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A molecular basis explanation of the dynamic and thermal effects of vinblastine sulfate upon dipalmitoylphosphatidylcholine bilayer membranes

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Abstract

Differential scanning calorimetry has been employed to study the thermal effects of vinblastine sulfate upon aqueous, single and multiple bilayer dispersions of 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC). The calorimetric results summarized to an increase in the gel to liquid–crystalline phase transition enthalpy and the abolishment of the L'_{β} (gel phase) to P'_{β} (ripple phase) pretransition for the uni- and multilamellar dispersions, as well as an increase in the transition temperature T_m and the transition cooperativity for single bilayer DPPC/vinblastine mixed vesicles, are consistent with an induced, partially interdigitated, gel phase. Computational analysis has been successfully applied to clarify the intermolecular effects and verify the feasibility of the proposed interdigitation for the vinblastine sulfate molecules and also for the ursodeoxycholic acid (UDCAH) and bromocylated taxanes, which have been shown to induce an interdigitated gel phase in DPPC bilayers.

Keywords: Vinblastine; DSC; DPPC; Bilayer; Liposome

1. Introduction

Vinblastine (Fig. 1) is a mitotic inhibitor isolated from the plant *Catharanthus roseus G. Don.* (Apocynaceae). It is used clinically in the treatment of leukemia, Hodgkin's disease, breast carcinoma, Wilm's tumor, Ewing's sarcoma and small-cell lung cancer, either alone or in combination with other chemotherapeutic agents [1]. Vinblastine enters into animal cells by diffusion through the plasma membrane. The permeability of the membrane depends on the molecular order and thus the mobility of the membrane lipids [2–4]. Interactions of water-soluble drugs with phospholipid membranes have been studied extensively. Their encapsulation gives rise to domain formation and thus an alteration of the lipid molecular order in the gel phase, by inducing fluidity [5]. The domain interface fluidity within the bilayer leads to an analogous increase of its permeability and eventually to a leaky bilayer. The macroscopic thermodynamic parameters are also directly affected. The broadening of the heat capacity peak (C_p) at the gel/liquid–crystalline phase transition, the lowering of the transition temperature, and the decrease of the total enthalpy with increasing concentration of the encapsulated drug, are often encountered.

Previous studies of the interactions of vinblastine sulfate with phospholipid membranes were focused on the thermal effects of this antitumor agent upon dipalmytoylphosphatidylcholine (DPPC) multilamellar vesicles (MLVs). Interestingly, the encapsulation of vinblastine was found to be followed by an increase in the total enthalpy change of the main lipid phase transition and depending on the experimental conditions used, an increase of the transition temperature [2–4]. While these results indicate a potentially interesting mechanism, which possibly reduces lateral heterogeneity of the membranes and enhances bilayer adhe-

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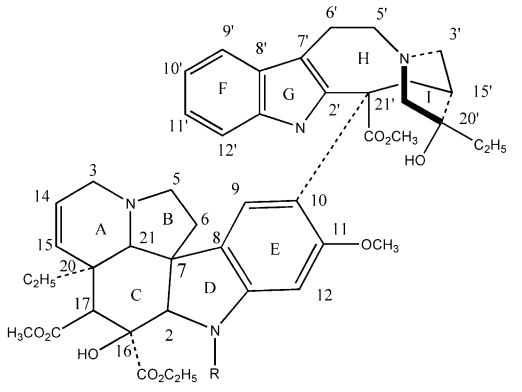
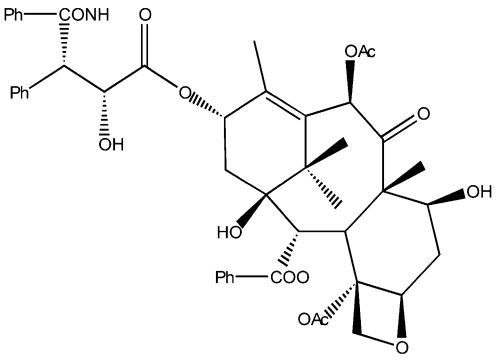


VINBLASTINE



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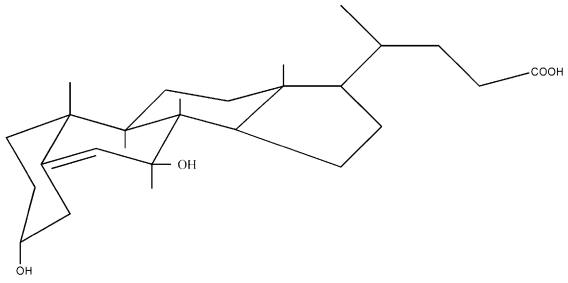

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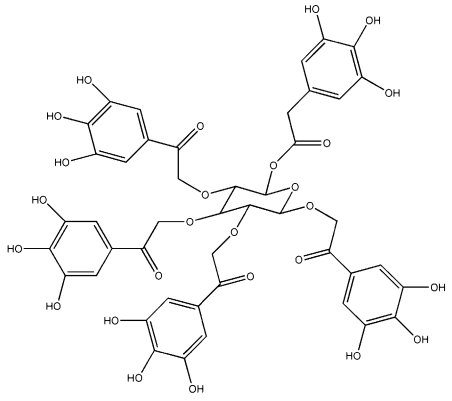
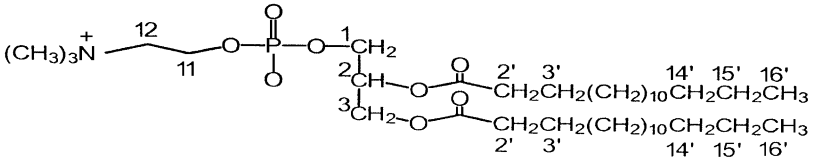


POGAD



DPPC

Fig. 1. Chemical structures of DPPC, vinblastine, UDCAH, paclitaxel, and penta-*O*-galloyl-a-D-glucose.

sion, the main lipid phase transition in MLVs was nevertheless characterized by reduced molecular cooperativity and no clear understanding of the DPPC/vinblastine sulfate interaction could thus have been achieved.

The detailed study of vinblastine interactions with phospholipids may lead to an improved design of liposomal formulations, for most efficient vinblastine delivery, and may as well be used to evaluate the way vinblastine interacts with the membranes of living cells. For this reason, the present calorimetric study was undertaken. In particular, the thermal effects of vinblastine sulfate upon single as well as multiple bilayer vesicles have been investigated in the present study.

Single bilayers are characterized by lower dimensionality (approach single layer and two dimensional structure) in comparison to multibilayers. This low dimensionality of a lipid membrane is important for the dynamic heterogeneity (existence of domains, i.e. areas in membrane which differ in lipid composition) and thus the macroscopic physicochemical properties of the bilayers, which in turn may be very important for biological functions.

Recent, calorimetric and structural studies on the interaction of the bile ursodeoxycholic acid (UDCAH), of synthetic polyphenols, of paclitaxel, as well as other bulky, amphoteric molecules with DPPC have demonstrated the formation of a thermodynamically stable, interdigitated gel

phase. The most characteristic calorimetric feature associated with this phase is a pronounced increase in the molar enthalpy of the main lipid phase transition [6–8]. Since the calorimetric results of the present study are comparable to those reported in the literature, especially in the case of UDCAH, and thus consistent with the formation of an interdigitated DPPC/vinblastine gel phase (for vinblastine concentrations $x = 17$ mol%), molecular modeling has been used for the vinblastine sulfate as well as for the UDCAH molecules and bromocylated taxanes in order to better understand the possible interdigitation mechanisms of the acyl chains. The results indicate that both molecules favor locations near the interfacial region of the membrane, while the van der Waals interactions of the hydrophobic groups are enhanced.

2. Materials and methods

2.1. Materials

DPPC was obtained from Avanti Polar Lipid Inc.; vinblastine sulfate was obtained from Eli Lilly Company; the organic solvents, i.e., chloroform ($CHCl_3$) and methanol (CH_3OH), used, were of spectroscopic grade.

2.2. Preparation of liposomes and encapsulation of vinblastine sulfate

Vinblastine containing DPPC liposomes were prepared by dissolving the lipid mixture in chloroform. The solvent was subsequently slowly evaporated in a rotary evaporator. MLVs were formed by adding ammonium sulfate 150 mM (pH = 5.3, 300 mOs). The preparation was then treated by freeze–thaw for 15 times. The resultant vesicles were extruded 10 times through two polycarbonate membranes of 200-nm pore size diameter using an extruder by Lipex Biomembranes Inc., heated at 50 °C [2,3]. The final lipid concentration of the formulations was 10 mg/ml. The vesicle size distribution for the DPPC liposomes was determined using the Mastersizer by Malvern Instruments Ltd. Repetitive extrusion of MLVs through two-stacked polycarbonate filters with 100-nm pore size after 10 freeze–thaw cycles resulted in large unilamellar vesicles (LUVs) with a size distribution of 180 nm in diameter. Subsequently, the LUV vesicles were passed through Sephadex G-75, pre-equilibrated with 100 mM TES + 100 mM NaCl (pH = 7.5, 300 mOs), thus, a transmembrane pH gradient was created. LUVs were incubated with the vinblastine sulfate at 60 °C for 5 min. The mixed DPPC/vinblastine vesicles with encapsulated vinblastine were separated from untrapped vinblastine by filtration through a Sephadex G-75 column. Vinblastine was assayed by UV spectroscopy at 262 nm and the drug/phospholipid molar ratio was determined by phosphate assay [4] from 0.13 to 0.18. The rate of vinblastine uptake was fast and efficient to more than 98% within 5 min.

2.3. Methods

2.3.1. Differential scanning calorimetry

High precision microcalorimetry using the VP-DSC microcalorimeter by Microcal Inc. was employed for the study of LUVs as well as mixtures of LUVs and MLVs prepared by the method described above. Heating and cooling scans were carried out at 15 K/h in order to study thermal history and hysterisis effects. In addition, a conventional DSC technique was applied for the study of MLV samples using a Perkin Elmer DSC-7 calorimeter. DPPC with or without vinblastine were dissolved in chloroform. The solvent was then evaporated by rotavapore under vacuum (0.1 mm Hg) at temperature above the transition temperature of the phospholipid. For measurements this dry residue was dispersed in appropriate amounts of bi-distilled water by vortexing. An aliquot of the samples (ca. 5 mg) was sealed into stainless steel capsules obtained from Perkin Elmer. Thermograms were obtained on a Perkin Elmer DSC-7 calorimeter. Prior to scanning, the samples were held above their phase transition temperature for 1–2 min to ensure equilibration. All samples were scanned at least twice until identical thermograms were obtained using a scanning rate of 2.5 °C/min. The temperature scale of the calorimeter was calibrated using indium ($T_m = 156.6$ °C) as the standard reference sample.

2.3.2. Molecular modeling

DPPC bilayers and minimized drug molecules were first minimized using S/G O2 and algorithms embedded in the QUANTA software. Details of these techniques are described in previous publication [9].

3. Results

3.1. Differential scanning calorimetry

The high-precision microcalorimetric results for the DPPC/vinblastine sulfate single and multiple bilayer, mixed vesicles are displayed in Fig. 2. In Fig. 2A, C the C_p vs. temperature (T) trace for pure DPPC vesicles is shown for a sample that contains a non-interacting mixture of 88 ± 4% LUVs and 12 ± 4% MLVs. For unilamellar systems, the membrane dynamic lateral heterogeneity is inherent due to the low dimensionality of the system [10]. This is emphatically demonstrated in the C_p peak describing the main lipid phase transition for DPPC, i.e. the transition from the ripple, gel phase (P_β') to the liquid–crystalline (L_α) phase. For the unilamellar system, this transition is described by a broadened C_p peak with a maximum at $T_{m/u} = 41.1$ °C and a total enthalpy change ($\Delta H = 7.40 \pm 0.40$ kcal/mol) that is characterized by low molecular cooperativity [11]. On the other hand, for MLVs, the transition temperature (T_{mm}) is at 42.3 °C while the transition is characterized by a sharp C_p peak with a molar enthalpic content $\Delta H = 8.5 \pm 0.40$ kcal/

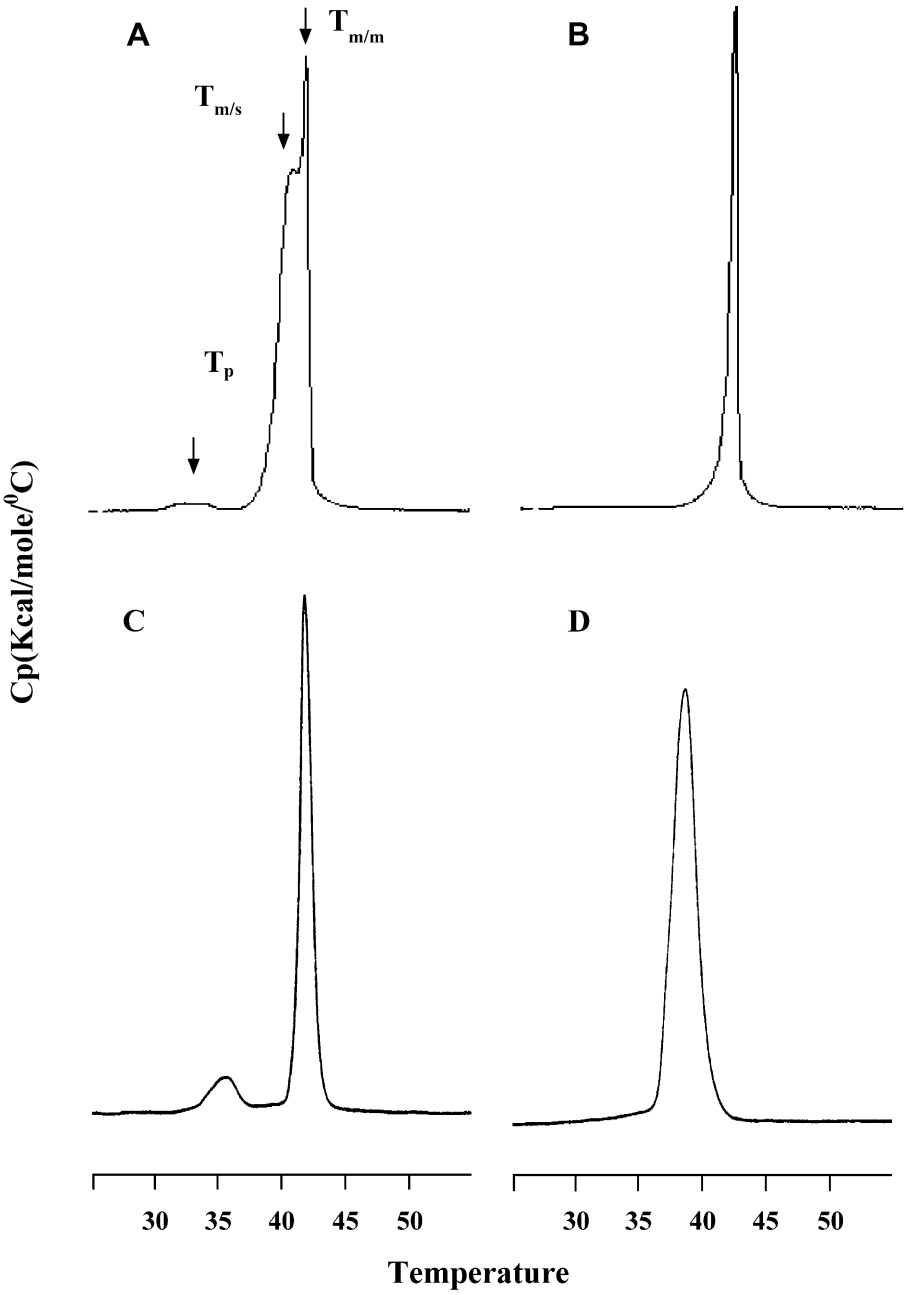


Fig. 2. (A, C) C_p vs. T data for a mixture of single- and multiple bilayer DPPC vesicles; T_{pu} and T_{pm} are the L'_β to P'_β pretransition temperatures for the uni- and multibilayer systems. Accordingly, T_{mu} and T_{mm} are the temperatures for the main-lipid phase transition of the two systems. (B, D) C_p vs. T data for the same mixture as in (A), with vinblastine sulfate incorporated in the DPPC membranes at molar concentration $x = 0.17$.

mol. The pretransition from the gel (L'_β) to the P'_β phase also depicts differences between the uni- and multilamellar systems. For single-membrane vesicles, it is characterized by a weak C_p anomaly at $T_{p/u} = 32.5$ °C with ($\Delta H = 0.3 \pm 0.2$ kcal/mol), while for DPPC MLVs the pretransition temperature is higher, at $T_{p/m} = 34.3$ °C and $\Delta H = 0.7 \pm 0.3$ kcal/mol. Conventional DSC calorimetry on multilamellar aquatic dispersions of DPPC demonstrates analogous thermal behavior. The main transition occurs at $T_m = 42.3$ °C with $\Delta H = 8.1 \pm 0.3$ kcal/mol and the pretransition at $T_p = 35.4$ °C with $\Delta H = 1.0 \pm 0.3$ kcal/mol [12].

The encapsulation of vinblastine sulfate within the DPPC membranes at a molar ratio $x = 0.17$ causes significant changes in their thermotropic phase behavior. As it can be seen from the C_p trace displayed in Fig. 2B, D, the weak $L'_\beta \rightarrow P'_\beta$ peak can no longer be detected and both the uni- and multi lamellar peaks appear to have merged in a single, sharp anomaly at 41.8 °C characterized by a molar enthalpy change $\Delta H = 8.8 \pm 0.3$ kcal/mol, which is larger than ΔH for either the unilamellar or the multilamellar pure DPPC system (Table 1). Analogous results have been obtained by conventional DSC calorimetry on multilamellar DPPC

Table 1
Calorimetric results for the thermotropic behavior DPPC and DPPC/vinblastine-sulfate mixed vesicles

Single bilayer vesicles					
	Pretransition		Main lipid phase transition		
	T_m (°C)	ΔH (kcal/mol)	T_m (°C)	ΔH (kcal/mol)	C.U. (# of DPPC molecules)
DPPC	32.5	0.3 ± 0.2	41.1	7.4 ± 0.4	92
DPPC/vinblastine-sulfate (x = 0.17 mol%)	–	–	41.8	8.8 ± 0.3	270
Multilamellar dispersions					
	Pretransition		Main lipid phase transition		
DPPC (high-precision DSC)	34.3	0.7 ± 0.3	41.8	8.5 ± 0.4	–
DPPC (conventional DSC)	35.4	1.0 ± 0.3	42.3	8.2 ± 0.4	–
DPPC/vinblastine-sulfate (x = 0.045 mol%) (conventional DSC)	–	–	41.2	10.0 ± 0.4	
DPPC/vinblastine-sulfate (x = 0.17 mol%) (conventional DSC)	–	–	40.5	10.4 ± 0.4	–

aqueous dispersions. The partitioning of vinblastine sulfate caused the abolishment of the pretransition, an abrupt increase in $\Delta H = 10.4 \pm 0.4$ kcal/mol with a simultaneous broadening of the C_p peak, and the lowering of the transition temperature (Table 1). As a measure of the molecular cooperativity within the bilayers, and thus of the dynamic heterogeneity, the ratio of ΔH to the van't Hoff enthalpy (ΔH_{vH}) has been calculated, which approximately gives the average number of domain-correlated molecules within the bilayer undergoing the transition [13]. The number of molecules within a cooperative unit (C.U.) along with other thermodynamic parameters is listed in Table 1. The conclusions that can be drawn from the thermal results presented in Table 1 is that the partitioning of $x = 0.17$ mol% of vinblastine sulfate in DPPC aqueous dispersions induces the abolishment of the pretransition and a sharp, 15–20% increase of molar ΔH for either LUVs or MLVs. Moreover, for the single bilayer DPPC vesicles, an increase in T_m is also induced along with an increase in the size of the C.U., which triples in the presence of vinblastine. For MLVs, the increase in ΔH is accompanied by a decrease in T_m and the broadening of the C_p peak.

3.2. Molecular modeling

To further elucidate the calorimetric results for the partitioning of vinblastine sulfate within DPPC bilayers, molecular modeling has been applied. Vinblastine sulfate was inserted into DPPC molecules after being subjected to minimization using various first order and second order algorithms embedded in QUANTA/Charmm algorithm. We used for our work the DPPC bilayer published by Tieleman et al. [14]. This bilayer contains 128 DPPC ligands and 3910 water molecules.

Our findings show that vinblastine molecules favor positioning near the polar region of the membrane lipids while extending their hydrophobic moieties towards the interior of the bilayer within the alkyl-chain region. In particular, the water-soluble amphoteric bulky molecule of vinblastine can be associated with different molecular interactions of DPPC bilayers. The HSO_4^- can interact electrostatically with $N^+(CH_3)_3$ head-group, the positively charged molecule with the PO_4^{3-} of the polar group, and the hydrophobic segments of vinblastine can be associated with van der Waals interactions of acyl chains of the phospholipid. Hydrogen bondings between A, B, and C rings of vinblastine and water and phosphate groups of DPPC bilayers were observed. In conclusion, the major core of vinblastine locates in the interface and its hydrophobic moieties point towards the alkyl chain by enhancing partial interdigitation.

4. Discussion

The calorimetric results obtained in the present study can be interpreted by considering the amphoteric character of the intermolecular interactions between vinblastine sulfate and DPPC. Molecular modeling clearly indicates that the amphoteric character of the vinblastine interactions govern its association with model membrane bilayers.

Thermotropic behavior analogous to what is encountered in the present study has been recently reported in the literature for a number of different additives. Of particular interest for the present study is the calorimetric (DSC) study on the effects of UDCAH and ursodeoxycholate ($UDCA^-$) upon the thermotropic phase behavior of aqueous bilayer dispersions of DPPC buffered at pH 7.0 [5]. For $UDCA^-$ concentrations of 25–60 mol%, thermal effects comparable to those observed for the vinblastine/DPPC ($x = 0.17$ mol%) dispersions have been recorded, i.e. the abolishment of the $L'_\beta \rightarrow P'_\beta$ pretransition, along with an increase in ΔH. The disappearance of the C_p pretransition peak can be attributed to interfacial interactions between the UCDA polar groups and the DPPC head groups. The increase of ΔH was interestingly attributed to an induced interdigitated gel phase L_{gi}, stabilized by the DPPC:UDCA molecular interactions. The enhanced van der Waals interactions between the hydrocarbon chains and the $UDCA^-$ hydrophobic groups give rise to more rigid bilayers, and thus larger values for the molar, main transition enthalpy change. For concentrations higher than 60% mol, the L_{gi} phase was shown to exist

in equilibrium with micelles of various UDCA:DPPC molar ratios whose structures remain to be elucidated. Similarly, in a DSC study of phosphocholines mixed with paclitaxel and its bromacylated taxanes [7] as well as in a calorimetric/X-ray scattering study of synthetic polyphenols investigating conditions for increasing bilayer adhesion [8], it was shown that these bulky water-soluble molecules encapsulated in DPPC bilayers exert similar thermotropic behavior as vinblastine sulfate. Once again, the measured increase of ΔH was interpreted as a possible increase in the van der Waals interactions in the interior of the membrane bilayers. The possibility that taxanes could form micelles or emulsions with phospholipids which are not detectable under DSC conditions was also discussed [7]. Another literature example is the ethylene oxide containing lipopolymers and triblock copolymers on lipid bilayers of palmitophosphatidylcholine [6,15].

A comparison of vinblastine with other molecules that exert similar thermal effects on DPPC bilayers reveals that they exert similar molecular interactions with phospholipid environments. In particular, these are amphiphilic bulky molecules that span the head-groups and the upper part of the alkyl chains.

The significance of this report is attributed to the understanding in molecular level the thermal effects of a vinblastine in membrane and the support of findings through literature. This study shows that similar stereoelectronic requirements in drug molecules govern their thermal effects. Jain et al. [16,17] made an effort to classify the thermal effects of different organic molecules in DPPC bilayers using as diagnostic parameters the lowering of the phase transition temperature and the half-width. However, molecular factors are not discussed in details in these articles, which in our opinion are the decisive ones that govern their thermal effects. In addition, effects such as the increase of ΔH while as it is mentioned is reported in the literature, no attempt was made to correlate this increase of ΔH with the stereoelectronic properties of a molecule.

It is apparent from this discussion that embedded molecules in membrane bilayers have the following major molecular features in order to cause increase of ΔH: (a) are bulky; (b) are amphoteric; and (c) have a tendency to form micelles. These properties force the phospholipid bilayers to open the space between the head-group regions in an attempt to accommodate themselves. In addition, their lipophilic part which is embedded between the alkyl chains associates with them and increases their flexibility.

Reported studies of vinblastine thermal effects in bilayers show that this molecule exerts specific effects. Its effects are depending on: (a) concentration; (b) phospholipid head-group specificity; (c) alkyl chain specificity; and (d) concentration of cholesterol. This is anticipated from the above discussion. These molecules have a net effect which results from their mixture of electrostatic, polar, and hydrophobic interactions. The change of the head-group or the length of the alkyl chain will have tremendous consequences on drug:membrane interactions. In addition, the increase of complexity in the system like the addition of cholesterol will affect the thermal properties of vinblastine [18]. Cholesterol is a relatively bulky molecule that is well known to incorporate in membrane bilayers. It is anticipated to interfere with vinblastine's molecular interactions and alternate its thermal effects. These effects are also governed by the concentrations of drug and or cholesterol. The phospholipid specificity may be explained due to the different degree to form micelles with vinblastine. The concentration may also be an important factor to the formation of micelles.

Acknowledgements

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