This approach was made possible by the fact that our preparation of purified Pgp, unlike several others reported in the literature (*9*, *43*), is entirely free of exogenously added lipids. About 55 lipids remain tightly bound to Pgp purified using the detergent CHAPS (*11*), and their removal results in inactivation of the ATPase activity of the protein (*32*). The use of sodium dodecyl sulfate has been reported to result in essentially lipid-free Pgp, but the protein did not display ATPase activity unless exogenous lipids were added (*44*). Quantitation of drug binding to Pgp has proved difficult to address using classical biochemical methods. The technique used most often has been photoaffinity labeling with a variety of radiolabeled substrate analogues, such as azidopine and prazosine. Unfortunately, the results of such experiments are difficult to interpret, since photolabeling is a nonequilibrium technique with very low labeling stoichiometries (<0.5%) (*14*), which is carried out at low drug concentrations, well below saturation. Direct measurement of the equilibrium binding of radiolabeled vinblastine to Pgp, both purified and in native plasma membrane, has been reported (*43*, *45*). However, more general application of this technique to other Pgp substrates is limited by the availability and cost of a particular drug in radiolabeled form, and it is unsuited for use in proteoliposome systems because of the high level of partitioning of hydrophobic compounds into lipid bilayers.

We have recently developed a fluorescence quenching method that is able to directly measure the equilibrium binding to purified Pgp of various substrates, including drugs, chemosensitizers, and hydrophobic peptides (*13*, *23*, *24*, *33*). In the present study, we used this technique to examine the binding to purified Pgp of three representative transported compounds, the chemotherapeutic drugs vinblastine and daunorubicin, and the chemosensitizer verapamil. For both vinblastine and verapamil, biphasic quenching curves were observed, suggesting the existence of two drug-binding sites with differing affinities. A recent study which measured binding to Pgp in permeabilized Caco-2 cells of ^{3}H-verapamil, and its competition by other putative Pgp substrates also noted biphasic, two-affinity binding of verapamil and several other drugs, including vinblastine, vincristine, quinidine, rhodamine 123, and chlorpromazine, but only single-affinity binding for daunorubicin (*35*). Our data are consistent with this report. In addition, positively cooperative effects of one drug on the transport of another (*36*, *38*) have been interpreted by Shapiro and Ling as indicating the existence of at least two drug-binding sites, the H-site and the R-site. In this model, daunorubicin was postulated to interact only with the R-site, and vinblastine was proposed to interact with both the R-site and the H-site (verapamil was not tested). The results of the present study support such a model, in that we observed single-site binding for daunorubicin and two-site binding for vinblastine. Recently, Dey et al. proposed the existence of two nonidentical binding sites for the photoactive drug analogue [^{125}I]-iodoarylazidoprazosin (*39*). Thus, there is substantial evidence to suggest that Pgp contains two drug-binding sites with different affinities, and that a particular drug may interact with one or both of these sites.

In the present study, K_{d1} for high-affinity binding of vinblastine to purified Pgp in egg PC (which will most closely mimic a native plasma membrane) was ~50 nM, which agrees well with the values of 36 and 65 nM for CH^R-B30 plasma membrane and purified Pgp, respectively, reported by Callaghan et al. using equilibrium binding of radiolabeled drug (*43*). Vinblastine is known to be a substantially higher-affinity substrate for Pgp than daunorubicin. For example, vinblastine inhibits drug transport in plasma membrane vesicles half-maximally at 1 μM, whereas the IC_{50} value for daunorubicin is 14 μM (*46*). *Vinca* alkaloids were also much more potent than anthracyclines in inhibiting [^{125}I]iodomycin and [^{3}H]azidopine photolabeling of Pgp (*47*). In addition, we previously reported K_d values for these two drugs using the phospholipid mixture asolectin (*13*), which are comparable to those measured in the present work. The K_d values determined for a large number of Pgp substrates in widely differing structural categories are very highly correlated with the ability of the compounds to block drug transport in a vesicle system, indicating that the K_d values are indeed relevant to Pgp function (*24*).

How does the level of resistance to a particular drug relate to its affinity of binding to Pgp? On the basis of our substantial experience in this area, we can make the following statements. First, the level of resistance that can be achieved against a particular drug is not correlated with its binding affinity. For example, MDR cells often show substantial levels of resistance to colchicine (the CH^{R}B30 cell line displays >600-fold resistance), yet the K_d of 158 μM makes it one of the lowest-affinity Pgp substrates (*4*, *13*, *24*). Resistance to vinblastine is comparable to that of colchicine, indicating that cells can show similar levels of resistance to drugs with widely differing binding affinities. Cell lines have been selected with high levels of resistance to the hydrophobic tripeptide ALLN (*48*), which is also a low-affinity substrate (K_d = 138 μM) (*23*). The level of resistance displayed by MDR cells likely reflects the ability of Pgp to maintain a concentration gradient of that compound across the plasma membrane. We have shown that gradients of comparable size are generated for vinblastine, colchicine, and the tripeptide NAc-LLY-amide in vesicle systems (*36*, *41*, *46*).

The high-affinity component of vinblastine binding to purified Pgp was highly dependent on several different properties of the lipid environment surrounding the protein. Changing the lipid headgroup from choline to serine to ethanolamine, with similar acyl chains, resulted in very large changes in the value of K_{d1}. All of the drugs bound to Pgp with the highest affinity in egg PC; in the case of vinblastine, the binding affinity was 17-fold higher in egg PC compared to egg PE. The nature of the acyl chains also affected drug binding. On changing the acyl chain composition from unsaturated (egg PC) to 14C and 16C saturated (DMPC and DPPC, respectively), large changes in binding affinity of up to 15-fold were noted (Table 2).

Since Pgp is proposed to extract compounds directly from the lipid bilayer, rather than the aqueous phase, the lipophilicity of its substrates becomes potentially important. The vacuum cleaner model of Pgp action predicts that the effective concentration of drug substrate in the lipid bilayer

is important for interaction with the transporter. *n*-Octanol−water partition coefficients (P_{ow}) have been a commonly used indicator of hydrophobicity. However, phospholipid bilayers are ordered structures and do not resemble a homogeneous solvent. In addition, they possess two charged, highly ordered polar headgroup regions, between which is sandwiched a partly ordered hydrocarbon region. Like many Pgp substrates, the three drugs employed in the present study are all positively charged. Hydrophobic drugs that also carry positive charge can interact electrostatically with the phosphate and alcohol moieties of the headgroup region of the bilayer, while the nonpolar portion inserts into the hydrophobic core. The overall result is interfacial partitioning of the drug (*49*, *50*). For this reason, P_{ow} values were not found to accurately describe the partitioning into membranes of charged molecules, especially those carrying protonated amino groups, which displayed affinity much higher than expected for membranes both in model bilayer systems (*50*−*52*) and in vivo (*53*). Such favorable interactions are more important for 1° amines (daunorubicin) than 3° amines (vinblastine, verapamil) (*50*). In addition, negatively charged lipids, such as PS, may undergo enhanced interaction with positively charged drugs (*54*). Drugs are also expected to partition differently into the highly ordered rigid gel phase relative to the more fluid liquid-crystalline phase (*30*).

Therefore, to obtain an accurate measure of the ability of Pgp substrates to interact with lipid bilayer membranes, we determined partition coefficients individually for the three drugs in liposomes composed of purified PC species, differing only in acyl chain composition. All three drugs showed substantial partitioning into PC bilayers. In general, the drugs partitioned much more readily into bilayers with unsaturated acyl chains (egg PC) compared to synthetic lipids with saturated chains (DMPC and DPPC). The binding affinity of the three drugs was found to correlate highly with the lipid−water partition coefficient, that is, the higher P_{lip}, the higher the binding affinity, and the lower the value of K_d. These results suggest that Pgp does in fact recognize its transport substrates in the context of the lipid bilayer and provides support for the vacuum cleaner model of Pgp action. Binding of the three drugs showed differing dependencies on partition coefficient. The correlation was most striking for vinblastine (slope of the line in Figure 4A = −1.70), which showed the largest differences in K_{d1} in the various PC species, and least obvious for daunorubicin (slope of the line in Figure 4C = −0.33), which displayed the smallest changes in K_{d1} (Table 2), whereas verapamil showed an intermediate dependence on P_{lip} (slope of the line in Figure 4B = −0.89). These differences may reflect the exact location within the bilayer into which each drug partitions and the relative efficiency with which it can access the binding site on the protein from this region. Daunorubicin is known to localize in the interfacial region, since it perturbs the first 8 carbons in the so-called cooperativity region of the bilayer (*49*); however, nothing is known about the membrane localization of vinblastine or verapamil.

Saeki et al. (*15*) reported that Pgp expressed in the yeast *Saccharomyces cerevisiae* did not confer drug resistance and displayed a marked decrease in photolabeling by the substrate azidopine. They found that the major yeast sterol, ergosterol, had a large inhibitory effect on azidopine photolabeling of Pgp in plasma membrane vesicles from mammalian MDR cells, while cholesterol had little effect. Later work using a reconstituted Pgp-β-galactosidase fusion protein (*16*) indicated that, as the cholesterol content of an egg PC-cholesterol mixture was increased, specific binding of azidopine reached a maximum at 20% (w/w) sterol. In contrast, we found that the addition of cholesterol in DMPC bilayers had little effect on the binding affinity of verapamil and daunorubicin, whereas it decreased the binding affinity for vinblastine by up to 10-fold at 20% (w/w). Clearly, the effects of cholesterol are complex, and different for individual drugs. There are two possible explanations for the differential effects of cholesterol on binding of vinblastine relative to verapamil and daunorubicin. Cholesterol may have effects on the conformation of Pgp which could selectively affect its ability to interact with certain drugs, since they may physically contact different regions of the protein. The second explanation may be, as mentioned earlier, that different drugs partition into different regions of the bilayer. Cholesterol also inserts into phospholipid bilayers in a very particular way, and will modify the properties of different regions of the membrane selectively. If cholesterol affects the region of the bilayer where a drug is located, it may change the partitioning of that drug but have no effect on another compound that is located in a different region of the bilayer. Thus the presence of cholesterol could differentially alter the partitioning of drugs into the membrane and modulate their measured binding affinity.

Differences in the affinity of drug binding to Pgp at 20 °C (below the phase transition) and 30 °C (above the phase transition) were also noted (Table 3). These observations are not easy to interpret, since they may reflect changes resulting from the difference in temperature, the change in bilayer phase state, or alterations in the conformation and activity of the protein itself. In addition, drugs display complex changes in lipid partitioning above and below the phase transition temperature (*30*). We are currently carrying out a detailed study of the effect of the bilayer phase state on Pgp drug binding and transport function in an attempt to dissect the contribution of these various parameters.

The biphasic profiles observed for modulation of Pgp ATPase activity by many drugs and chemosensitizers have been attributed to the existence of two drug-binding sites, one stimulatory and one inhibitory (*42*). Urbatsch and Senior previously reported that the profiles for stimulation or inhibition of Pgp ATPase activity were also affected by the membrane environment, using lipids that differed in both their acyl chains and polar headgroup (*14*). In the present study, we have made similar observations using PC species differing only in acyl chain composition and have attempted to link the noted changes to differences in binding affinity at the two drug-binding sites. Of the three drugs, verapamil showed the largest changes in the ATPase stimulation profile in the presence of lipids with different acyl chain composition. It can be hypothesized that these changes in the level of ATPase stimulation reflect binding of drug to the high-affinity site (K_{d1}). Vinblastine shows essentially no change in inhibition profile in the three lipids, which suggests that ATPase inhibition may arise from drug interaction with the low-affinity site, since differences in K_{d2} are small when the lipid is varied, whereas K_{d1} shows large changes in affinity. Daunorubicin also shows little change in inhibition profile with lipid type. Since this drug apparently interacts with Pgp

at only a single site and binding is not greatly affected by lipids, we suggest that this site is equivalent to the low-affinity site also occupied by vinblastine and verapamil; that is, K_{d2} for the latter two drugs reflects binding to the same site as K_{d1} for daunorubicin.

An additional complicating factor when interpreting the effects of lipids on drug-binding affinity is that they may actually be Pgp substrates. Earlier work by van Helvoort and co-workers indicated that Pgp acted as a broad specificity flippase for short chain species of PC, sphingomyelin and glucosylceramide (*55*, *56*), and Bosch et al. reported that fluorescent derivatives of both PC and PE (but not PS) behaved like Pgp substrates (*57*). Pgp and the a-factor exporter STE6 were also shown to interact with the short chain alkyl PC analogue edelfosine (*58*, *59*). Membrane lipids and related species may be low-affinity Pgp substrates, thereby giving rise to its observed constitutive ATPase activity, which is completely dependent on the presence of lipids (*32*). The apparent affinity of the binding of drugs and chemosensitizers would thus depend on their ability to displace the low-affinity lipid substrates at the drug-binding site(s) within the Pgp molecule. A lipid that interacted weakly with Pgp would be more easily displaced, giving a higher apparent affinity for binding of the drug (e.g., egg PC), whereas tighter binding lipids would be more difficult to displace, leading to a lower apparent drug-binding affinity (e.g., egg PE).

The high dependence of Pgp drug binding on lipid environment suggests that the ability of the protein to interact with and transport drugs may vary in different tissues, depending on the lipid composition and properties of their plasma membranes. This may be an important factor in clinical strategies to overcome MDR with high-affinity chemosensitizers, where it would be desirable to promote the interaction of Pgp with these compounds in the tumor, but not in the blood brain barrier. It also indicates that modulation of the physical state of the membrane, for example, by the use of nontoxic membrane fluidizing agents, may be a useful strategy to modulate MDR. In this regard, Sinicrope et al. reported that the rate of daunomycin transport in rat liver canalicular vesicles was modulated by membrane fluidizers (*60*), and several surfactant-type compounds (Cremaphor EL, Solutol HS) are known to modulate MDR (*61*).

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