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Verapamil prevents the effects of daunomycin on the thermotropic phase transition of model lipid bilayers

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High-sensitivity differential scanning calorimetry and fluorescence-depolarization techniques were used to study how the presence of daunomycin and/or verapamil affect the thermotropic behaviour of dipalmitoyl phosphatidylcholine (DPPC) vesicles. Daunomycin, a potent anti-cancer agent, perturbs the thermodynamic parameters associated with the lipid phase transition: it decreases the enthalpy change, lowers the transition temperature and reduces the co-operative behavior of the phospholipid molecules. Verapamil, on the other hand, produces smaller alterations in the lipid phase transition. However, when daunomycin and verapamil are present simultaneously in the DPPC vesicles, it is observed that verapamil prevents, in a concentration-dependent manner, the alteration in the phospholipid phase transition expected from the presence of daunomycin in the bilayer. Furthermore, drug-binding studies suggest that the observed interference of verapamil in the daunomycin/phospholipid interaction occurs without a decrease in the amount of daunomycin bound to the lipid bilayer and without the formation of a daunomycin–verapamil complex. Because of the importance of drug–membrane interactions in anthracycline cytotoxicity, we speculate that the lipid bilayer of biological membranes may provide appropriate sites at which the presence of verapamil influences the activity of daunomycin.

INTRODUCTION

Tumour cells having the multi-drug-resistance (MDR) phenotype exhibit a reduced sensitivity to cytotoxic drugs when compared with the parental drug-sensitive cells [1,2]. An increased drug efflux has been used to explain this phenomenon on the basis of the overexpression of certain membrane glycoproteins (the P-glycoprotein family) responsible for actively pumping the drugs out of the cells [3,4]. On the other hand, several membrane-active agents, including well-known calcium-channel blockers such as verapamil (VRP), have been shown to increase the sensitivity of MDR cells to the drugs, thus reverting drug-resistance (see [5] and references therein). Furthermore, on the basis of photoaffinity labelling with VRP-photoactivatable analogues [6–10] and on drug-binding studies [5,11,12], it has been suggested that the drug-enhancing activity of VRP occurs by competition with the anti-tumour drugs for binding to common sites on the P-glycoproteins and not through blockade of calcium channels [5]. The overall conclusion emerging from the above observations is that although overexpression of P-glycoproteins confers drug-resistance, the presence of compounds able to compete for drug-binding sites on the P-glycoproteins increases drug-sensitivity by preventing an efficient elimination of the drug from the cells. Nonetheless, there are MDR cells lines and tumours which seem not to express P-glycoproteins [13–19], and therefore it should be expected that mechanisms other than those mediated by P-glycoproteins are also involved in conferring drug-resistance to tumour cells and in producing the cellular response to VRP.

In the present paper we describe how the interaction of a potent antitumour drug, the anthracycline daunomycin (DNM), with a model lipid bilayer, is prevented by the presence of VRP. The rationale for doing such experiments is that it has been demonstrated that drug–membrane interactions play a key role in anti-cancer-drug cytotoxicity (reviewed in [20]) and that the membrane lipid bilayer is mostly responsible for the interaction with the drugs [21–23].

MATERIALS AND METHODS

Dipalmitoyl phosphatidylcholine (1,2-dipalmitoyl-3-*sn*-glycerophosphocholine; DPPC) was obtained from Avanti Polar Lipids and used without further purification. DPPC was shown to be pure when analysed by t.l.c. DNM hydrochloride and VRP hydrochloride were from Sigma. Stock solutions of DPPC, DNM and VRP were prepared in chloroform and stored under N_2 and in the dark at −20 °C. The DNM concentration in dilute solutions was determined spectrophotometrically [24]. The concentration of lipid phosphorus was determined as described by Kyaw *et al.* [25], with the modifications described previously [23].

Stock solutions of 1,6-diphenylhexa-1,3,5-triene (DPH) (Molecular Probes) were prepared in tetrahydrofuran and stored under N_2 in the dark at 4 °C.

Preparation of lipid vesicles

Appropriate amounts from stock solutions of DPPC, DNM and VRP in chloroform were combined and the solvent was driven off by a stream of N_2 and under vacuum. To prepare large multilamellar vesicles for differential scanning calorimetry (d.s.c.) measurements, a volume of 10 mM-Hepes buffer, pH 7.4, containing 100 mM-NaCl and 1 mM-EDTA, was added to the dry mixtures from the procedure described above to give a final concentration of 2 mM in terms of lipid phosphorus. The samples were kept for 90 min at about 50 °C and vortex-mixed until all lipids were suspended. The liposomes were stored overnight, at 4 °C, to assure a complete hydration of the sample before the d.s.c. experiments.

To prepare small unilamellar vesicles for fluorescence-depolarization studies, the dry mixtures containing DPPC, with or without DNM and/or VRP, were resuspended in 10 ml of the same buffer to give a final lipid phosphorus concentration of 50 μM. The samples were kept at 50 °C for 90 min, frequently vortex-mixed and stored overnight, at 4 °C, to achieve a complete equilibration of the sample with ions and water. The vesicle

Abbreviations used: MDR, cellular multi-drug-resistance; DNM, daunomycin; VRP, verapamil, DPPC, dipalmitoyl phosphatidylcholine; DPH, 1,6-diphenylhexa-1,3,5,-triene; d.s.c., differential scanning calorimetry.

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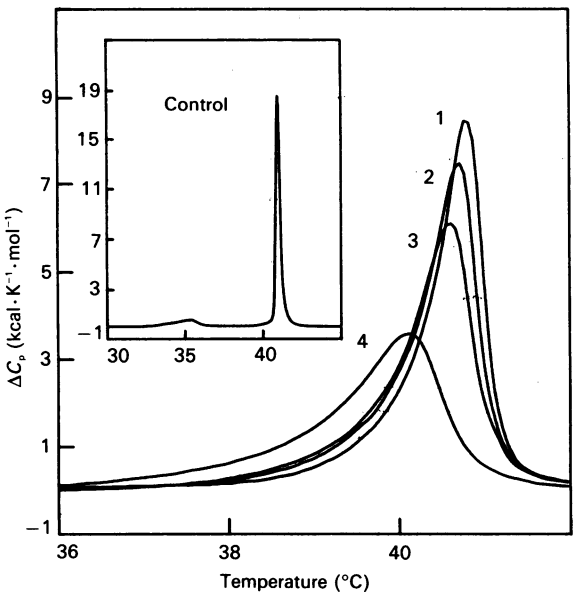

Fig. 1. Representative d.s.c. thermograms of large multilamellar DPPC vesicles in the absence (control shown in the inset) and in the presence of DNM at 25 (1), 37.5 (2), 50 (3) and 137.5 (4) μM

The DPPC concentration was maintained at 2 mM (in terms of lipid phosphorus) in all the experiments. ΔC_p is excess heat capacity; 1 kcal = 4.184 kJ (SI unit).

dispersions were then sonicated in a probe-type Soniprep 150 apparatus, for a total of 15 min, and kept for 1 h at 50 °C.

D.s.c.

Thermograms were recorded in a high-resolution Microcal MC-2 differential scanning microcalorimeter, equipped with the DA-2 digital interface and data acquisition utility for automatic data collection, as previously described [26], except that differences in the heat capacity between the sample and the reference cell were obtained by raising the temperature at a constant rate of 45 °C/h, which is an adequate heating rate to describe lipid phase transitions [27]. The lipid dispersions (2 mM in terms of lipid phosphorus) and the corresponding buffer in the reference cell were thermally equilibrated in the microcalorimeter at 18 °C for 45 min, before heat was applied. Transition temperatures and enthalpies were calculated by fitting the observed transitions to a single van't Hoff component. Reported transition temperatures correspond to those at which there is a maximum differential heat capacity, as observed in the thermograms.

Fluorescence-polarization measurements

Steady-state fluorescence-polarization experiments were performed as described by Lakowicz [28] for 'T-format' instruments (double-channel detection), using a SLM-8000 C spectrofluorimeter, with Glan–Thompson calcite prism polarizers in the excitation and emission beams. Temperature was controlled by a Haake waterbath circulator connected to the fluorimeter cuvette holder. The concentration of lipid vesicles was 50 μM in terms of lipid phosphorus, and the molar ratio of DPH to DPPC was 1:500. Under these conditions, fluorescence-polarization values do not depend on the DPH concentration [29]. Furthermore, at this probe-to-lipid ratio, DPH does not cause significant effects on the DPPC bilayer, as reported by d.s.c. [30]. Samples were excited at 360 nm, and the emission of DPH fluorescence was monitored by using Corning 3–74 cut-off filters to eliminate scattered light.

Equilibrium binding of DNM

Binding of DNM to DPPC vesicles was determined by using the ultracentrifugation or the fluorescence-anisotropy procedures described previously [23]. Klotz & Hunston plots [31] of the binding data were used to determine the stoichiometry and the equilibrium constant of the drug-binding process.

C.d.

C.d. spectra were taken in a Jovin Yvon Mark III dichrograph at a scanning speed of 0.2 nm/s, using 5-cm-path-length quartz cuvettes. C.d. results were the mean values of at least three determinations and were expressed as molar ellipticities (degrees·cm^2·dmol^{-1}).

RESULTS

Typical d.s.c. scans of multilamellar DPPC vesicles obtained in the presence of increasing concentrations of DNM are shown in Fig. 1. Pure DPPC vesicles used in this work exhibit a main thermal transition and a pretransition at approx. 40.9 and 35.2 °C respectively, which correspond to the characteristic phase-transition temperatures for this phospholipid (for a recent review, see [32]). Thermodynamic data for the pure DPPC vesicles were as follows: transition temperatures: $T_{m_1} = 35.22$ °C; $T_{m_2} = 40.97$ °C; transition enthalpies: $\Delta H_1 = 5.26$ kJ (1.26 kcal)/mol; $\Delta H_2 = 36.32$ kJ (8.69 kcal)/mol; transition entropies: $\Delta S_1 = 17.05$ J (4.08 cal)/K·mol; $\Delta S_2 = 115.62$ J (27.66 cal)/K·mol, where the subscripts 1 and 2 refer to the pretransition and main transition respectively. In the presence of DNM it is observed that the pretransition is completely abolished, even at the lowest DNM concentration used, and that the main transition is progressively broadened as the DNM/DPPC molar ratio is increased. Thus the half-height width of the pure phospholipid transition is ~0.4 °C, whereas at DNM/phospholipid ratio of, for instance, 11:160 it is more than 1.3 °C. This broadening of the phase transition indicates a loss in the co-operative behaviour exhibited by the phospholipid molecules. In the pure phospholipid vesicles the co-operative

Table 1. Effects of DNM and VRP on thermodynamic parameters of the gel-to-fluid-phase transition of DPPC vesicles

The concentration of DPPC was 2 mM in all samples. T_m is the transition temperature at which there is a maximum differential heat capacity; $\Delta C_{p,max.}$ is excess heat capacity at the T_m and ΔH_o is enthalpy change. Note 1 kcal = 4.184 kJ (SI unit).

Drug and concentration (μM)	T_m† (°C)	$\Delta C_{p,max.}$ (kcal·mol^{-1}·K^{-1})	ΔH_o (kcal·mol^{-1})
None	40.90	17.51	8.69
DNM			
20.0	40.82	10.09	9.01
25.0	40.82	8.55	7.54
32.5	40.77	7.56	7.16
37.5	40.72	7.48	7.12
50.0	40.62	6.14	6.57
75.0	40.07	4.84	6.60
137.5	40.12	3.59	6.55
200.0	39.97	3.82	5.98
300.0	39.92	3.46	5.46
VRP			
25.0	40.92	10.50	8.67
37.5	40.92	9.80	8.88
50.0	40.87	9.80	8.93
137.5	40.80	9.80	9.02

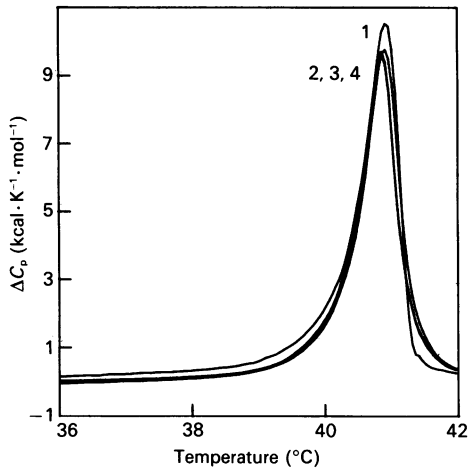

Fig. 2. Representative d.s.c. thermograms of large multilamellar DPPC vesicles in the presence of VRP at 25 (1), 37.5 (2), 50 (3) and 137.5 (4) μM

The DPPC concentration was maintained at 2 mM (in terms of lipid phosphorus) in all the experiments. ΔC_p is excess heat capacity; 1 kcal = 4.184 kJ (SI unit).

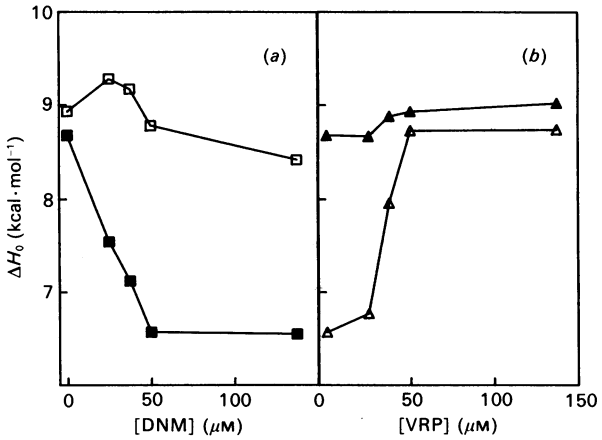

Fig. 3. Effect of the simultaneous presence of DNM and VRP on the enthalpy change corresponding to the DPPC gel to fluid-phase transition

(*a*) Large multilamellar DPPC vesicles were prepared in the presence of increasing concentrations of DNM and in the absence (■) or in the presence (□) of a fixed concentration (50 μM) of VRP. (*b*) DPPC vesicles prepared in the presence of increasing concentrations of VRP and in the absence (▲) or in the presence (△) of a fixed concentration (50 μM) of DNM. The concentration of DPPC was 2 mM, in terms of lipid phosphorus, in all samples.

unit size is about 247 molecules, whereas in the presence of DNM at a DNM/phospholipid ratio of 11:160, it becomes 78 phospholipid molecules. Parallel with the observed broadening, the presence of DNM shifts the transition temperature to slightly lower values and decreases most significantly the magnitude of the enthalpy change associated with the phospholipid phase transition. The effects of DNM on the transition temperature, excess heat capacity and enthalpy change are shown in Table 1.

Experiments similar to those described above were carried out in the presence of VRP instead of DNM, at VRP/DPPC molar ratios which were within the range of DNM/DPPC molar ratios used previously (Fig. 2). A comparison between Figs. 1 and 2 indicates that presence of VRP at low concentrations (curve 1 in

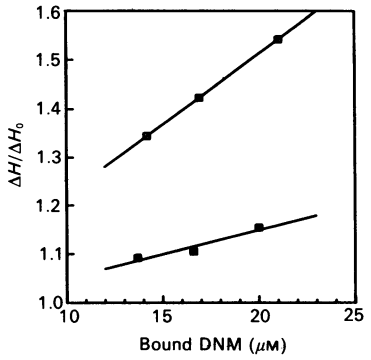

Fig. 4. Relative decrease in the enthalpy change associated with the gel-to-fluid-phase transition of DPPC as a function of the concentration of bound DNM

Experiments were conducted in the absence (□) and in the presence of 50 μM-VRP (■). The number of DPPC molecules prevented from undergoing the phase transition per DNM molecule (N_a) was calculated according to the expression described in [33]:

$$\frac{\Delta H}{\Delta H_0} = 1 - \left(N_a \cdot \frac{[\mathrm{DNM}]_b}{[\mathrm{DPPC}]} \right)$$

where $[\mathrm{DNM}]_b$ represents the concentration of DNM bound to the DPPC vesicles (calculated from binding data shown in Fig. 6) and ΔH_0 and ΔH are the calorimetric enthalpy changes in the absence and in the presence of DNM, respectively. Note: 1 kcal = 4.184 kJ (SI unit).

Fig. 2) produces changes in the thermograms that are comparable with those induced by the presence of similarly low concentrations of DNM (curve 1 in Fig. 1). However, further increasing the VRP concentration is much less effective than comparable increases in the concentration of DNM in producing a decrease in the co-operativity (broadening) or in the change in heat capacity associated with the phospholipid phase transition. The effects of VRP on thermodynamic parameters of the DPPC phase transition are shown in Table 1.

Several of the concentrations of DNM and VRP tested in the above experiments were chosen to produce ternary DPPC/DNM/VRP mixtures to be analysed by d.s.c. These mixtures were prepared in such a way that, in addition to having a fixed concentration of DPPC, they contained either a fixed concentration of DNM and increasing concentrations of VRP or a fixed concentration of VRP and increasing concentrations of DNM. The effects of the simultaneous presence of DNM and VRP on the thermotropic behaviour of DPPC are illustrated in Fig. 3, in terms of the alterations observed on the enthalpy changes associated with the gel-to-liquid-phase transition. In the presence of a constant concentration of VRP, increasing the concentration of added DNM does not cause a significant alteration in the enthalpy change of the lipid transition other than that already caused by the presence of VRP in the absence of DNM (Fig. 3*a*). Furthermore, the decrease in the enthalpy change observed when the DPPC vesicles are prepared in the presence of DNM is progressively cancelled by the presence of increasing concentrations of VRP (Fig. 3*b*).

The dependence of the enthalpy change on the concentration of DNM in the presence and in the absence of VRP was analysed as described by Rigell *et al.* [33] (Fig. 4). We estimate that, in the absence of VRP, each DNM molecule prevents an average of 35 phospholipid molecules from undergoing the phase transition. On the other hand, the presence of VRP, at a molar ratio identical with that used in Fig. 3*a*, reduces that number to only 12 phospholipid molecules per DNM molecule.

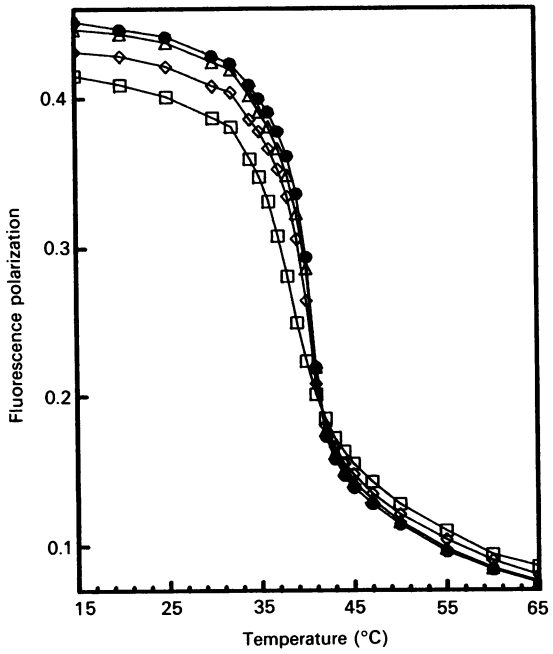

Fig. 5. Temperature-dependence of the steady-state fluorescence polarization of DPH in small unilamellar DPPC vesicles prepared in the absence (●) and in the presence of the following: 2.5 μM-DNM (□) (DNM/DPPC molar ratio 1:20); 2.5 μM-VRP (△) (VRP/DPPC molar ratio 1:20); 2.5 μM-DNM and 2.5 μM-VRP present simultaneously (◇)

The thermotropic behaviour of the vesicles in the presence of DNM, VRP or both was also examined by steady-state fluorescence-polarization measurements using DPH as a probe (Fig. 5). In agreement with the calorimetric results, the temperature-dependent fluorescence depolarization of DPH also shows that DNM produces a downward shift in the transition temperature and a broadening of the transition, whereas VRP produces much smaller changes in such parameters. Moreover, when DNM and VRP are present simultaneously, it is observed that VRP partly prevents the alteration in the phospholipid phase transition expected from the presence of DNM in the DPPC bilayer, which is also in agreement with the observations made using d.s.c.

Since DNM partitions between the lipid bilayer and the aqueous medium, studies of DNM binding to DPPC vesicles are needed to determine the fraction of the added DNM that became incorporated into the lipid vesicles under our experimental conditions. Equilibrium binding data for the interaction between DNM and DPPC vesicles were obtained, at 25 °C, by using the fluorescence anisotropy or the ultracentrifugation procedures described previously to study drug binding [23] (results not shown). Klotz & Hunston [31] plots of the binding data were always linear, suggesting the existence of either a single class of drug binding sites or multiple classes of non-interacting sites with identical binding constants. The estimated values for n (the apparent maximum number of drug-binding sites per phospholipid molecule), $K_{app.}$ (the apparent binding constant) and K_s (the overall association constant, equal to $n \times K_{app.}$) were 9×10^{-3}, 3.9×10^5 M^{-1} and 3540 M^{-1} respectively, and they are similar to those reported by other authors for the binding of anthracyclines to either gel or fluid-phase lipid bilayers [34]. Experiments involving DNM binding to DPPC vesicles were also conducted in the presence of VRP under conditions identical with those used in the d.s.c. (Table 2) or in the fluorescence-polarization

Table 2. Effect of VRP on DNM binding to large multilamellar DPPC vesicles (a)

The experiments were conducted under conditions identical with those employed in the d.s.c. experiments used in Fig. 3(*a*). The ultracentrifugation binding assay described by Escriba *et al.* [23] was used to distinguish between free and bound DNM. The results are expressed in terms of the molar fraction of DNM bound to the DPPC vesicles (2 mM, in terms of lipid phosphorus), in the absence and in the presence of 50 μM-VRP. Experimental error was less than ± 10 %

Total [DNM] (μM)	Fraction of DNM bound	
	Without VRP	With VRP (50 μM)
25	0.57	0.55
37	0.45	0.44
50	0.42	0.40
137	0.30	0.31

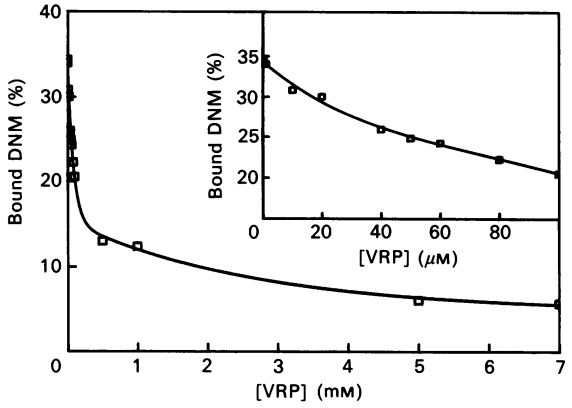

Fig. 6. Effects of VRP on DNM binding to small unilamellar DPPC vesicles

The fluorescence-anisotropy assay described by Escriba *et al.* [23] was used in the experiments. The results are expressed in terms of the percentage of DNM bound to the DPPC vesicles as a function of VRP concentration. The concentration of DNM and DPPC used in the assays were 2.5 μM and 50 μM respectively, which are identical with those used in the DPH fluorescence-polarization experiments. The inset shows in more detail the region of the plot corresponding to low concentrations of VRP, including that (2.5 μM) used in the fluorescence-polarization experiments in Fig. 5.

(Fig. 6) measurement. Under either of those conditions, VRP does not change significantly the amount of DNM bound to the DPPC vesicles. Nevertheless, Fig. 6 shows that, when the VRP-to-DNM molar ratio is further increased, there is a progressive decrease in the amount of drug that is able to bind to the lipid vesicles.

The observed effects of VRP on the DNM–lipid interaction could perhaps be explained if a complex between DNM and VRP had been formed. To test this possibility, we used c.d., which has been shown to be useful in describing the formation of complexes between DNM and other substances such as DNA or nucleotides [24]. However, c.d. spectra of samples containing DNM (5 μM) and VRP (from 5 to 100 μM), both in aqueous solution and in the presence of DPPC vesicles at concentrations ranging 25 to 100 μM, were not distinguishable from that of DNM alone (results not shown) [24] and therefore we have no evidence to substantiate the possible existence of such complexes.

DISCUSSION

The present paper reports on how the presence of VRP modulates the effects of DNM on the thermotropic phase transition of a model lipid bilayer. To illustrate this, we have first determined the effects of DNM and VRP alone and, secondly we have studied how such individual effects are modified as a consequence of the simultaneous presence of both compounds. D.s.c. studies of DPPC multilamellar vesicles in the presence of various anthracyclines have been reported previously [35,36]. The report by Constantinides *et al.* [36] used a high-resolution instrument, but it did not include studies on DNM. These authors concluded that the different anthracyclines tested were more or less capable of altering the thermotropic behaviour of the multilamellar vesicles, depending upon their ability to partition into the bilayer. For instance, adriamycin, an analogue of DNM, had a low partitioning into membranes and, consequently, this drug had little effect on the lipid phase transition. The presence of AD-32, a strongly hydrophobic anthracycline analogue, produced multi-peak transitions, which were interpreted as the result of acyl-chain interdigitation caused by the presence of the drug. On the other hand, Goldmann *et al.* [35] included DNM in their studies, but only a single DNM/DPPC molar ratio of 1:7 and using a low-sensitivity instrument and high scan rates, thus providing limited information. Nevertheless, they concluded that DNM was seemingly among the most effective anthracyclines in altering the transition temperature and enthalpy change of DPPC bilayers, which is similar to our findings using a high-sensitivity microcalorimeter. In our hands, the most noticeable change in the thermodynamic parameters of the phase transition occurs in the enthalpy change, which is reduced from 36.4 J ($\sim$ 8.7 kcal)/mol of the pure DPPC phase transition to 27.2 J ($\sim$ 6.5 kcal)/mol in the presence of DNM at a DNM/DPPC molar ratio of 11:160. From the above comments it is clear that the structure of the anthracycline is an important factor in determining the interaction with a lipid bilayer and, also, that d.s.c. constitutes a powerful and sensitive approach to monitor the effects of DNM on model membranes.

The most intriguing findings reported here relate to the effects of VRP on the interaction of DNM with the bilayer. Drug–membrane interactions are considered important for the biological activity of anthracyclines and, for instance, it has been reported that the anthracyclines can be cytotoxic without entering the cells [20], that is, through interaction with the plasma membrane. Furthermore, complexation of anthracyclines with acidic phospholipids also seems to be critical for the anthracycline cardiotoxicity observed in heart muscle (see [37] and references therein). We have also reported that DNM binds with different affinity to plasma membranes from drug-sensitive or drug-resistant P388 cells and that the lipid components of those membranes are mostly responsible for the observed drug binding [23]. Moreover, the ability of VRP to increase the sensitivity of drug-resistant tumour cells to anti-cancer drugs, which has been shown both in tissue culture and in cancer patients (see references in [5]), is also shared by other amphipathic compounds having in common a lipid solubility and a positive charge at physiological pH [38]. These amphipathic compounds, as DNM itself does, are expected to interact with membranes and this led us to study whether VRP modifies the interaction of DNM with a well-known model lipid bilayer. Our results clearly indicate that the presence of VRP prevents DNM from altering the thermodynamic parameters of the phospholipid phase transition. We do not know the significance of this observation, if any at all, in regard to the effects of VRP in increasing drug-sensitivity in MDR cells. To date such effects of VRP have only been explained based on either (i) the interference of VRP with drug binding to P-glycoproteins (see [4] for a review) or (ii) the drug redistribution resulting from the alteration by VRP of the hydrophobic–hydrophilic solubility of the drug in MDR cells [39]. However, given the importance of drug–membrane interactions in the biological activity of anthracyclines or other drugs, including local anaesthetics [40–42], anti-arrhythmics [41], tranquillizers [43,44] and antibiotics [35], it is possible that the interference of VRP with the effects of DNM on the bilayer could be of some relevance to the antineoplastic activity of the latter.

The report by Hindenburg *et al.* [39] on the distribution of DNM in the two-phase system of Folch (non-miscible hydrophilic and lipophilic solvents) shows that DNM alone partitions mostly into the lipophilic compartment. In the presence of VRP at millimolar concentrations, however, they found that the anthracycline associates almost exclusively with the hydrophilic compartment. In our binding assays (Fig. 6), use of millimolar concentrations of VRP also decreases dramatically the binding of DNM to the DPPC liposomes. Nonetheless, under the conditions used in both the d.s.c. and the fluorescence-polarization experiments, the amount of DNM bound to the bilayer remains practically unperturbed in the presence of VRP and, therefore, DNM release from the membrane cannot be invoked to explain our observations on the effects of VRP on the thermodynamics of the DNM–lipid interaction.

We have also considered the possible formation of a complex between DNM and VRP to explain the effects of VRP on the DNM–lipid interaction. However, c.d. experiments do not support this possibility, since the c.d. spectra of DNM, in either the presence or absence of DPPC vesicles, are not affected by the presence of VRP.

In conclusion, to our knowledge this is the first time that VRP has been shown to interfere with the interaction between DNM and a lipid membrane bilayer. Caution should always be exercised in extrapolating the results from studies in simple model liposomes to the much more complex natural membranes. However, since the lipid bilayer of the plasma membrane and drug–lipid interactions play a definitive role in anthracycline cytotoxicity, it is tempting to speculate that our observations could be an indication that the lipid bilayer constitutes an appropriate locus to account, at least in part, for the modulation of such cytotoxic activity by VRP.

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