Biochimica et Biophysica Acta, 1153 (1993) 225–236

BBAMEM 76149

The role of drug–lipid interactions in the biological activity of modulators of multi-drug resistance

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(Received 8 April 1993)
(Revised manuscript received 13 August 1993)

Key words: Multidrug resistance; Membrane; Modulators; Lipid; Micelle; Fluorescence

Of the compounds that have now been shown to circumvent acquired cellular multidrug resistance, little or no structure-activity relationship has been found, although their proposed mechanism of action is through modulation of function of p-glycoprotein. While it has been suggested that this inhibition is a direct binding to p-glycoprotein, we show here that such a model seriously neglects the effects many of these compounds have on lipid physical properties. We have characterized the interactions between 16 structurally diverse pharmacological agents (nine of which are known to reverse multidrug resistance) and a variety of lipids. Potent modulators inhibit the membrane binding of rhodamine 6G, and we have observed a correlation of the measured K_i values with the effectiveness of the compounds in situ. We have determined the effects of the compounds on detergent micellization, and have shown substantial changes on the critical micelle concentration of detergents in the presence of modulators. Finally, we have examined the changes in model membrane 'viscosity' induced by the compounds. These results indicate that both direct p-glycoprotein and indirect lipid interactions of modulators should be considered in the mechanism by which these compounds reverse multidrug resistance.

Introduction

Cellular resistance to a wide spectrum of cytotoxic agents has been a subject of increasing interest to those concerned with the clinical treatment of a wide variety of cancers [1]. Of the various forms of resistance, the phenomenon of multidrug resistance (MDR) has received the most attention. As a biochemical phenomenon, MDR is a curiosity, especially the form of 'typical' MDR, which has been linked to the function of a single 130–190 kDa (depending on the degree of glycosylation and/or phosphorylation) membrane glycoprotein known as p-glycoprotein (pgp) [2]. This protein is proposed to act as a pump to remove cytotoxic agents from the interior of cells, and its detection in normal human tissues, particularly those of the gastrointestinal tract, blood-brain barrier, adrenals, and kidneys, would seem to indicate its role in a cellular protective mechanism or in secretion. However, the exact function of this protein is unknown, and its ablity to confer resistance to a wide variety of structurally and chemically unrelated compounds remains puzzling.

The initial findings of Skovsgaard [3] showing reversal of daunorubicin resistance by the noncytotoxic analogue *N*-acetyldaunorubicin, and subsequently by Tsuruo et al. [4] that MDR could be reversed by co-incubation of resistant cells with the structurally dissimilar calcium channel antagonist verapamil has sparked considerable interest in development of agents that can overcome or prevent MDR (for reviews, see Refs. 5–7). Currently, an extremely wide variety of structurally and chemically unrelated compounds have been found to modulate MDR. These agents cause otherwise resistant cell lines to accumulate cytotoxic drugs as their drug-sensitive counterparts. However, the mode(s) of activity of modulators of MDR is unclear.

An aspect of modulation of MDR that remains difficult to explain is the absence of a structure–activity relationship among modulators. In part, this may be

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Abbreviations: MDR, multi-drug resistance; pgp, p-glycoprotein; cmc, critical micelle concentration; SDS, sodium dodecyl sulfate; LSB, lauryl sulfobetaine (*N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate);Rh6G, rhodamine 6G; NPN, *N*-phenyl-1-naphthylamine; PC, egg phosphatidylcholine; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; ^{3}H-VLB, [^{3}H]vinblastine; ^{3}H-VCR, [^{3}H]vincristine; ^{3}H-AMD, [^{3}H]actinomycin D; ^{3}H-DNM, [^{3}H]daunomycin.

a consequence of experimental designs used, different cytotoxic agents being modulated, and different endpoints used for evaluations (see Ref. 7). However, only two factors seem to correlate to any degree with a compounds' ability to modulate MDR [8,9]: (i) the partition coefficient between buffer and octanol or other hydrophobic alcohols, and (ii) a similar volume element for the modulators (although in actuality these two properties are not independent). The dependence on the hydrophobicity of a compound as a criterion for modulator activity has led us to consider that the interactions between the hydrophobic, membrane-spanning regions of pgp and modulators may be the parameter most critical to the reversal of MDR. Several studies have reported that membrane alterations exist between MDR and sensitive cell lines [11–13], but whether these differences exist in the plasma membrane or organelle membranes is not always clear, as the results seem to depend on the cellular distribution of the reporter molecule used [11]. It has also been suggested that some modulators of MDR may function by altering the lipid structure of MDR cells rather than by direct binding to pgp [14], especially since many modulators of MDR, including propranolol, chlorpromazine, promethazine, and trifluoperazine, are known to significantly alter membrane properties [15,16], while modulators such as verapamil can eliminate plasma membrane ultrastructural alterations found in MDR cells [17].

Recent work has shown that there is a direct link between membrane lipid composition and MDR. Incubation of MDR cells with anionic phospholipids [18], fatty acids [19], or compounds known to affect membrane fluidity [19,20] results in reversal of resistance. A rationale for these effects is that pgp ATPase function is highly dependent on the lipid environment in which it is found. Regulation of membrane 'fluidity' with cholesterol results in a range of lipid/cholesterol ratios that allow photolabeling of pgp with the calcium channel antagonist azidopine [21]. Ratios outside this range result in a loss in photolabeling efficiency. Likewise, the electrical makeup of the membrane is important, as pgp ATPase activity can be regulated by the presence of phosphatidylethanolamines in the membrane [22]. Additionally, the cholesterol and anionic lipid content of membranes influences the membrane binding of drugs known to be recognized by MDR [23], suggesting a dual role for the plasma membranes of MDR cells.

In the studies described here, we show that the most potent modulators of MDR significantly affect the structure and properties of a variety of lipids, in the absence of any pgp or other proteins. We have examined 16 structurally diverse compounds, both modulators and non-modulators of MDR, to see whether any general effect on lipid physical properties could be detected with those compounds shown to reverse MDR. We have measured the inhibition of binding of rhodamine 6G to phospholipid membrane vesicles in the presence of verapamil, bepridil, clindamycin, atropine, pirenzepine, quinidine, chlorpromazine, quinine, cefotaxime, colchicine, prenylamine, amitriptyline, pentazocine, lidocaine, furosemide, and promethazine. Resistance to rhodamine 6G (Rh6G), as characterized by decreased cellular accumulation, has been shown to be part of the MDR phenotype [24–26], which includes *vinca* alkaloids and anthracyclines. Rh6G is also hydrophobic, having a high binding affinity for phospholipid membranes, and thus responds well to significant changes in membrane properties. We show here that those compounds most effective in inhibiting Rh6G binding are also highly effective in modulating MDR. We also examined the ability of these 16 compounds to affect the critical micelle concentration (cmc) of the detergents sodium dodecyl sulfate (SDS) and lauryl sulfobetaine (LSB), a zwitterionic analog of SDS. Again, those agents most effective in altering SDS cmc values were also most effective in modulating MDR. Finally, we have examined the effect of the drugs on lipid structure by examining the environmentally sensitive fluorescence of *N*-phenyl-1-napthylamine (NPN) in phospholipid membranes, and have found that the most effective modulators perturb the order/viscosity within these membranes. We consider here the implications for each of these results in the mechanism of modulator reversal of MDR.

Materials and Methods

Rhodamine 6G-membrane binding assays. Rhodamine 6G was purchased from Eastman Fine Chemicals (Rochester, NY), while the 16 test compounds were purchased from Sigma (St. Louis, MO). Phosphatidylcholine (PC) was purchased from Avanti Polar Lipids (Alabaster, AL) as a lyophilized powder, which was subsequently suspended in phosphate-buffered saline (PBS; 20 mM phosphate, 150 mM NaCl, pH 7.4) to 25 mg/ml. The vesicles were carried through 8 freeze-thaw cycles using liquid nitrogen followed by sonification for 30 s in an E/MC ultrasonic bath cleaner. These suspensions were stored under argon until ready for use. Incubations of Rh6G, modulator, and phospholipid were conducted by mixing 0.7 μM Rh6G, 620 μM phospholipid (assuming an average molecular weight of 786 for PC), and increasing amounts of concentrated modulator-DMSO solutions in glass vials. The mixture was vigorously mixed every 10 min for 40 min, and then subsequently spun at $10000 \times g$ in a Sorvall ultracentrifuge for 30 min to pellet the membranes. A 100 μl aliquot of the supernatant was removed, mixed with 2 ml of methanol/water (3:1), and the concentration of the supernatant

(free Rh6G) determined by comparing the fluorescence from the sample with a standard curve.

For the binding of Rh6G to PC, a simple model was used to describe the binding observed. We describe the equilibrium situation as a binding of a single Rh6G to a 'binding site'. Thus,

$$K_d = [P][L]/[PL] = ([P])([L]_o - [PL])/[PL] \quad (1)$$

where [P] is the concentration of free phospholipid sites, [L] is the free Rh6G concentration, $[L]_o$ is the total Rh6G concentration, and [PL] is the molar concentration of complex. To convert the molar concentration of PC into concentrations of sites, we use n, where:

$$[P] = n[P]_o - [PL] \quad (2)$$

and $[P]_o$ is the molar concentration of phospholipid. The equation resulting from substitution of Eqn. 2 into Eqn. 1 was fit by the commercial software package Kaleidagraph (Synergy Software, Reading, PA) using K_d and n as adjustable parameters.

To examine the effects of 16 compounds on the binding of Rh6G to PC, a simple, competitive inhibitor model was used, where:

$$P + L \rightleftharpoons PL \quad (3)$$

and

$$P + M \rightleftharpoons PM \quad (4)$$

where P, L, and M represent phospholipid site, Rh6G, and modulator, respectively. By a treatment analogous to Eqn. 1, the inhibitor constant for the situation arising in Eqns. 3 and 4 can be calculated from the n and K_d values obtained from Eqn. 1.

Critical micelle concentration determination. The critical micelle concentrations for SDS and LSB in PBS were determined using the method of Brito and Vaz [27]. Briefly, an aqueous solution of 0.35 μM NPN was titrated with a stock solution of SDS. The fluorescence at 410 nm (excitation at 356 nm) was monitored after each addition of SDS. The cmc was determined from the intercept of a line drawn through the pre-micelle concentration range of detergent and a line drawn through the initial slope occurring at the onset of fluorescence enhancement. The fluorescence spectra of quinidine and quinine overlap that of NPN. For these two modulators, Coumarin 153 (Eastman Fine Chemicals, Rochester, NY) was substituted for NPN, and the emission at 532 nm (excitation at 423 nm) was monitored as for NPN. The effects of modulators on the cmc values were determined by adding appropriate volumes of DMSO stock solutions to the cuvette prior to addition of detergent.

Modulator effects on membrane-bound NPN fluorescence. The effects of modulators on the fluorescence spectrum of NPN incorporated into PC vesicles was examined using varying concentrations of modulator. NPN fluorescence is highly dependent on the viscosity of the surrounding medium, where more rigid, viscous environments produce both an increase in the fluorescence intensity of the probe and a hypsochromic shift in the emission wavelength [28]. NPN (0.35 μM) was added to a 250 μg/ml suspension of PC or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC; Avanti Polar Lipids, Alabaster, AL) that had been passed repeatedly through a polycarbonate filter of 0.1 μm pore size, as described by MacDonald et al. [29]. The resulting vesicles are unilamellar and have an average diameter of 80 nm. Fluorescence emission spectra were recorded in the absence of modulator over the range 360–480 nm (excitation at 350 nm) using an SLM Aminco LS8000 fluorimeter. Spectra were recorded again following incubation for 20 min at 25°C with 50, 100, and 200 μM modulator.

Accumulation studies. The accumulation of radiolabeled antitumor drugs was performed as described in detail by Thimmaiah et al. [30] except that the incubation media containing labeled drug and modulator was serum-free RPMI-1640 buffered by 15 mM β-glycerophosphate, pH 7.4. Briefly, KB-Ch^R-8-5 cells (KB-8-5), a HeLa line that overexpresses *mdr1* and are positive for pgp [31], were obtained from Dr. M. Gottesman (NIH, Washington, DC) and diluted into 6-well plates to a final concentraton of approx. $1 \cdot 10^6$ cells/well. The cells were allowed to attach overnight, and were then incubated for 2 h with [^{3}H]vinblastine (^{3}H-VLB), [^{3}H]vincristine (^{3}H-VCR), [^{3}H]actinomycin D (^{3}H-AMD) (obtained from Moravek Biochemicals, Brea, CA), or [^{3}H]daunomycin (^{3}H-DNM) (DuPont/NEN, Boston, MA) in the presence of 10, 50, and 100 μM modulator. The cells were then removed from the plates with trypsin-EDTA and counted using a Coulter counter (Hialeah, FL), and the amount of accumulated drug determined by scintillation counting. The control for these studies was accumulation under the same conditions in the absence of added modulator. Data shown is the average of triplicate determinations.

Results

Accumulation studies

The results of the concentration-dependent effectiveness of 16 compounds on the 2 h accumulation of 4 cytotoxic agents into KB-8-5 cells are shown in Table I. Of these 16, atropine, clindamycin, pirenzepine, cefotaxime, colchicine, pentazocine, quinine, lidocaine, and furosemide showed little or no effect at 10, 50, or 100 μM concentrations, in agreement with previous reports on the ability of these compounds to influence drug

TABLE I

Two hour accumulation of labeled antitumor drugs into KB-8-5 cells in the presence of amphiphilic compounds (expressed as % control [a])

Compound	VLB	VCR	DNM	AMD
Verapamil				
10 μM	229(10)	179(37)	152(8)	80(7)
50 μM	519(18)	178(30)	185(6)	96(10)
100 μM	666(60)	203(34)	179(8)	92(5)
Bepridil				
10 μM	176(6)	140(26)	177(13)	99(6)
50 μM	708(17)	351(69)	213(11)	170(16)
100 μM	1081(37)	699(125)	264(6)	432(27)
Clindamycin				
10 μM	118(3)	100(18)	112(5)	103(7)
50 μM	147(12)	83(14)	155(8)	114(8)
100 μM	146(6)	123(25)	121(21)	91(9)
Atropine				
10 μM	136(10)	101(18)	100(3)	93(6)
50 μM	121(15)	114(22)	88(3)	109(7)
100 μM	134(14)	108(20)	86(2)	62(6)
Pirenzepine				
10 μM	103(6)	107(23)	74(5)	126(13)
50 μM	98(6)	83(16)	81(7)	158(15)
100 μM	112(6)	76(17)	94(1)	154(14)
Quinidine				
10 μM	230(2)	167(33)	112(2)	128(10)
50 μM	435(3)	195(44)	137(1)	152(19)
100 μM	654(10)	198(34)	156(3)	156(13)
Chlorpromazine				
10 μM	112(3)	172(32)	112(5)	118(12)
50 μM	674(18)	404(69)	176(8)	190(10)
100 μM	4815(398)	826(52)	255(10)	253(33)
Quinine				
10 μM	115(4)	97(17)	115(7)	118(10)
50 μM	157(5)	119(23)	138(3)	113(10)
100 μM	209(16)	141(26)	134(6)	101(8)
Cefotaxime				
10 μM	115(8)	79(21)	88(1)	102(21)
50 μM	91(8)	89(16)	90(5)	120(14)
100 μM	95(7)	99(17)	89(1)	139(9)
Colchicine				
10 μM	112(9)	85(20)	69(3)	130(15)
50 μM	130(15)	108(18)	66(1)	120(19)
100 μM	121(6)	121(20)	73(3)	116(15)
Prenylamine				
10 μM	193(9)	116(21)	126(4)	137(8)
50 μM	416(9)	190(32)	184(7)	244(24)
100 μM	675(6)	368(63)	189(7)	545(29)
Amitriptyline				
10 μM	102(4)	67(13)	114(5)	125(9)
50 μM	118(5)	101(19)	170(1)	145(14)
100 μM	184(12)	110(19)	205(4)	199(23)
Pentazocine				
10 μM	121(7)	75(14)	115(4)	91(13)
50 μM	88(8)	77(14)	130(11)	99(7)
100 μM	119(3)	105(24)	155(9)	126(12)
Lidocaine				
10 μM	118(5)	97(19)	98(2)	95(8)
50 μM	93(5)	87(15)	91(5)	100(12)
100 μM	102(11)	79(13)	102(2)	94(6)
Furosemide				
10 μM	119(7)	102(25)	132(6)	77(14)
50 μM	120(11)	95(17)	104(2)	107(6)
100 μM	130(2)	89(16)	95(6)	117(13)
Promethazine				
10 μM	122(9)	134(25)	159(2)	109(14)
50 μM	178(4)	213(44)	215(11)	119(14)
100 μM	352(7)	357(63)	212(4)	180(18)

[a] The percentage of control (no modulator) is shown for each cytostatic drug, with standard errors in parenthesis. The results are for approx. 10^6 cells with 50 nM ^{3}H-VLB, 14 nM ^{3}H-VCR, 180 nM ^{3}H-DNM, or 90 nM ^{3}H-ADM. Data shown are the average of a triplicate determination, with errors representing the range of values obtained.

accumulation [32]. However, verapamil, bepridil, quinidine, chlorpromazine, prenylamine, promethazine and amitriptyline all gave varying levels of modulation of drug accumulation, in agreement with their known abilities to reverse MDR [7].

At 10 μM modulator (Table I), enhanced accumulation is slight for all compounds tested, generally < 2-fold accumulation vs. cells in the absence of modulator. Accumulation by ^{3}H-VLB was the most affected of the four antitumor drugs examined. Comparison with accumulation of ^{3}H-VCR shows a differential modulation of the two *vinca* alkaloids, as has been reported elsewhere [30]. Accumulation of ^{3}H-DNM was enhanced approx. 1.5-fold for verapamil, bepridil, quinidine, chlorpromazine, and promethazine. However, ^{3}H-AMD accumulation was not significantly affected by any of the compounds at 10 μM.

Increasing the concentrations of the 16 compounds to 50 μM (Table I) clearly indicates the most effective modulators. Prenylamine, bepridil, and chlorpromazine exhibit the greatest influence on accumulation of all four antitumor drugs, leading to accumulations > 2-fold more than that observed without modulators. Quinidine, verapamil, and promethazine also lead to significant increases in the quantity of all antitumor drugs accumulated, whereas quinine shows almost no effectiveness, and amitriptyline is effective only against the two DNA-binding drugs ^{3}H-DNM and ^{3}H-AMD. Once again, ^{3}H-VLB shows the greatest response to modulator activity, increasing to around 7-fold that of the control cells with both bepridil and chlorpromazine, whereas ^{3}H-VCR is accumulated 3-fold and 3.5-fold for these two modulators, respectively.

When modulator concentration was raised to 100 μM (Table I), further enhancement of accumulation was observed for all four antitumor drugs in the presence of verapamil, bepridil, prenylamine, and promethazine. Amitriptyline was also found to double the accumulation ^{3}H-VLB, ^{3}H-DNM, and ^{3}H-AMD, while exhibiting no affect on ^{3}H-VCR. At 100 μM, quinine did enhance accumulation of ^{3}H-VLB, but had little or no effect (< 1.5-fold) on the other 3 agents tested.

Inhibition of rhodamine 6G binding

One of the most characterized phenomenon in studies of MDR is the ability of many modulators to reduce the photolabeling of pgp in membrane suspensions (reviewed in Ref. 33). This effect is typically measured by incubating membrane suspensions with increasing concentrations of modulators in the presence of a fixed concentration of labeling reagent. We have modeled our studies after such experiments, using only purified PC (containing no protein) and the fluorescent dye Rh6G instead of a photolabeling reagent. Fig. 1 shows the binding of Rh6G to 620 μM PC in the absence of added drug and in the presence of 10 μM (Fig. 1B) and 100 μM (Fig. 1A) drug. The compounds found to enhance accumulation of radiolabeled drug also inhibited binding of Rh6G to the membranes, with the most potent modulators also decreasing the amount of Rh6G bound to PC by as much as 25% at concentrations of 10 μM (Fig. 1B). At 100 μM, several modulators, verapamil, bepridil, chlorpromazine, prenylamine, amitriptyline, and promethazine, reduced the amount of Rh6G bound to membranes by > 50% with membrane concentration 620 μM. When the lipid content was raised to 1.9 mM (Fig. 1C), both chlorpromazine and prenylamine were still able to reduce the amount of Rh6G bound by over 50% at 100 μM, while several other drugs also proved to significantly decrease the amount of Rh6G bound to the membranes. Additionally, at 100 μM, bepridil, chlorpromazine and prenylamine caused a lowering of membrane density, making the lipid suspensions more difficult to pellet (data not shown).

To further characterize the concentration dependent effects of modulators on membrane structure, the binding of Rh6G was measured in the presence of increasing amounts of these compounds. In the absence of added hydrophobic drugs, the binding of Rh6G to PC vesicles gives a K_d of 9.2 ± 2.4 μM with an n value of 0.012 ± 0.002.

Addition of hydrophobic drugs led to, in several cases, a marked decrease in the ability of Rh6G to bind to PC. By modeling this as a competitive inhibition of lipid binding sites, we calculated K_i for the series of compounds showing any ability to inhibit Rh6G binding. Due to the larger uncertainty in the value of K_d calculated above, the value of n was fixed at 0.012 and the K_d value re-fit for each modulator. The results of these fits are shown in Fig. 2, with the calculated values of K_i recorded in Table II. In all cases, K_d remained at 9.2 ± 2.4 μM.

Prenylamine and chlorpromazine proved to have the lowest K_is, with promethazine and amitriptyline next, followed by bepridil and verapamil. Figs. 2C and 2D also show the dependency of the K_i values on lipid concentration. This dependency most likely results from our assumptions about the binding interactions of

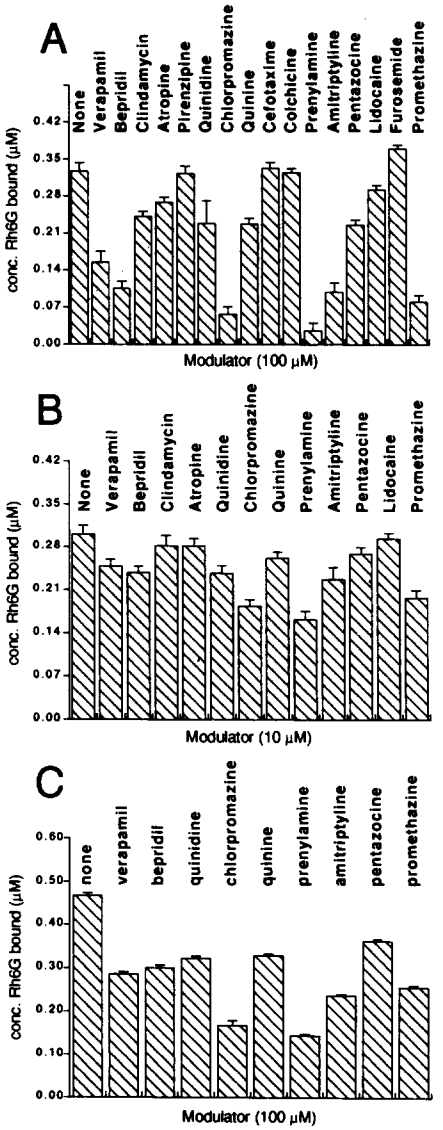

Fig. 1. Displacement of rhodamine 6G from phosphatidylcholine vesicles by modulators. The concentration of Rh6G bound to PC following centrifugation (as described in Materials and Methods) is shown. (Panel A) Addition of 100 μM drug to 620 μM of PC in the presence of 0.7 μM Rh6G. (Panel B) Those compounds showing displacement of Rh6G at 100 μM are shown here at 10 μM. (Panel C) The concentration of PC was increased to 1.9 mM while the modulator concentration was 100 μM (0.7 μM Rh6G). The column marked 'None' indicates the binding of Rh6G in the absence of any added modulator. Data shown is the average of duplicate determinations with error bars representing the range of values determined.

Rh6G and PC membranes, resulting in changes for values of K_d and K_i (at fixed n) at higher lipid/modulators ratios. Therefore, the values recorded in Table II should be viewed as approximate rather than absolute numbers, as K_i for chlorpromazine and prenylamine range from 4.2–10.9 μM and 4.9–11.5 μM, respectively, depending on PC concentrations. Likewise, the K_d values for Rh6G binding become greater (Figs. 2C and 2D), probably reflecting alterations in the values for n at higher lipid concentrations.

SDS critical micelle concentration (cmc) determinations

In order to more fully understand the interactions of MDR modulators with lipids, we examined the nature of the interaction of the 16 compounds with the detergent SDS. Using the fluorescence enhancement of NPN, we determined the change in cmc in the presence of 10 μM concentrations of hydrophobic drugs. The results of these studies are shown in Fig. 3, and the induced changes in the values of the cmc are recorded in Table III. In the absence of added drug, the cmc of SDS in PBS was found to be 0.84 ± 0.05 mM, in good agreement with other studies (Ref. 34 and the references therein). A wide variety of amphiphilic compounds are known to decrease the cmc of SDS [35,36]. Similarly, all compounds found to be effective modulators of antitumor drug accumulation were able to induce distinct decreases in the cmc for SDS, even at concentrations of 10 μM (Table III). Only two compounds of the 16 examined, clindamycin and pentazocine, lowered the cmc of SDS without significantly enhancing cellular drug accumulation, although pentazocine did modulate accumulation slightly at 100 μM (Table I).

At 50 μM of added drug, the cmc of SDS was depressed even further by modulators of drug accumulation. Although quinidine and amitriptyline showed approximately equivalent abilities to modulate cytostatic drug accumulation at 100 μM (Table I) they

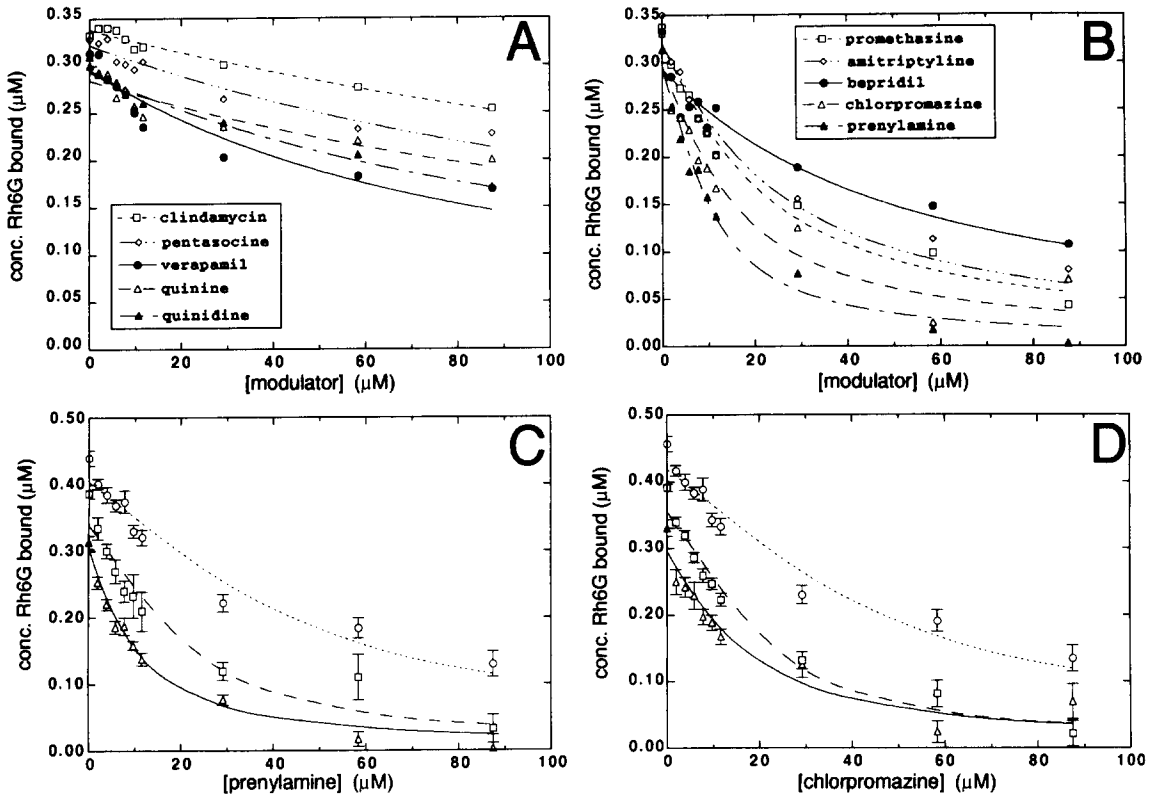

Fig. 2. Inhibition of Rh6G binding to egg PC by modulators. The concentration of Rh6G bound to PC is shown as a function of increasing concentration of modulators. Those compounds shown to inhibit Rh6G binding at 100 μM (Fig. 1A) were tested. The lines drawn are computer fits to the simple single-site inhibitor model described by Eqns. 3 and 4 in Materials and Methods, with the K_i values determined by this method recorded in Table II. (Panel A) Several compounds were less effective at displacing Rh6G. (Panel B) Potent modulators of MDR (Table I) were also shown to be highly effective in displacing Rh6G from PC membranes. (Panel C) The Rh6G displacement by prenylamine was somewhat dependent on PC concentration. The data were fit to the inhibitor model (see Materials and Methods) maintaining n at 0.012 and allowing K_d and K_i to change. The lines represent the resulting fits: $K_d = 10.4 \pm 0.6$ μM and $K_i = 3.3 \pm 0.6$ for 620 μM PC (triangles); $K_d = 16.0 \pm 1.3$ and $K_i = 4.9 \pm 1.7$ μM for 1.2 mM PC (squares); $K_d = 16.5 \pm 0.9$ μM and $K_i = 11.5 \pm 2.3$ μM for 1.9 mM PC (circles). (Panel D) Chlorpromazine displacement of Rh6G from PC as in Panel C: $K_d = 10.3 \pm 0.9$ μM and $K_i = 6.5 \pm 1.9$ μM for 620 μM PC (triangles); $K_d = 14.6 \pm 0.8$ and $K_i = 4.2 \pm 1.0$ μM for 1.2 mM PC (squares); $K_d = 15.1 \pm 0.9$ μM and $K_i = 10.9 \pm 2.3$ μM for 1.9 mM PC (circles). Data shown is the average of duplicate determinations with error bars representing the range of values obtained.

TABLE II

Summary of modulator-lipid interactions examined

Compound	Reverse MDR [a,b]	K_i for Rh6G inhibition (μM) [c]	SDS cmc [b] (mM)	Effect on NPN fluorescence in PC [d]
Verapamil	intermediate	46.7 ± 10.0	0.31 ± 0.07	blue shift (λ_{max} 416 nm)
Bepridil	high	27.1 ± 6.0	0.02 ± 0.01	blue shift (λ_{max} 418 nm)
Clindamycin	none	128.0 ± 10.9	0.45 ± 0.05	none
Atropine	none	none [e]	0.66 ± 0.02	none
Pirenzepine	none	none [e]	0.74 ± 0.02	red shift (λ_{max} 422 nm)
Quinidine	intermediate	67.1 ± 8.6	0.50 ± 0.03	NA [f]
Chlorpromazine	high	6.5 ± 1.9	0.07 ± 0.06	red shift (λ_{max} 425 450 nm)
Quinine	low	103.3 ± 20.7	0.41 ± 0.06	NA [f]
Cefotaxime	none	none [e]	0.73 ± 0.02	quenches
Colchicine	none	none [e]	0.79 ± 0.09	quenches
Prenylamine	high	3.3 ± 0.6	< 0.02 mM	blue shift (λ_{max} 415 nm)
Amitriptyline	low	11.3 ± 2.3	0.08 ± 0.01	none
Pentazocine	none	88.3 ± 11.9	0.51 ± 0.04	none
Lidocaine	none	none [e]	0.72 ± 0.03	none
Furosemide	none	none [e]	0.72 ± 0.03	quenches
Promethazine	intermediate	9.4 ± 1.3	0.10 ± 0.01	reduced (quenched?)

[a] General ability to enhance accumulation (> 2-fold) of any cytostatic drugs from Table I.
[b] At 50 μM modulator. Values for the cmc represent the average of duplicate experiments except that for DMSO, which is the average of four determinations. Errors represent the range of values determined.
[c] Average of duplicate determinations, with errors representing the range of values determined.
[d] At 200 μM modulator. Representative of duplicate experiments.
[e] $K_i > 128$ μM.
[f] Not available due to fluorescence spectral overlap with NPN.

showed diverse effects on cmc depression, with amitriptyline being the most effective in this function. Chlorpromazine is unusual in its ability to alter cmc values (Fig. 3B) in that a somewhat biphasic curve results. This becomes more pronounced at 50 μM, and thus we consider the cmc for SDS in the presence of this compound to be 0.78 ± 0.04 mM at 10 μM drug and 0.07 ± 0.06 mM at 50 μM drug, although these values may in fact represent two independent micelle environments.

The nature of the abilities of the MDR modulators to depress cmc values was probed using the zwitterionic analog of SDS, lauryl sulfobetaine. None of the 16 compounds significantly affected the cmc of this detergent. This is illustrated in Fig. 3D, where the compounds that extensively affected SDS micelle formation (bepridil, verapamil, and prenylamine) induced little or no change in the cmc of LSB.

NPN fluorescence in phosphoplipid vesicles

We also utilized the fluorescence properties of NPN to examine the effects of modulators on phospholipid vesicles. This is shown in Figs. 4A–E, where unilammellar vesicles of PC containing NPN were mixed with increasing concentrations of modulators. Only those compounds exhibiting MDR-reversing activity blue-shifted the fluorescence of NPN. As shown in Figs. 4A, 4B and 4E, verapamil, prenylamine, and bepridil all induced both an enhancement in the intensity of NPN fluorescence as well as a hypsochromic shift in the emission spectrum, indicating both a rigidification of the environment in which the NPN was located, as well

TABLE III

SDS critical micelle concentrations in PBS[a] in the presence of amphiphilic compounds

Standard errors were calculated from the graphical determination of cmc (Ref. 27) and represent the range of values determined. The number of experiments are shown in parentheses.

Compound	cmc (mM) + 10 μM modulator	cmc (mM) + 50 μM modulator
None	–	0.84 ± 0.05 (4)
Verapamil	0.46 ± 0.02 (2)	0.46 ± 0.02 (2)
Bepridil	0.08 ± 0.02 (2)	0.02 ± 0.01 (2)
Clindamycin	0.71 ± 0.04 (2)	0.45 ± 0.05 (2)
Atropine	0.79 ± 0.08 (1)	0.66 ± 0.02 (2)
Pirenzepine	0.78 ± 0.09 (1)	0.74 ± 0.02 (2)
Quinidine	0.60 ± 0.05 (2)	0.50 ± 0.03 (2)
Chlorpromazine	0.78 ± 0.04 (2)	0.07 ± 0.06 (2)
Quinine	0.69 ± 0.02 (2)	0.41 ± 0.06 (2)
Cefotaxime	0.77 ± 0.18 (1)	0.73 ± 0.02 (2)
Colchicine	0.77 ± 0.18 (1)	0.79 ± 0.09 (2)
Prenylamine	0.01 ± 0.01 (2)	< 0.02 (2)
Amitriptyline	0.24 ± 0.01 (2)	0.08 ± 0.01 (2)
Pentazocine	0.76 ± 0.04 (2)	0.51 ± 0.04 (2)
Lidocaine	0.79 ± 0.10 (1)	0.72 ± 0.03 (2)
Furosemide	0.77 ± 0.10 (1)	0.72 ± 0.03 (2)
Promethazine	0.29 ± 0.04 (2)	0.10 ± 0.04 (2)

[a] PBS: 150 mM NaCl, 20 mM phosphate (pH 7.4).

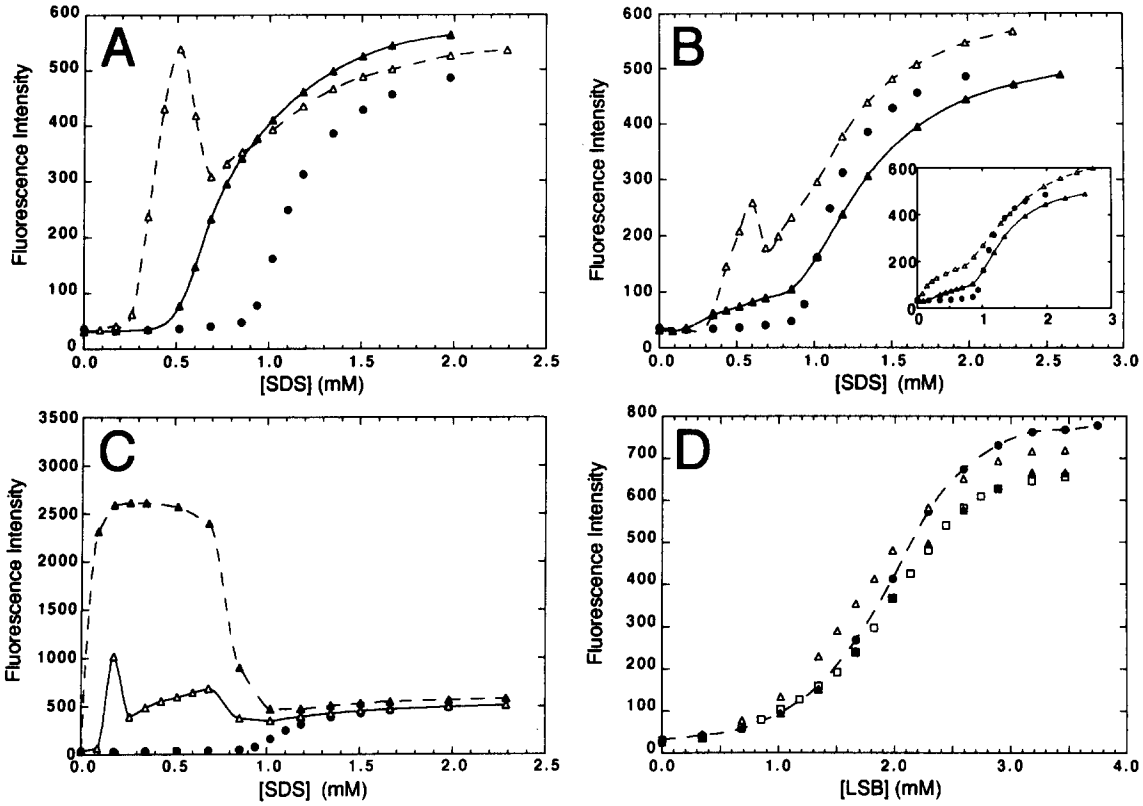

Fig. 3. Effects of modulators on SDS critical micelle concentration. The fluorescence of 0.35 μM NPN in PBS is plotted vs. increasing SDS concentration in the presence or absence of modulators. In all panels, the fluorescence change without modulator is shown as closed circles. The new values of the cmc are recorded in Table III. (Panel A) The addition of 10 μM verapamil (closed triangles) or amitriptyline (open triangles) is shown. (Panel B), changes induced by 10 μM chlorpromazine (closed triangles) or promethazine (open triangles) are shown. The concentration dependent effect of chlorpromazine is indicated in the insert where both 10 μM (closed triangles) and 50 μM (open triangles) are shown. (Panel C), both 10 μM prenylamine (closed triangles) and bepridil (open triangles) lead to significant enhancement of the fluorescence of NPN. (Panel D), the cmc of lauryl sulfobetaine was not significantly affected by the compounds interacting strongly with SDS. Shown are bepridil (open triangles), prenylamine (closed triangles), and verapamil (open squares). The dashed line indicates the changes occurring in the absence of modulator. Data shown is representative of duplicate determinations.

as an increased hydrophobic environment (decreased polarity). This effect was dependent on the phospholipids used. Fig. 4E shows the effect of increasing concentrations of prenylamine on PC vesicles, while Fig. 4F shows the artificial POPC vesicles under the same conditions. While NPN in PC shows an increase in fluorescence at shorter wavelengths (Fig. 4E), in POPC there is an increase in fluorescence intensity at longer wavelengths also (Fig. 4F).

Interestingly, amitriptyline has a lesser effect on NPN fluorescence than the other modulators examined, even at 200 μM (Fig. 4C). In contrast to prenylamine, verapamil, or bepridil is the effect of chlorpromazine on NPN fluorescence (Fig. 4D). Unlike the other modulators studied, chlorpromazine results in both a decrease in fluorescence intensity as well as a bathochromic shift in the spectrum.

Discussion

The often-proposed mechanism of inhibition of MDR, and particularly of p-glycoprotein, by a wide variety of structurally and physically unrelated compounds is one in which hydrophobic compounds bind to pgp 'binding sites' and inhibit the binding of cytotoxic agents [5–7]. However, the absence of any sort of structure-activity relationship among modulators, nor among the cytotoxic agents proportedly transported by pgp, has been a puzzle. The only common property modulators have been shown to share is one of hydrophobicity [8,9]. This is curious, in that many of the drugs that are part of the MDR phenotype, including actinomycin D, colchicine, rhodamine 123, and doxorubicin, are not particularly hydrophobic, having partition coefficients that often favor aqueous buffer over organic solvents [8,26,37]. What, then, is the necessity for hydrophobicity in a modulating agent? To gain insight into this question, it must be recalled that pgp is a membrane-spanning protein, and is therefore directly affected by membrane compostion and physical properties. Any change in the nature of the lipid environment surrounding pgp will lead to alterations in protein function [19,22].

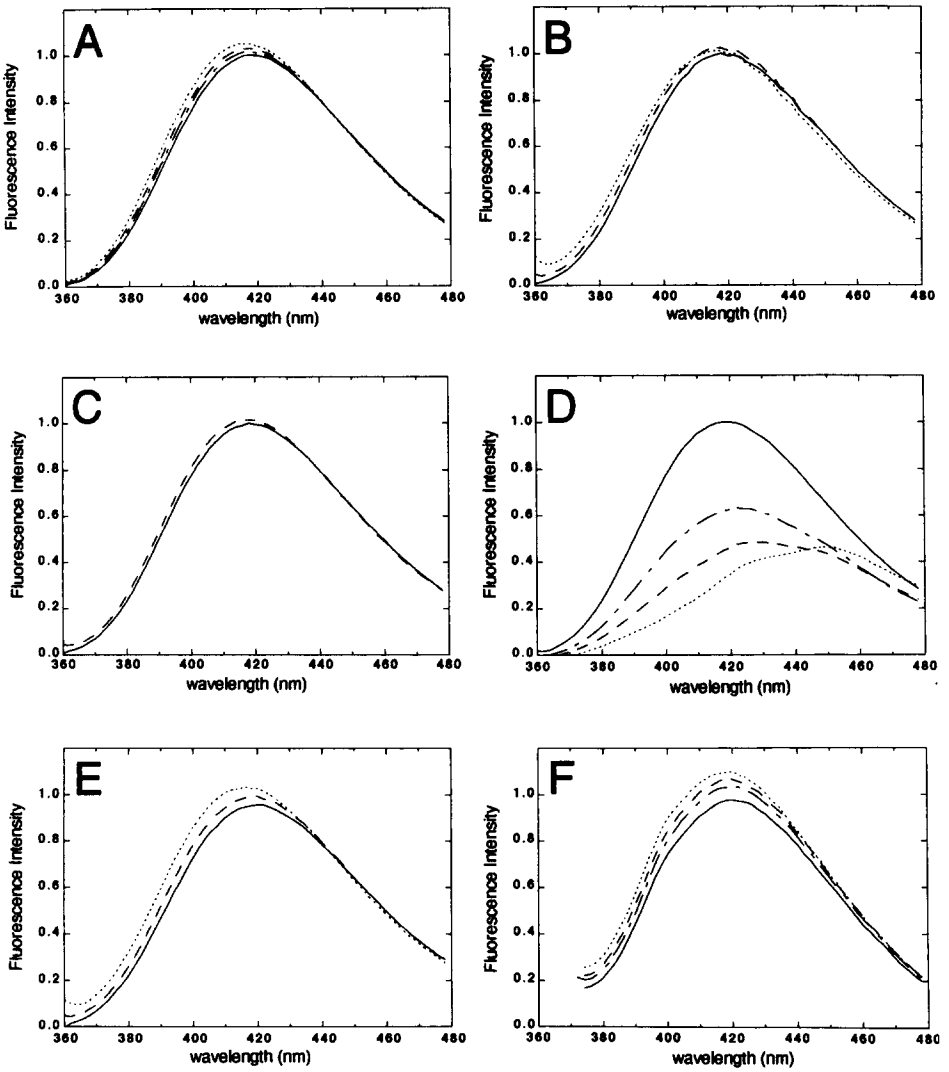

Fig. 4. The fluorescence of NPN in membrane vesicles upon addition of modulators. The emission spectrum of 0.35 μM NPN (λ_{ex} = 350 nm) in 620 μM unilamellar phospholipid vesicles is shown in the absence of modulators (solid line in all panels, λ_{max} 420 nm), as well as with increasing amounts of drug. (Panel A) NPN in PC with addition of verapamil: 50 μM (– – –), 100 μM (– – –), and 200 μM (– – –). (Panel B) NPN in PC with addition of bepridil: 50 μM (– – –), and 200 μM (– – –). (Panel C) NPN in PC with addition of amitriptyline, 200 μM (– – –). (Panel D) NPN in PC with addition of chlorpromazine: 50 μM (– – –), 100 μM (– – –), and 200 μM (– – –). (Panel E) NPN in PC with addition of prenylamine: 50 μM (– – –) and 200 μM (– – –). (Panel F) NPN in POPC with addition of prenylamine: 50 μM (– – –), 100 μM (– – –), and 200 μM (– – –). Both quinine and quinidine have spectral overlap with NPN and could not be used in this assay. Additionally, furosemide, cefotaxime, promethazine, and colchicine quench NPN fluorescence, and their effects on membrane structure cannot be unambiguously ascertained. Pirenzepine gave a slight red shift in NPN fluorescence (emission λ_{max} = 422 nm), whereas the remaining compounds (clindamycin, atropine, lidocaine, and pentazocine) resulted in no alterations in NPN fluorescence at up to 200 μM. Experiments were performed in duplicate, and representative spectra are shown.

The experiments presented here have attempted to discern any general trends or properties that result when modulators of MDR interact with lipids. We have examined a series of 16 drugs, varying in structure and biological activity. Our results clearly indicate that it is dangerous to assign the effectiveness of a modulator of MDR strictly due to its ability to bind to a protein (particularly pgp) since even in the absence of this target, significant alterations in membrane properties occur that could radically affect the distribution of drugs within cellular subcomponents. These properties are summarized in Table II.

Of the 16 compounds studied, pirenzepine, cefotaxime, colchicine, lidocaine, and furosemide showed no ability to modulate accumulation of ^{3}H-VLB, ^{3}H-VCR, ^{3}H-DNM, or ^{3}H-AMD (Table I), in good agreement with previous studies [32]. The effects of these compounds on lipid structure were also negligible. They did not displace Rh6G, a compound associated with the MDR phenotype, from membranes even at concen-

trations of 100 μM drug (Fig. 1), nor did they significantly affect, at up to 50 μM, the critical micelle concentration of SDS. Pirenzepine and lidocaine had no affect on phospholipid bilayer structure, as detected by NPN fluorescence. These results are not directly related to the lipid solubility of these compounds, as lidocaine is highly hydrophobic, whereas pirenzepine is practically insoluble in non-aqueous media. Colchicine, cefotaxime, and furosemide are moderately lipophilic.

Other poor modulators of drug accumulation include clindamycin, atropine, quinine, and pentazocine. The effects of these compounds on lipid structure varied somewhat. All four compounds displaced Rh6G from PC vesicles, but were effective in this function only at concentrations near 100 μM (Fig. 1). The K_i values for these compounds were > 50 μM (Fig. 2, Table II), indicating that they had limited effectiveness as membrane perturbing agents. At 10 μM, these three compounds showed slight or no ability to depress the cmc of SDS (Table III). However, at 50 μM, clindamycin, quinine, and pentazocine were able to depress the cmc to ~ 0.45 mM. Clindamycin, atropine, and pentazocine had no effect on NPN fluorescence in PC vesicles, but due to overlapping fluorescence emission spectra, the effect of quinine on this reporter molecule could not be determined.

Verapamil proved to be intermediate in both its ability to enhance antitumor drug accumulation and its membrane perturbing activity. Verapamil does enhance accumulation of labeled drugs (Table I), although at equimolar concentrations is less effective than bepridil, chlorpromazine, prenylamine, or promethazine. It is also moderately effective in displacing Rh6G from PC vesicles, is effective in lowering the cmc of SDS (Table III) even at 10 μM, and also imparts a blue shift to NPN fluorescence in PC vesicles (Fig. 4A).

Four compounds proved highly effective in modulating accumulation of cytostatic drugs (Table I): bepridil, chlorpromazine, prenylamine, and promethazine. Each of these compounds greatly affected membrane structure, as determined by the methods of study used here. They were highly effective at displacing Rh6G from PC vesicles and strongly affected the ability of SDS to form micelles (Table III). Both prenylamine and bepridil were able to condense SDS, resulting in significant changes in the viscosity within the micelle, and leading to the intense fluorescence of NPN shown in Fig. 3C. Finally, three of the four compounds affected NPN fluorescence in PC vesicles (Fig. 4). Bepridil and prenylamine induced blue shifts in the NPN emission spectrum, indicating an increased hydrophobicity in the environment near the fluorophore. Chlorpromazine caused a decrease in fluorescence of NPN, as well as a bathochromic shift in the emission maximum, indicating NPN was associated with a highly polar environment, suggesting a dissolution of the PC bilayer by this drug. This would not be surprising, as chlorpromazine is known to act much like a detergent, forming mixed micelles with phospholipids at concentrations > 50 μM [38].

The modulation of drug accumulation by amitriptyline is less than would be predicted by consideration of its effects on lipids, although effective modulation is accomplished at 100 μM, in good agreement with the work of Hofsli and Nissen-Meyer [32], who found it effective in doubling ^{3}H-AMD accumulation at 160 μM. Amitriptyline is a good inhibitor of Rh6G binding to PC vesicles (Table II), significantly lowers the cmc for SDS (Table III), but has virtually no effect on the fluorescence of NPN in PC vesicles (Fig. 4C), even at 200 μM. Thus, it would appear that unlike bepridil, prenylamine, and promethazine, amitriptyline does not induce membrane rigidity or enhance the hydrophobic environment of NPN. It may be, based on the lesser effectiveness of amitriptyline as a modulator of drug accumulation, that alterations in membrane rigidity may be the most critical factor in determining which drug-induced membrane perturbations lead to modulation of MDR. An increased rigidity or viscosity at the membrane level might deter the 'pumping' or 'channel' activity of pgp due to increased membrane resistance to protein conformational changes. Such regulation in membrane fluidity/rigidity by addition of cholesterol or other agents affects the membrane association of cytotoxic drugs [23], the photolabeling efficiency of pgp by azidopine [21], and the pgp-mediated transport of ^{3}H-VLB and ^{3}H-DNM [20].

The effects on membrane structure by quinidine, and its relationship to modulation of cytotoxic drug accumulation are not clear. In its ability to perturb membrane structure, quinidine mimics quinine in every way, as might be expected from stereoisomers. However, quinidine is much more effective at enhancing accumulation of antitumor drugs than is quinine (Table I). This result may indicate that quinidine is capable of interacting with a specific target in the MDR cells, such as p-glycoprotein, as well as expressing the non-specific, membrane altering effects described here. Quinidine's direct interaction with pgp would also explain its ability to inhibit [^{3}H]azidopine photolabeling of pgp to a greater extent than quinine [39].

The non-specific, membrane perturbing properties of MDR modulators characterized in this report may explain why no structure-activity relationship for modulators has emerged. An analogous scenario exists with the broad series of compounds that serve as calcium antagonists, where it appears that many exert their activity through non-specific, membrane interactions [40]. As many of these calcium channel antagonists are also MDR modulators (e.g., nifedipine, verapamil, prenylamine), it should not be surprising that general

membrane interactions may be the mechanism by which modulators manifest their MDR reversing activity. It is easy to envision a broad spectrum of ways in which MDR modulators function. On one end of the spectrum, there may be specific interactions with, for example, pgp. This would be expected when the modulators reverse MDR at concentrations much lower than that at which they affect membrane structure. Of the modulators examined here, quinidine is potentially such a compound.

On the other end of the spectrum lie those compounds whose only cellular effect is to disrupt membrane function. This can be done in several ways. Cationic modulators may interact with negatively charged lipids, as in the SDS experiments, to reduce total membrane charge, increase lipid packing density, and/or release counterions critical for charged drug transport. Where regions of anionic phospholipids exist, the cationic modulators may induce condensation of the lipid phase, as bepridil and prenylamine do with SDS (Fig. 3C). Condensation would lead to a higher membrane viscosity, and consequently a lowering of molecular diffusion coefficients for species within the membrane regions. This would explain why bepridil and prenylamine are able to increase [^{3}H]azidopine labeling of pgp [41,42], as the photoactivated form of the drug would be less able to diffuse away from its binding site on pgp.

Some amphiphilic modulators may act in a fashion similar to a detergent, essentially dissolving membranes. This has been noted for chlorpromazine [38] and seems verified by our experiments with NPN in PC vesicles (Fig. 4D). Such a mechansim may explain why chlorpromazine fails to inhibit photolabeling of pgp by [^{3}H]azidopine [39].

Amphiphilic compounds may also act to alter membrane ordering, as has been observed with verapamil [43], leading to a higher membrane surface density and more viscous membrane environment. This effect leads to a decrease in membrane partition coefficients for other drugs, such as daunomycin [23] and rhodamine 6G (described above). This may prevent antitumor drugs from partitioning into membrane regions near pgp, a result that would have serious consequences if the 'flippase' model of pgp were accurate [44]. Furthermore, this may be linked to the reasons why modulators affect ^{3}H-VLB accumulation to a greater extent than ^{3}H-VCR, ^{3}H-DNM, or ^{3}H-AMD (Table I). VLB has a partition coefficient (1-octanol/buffer) of $> 1.6 \cdot 10^4$; while for VCR it is 158; for DNM, 6; and for AMD, 3.3 [8,37,45]. Thus, at equivalent intracellular concentrations of each drug, VLB should be predominantly associated with membrane components, while the other three drugs would be associated to a greater extent with aqueous regions of the cell. Consequently, the cellular accumulation, trafficking, and localization of VLB is more dependent on lipid structure than are the other three drugs examined. In several models of pgp, VLB is proposed to bind to the protein at a site distant to the binding site of more hydrophilic drugs such as DNM, AMD, or colchicine (see, e.g., Ref. 5). Due to the hydrophobicity of VLB, and the fact that aryl azides label pgp in the hydrophobic, transmembrane regions of the protein [46] it may be that membrane alterations like those described here affect certain drug binding sites (such as that for VLB) more than others (such as those for colchicine).

The middle of the modulator spectrum would consist of those compounds that may interact with pgp as well as altering membrane structure. Their relative effectiveness at MDR reversal may be governed by either property, and de novo predictions about the mechanism of MDR reversal by these compounds is difficult. However, insight might be gained by examining the concentration dependence of both their membrane-altering effects with their ability to directly interact with pgp, possibly as measured by inhibition of [^{3}H]azidopine (or other photolabel) binding. Clearly, the effects on lipid structure, as described in this report for several effective modulators of MDR, should not be neglected as a mechanism through which they exert their biological action. This may, in part, explain the differences reported for reversing MDR in different cell lines having apparently wild-type pgp (e.g., Ref. 30).

Acknowledgements

This work was supported by PHS award CA 23099 and the Cancer Center Support (CORE) Grant CA21765 from the National Cancer Institute and American Lebanese Syrian Associated Charities (ALSAC). The authors wish to thank G. Germain, D. Carrigan, and L.S.H. Carboy for excellent technical assistance.

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