

A Differential Scanning Calorimetry Study of Phosphocholines Mixed with Paclitaxel and Its Bromoacylated Taxanes

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ABSTRACT High sensitivity differential scanning calorimetry (DSC) was used to investigate the thermotropic phase properties of binary mixtures of disaturated phosphocholines (PCs) and α -bromoacyl taxane derivatives. The α -bromoacyl taxanes were synthesized as hydrolyzable hydrophobic prodrugs of paclitaxel. The PCs used were 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC). The bromoacyl chain lengths of the taxane prodrugs were varied from 6 to 12 or 16 carbons. For comparison, paclitaxel and PC mixtures were also examined. DSC data from DPPC and bromoacyl taxane mixtures showed a complete abolition of the pretransition and significant broadening of the main phase transition with increasing amounts of bromoacyl taxane prodrugs. The effects were more pronounced with the long-chain compared to the short-chain prodrugs. Under equivalent DSC conditions, the short-chain DMPC showed greater changes in thermotropic phase behavior than with DPPC on taxane addition, suggesting an enhanced degree of association with the fluid-type bilayers. Under similar conditions, the long-chain DSPC bilayers showed a far less significant change in phase behavior on taxane addition than DPPC. These changes were also chain length-dependent for both the PCs and the taxane prodrugs. In contrast, PC and paclitaxel (lacking the acyl chain) mixtures under similar conditions showed insignificant changes in the endotherms, suggesting only slight insertion of the molecule into the PC bilayers. From the DSC data it is apparent that taxane prodrugs solvated in DMPC bilayers more than in DPPC and DSPC bilayers, and taxane prodrugs with longer acyl chains were able to associate with PCs better than those with shorter chain prodrugs. DSC data also suggest that paclitaxel was poorly associated with any of the PCs. In general, the amount of taxane association with bilayers decreased in order: DMPC > DPPC >> DSPC. In contrast, the transition enthalpy (ΔH) of DMPC, DPPC, and DSPC mixtures with paclitaxel showed significantly lower enthalpies than with taxane prodrugs. Taken together, the DSC data suggest that the acyl chains of paclitaxel prodrugs have some access into the bilayers via alignment with the acyl chain of the PC component.

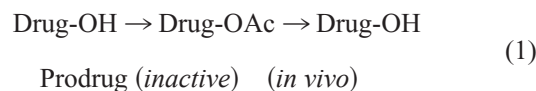
INTRODUCTION

Paclitaxel formulated in Cremophor EL as Taxol is used for the treatment of several types of cancer (for reviews, see Straubinger, 1995; Suffness, 1993). Taxol causes side effects in humans (Woodburn and Kessel, 1994; Weiss et al., 1990) and, although these effects are clinically manageable, there is a continued interest in finding excipients that can be administered easily and safely.

There is an interest from other laboratories in finding paclitaxel prodrugs with increased water solubility, low acute toxicity, and yet therapeutic efficacy. Water-soluble paclitaxel derivatives have been prepared and their structure-activity relationships have been investigated (Li et al., 1996; Greenwald et al., 1994; Deutsch et al., 1989). Some of these derivatives have anticancer activities in animals similar to those of paclitaxel (Rose et al., 1997). Alternatively, paclitaxel alone has been encapsulated in biodegradable polymers (Zhang et al., 1996; Sharma et al., 1996; Burt et

al., 1995) and associated with liposomes, micelles, and emulsions in attempt to reduce the toxic effects of Taxol (Sharma et al., 1993; Tarr et al., 1987). However, the amount of paclitaxel that can be incorporated into lipid bilayers is limited (Sharma et al., 1998; Shieh et al., 1997; Balasubramanian and Straubinger, 1994). It is therefore of interest to investigate the interactions of lipid-based prodrugs into lipid bilayers.

A prodrug is defined as a pharmacologically inactive compound that is activated upon exposure into the biological environments (Silverman, 1992). As shown in Eq. 1, the hydroxyl group of a drug can be functionalized with a cleavable ligand or carrier-linked, which in turn could be hydrolyzed by chemical and/or enzymatic means. When the prodrug is administered the aim is to release the drug more efficiently and effectively at its site of action, reducing toxicity and enhancing activity compared with the parent molecule.



We initiated an effort to synthesize prodrugs of paclitaxel of the lipophilic nature that were approximately as active as paclitaxel *in vivo*, and that could lead to a greater degree of association with PC bilayers than the parent molecule. Thus, we synthesized a series of prodrugs with α -bromine substituted acyl chains at the 2'-position of the C-13 paclitaxel

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side chain. The aim was that the heteroatom at the α -position would facilitate the hydrolysis of the hydrophobic chain in the biological environment with release of paclitaxel in the tumor site (Mayhew et al., 1997). Here we report the mixing behavior of paclitaxel and its bromoacylated C-6, C-12, and C-16 derivatives with PCs liposomes using differential scanning calorimetry (DSC). Liposomes were formed from DMPC, DPPC, and DSPC, which had saturated di-C-14:0, di-C-16:0, and di-C-18:0 acyl chains, respectively. DSC data from the present study indicate that PC liposomes had limited ability to accommodate paclitaxel because of its bulky taxane structure. From the data it is also clear that the preferential association of modified paclitaxels with hydrophobic moieties varied with the PC's bilayer compositions and the prodrug's acyl chain lengths. Furthermore, the DSC results suggest that relatively long acyl chains attached to the taxane may facilitate a greater degree of association with fluid-type rather than gel-type bilayers and may, therefore, assist in increasing the amount of prodrug association with the PC's bilayers. Although the mechanism of paclitaxel interactions with phospholipid membranes is poorly understood (Balasubramanian and Straubinger, 1994; Wenk et al., 1996), this report sheds light on how acylated taxane prodrugs may interact with model and biological membranes.

MATERIALS AND METHODS

Paclitaxel of purity >99%, was purchased from Hauser Chemical Research, Inc. (Boulder, CO). 1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC) were from Nippon Oil and Fats Co. Ltd. (Japan). DSPC was also purchased

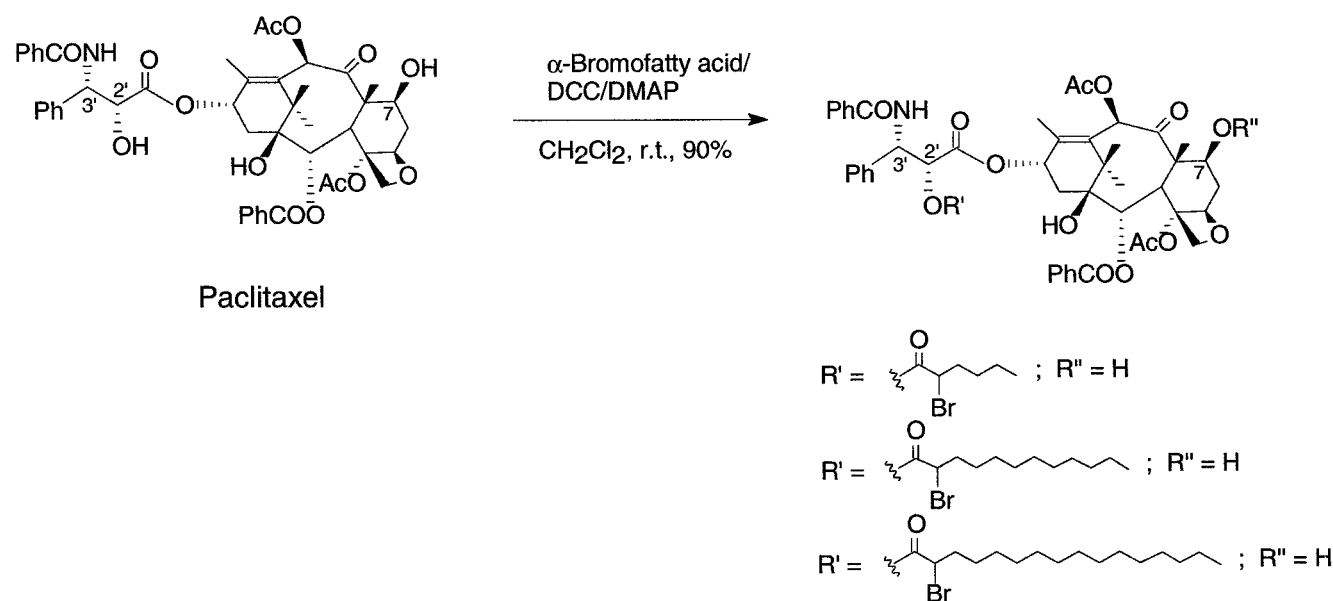
from Sigma (St. Louis, MO). The α -bromofatty acids were from either Aldrich or Fluka Chemicals. 1,3-Dicyclohexylcarbodiimide (DCC) and 4-dimethylamino-pyridine (DMAP) were obtained from Fluka and used without purification. The products were purified using preparative thin layer chromatography (TLC) plate (1000 μ , Analtech, Newark, DE) using CHCl_3 :MeOH (95:5) or EtOAc:Hexane (40:60).

Synthesis

Scheme I shows the general route to the synthesis of α -bromoacyl taxane prodrugs.

General procedure for the preparation of 2'-(\pm)-(α)-bromohexanoyl taxane

Paclitaxel (500 mg, 0.586 mmol) and the catalyst 4-dimethylaminopyridine (71.5 mg, 0.586 mmol) were added to a 10 min stirred solution of (\pm)- α -bromohexanoic acid (229 mg, 1.17 mmol) and 1,3-dicyclohexylcarbodiimide (241 mg, 1.17 mmol) in 30 ml of dry CH_2Cl_2 . After 5 min of reaction, the white precipitate of dicyclohexyl urea was filtered through a Celite pad. The filtrate was evaporated under vacuo and the residue obtained was purified by preparative TLC in CHCl_3 :MeOH (95:5) to give the desired product (R_f = 0.58). After passing through a Metrical filter (0.1 μ) to remove fine silica gel from CHCl_3 solution, the product was lyophilized from cyclohexane to yield 507 mg (84%) as a white solid. ^1H NMR (CDCl_3 , 300 MHz) chemical shifts of some of the characteristic peaks at δ (in ppm) were: 8.14 (d, J = 7.3 Hz, 2H, aromatic), 7.72 (d, J = 7.3 Hz, 2H, aromatic), 7.61 (m, ^1H , aromatic), 7.54–7.48 (m, 3H, aromatic), 7.42–7.36 (m, 7H, aromatic), 6.87 (dd, J = 2.4 Hz, 3.4 Hz, ^1H , NH), 6.29 (m, 2H, H-10 and H-13), 6.0 (m, ^1H , H-3') 5.68 (d, J = 6.9 Hz, ^1H , H-2b), 5.50 (dd, J = 1.4 Hz, 1.0 Hz, ^1H , H-2'), 4.97 (d, J = 7.8 Hz, ^1H , H-5), 4.45 (m, ^1H , H-7), 4.32 (d, J = 7.3 Hz, ^1H , H-20a), 4.28 (m, ^1H , CH(Br)), 4.20 (d, J = 8.3 Hz, ^1H , H-20b), 4.0 (br, OH), 3.81 (d, J = 6.9 Hz, ^1H , H-3), 0.86 (app. t, 3H, ω - CH_3). FABMS: (M.H) $^+$ 1030. Analysis calculated for $\text{C}_{53}\text{H}_{60}\text{O}_{15}\text{NBr} \cdot 0.5 \text{H}_2\text{O}$: C, 61.21; H, 5.91; N, 1.34; Found: C, 61.35; H, 5.72; N, 1.96.



Scheme I.

2'-(\pm)-(α)-Bromododecanoyl (C-12) and bromohexadecanoyl (C-16) taxane prodrugs were prepared (in 80–90% yield) using the procedure as described above and were identified by ^1H NMR, mass spectral, and elemental analyses.

Liposome preparations

Stock solutions of taxol derivatives and phospholipids were prepared and stored in chloroform at -20°C . The phospholipid concentration was determined by the procedure of Bartlett (1959). For preparation of liposomes, aliquots of stock solutions of mixtures of each component were thoroughly mixed in 10-ml tubes, and CHCl_3 was evaporated under a stream of nitrogen gas (N-EVAP). The resulting thin film was dried under vacuum for several hours and then hydrated with 150 mM NaCl or PBS buffer at pH 7.4, unless indicated otherwise. The mixture was vortexed and was heated (above the phase transition temperatures of the lipids) and cooled (0°C) several times. The resulting multilamellar vesicles (MLVs) were used in the subsequent experiments. The sucrose gradient column (Perkins, personal communication) and gel exclusion column (Qiu and Pidgeon, 1994) were performed to separate C-16 taxane-associated liposomes from free prodrug. The samples for the column chromatography were prepared in the same manner as those prepared for the DSC run.

Differential scanning calorimetry (DSC)

High resolution DSC measurements were performed using an MC-2 calorimeter (MicroCal, Amherst, MA) equipped to perform ascending and descending temperature mode operations. The lipid concentration used was 4–5 mg/ml and the temperature of the sample and reference cells was controlled by a circulating water bath. The scan rate was 20°C/hr for both heating and cooling scans. Data were analyzed using ORIGIN software provided by MicroCal. After DSC run the samples were checked for decomposition by TLC. No decomposition was observed. Samples were scanned several times to ensure the reproducibility of the endotherms.

RESULTS

The aim of this study was to investigate how paclitaxel and its bromoacylated taxane prodrugs affect the thermotropic phase behavior and packing of the bilayers in model membranes. As DSC data from the heating curves reflected essentially the same changes as those from the cooling curves, only endothermic curves were used to characterize the thermotropic phase transitions of the binary mixtures of PCs and paclitaxel or bromoacyl taxane prodrugs.

The effects of paclitaxel addition on DMPC bilayers

Fig. 1 shows the endothermic phase transitions for binary mixtures of DMPC and paclitaxel. The multilamellar bilayers of DMPC alone (curve *a*) showed a pretransition at 13.1°C ($\Delta T_{1/2} = 1.9^\circ\text{C}$ and $\Delta H = 1.1$ kcal/mol) and a main phase transition (T_m) at 23.2°C ($\Delta T_{1/2} = 0.4^\circ\text{C}$ and $\Delta H = 7.1$ kcal/mol). The pretransition indicates a transformation from a tilted to a rippled chain gel phase ($L_{\beta'} \rightarrow P_{\beta'}$) and at the T_m there is a transformation from the gel to the liquid crystalline phase ($P_{\beta'} \rightarrow L_\alpha$). In general, paclitaxel addition in the range investigated (from 1 to 9.1 mol%) caused some

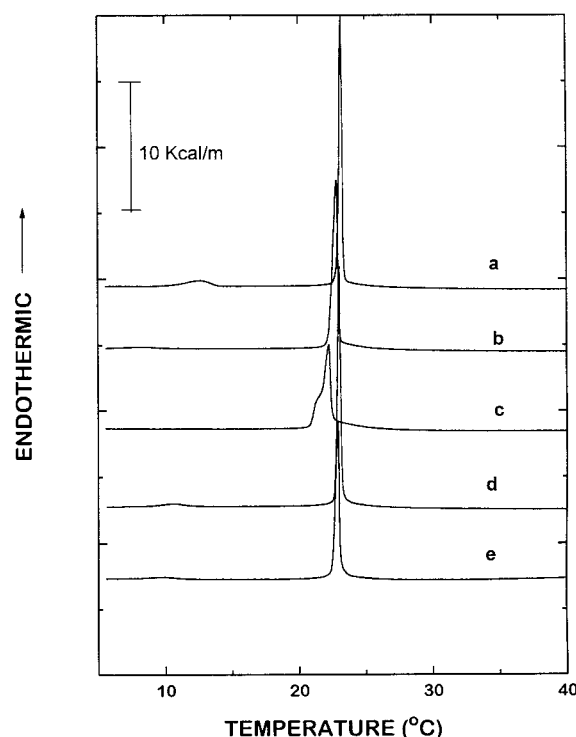


FIGURE 1 The DSC endotherms of binary mixtures of DMPC with varying amounts (in mol%) of paclitaxel: 0 (curve *a*), 1 (curve *b*), 3 (curve *c*), 4.8 (curve *d*), and 9.1 (curve *e*).

degree of perturbation in the DMPC bilayers. At 1 mol% paclitaxel addition the pretransition broadened but did not disappear and was shifted to a lower temperature ($\sim 8^\circ\text{C}$), while the main transition temperature was shifted slightly to a lower temperature (22.8°C) and remained symmetric (curve *b*). At 3 mol% paclitaxel, the pretransition disappeared and the main phase transition was broadened significantly with a broad shoulder at a lower temperature ($\sim 21^\circ\text{C}$) and a distinct sharp peak at a higher temperature ($\sim 22^\circ\text{C}$) (curve *c*). This change in the main transition was reproducible independent of sample preparations. This result clearly indicates that there may be complex formation or inhomogeneous mixing of paclitaxel with DMPC. At 4.8 mol% paclitaxel concentration in DMPC, the endotherm was sharp and symmetrical with a noticeable pretransition (curve *d*), essentially the same as curve *b*. Increasing amount of paclitaxel to 9.1 mol%, however, progressively shifted the endotherm (curve *e*) in pretransition to a lower temperature. The main phase transition temperature, however, remained symmetrical, suggesting that paclitaxel had a limited access to bilayer interiors at higher concentrations.

The effects of α -bromoacyl taxane prodrugs addition on DMPC bilayers

Fig. 2A shows the endothermic DSC profile for binary mixtures of DMPC and C-6 taxane. In general, all the DSC

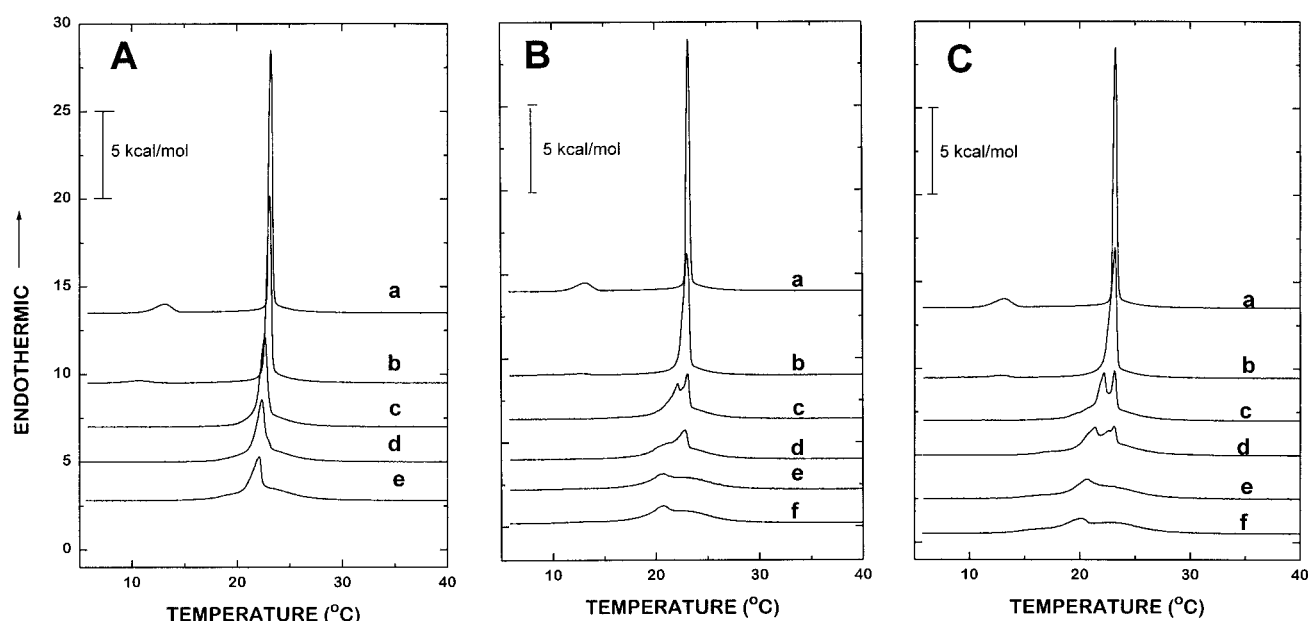


FIGURE 2 The DSC endotherms of binary mixtures of DMPC with varying amounts (in mol%) of bromoacyl taxane prodrugs: 0 (curve *a*), 1 (curve *b*), 3 (curve *c*), 4.8 (curve *d*), 9.1 (curve *e*), and 16.7 (curve *f*). (A) DMPC and C-6 taxane; (B) DMPC and C-12 taxane; (C) DMPC and C-16 taxane mixtures.

endotherms showed addition of taxane to DMPC bilayers caused a broadening and a decrease in the peak height of the of main phase transition of DMPC. For example, addition of taxane at concentrations of 1 to 3 mol% nearly abolished the pretransition and lowered the main transition temperature (curves *b* and *c*). Furthermore, the shape of endotherms remained broad and symmetric, suggestive of ideal mixing. At C-6 taxane concentrations of 4.8 mol%, the pretransition completely disappeared and the lamellar phase transition broadened further (curve *d*). Addition of C-6 taxane at concentrations 9.1 mol% (highest concentration used) resulted in a similar change (curve *e*) as the curve *d* except the endotherm was broadened further.

Increasing the taxane acyl chain length to 12 carbons increased association with DMPC (Fig. 2 *B*). The addition of C-12 taxane to DMPC caused a significant broadening of the gel to liquid crystalline lamellar phase transition. For example, the addition of C-12 taxane at as little as 1 mol% (curve *b*) nearly abolished the pretransition and significantly broadened the main phase transition. At 3 mol%, the pretransition was completely abolished and the main phase transition had two inseparable peaks (curve *c*) at about 24°C, 1°C apart, indicative of an initiation of inhomogeneous mixing and possibly of the phase separation in the gel phase. Increasing the taxane content to 4.8 mol% resulted in coalescence of the two separate peaks into a relatively broad peak with a low temperature shoulder at ~21°C (curve *d*). At 9.1 mol% taxane the endotherm was further broadened and there was a low temperature shoulder at ~21°C (curve *e*). At taxane concentrations 16.7 mol% no further change in the endotherm was observed (curve *f*), suggesting that the broadening was saturable at or above 9 mol%.

Like C-12 taxane, C-16 taxane addition in DMPC (Fig. 2 *C*) resulted in the same changes in endotherms as those shown in Fig. 2*B*. Accordingly, increasing amounts of C-16 taxane (ranging from 1 to 3 mol%) showed an endotherm with $\Delta T_{1/2} \sim 1.75^\circ\text{C}$ comparable to a $\Delta T_{1/2} \sim 1.7^\circ\text{C}$ for C-12 taxane at the equivalent concentrations (curves *c*, Fig. 2 *C* vs. 2 *B*). With an increase in C-16 taxane to 4.8 mol%, the $\Delta T_{1/2}$ increased to 3°C , comparable to $\Delta T_{1/2} \sim 2.5^\circ\text{C}$ for C-12 taxane (Fig. 2 *B*). With further increases in C-16 taxane to 9.1 mol% and 16.7 mol%, there was no apparent change in the shape of the endotherms with $\Delta T_{1/2} \sim 5\text{--}6^\circ\text{C}$ (curves *e* and *f*, Fig. 2 *C*), the same as those observed for C-12 taxane at equivalent concentrations (Fig. 2 *B*). At higher taxane concentrations (9.1 mol% and above), the endotherms remained broad with shoulders at temperature slightly higher than DMPC main phase transition, probably indicating the saturation level of C-16 taxane in DMPC with inhomogeneous mixing or domain formation.

Similar experiments to those described for DMPC were carried out using DPPC and DSPC.

The effects of paclitaxel addition on DPPC bilayers

Fig. 3 shows the DSC endothermic profile for binary mixtures of DPPC and paclitaxel. DPPC alone (curve *a*) showed endotherm comprised of a pretransition ($L_{\beta'} \rightarrow P_{\beta'}$) at 34.5°C ($\Delta T_{1/2} = 1.9^\circ\text{C}$ and $\Delta H = 1.97 \text{ kcal/mol}$), and a $P_{\beta'} \rightarrow L_{\alpha}$ main phase transition (T_m) at 42°C ($\Delta T_{1/2} = 0.25^\circ\text{C}$ and $\Delta H = 8.5 \text{ kcal/mol}$). At 1 mol% paclitaxel addition (curve *b*), the pretransition and the main phase

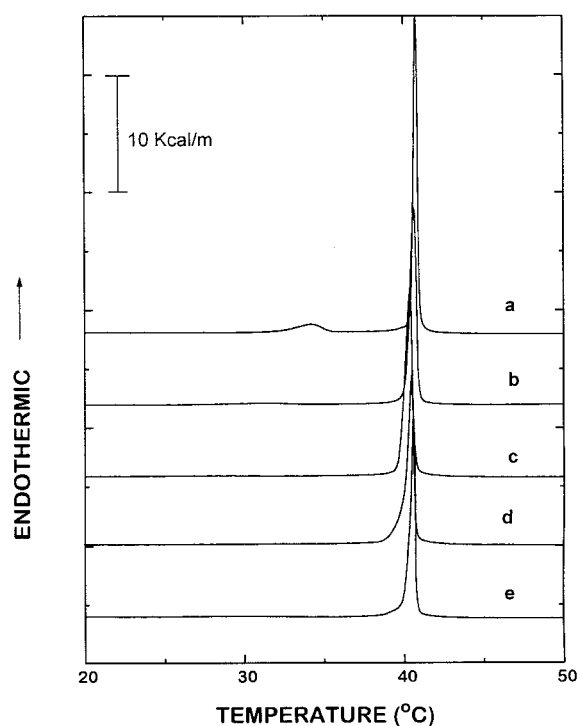


FIGURE 3 The DSC endotherms of binary mixtures of DPPC with varying amounts (in mol%) of paclitaxel: 0 (curve *a*), 1 (curve *b*), 3 (curve *c*), 4.8 (curve *d*), and 9.1 (curve *e*).

transitions were significantly broadened. At 3 to 4.8 mol%, the pretransition was abolished and the main phase transition remained symmetric (curves *c* and *d*). At paclitaxel concentration 9.1 mol% the main phase transition remained

symmetric and relatively sharp (curve *e*), indicating at all concentrations the bilayers were less perturbed, probably due to lack of DPPC ability to solvate paclitaxel. Interestingly, at low mol% of paclitaxel, the DPPC was less perturbed compared to DMPC (Fig. 3 vs. Fig. 1). The data suggests that, unlike DPPC, DMPC fluid-type bilayers allow some access for intrusion of part of the paclitaxel molecule but not for the ideal mixing. It is possible that paclitaxel at 1 to 4.8 mol% in DPPC preferentially favors the phase-separated domains at the membrane-water interface with little or no influence on the bilayers phase behavior. However, at higher mol% DPPC showed a similar effect as observed with DMPC under equivalent conditions, where the endotherms were far less perturbed but remained symmetric (Figs. 1 and 3) with reduced transition enthalpy (ΔH).

The effects of α -bromoacyl taxane prodrugs addition on DPPC bilayers

Fig. 4 *A* shows the DSC profile for the binary mixtures of DPPC and C-6 taxane. Like DMPC/C-6 taxane mixtures (Fig. 2 *A*), the addition of C-6 taxane into DPPC at concentrations 1–3 mol% abolished the pretransition. The main phase transition remained broad and symmetric (curves *b* and *c*). As the taxane increased to 4.8 mol% (curve *d*), the main phase transition was broadened further but remained symmetric. Increasing C-6 taxane content to 9.1 or 16.7 mol% (curves *e* and *f*) did not influence the main phase transition, indicating a lesser tendency of DPPC than DMPC in accommodating C-6 taxane, in part, due to a strong lateral chain-chain interaction in the bilayers.

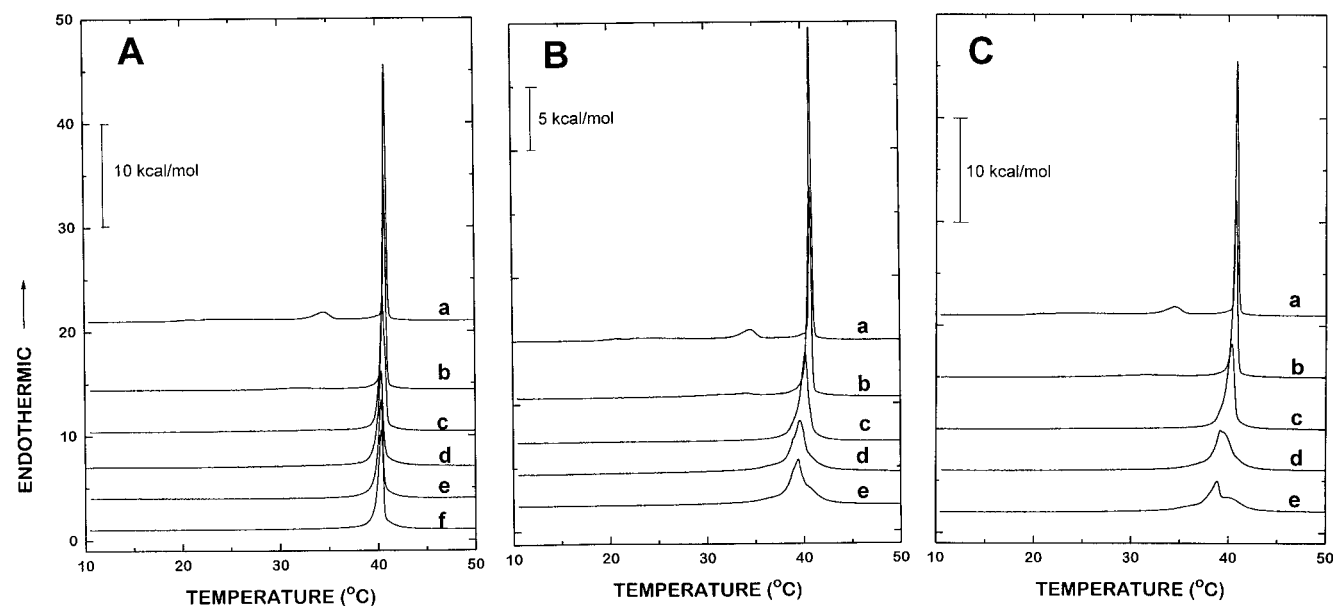


FIGURE 4 The DSC endotherms of binary mixtures of DPPC with varying amounts (in mol%) of bromoacyl taxane prodrugs: 0 (curve *a*), 1 (curve *b*), 3 (curve *c*), 4.8 (curve *d*), 9.1 (curve *e*), and 16.7 (curve *f*). (*A*) DPPC and C-6 taxane; (*B*) DPPC and C-12 taxane; (*C*) DPPC and C-16 taxane mixtures.

The DSC profile for mixtures of DPPC and C-12 taxane is shown in Fig. 4 *B*. At low concentrations, the effects of C-12 taxane on the DPPC were the same as for DMPC and C-12 taxane mixtures (Fig. 2 *B*). For instance, prodrug addition at 1–3 mol% (curves *b* and *c*) abolished the pretransition and broadened the main phase transition, suggestive of a noncooperative and inhomogeneous mixing of C-12 taxane in DPPC. At 4.8 mol% taxane (curve *d*), the endotherm remained symmetric and was relatively less perturbed than DMPC under similar conditions. With further increasing amounts of taxane prodrug to 9.1 and 16.7 mol% (curves *e* and *f*), the DSC endotherms remain unchanged, as was the case with C-6 taxane in DMPC at the same mol% (Fig. 2 *B*). This suggests the point of saturation at higher mol% by limiting solvation ability of the bilayers for the acyl chain of the prodrug.

DSC endotherms for DPPC and C-16 taxane are shown in Fig. 4 *C*. Similar to C-12 taxane, increasing C-16 taxane content to 1 to 3 mol% abolished the pretransition and broadened the main phase transition (curves *b* and *c*). As the C-16 taxane concentrations were increased to 4.8 and 9.1 mol% (curves *d* and *e*), DPPC MLVs were relatively more perturbed than for C-12 addition under similar conditions (curves *d* and *e*; Fig. 4 *B*). At 9.1 mol% C-16 taxane, the endotherm showed a high temperature shoulder, unlike the C-12 taxane, possibly indicative of inhomogeneous mixing or domain formation.

The effects of paclitaxel addition on DSPC bilayers

The effects of paclitaxel addition on the thermotropic phase behavior of DSPC were different than on DMPC and DPPC. Fig. 5 shows endotherms of DSPC and paclitaxel mixtures. DSPC alone showed a pretransition at 48.1°C ($\Delta T_{1/2} = 3.7^\circ\text{C}$ and $\Delta H = 2.1$ kcal/mol) and a main phase transition (T_m) at 53.4°C ($\Delta T_{1/2} = 0.5^\circ\text{C}$ and $\Delta H = 11.5$ kcal/mol). Paclitaxel addition in the range of 1 to 9.1 mol% caused insignificant changes in the pretransitions and main phase transitions of the DSPC bilayers, except that the pretransitions were broadened with increasing amounts of paclitaxel (curves *b–e*). In addition, at paclitaxel concentrations as low as 1 mol% a new phase appeared apart from the main phase transition temperature at a lower temperature ($\Delta T_m = 1^\circ\text{C}$). This peak is referred to as the twin peak, which arises from the effects of osmotic stress on DSPC bilayers in the presence of sugars and salts (Perkins et al., 1997).

The effects of α -bromoacyl taxane prodrugs addition on DSPC bilayers

Unlike DMPC/C-6 and DPPC/C-6 taxane mixtures, the addition of C-6 taxane caused little change in the shape of the endotherms between 1 and 16.7 mol% (Fig. 6, curves

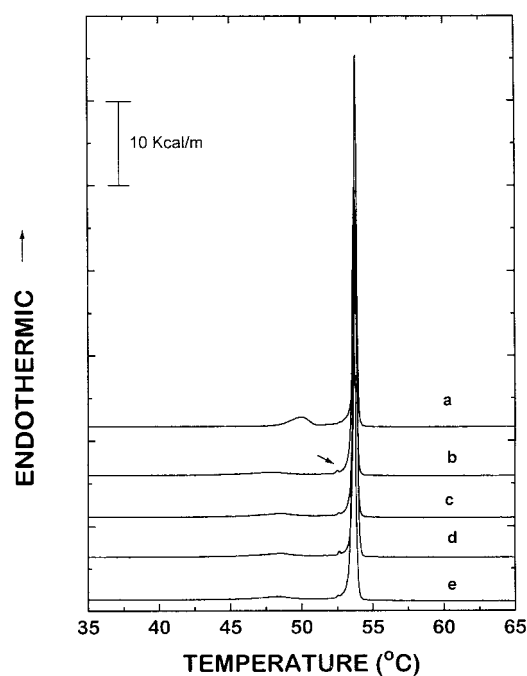


FIGURE 5 The DSC endotherms of binary mixtures of DSPC (Sigma) with varying amounts (in mol%) of paclitaxel: 0 (curve *a*), 1 (curve *b*), 3 (curve *c*), 4.8 (curve *d*), and 9.1 (curve *e*). Arrow indicates appearance of a distinct peak next to the main phase transition (Perkins et al., 1997).

a–f), indicating that DSPC bilayers were less perturbed than those composed of DMPC and DPPC (Figs. 2 *A* and 4 *A*, curves *a–f*). The pretransition was evident at all mol% values but was slightly shifted and broadened nearly to baseline at 9.1 and 16.7 mol%, suggestive of little or insignificant inclusion of the taxane into DSPC bilayers.

As illustrated in Fig. 6 *B*, DSPC/C-12 taxane mixtures showed a similar behavior to DSPC/C-6 taxane mixtures (Fig. 6 *A*). Thus, from 1 to 16.7 mol% C-12 taxane, the endotherms (curves *a–f*) showed an insignificant broadening of the main phase transition ($\Delta T_{1/2} \sim 1^\circ\text{C}$) and near abolishment of the pretransition.

DSPC and C-16 taxane binary mixtures behaved somewhat similarly to DSPC/C-12 taxane mixtures. Like C-12 taxane, the pretransition of DSPC was abolished at lower C-16 taxane concentration (~ 1 mol%), as was observed for C-12 taxane (Fig. 6 *C*, curve *b*). The main phase transitions were less perturbed and significantly less broadened ($\Delta T_{1/2} \sim 1^\circ\text{C}$; curves *c–f*).

The effects of paclitaxel and bromoacyl taxane prodrugs on transition enthalpy (ΔH)

In order to understand the interactions of PCs with paclitaxel and bromoacylated taxane prodrugs, we examined the transition enthalpy (ΔH) from each of the binary mixtures. In general, except for DSPC, the ΔH of DMPC and DPPC was significantly lower for addition of paclitaxel compared

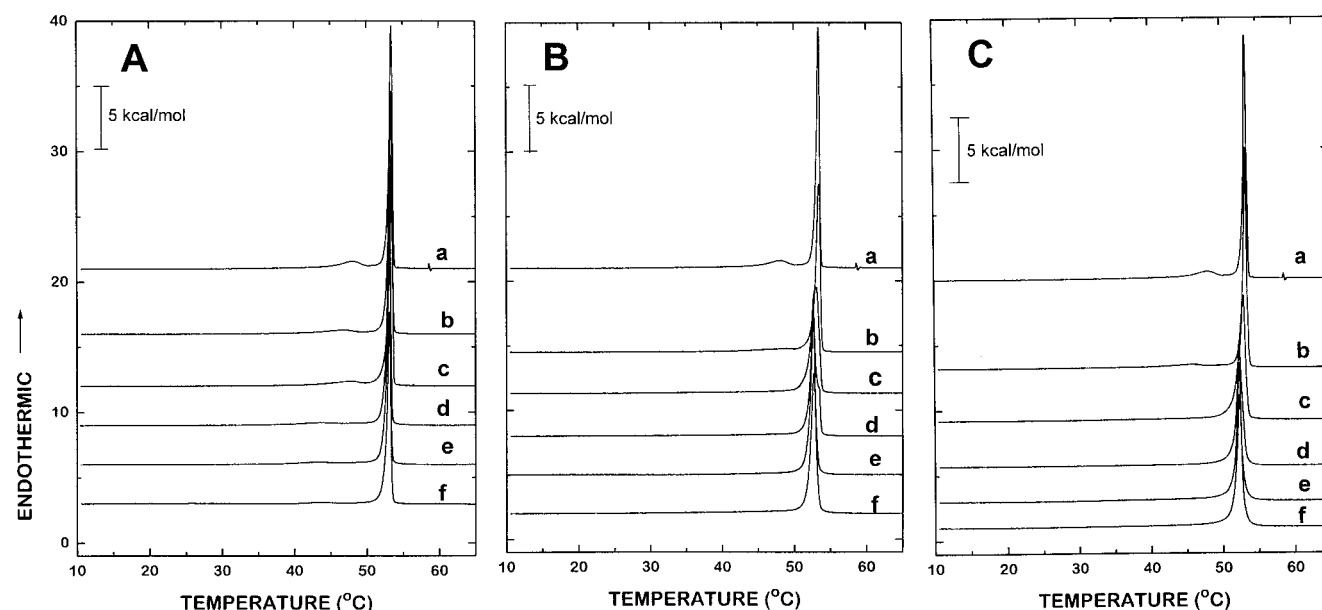


FIGURE 6 The DSC endotherms of binary mixtures of DSPC with varying amounts (in mol%) of bromoacyl taxane prodrugs; 0 (curve *a*), 1 (curve *b*), 3 (curve *c*), 4.8 (curve *d*), 9.1 (curve *e*), and 16.7 (curve *f*). (A) DSPC and C-6 taxane; (B) DSPC and C-12 taxane; (C) DSPC and C-16 taxane mixtures.

to taxane prodrugs in the concentration range investigated. It is worth noting that there was a biphasic response in transition enthalpy on paclitaxel or taxane prodrug additions to PCs showed; at low paclitaxel concentrations (1–3 mol%) ΔH did not decrease, but did increase significantly with the prodrugs at equivalent concentrations. However, there was a decrease at 4.8 mol% with taxane, which reached a minimum at or above 9.1 mol%.

Fig. 7 *A* shows the effects of addition of paclitaxel or taxane prodrugs on the ΔH of DMPC. Although there was a decrease in ΔH from 0 to 1 mol% paclitaxel, we think this could be due to sample preparation. Paclitaxel addition at concentrations ranging from 1 to 4.8 mol% did not result in a significant change in the transition enthalpy of DMPC

(curve *a*, Fig. 7 *A*). However, as the paclitaxel concentration increased to 9.1 mol% (the highest concentration used), the ΔH decreased by $\sim 20\%$. This decrease in ΔH values suggests that increasing amounts of paclitaxel in DMPC not only perturbed the bilayers but also significantly reduced the intermolecular interactions between bilayer interiors, probably caused by the disruption of hydrogen bonding at the lipid-water interface. In contrast, addition of taxane prodrugs in the 1 to 3 mol% range, regardless of their acyl chain lengths, caused relatively a higher ΔH than paclitaxel. This effect was more obvious with prodrugs bearing long acyl chains. For example, ΔH increased by $\sim 10\%$ with C-12 and C-16 taxanes (curve *c* and *d*), whereas it did not increase significantly with C-6 (curve *b*) taxane prodrug at

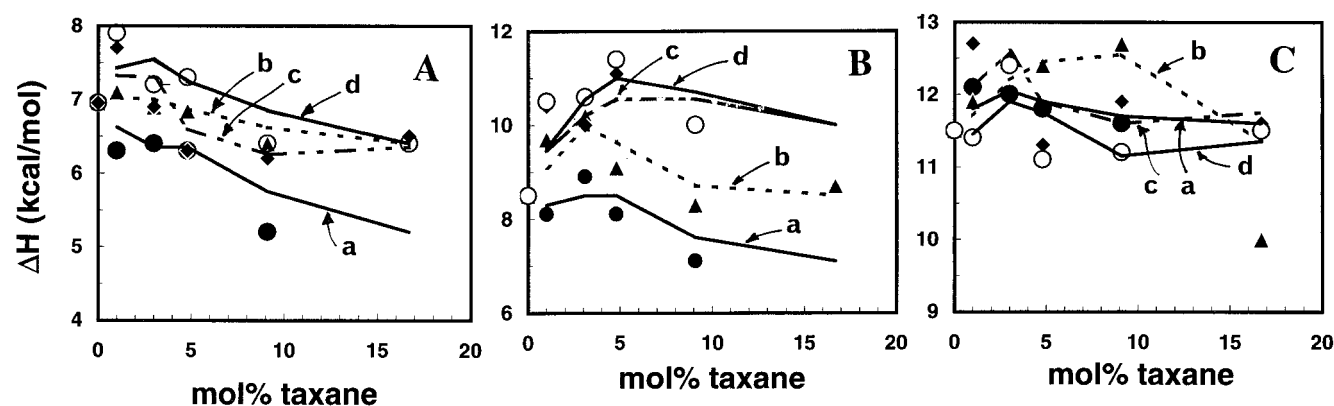


FIGURE 7 The transition enthalpy, ΔH (kcal/mol) as a function of paclitaxel and taxane prodrug concentrations: Panel A: DMPC; Panel B: DPPC; Panel C: DSPC; shown are paclitaxel (curve *a*, ● with solid line), C-6 taxane (curve *b*, ▲ with dashed line), C-12 taxane (curve *c*, ◆ with dash-dot line), and C-16 taxane (curve *d*, ○ with solid line).

lower concentrations. This change in ΔH after addition of taxanes suggests that long-chain taxane prodrugs associated with bilayer hydrophobic residues better than short-chain prodrugs. At concentrations 9.1 mol% with either taxane prodrug, however, there was a plateau in the ΔH , which presumably reflects prodrug saturation in DMPC bilayers.

The effects of paclitaxel and taxane prodrugs on the ΔH of DPPC are shown in Fig. 7 *B*. Like DMPC/paclitaxel mixtures, there was no significant change in ΔH at lower concentrations (1–4.8 mol%) of paclitaxel. However, at higher paclitaxel concentrations, the transition enthalpy started to decrease and it decreased by $\sim 20\%$ at 9.1 mol% of paclitaxel (curve *a*). A similar trend was also observed for DMPC and paclitaxel binary mixtures as shown in Fig. 7 *A* (curve *a*). At low taxane concentrations, with increasing amount of prodrugs from 1 to 4.8 mol%, there was a continuous increase in ΔH , but more so with long-chain than short-chain taxane prodrugs. This increase in ΔH suggests that long-chain prodrugs increase the van der Waals interactions by associating better with bilayer interiors than do short-chain prodrugs. For instance, ΔH at 4.8 mol% C-6 taxane prodrug (curve *b*) showed a less than 5% increase compared to $\sim 20\%$ increase with C-12 (curve *c*) and C-16 (curve *d*) taxane prodrugs. Similar to DMPC/taxane mixtures (Fig. 7 *A*), at 9.1 mol% prodrug concentrations there was a plateau in ΔH , suggesting saturation of prodrugs in DPPC bilayers.

Fig. 7 *C* shows the effects of paclitaxel and taxane prodrugs addition on the ΔH of DSPC. The transition enthalpy of DSPC after paclitaxel addition showed the same trend as those of DMPC (Fig. 7 *A*) and DPPC (Fig. 7 *B*). At low paclitaxel concentrations ranging from 1 to 4.8 mol%, ΔH remained constant or changed insignificantly. However, at a higher paclitaxel content (9.1 mol%) in DSPC, the transition enthalpy decreased by $\sim 10\%$ (curve *a*, Fig. 7 *C*) compared to a $\sim 20\%$ decrease in DMPC and DPPC at equivalent paclitaxel concentrations (curve *a*, Figs. 7, *A* and *B*). In contrast to C-6 taxane prodrug, C-12 and C-16 prodrugs showed a similar ΔH profile for the addition in DSPC as those observed for DMPC and DPPC. The ΔH value did not change significantly with C-6 taxane at low prodrug concentrations (1–4.8 mol%), while it increased by ~ 5 to 10% with C-12 and C-16 taxanes at equivalent concentrations. With increasing amounts of C-6 taxane, ΔH showed somewhat a similar decreasing trend as those of C-12 and C-16 taxane prodrugs before attaining a plateau at higher taxane contents (Fig. 7 *C*, curves *c* and *d*). This again may indicate the point of saturation of the hydrophobic core of the PCs with paclitaxel or taxane prodrugs.

Taxane prodrug association with phosphocholine bilayers

DSC data, as shown in Figs. 1, 3, and 5, suggest that paclitaxel lacking the hydrophobic residue is least associ-

ated with the lipid bilayers, presumably due to lack of its penetrating ability. Alternatively, degree of insertion of the taxane prodrugs bearing short and long acyl chains attached to paclitaxel with PC bilayers varied depending upon their hydrophobic chain lengths. The DSC data indicate that the long-chain C-16 taxane prodrug was associated with PCs better than the short-chain taxane prodrugs (C-6 and C-12 derivatives), as indicated by the perturbation on the lipid bilayers. We selected C-16 taxane prodrug to examine the prodrug's association with the PC bilayers. Thus, we prepared DMPC, DPPC, and DSPC binary mixtures with C-16 taxane prodrug at a maximal concentration of 16.7 mol% used in the experiments. The fractions from the sucrose gradient column were analyzed for their taxane and phospholipid contents (data not shown), and the ratios of the two were estimated. It was found that first few liposomal fractions contained ~ 5 to 6 mol% of C-16 taxane prodrug. Fig. 8 exhibits the endotherms of pooled liposomal fractions of PC/taxane mixtures. It was obvious from the endotherms that taxane prodrug as high as 16.7 mol% in all three PCs abolished the pretransition and significantly broadened the main phase transition of each of the PCs in the order: DMPC > DPPC >> DSPC. These endotherms support the DSC data for the PCs examined in Figs. 2, 4, and 6. From these data it was apparent that association of acylated taxane prodrug was independent of the amount of taxane addition. It can also be suggested that taxane ring was being excluded from intercalating into bilayer because of its bulky size, as was the case with paclitaxel and PC mixtures (Figs. 1, 3, and 5). Thus, it is tempting to speculate that the amount of C-16

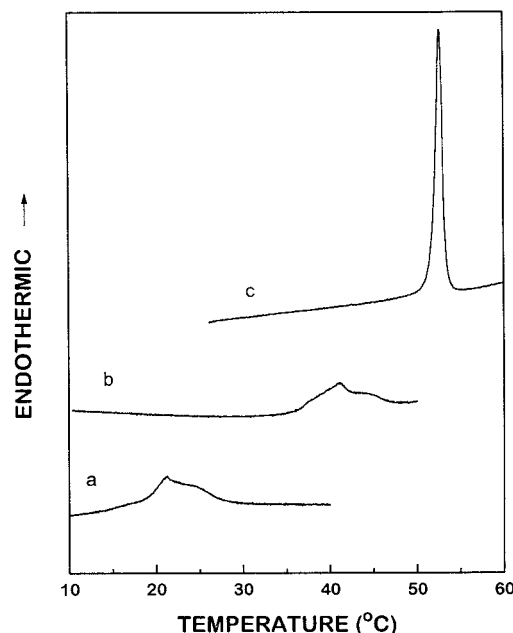


FIGURE 8 DSC endotherms of C-16 taxane prodrug associated with DMPC (curve *a*), DPPC (curve *b*), and DSPC (curve *c*) liposomes obtained after sucrose gradients.

taxane prodrug would have been greater than 5 to 6 mol% if the bulky taxane had been fully incorporated into PC bilayers. Furthermore, the perturbation of main phase transition, most notably in DMPC and DPPC with shoulders at low and high temperatures, indicates the intercalation of prodrug's acyl chain into PC, which may result in an inhomogeneous mixing and possibly the phase separation (Fig. 8).

DISCUSSION

The DSC results demonstrate that the chain lengths of acylated taxane prodrugs play an important role in the mixing behavior of the derivatives with phosphocholines varying in acyl chain composition. For instance, taxane prodrugs with long chains are incorporated relatively effectively into the short-chain bilayers. In contrast, the short-chain taxane prodrugs are incorporated relatively poorly into the long-chain bilayers. Thus, the acyl chain composition of the lipid matrix plays an important role in the solvation ability of the taxane prodrugs varying in acyl compositions. The DSC data for PCs mixed with acylated taxane prodrugs clearly showed that the gel-to-liquid crystalline lamellar phase transition was shifted to lower temperatures and broadened as the taxane prodrug content increased. The effect was more pronounced with DMPC than DPPC or DSPC. These effects possibly result from intercalation of the taxane acyl chains into phospholipid bilayers in order to maximize the van der Waals interactions. The depth of acyl chain penetration into the bilayers remains unknown at present. However, the DSC data (Figs. 2 and 4) suggest that the C-16 chain of the taxane derivative could span the entire width of PC monolayer in liposomes. This type of association perturbs the phospholipid bilayers as observed by a decrease in the onset and an increase in completion temperatures of the L_α phase. As shown in Figs. 2 *A* and 4 *A*, the C-6 taxane had limited access to the hydrophobic interiors of the PC compared to C-12 and C-16 taxane prodrugs and the amount of association was greater with the long-chain compared to the short-acyl chain prodrugs. At 9 mol% or greater C-16 taxane prodrug concentrations in DMPC and DPPC, the bilayers perturbation was larger with C-16 taxane than those with C-6 and C-12 taxane prodrugs. Thus, a taxane prodrug with a longer chain could intercalate more deeply into the bilayer.

Taxane prodrug addition to DSPC bilayers resulted in apparent anomalous behavior compared to those of DMPC and DPPC as observed by only a slight change in the shape of the endotherms on taxane addition. This was, in part, due to a stronger intermolecular hydrophobic chain interaction than DMPC and DPPC. Increasing amounts of taxane prodrugs in all the PCs examined may form taxane-rich microdomains, which are undetectable in the endotherms, within the temperature range investigated. This can also suggest that paclitaxel alone or prodrugs may sequester at

the water-lipid interface in the MLVs. Another possibility is that taxanes could form micelles or emulsions with phospholipids, which are not detectable under the DSC conditions. Such structures have been reported for taxane prodrugs associated with PEG-lipids in high drug/lipid ratios (Perkins et al., 1999). It can be argued that the lipid bilayers have limited ability to solubilize paclitaxel because of bulky size of the taxane ring. Interestingly, the ΔH decrease of the PCs after paclitaxel addition at low and higher concentrations may not be associated with the intercalation of the molecule into bilayer interiors since symmetric and sharp endotherms were apparent at those concentrations. A similar decrease in ΔH was also found with prodrugs bearing hydrophobic moieties regardless of the acyl chain composition at higher drug lipid molar ratios. It is possible that accumulation of paclitaxel or taxane prodrugs at the bilayer surface, undetectable by DSC, may disrupt hydrogen bonding at the surface between the lipid molecules by diminishing the intermolecular interaction capabilities. However, DSC can not detect such a change. From the ΔH data it is also apparent that paclitaxel at low concentrations in PCs showed an anomalous behavior compared to taxane prodrugs. For example, PCs containing 1 to 4.8 mol% of taxane, showed the ΔH \sim 20% increase for DMPC and DPPC and \sim 5 to 10% for DSPC. This increase in ΔH indicates that there is a strong chain-chain interaction between prodrug and PC, presumably as a result of the taxane prodrug's acyl chain partitioning into the bilayer. Recently, Lehtonen et al. (1996) have shown that a heterocyclic daidzein compound, a protein tyrosine kinase inhibitor, when interacting with DMPC or DPPC, caused a \sim 15% decrease in ΔH values, albeit at higher concentrations than taxane, probably due to the smaller size of the molecule. Our data are consistent with the findings that solutes show preference for the low-temperature gel phase lipids possessing hydrophobic chain lengths approximately the same as the bilayer acyl chain lengths (McMullen and McElhaney, 1995).

Recently, Baemark et al. (1997) have shown that an amphiphilic polymer, DC₁₈PEO₄₅, had ΔH profile with PC's similar to those observed with the taxanes on PCs. Accordingly, at low polymer concentrations (3–5 mol%), ΔH increased as the polymer concentration increased, followed by a \sim 26% decrease at higher concentrations, a phenomenon similar to those observed in taxane/PC mixtures (Fig. 7). This ΔH increase is primarily due to an ordering effect of the polymer in forcing part of the molecule to align into the bilayer interior (Baemark et al., 1997). As the polymer concentrations increased to 10 mol% or higher, the polymer was forced to the exterior at the water-lipid interface with little or no change in the phase transitions of PC. It is clear from our data that dehydration or osmotic stress did not shift the phase transition of the mixtures to a higher temperature. Indeed there was a slight shift in PC's phase transition to a lower temperature, which was more noticeable at lower taxane than at higher taxane

prodrug concentrations. It is possible that intermolecular interactions may be diminished by the disruption of hydrogen bonding and with promotion of electrostatic interactions between the taxanes and lipid molecules at the water-lipid interface. A slight phase transition shift and a constant ΔH at higher concentrations suggest that the hydrophobic core of the PC bilayers are saturated with prodrugs. Ruocco and Shipley (1983) have shown that mixing DPPC bilayers with cerebrosides at concentrations beyond 23 mol% promotes the formation of clusters, by partial trapping of the phospholipid molecules in laterally segregated glycolipid domains. This domain formation results in a decrease in ΔH of the phospholipid-rich gel-to-fluid phase transition at higher cerebroside concentrations. We observed a similar ΔH decrease of the phospholipid-rich gel-to-liquid phase transition at higher concentrations of paclitaxel or taxane prodrug, which may be indicative of clustering or aggregation of the taxanes with and without acyl chains at the membrane surface.

It can be suggested that fluid-type or thin bilayers (e.g., DMPC) could solvate a larger percentage of taxane compared with gel-type bilayers (DPPC or DSPC). Thus, the extent of solvation of taxane into lipid bilayers may be a result of a balance between the type of lipid assembly and the orientation of the taxane hydrophobic residues. Our phosphocholines and taxane prodrugs DSC data supports the results of another study (Balasubramanian and Straubinger, 1994) where non-acylated paclitaxel had a limited access into DPPC bilayers because of the bulky taxane ring, as also we showed in Fig. 3. We found that it was also true for paclitaxel mixed with short-chain DMPC and long-chain DSPC (Figs. 1 and 5). In essence, having a prodrug with an acyl chain and a lipid with low chain melting temperature may facilitate association of the taxane with bilayers through anchoring of the taxane hydrophobic chain in the bilayers. This was indeed the case with C-16 taxane/PC liposomes, which showed 5 to 6 mol% of taxane incorporation.

Implications

The lipophilicity and poor water solubility of antineoplastic taxane prodrugs provide an opportunity to examine the lipid-prodrug interactions in the model membranes (lipid formulations), where the hydrophobic residues of the prodrug can be anchored into the bilayer interiors. The DSC technique used in this study helps understand drug solubilization in lipid assemblies. The DSC data can help identify and, to some extent, solve problems associated with drug induced changes in lipid assemblies, and provide information about concentration-dependent exclusion of drugs from bilayers. Importantly, this study provides evidence that taxane prodrugs bearing hydrophobic moieties varying in chain length may prefer one lipid matrix over others, due to

lipid-specific selective solvation. It has been shown previously that association of paclitaxel with liposomes reduces acute toxicity while maintaining anticancer activity in animals (Sharma et al., 1993, 1997), and it can therefore be suggested that association of acylated taxane prodrugs with liposomes may have similar effects. DSC alone can not determine how a drug is oriented in the lipid bilayer matrix and how the acyl chain composition of the taxane modulates the taxane-lipid interactions. The therapeutic utility of taxane prodrugs formulated in lipid membrane remains to be established, although these taxane prodrugs are therapeutically active in vivo when formulated in Cremophor EL (Ahmad et al., 1997). The DSC data, however, show that phospholipids having variable acyl compositions accommodate certain amounts of drug dependent upon the taxane acyl composition and that prodrugs with long acyl chains associate better than those with short chains. This study also suggest that other phospholipids with structures different than disaturated DMPC, DPPC, and DSPCs may be of value in formulating taxane prodrugs and other acylated drugs. For example, lipids that form interdigitated assemblies or have bilayer defects due to unsaturation could be used in formulating some drugs. However, much further study is needed for a full understanding of taxane interactions with lipid bilayers.

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