The Antimalarial Agent Halofantrine Perturbs Phosphatidylcholine and Phosphatidylethanolamine Bilayers: a Differential Scanning Calorimetric Study

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The interaction of halofantrine with phosphatidylcholine and phosphatidylethanolamine bilayers has been investigated by differential scanning calorimetry. Halofantrine caused a broadening of the gel to liquid crystalline phase transition endotherm of the phosphatidylcholines. A decrease in the transition temperature T_m and enthalpy (ΔH) of transition was also observed. This varied with the chain length of the phospholipid and was more pronounced with short chain members. Halofantrine-induced changes to the thermotropic characteristics of dipalmitoylphosphatidylcholine (DPPC)/cholesterol bilayers suggested that the penetration of halofantrine into the bilayer was diminished in the presence of cholesterol. A more complex calorimetric profile was observed in the interaction of halofantrine with phosphatidylethanolamines and the results suggested that halofantrine did not disrupt the cooperativity of the phosphatidylethanolamine bilayers to the same extent as that observed with the phosphatidylcholines. Halofantrine caused significant perturbation of phospholipids and this property might have an important bearing on its pharmacodynamic effects.

Key words antimalarial; halofantrine; differential scanning calorimetry; phosphatidylcholine bilayer; phosphatidylethanolamine bilayer

The antimalarial agent, halofantrine is widely prescribed for the treatment of acute malarial attacks caused by chloroquine-resistant and multi-drug resistant strains of *Plasmodium falciparum*.[1] Despite its wide usage, little is known of the mode of action of this drug.[2] Structurally, halofantrine is an amphiphile, *viz*., it has both hydrophobic and hydrophilic portions in the molecule (Fig. 1). The phenanthrene ring in halofantrine is largely hydrophobic and the aliphatic side chain which bears a hydroxyl group and a tertiary amino function (protonated at physiological pH) gives it hydrophilic character. Many amphiphilic molecules are known to interact specifically with phospholipid membranes, with important consequences to their pharmacology and toxicology.[3] Not unexpectedly, our earlier study has shown that halofantrine interacted with 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) bilayers[4] and in a manner which was quite different from that of other catamphiphilic antimalarial agents. The interaction of halofantrine with biological membranes is potentially significant as membrane-related processes might be implicated in the antimalarial action and toxicology of the drug. Physical disruption of acid intracellular parasitic vesicles, inhibition of the parasitic vesicle proton pump and accumulation within plasmodial infected erythrocytes are membrane related processes which are relevant to the mode of antimalarial action.[5,6] Halofantrine has been reported to cause life-threatening arrhythmia[7] which might arise from drug-induced disruption of the phospholipid bilayers located in the vicinity of sodium/calcium ion channels.[8] Therefore, it is of interest to investigate the interaction of halofantrine with biologically important phospholipids. In this study, we have used differential scanning calorimetry (DSC) to examine the action of halofantrine on two phospholipid classes (phosphatidylcholines, phosphatidylethanolamines) which are the major lipids found in mammalian plasma membranes. The effect of varying phospholipid chain length and the inclusion of cholesterol into DPPC layers on the halofantrine interaction are reported in this study.

Experimental

Materials Halofantrine hydrochloride was a gift from SmithKline Beecham (Hertfordshire, UK). The phosphatidylcholines (1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine DMPC, DPPC, 1,2-distearoyl-sn-glycero-3-phosphatidylcholine (DSPC) and cholesterol were purchased from Sigma Chemical Company (St. Louis, MO). The phosphatidylethanolamines (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine DMPE, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine DPPE, 1,2-dielaidoyl-sn-glycero-3-phosphoethanolamine DEPE) were obtained from Avanti Polar Lipids Inc. (Alasbaster, AL).

Preparation of Liposomes The phospholipid mixtures for calorimetry measurements were prepared according to reported methods.[4,9] Briefly, this involved combining an aliquot (5 ml) of chloroform containing 50 μmol of phospholipid with varying amounts of halofantrine in methanol to give final halofantrine: phospholipid mol ratios of 0.01 : 1 to 1.2 : 1 (equivalent to 1 to 120 mol% ratio of drug to phospholipid). The organic solvents were removed by evaporation *in vacuo* on a rotary evaporator and the resulting film was dried under vacuum overnight. Mutlilamellar liposomes were prepared by adding 1 ml of Tris buffer (0.05 M, pH 7.4) to the dried film, vortexing for 1 min, and rotating the flask with its contents on a rotary evaporator for 30 min at a temperature above the gel to liquid-crystalline phase transition temperature (T_m) of the lipid. The milky suspension was allowed to stand at room temperature for 30 min and subsequently centrifuged (80000×g, 40 min, 20 °C) on a Beckman L7 ultracentrifuge. The resulting pellets were decanted dry by inverting the centrifugation tubes containing the pellet and standing for 20 min. During this period, some liquid would flow out leaving a fairly compact pellet in the centrifugation tube, whose weight was determined by difference.

Experiments were also carried out in which the milky suspensions obtained from selected phospholipids (DPPC, DMPC) were equilibrated for varying periods of time (up to 6 h) at room temperature before centrifugation and DSC analysis. The experiment was also repeated in the presence of the

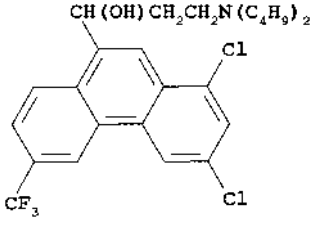

Fig. 1. Halofantrine (1,3-Dichloro-α-[2-(dibutylamino)ethyl]-6-trifluoromethyl-9-phenanthrenemethanol)

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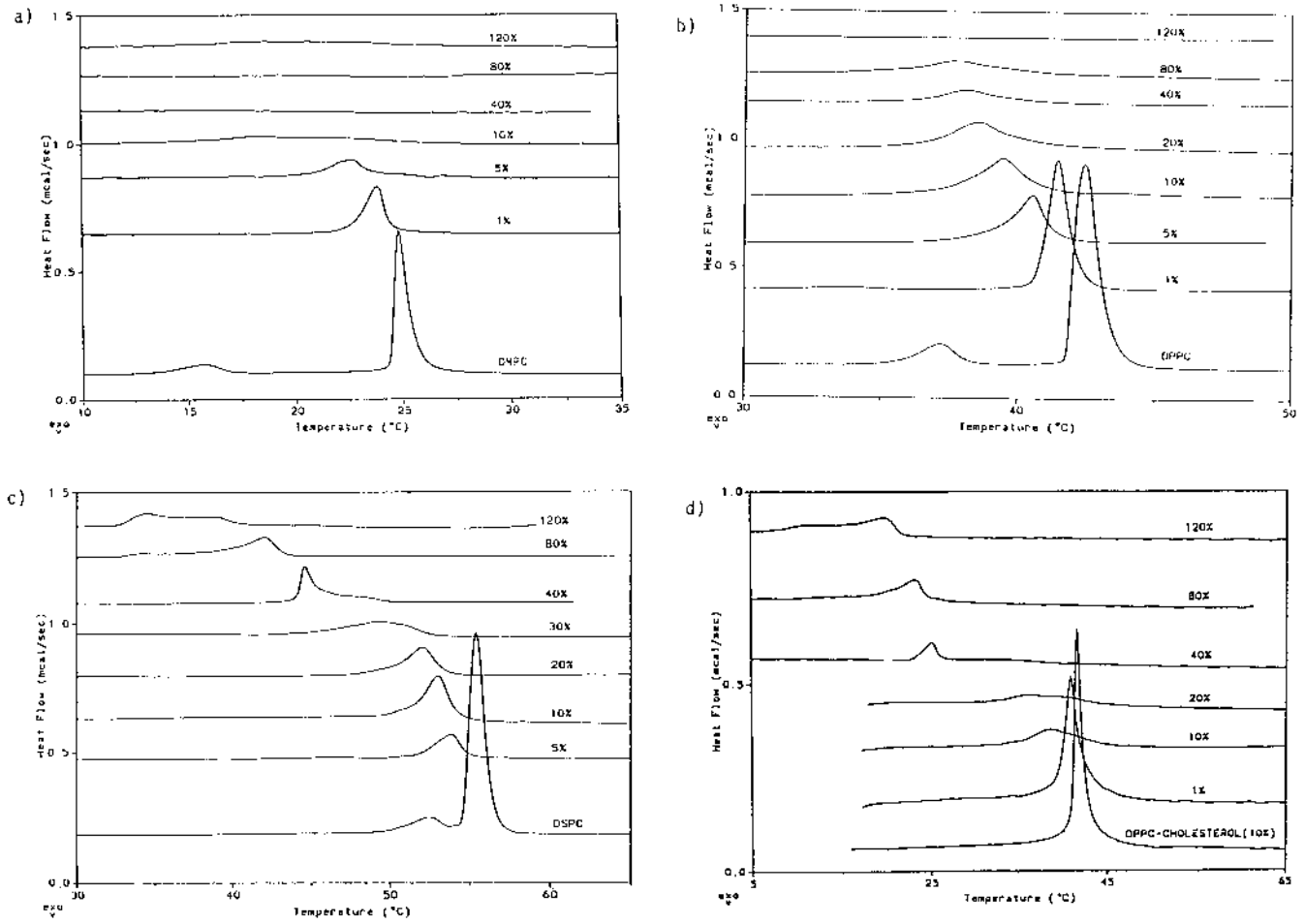

Fig. 2. DSC Thermograms for Mixtures of Halofantrine and the Following Phosphatidylcholines

(a) DMPC, (b) DPPC, (c) DSPC and (d) DPPC/10% cholesterol. % mol ratio of halofantrine is indicated on the curves. The profiles correspond to heating scans.

drug at 10 mol% ratio.

DSC Measurements A known amount of the pellet (3—12 mg) was weighed into an aluminium pan which was then hermatically sealed and scanned on a TA Instruments DSC 2010 differential scanning calorimeter, using empty hermatically sealed aluminium pans as reference. The heating rate was set at 2 °C per minute. The temperature scale and enthalpy measurements were calibrated using indium and cyclohexane as standards. The thermal data was analyzed using the TA Instruments Universal Analysis program to give the peak temperature T_m and enthalpy of the transition (ΔH) of the phospholipid admixture obtained in the absence/presence of halofantrine. Each analysis was repeated three times. Although hydration water was inevitably included in the sample weight, the results obtained for T_m and transition enthalpy were reproducible within acceptable limits (Tables 1,2). All T_m values had coefficients of variation which were less than 10%. A wider variation (>20% C.V.) was observed for enthalpy determinations, in particular where broad endotherms were encountered.

The period of equilibration (30 min) used for preparation of the liposomes also appears to be adequate as longer periods of equilibration (up to 6 h) gave thermograms which were not different from those obtained after a shorter equilibration period. Pure phospholipids treated in this way give T_m values which were comparable to those reported in the literature.[10—12)]

The amount of halofantrine and phospholipid in the pellet was determined by ultraviolet spectroscopy and the Steward assay,[13)] respectively. No halofantrine was detected in the supernatant. Halofantrine content is expressed as % mol ratio of the drug present to the number of moles of phospholipid in the following discussion.

Results and Discussion

The Effect of Halofantrine on Saturated Phosphatidylcholine Bilayers The DSC scans in Fig. 2a—c demonstrate the effect of incorporating increasing quantities of halofantrine on the main phase transition of phosphatidylcholines of varying chain lengths, *viz*. DMPC (C14), DPPC (C16) and DSPC (C18). It can be seen that concentrations of halofantrine as low as 5% mol introduced a significant perturbation in these phospholipids. The pretransition was completely abolished and the main transition broadened and shifted to lower temperatures. This effect is particularly pronounced in DMPC, where 10% mol of halofantrine caused the complete disappearance of the DMPC transition endotherm. In contrast, a much higher concentration of halofantrine (at least 80% mol) was required to abolish the transition endotherm of DPPC. In the case of DSPC, the transition endotherm was not abolished even at the highest concentration of halofantrine (120% mol). Figure 2c shows that the main transition endotherm of DSPC shifted to lower temperatures and showed considerable broadening with increasing halofantrine concentrations.

The observation that the transition endotherms of DMPC and DPPC are more affected than DSPC in the presence of high concentrations of halofantrine is not unexpected. Other investigators have also noted in their studies with suramin[14)] and α-tocopherol[15)] that short chain phospholipids were more susceptible to perturbation by the drug. For example, in the interaction of α-tocopherol with phosphatidylcholines, the transition endotherms of DMPC and DPPC showed a

Table 1. Transition Temperatures (T_m °C) and Enthalpies (ΔH kcal/mol) for the Gel to Liquid Phase Transition of Mixtures of Halofantrine and Phosphatidylcholines[a)]

Halofantrine % mol ratio	DMPC		DDPC		DSPC		DPPC/Cholesterol[b)]	
	T_m	ΔH	T_m	ΔH	T_m	ΔH	T_m	ΔH
0	24.10 (0.15)	5.94 (0.84)	42.09 (0.06)	9.30 (0.18)	55.31 (0.12)	13.32 (1.1)	41.55 (0.02)	7.25 (0.19)
1	23.93 (0.11)	5.52 (0.16)	39.77 (0.05)	6.32 (1.1)	—	—	40.88 (0.01)	5.77 (0.07)
5	22.62 (0.14)	2.27 (0.73)	—	—	53.86 (0.01)	8.83 (0.38)	39.43 (0.04)	4.66 (0.16)
10			39.43 (0.03)	4.19 (0.04)	53.11 (0.08)	10.37 (0.5)	39.14 (0.24)	4.78 (1.73)
20			38.89 (0.38)	4.24 (0.32)	52.14 (0.05)	9.17 (0.61)	36.36 (0.26)	3.27 (0.40)
30			—		49.25 (0.19)	8.96 (1.34)		
40			26.97 (0.08)	3.00 (0.29)	44.62 (0.05)	9.29 (0.75)	25.19 (0.01)	2.75 (0.17)
80			26.53 (0.09)	5.40 (0.73)	42.14 (0.03)	8.75 (0.46)	23.29 (0.04)	5.21 (0.17)
120			22.85 (0.19)	5.61 (0.04)	34.32 (0.26)	13.97 (0.72)	19.84 (0.01)	2.12 (0.10)

a) Values in parentheses represent S.D. for $n=3$ determinations. *b*) Bilayers contained 10 mol% of cholesterol.

greater downward shift than the DSPC endotherm at high concentrations of α-tocopherol. The susceptibility to perturbation is related to the different degrees of interaction between the phospholipid molecules. Phospholipids containing shorter acyl chains have lower net van der Waals interaction between the hydrocarbon chains. In addition, bilayer intermolecular spacing increases as the length of the acyl chain decreases. This would explain why a short chain phospholipid like DMPC is more susceptible to perturbing effects by an external agent like halofantrine than a longer chain phospholipid like DSPC.

Table 1 summarizes the changes in transition temperatures and enthalpies for the gel to liquid crystalline phase transition of mixtures of phosphotidylcholines and halofantrine at different drug concentrations. For each phospholipid, enthalpy decreases in a non-linear fashion as a function of halofantrine concentration. The greatest reduction in ΔH is seen for DMPC. For example, at 20% mol ratio of halofantrine, the reduction in ΔH was 5.94 kcal/mol (100%) for DMPC, 5.06 kcal/mol (55%) for DPPC and 4.15 kcal/mol (31%) for DSPC.

The results show that halofantrine exerts a strong perturbing effect on phosphatidylcholine bilayers. The phase transition of the phospholipid is modified to show a progressive broadening of the transition peak and a shift to a lower transition temperature. The extent of this change is dependent on the chain length of the phospholipid. In reports where an external agent produced similar changes,[15,16)] the results were interpreted to indicate the establishment of a molecular interaction between the phospholipid acyl chains and the external agent, resulting in the perturbation of the cooperative behavior of the lipid bilayer matrix. Halofantrine may have a similar effect in this context.

It is significant to note that halofantrine causes a decrease in ΔH of the main transition endotherm of all the phospholipids investigated. Many drugs like capsaicin[16)] and phenylbutazone[17)] cause a change in the transition temperature of phospholipid bilayers without changing the calorimetric enthalpy. The effect on the enthalpy of the transition depends on the localization of the molecule in the phospholipid bilayer. Where changes are observed in transition enthalpy, it is normally attributed to the location of the drug within the hydrophobic interior of the phospholipid array, and not superficially in the vicinity of the polar groups or at the interfacial region of the phospholipids.[3)] Additional investigations using fluorescence spectroscopy and nuclear magnetic resonance spectroscopy are needed to confirm the localization of halofantrine in the phospholipid interior.

The Effect of Halofantrine on DPPC-Cholesterol Bilayers In another set of experiments, the effect of halofantrine on the transition parameters of a DPPC-10% cholesterol admixture was investigated. Cholesterol is a major lipid component of most natural membranes. The content of cholesterol in membranes can vary from 0.1 to 1.0% mol ratio and it is known to act as a modulator of several physical properties of the phospholipid bilayer.[18)] Some investigators have reported that cholesterol inhibited the binding of antimalarial drugs to phospholipids found in the membrane of parasitized erythrocytes and suggestions have been made that drug resistance in malaria may be correlated to the content of cholesterol in parasitized erythrocytes.[19)] It is therefore of interest to investigate the effect of cholesterol on the perturbative action of halofantrine on phospholipid bilayers.

In this study, DPPC bilayers containing 10% mol cholesterol were prepared in the usual way. As seen in Fig. 2d, this gave rise to a sharp endotherm centred at 41.55 °C, which was not significantly different from the T_m of pure DPPC bilayers (42.09 °C). It is known that cholesterol has relatively little effect on the position of the phase transition, but is able to abolish completely the heat of transition at high concentrations (>50% mol). The usual pattern of characteristic broadening and downward shift in the transition endotherm with increasing halofantrine concentrations was observed in the cholesterol/DPPC bilayers. However at 40% mol halofantrine, an endotherm was observed at 25.19 °C , which was not observed with DPPC bilayers alone. This may be attributed to a lateral phase separation of a halofantrine-rich domain, possibly because the miscibility of the drug in the cholesterol/phospholipid matrix has been exceeded at this concentration (40% mol ratio) of halofantrine.

When cholesterol is incorporated into phospholipid bilayers, its hydroxyl group interacts with the acyl chain carboxyl group of the phospholipid, and the rigid, planar steroid nucleus is aligned alongside the first 10 or so carbons of the phospholipid chain.[18)] This has the effect of reducing the freedom of motion of these carbons while at the same time creating space for a wide range of movement for the remaining carbons towards the terminal end of the chain. As a result of this orientation, at temperatures above the phase transition temperature of the phospholipid, cholesterol causes the bi-

layer to "condense" with a reduction in area, closer packing and a decrease in fluidity and permeability.

As halofantrine is incorporated into the cholesterol/DPPC bilayers at temperatures above T_m, one can reasonably expect halofantrine to have a lesser permeability into the bilayer. However, because of the small proportion of cholesterol used (10% mol), the thermotropic characteristics of bilayers prepared from DPPC and DPPC/cholesterol in the presence of varying amounts of halofantrine were not different until higher concentrations (40% mol) of halofantrine were used. At this concentration, a low temperature endotherm with a smaller transition enthalpy appeared at 25.2 °C, which is about 16 °C lower than the original DPPC/cholesterol endotherm (Fig. 2d, Table 1). Such an observation was not seen in the interaction of halofantrine with pure DPPC bilayers and would suggest that the endotherm is due to the separation of a halofantrine-rich phase in DPPC/cholesterol bilayers as cholesterol diminishes the miscibility and incorporation of halofantrine into bilayers.

The Effect of Halofantrine on Phosphatidylethanolamine Bilayers Besides the saturated phosphatidylcholines, we have also investigated the interaction of halofantrine with phosphatidylethanolamines, which represents the other major glycerophospholipid species found in biological membranes. The balance of these two phospholipids is known to be critical to membrane function. For example, in erythrocyte membranes, the major phospholipids found in the outer and inner monolayers are different, being a phosophatidylcholine in the outer layer and a phosphatidylethanolamine in the inner monolayer.[20]

Both phosphatidylcholines and phosphatidylethanolamines are neutral phospholipids at physiological pH. The protonated amino headgroup in phosphatidylethanolamines is smaller than the bulky quaternary ammonium group in phosphatidylcholines. In addition, the amino (but not the quaternary ammonium) headgroup is able to take part in hydrogen bonding interactions with its neighbours in the membrane. These differences would obviously affect the interaction of halofantrine with these two classes of phospholipids. In this study, the interactions of halofantrine with two saturated (DMPE, DPPE) and one unsaturated (DEPE) phosphatidylethanolamines were investigated.

The effect of halofantrine on the thermotropic phase transition of DMPE is shown in Fig. 3a. Table 2 summarises the change in transition temperature and enthalpy in various mixtures of DMPE and halofantrine. Pure DMPE shows only a major endotherm corresponding to the gel to liquid crystalline bilayer transition at 50.73 °C and does not present a lamellar to H_{II} phase transition, which is commonly seen for many phosphatidylethanolamines. Other workers have reported that such a transition is not observed for DMPE at temperatures below 90 °C.[12] Concentrations of halofantrine (up to 20% mol) caused a small downward shift in the transition temperature which is also accompanied by a decrease in transition enthalpy. This is in contrast to the interaction of halofantrine with the corresponding phosphatidylcholine (DMPC) where a much smaller amount of halofantrine (5% mol) is sufficient to abolish the transition endotherm completely. This could arise from the strong intermolecular hydrogen bonding between the headgroups in DMPE which may hinder the penetration/orientation of halofantrine in the bilayer array. Thus, a larger amount of halofantrine is required to disrupt the DMPE bilayers.

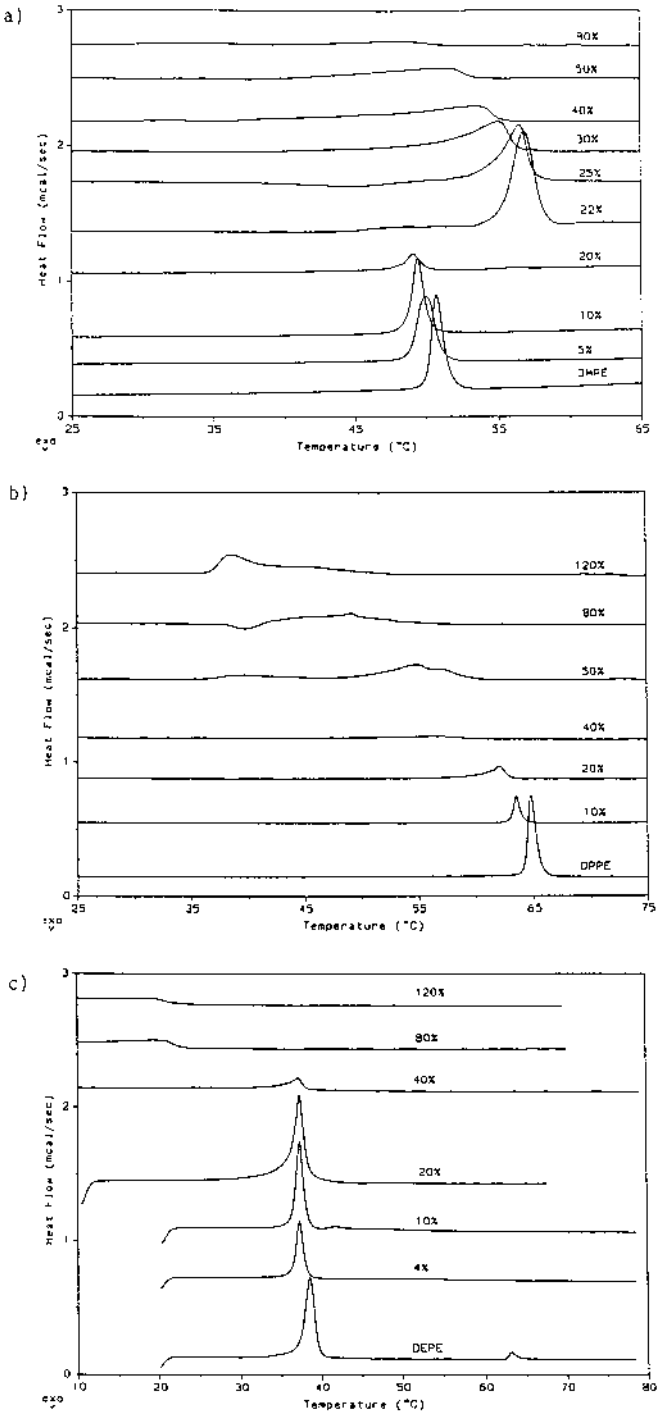

Fig. 3. DSC Thermograms for Mixtures of Halofantrine and the Following Phosphatidylethanolamines

(a) DMPE, (b) DPPE and (c) DEPE . % mol ratio of halofantrine is indicated on the curves. The profiles correspond to heating scans.

Figure 3a also shows that as the concentration of halofantrine is increased further, the transition endotherm was shifted up by about 4.6 °C above the transition endotherm of pure DMPE. As seen from Table 2, there is also a concurrent increase in the enthalpy of transition, suggesting the formation of stronger/greater intermolecular forces among the lipid molecules in the presence of halofantrine. This endotherm is

Table 2. Transition Temperatures (T_m °C) and Enthalpies (ΔH kcal/mol) for the Gel to Liquid Phase Transition of Mixtures of Halofantrine[a)]

Halofantrine % mol ratio	DMPE		DPPE		DEPE	
	T_m	ΔH	T_m	ΔH	T_m	ΔH
0	50.73 (0.055)	10.43 (0.46)	64.60 (0.16)	23.28 (1.34)	38.48 (0.11)	14.10 (0.50)
4					37.35 (0.09)	5.51 (0.40)
5	49.79 (0.21)					
10	49.54 (0.21)	8.37 (0.46)	63.54 (0.05)	4.87 (0.44)	35.59 (0.09)	6.81 (0.44)
20	49.12 (0.05)	6.93 (0.42)	62.06 (0.10)	7.01 (0.49)	37.44 (0.03)	9.29 (1.68)
22	55.29 (0.10)	20.06 (1.05)				
25	56.58 (0.10)	26.68 (1.17)				
30	55.19 (0.10)	21.44 (0.71)				
40	53.24 (0.2)	17.52 (1.57)	57.17 (0.10)	3.91 (0.31)	37.26 (0.12)	4.60 (1.18)
50			55.89 (0.09)	5.92 (0.40)		
80	47.99 (0.63)	24.20 (2.60)	49.10 (0.18)	11.88 (2.00)	20.68 (0.78)	4.41 (1.52)
120			39.21 (0.54)	5.62 (0.53)		

a) Values in parentheses represent S.D. for $n=3$ determinations.

gradually abolished as the concentration of halofantrine is increased beyond a 25% mol ratio. It would seem that at a certain critical concentration of halofantrine (≈22% mol), the presence of the drug enhances the cooperativity of the phospholipid array.

Unlike the contrasting effects of halofantrine in DMPC and DPPC bilayers, fewer differences were evident from the interaction of halofantrine with DPPC and DPPE (Fig. 3b, Table 2). Increasing concentrations of halofantrine caused an almost linear decrease in the temperature of transition and a simultaneous decrease in ΔH of DPPE bilayers. Interestingly, the interaction of halofantrine with DPPE results in the appearance of broad endotherms at concentrations of halofantrine greater than 50% mol. This rather complex calorimetric profile, not observed with DPPC, suggests a hydrophobic mismatch between the drug and phospholipid above a critical concentration.

Unlike the saturated phosphatidylethanolamines (DMPE, DPPE), DEPE undergoes a gel to liquid-crystalline phase transition in the lamellar phase as well as a lamellar to hexagonal H_{II} phase transition in the temperature range under study. The gel to liquid-crystalline phase transition was observed at 38.5 °C and the bilayer to hexagonal H_{II} phase transition occurred around 65.4 °C, as compared to other reports assigning values of 37 °C and 63 °C for these two transitions, respectively.[16)] The bilayer to hexagonal H_{II} phase transition has a much smaller transition enthalpy because of the fluid character of both lamellar and hexagonal H_{II} phases. The effect of increasing halofantrine concentrations on DEPE transitions is shown in Fig. 3c. Halofantrine completely abolished the bilayer to H_{II} phase transition when investigated at a 2% mol ratio. In contrast, the gel to liquid crystalline transition of DEPE was not affected by halofantrine until a much higher concentration of the drug (20% mol ratio and more) was used.

Based on the interaction of halofantrine with phosphatidylcholines, it is proposed that halofantrine is aligned within the phospholipid acyl chains. It is likely that the hydrophobic phenanthrene nucleus of halofantrine is involved in such an interaction. With such an alignment, the aliphatic side chain of halofantrine is likely to project towards the polar headgroup region of the bilayer. One might expect halofantrine to interact differently with the phosphatidylethanolamines due to its different headgroup. The differences observed in the endotherms of halofantrine/DPPE compared to halofantrine/DPPC, and the rapid disappearance of the DMPC (but not DMPE) transition endotherm in the presence of the drug may reflect this difference. However, other observations made with DMPE bilayers (shift of T_m to a higher temperature) and DEPE bilayers (disappearance of lamellar to hexagonal H_{II} phase transition) are less readily explained.

Conclusions

The results reported here demonstrate that the interaction of halofantrine with individual lipids is dependent on both the headgroup and the chain length of the particular lipid. The interaction of halofantrine with phospholipids is likely to involve the alignment of the phenanthrene ring with the phospholipid acyl chains. The packing of the phospholipids would be perturbed, thus resulting in a change in their thermotropic properties (downward shift in T_m, decrease in ΔH). It is also interesting to note that in its interaction with several phospholipids (DPPC/cholesterol, DSPC, DMPE, DPPE), separate domains were formed where the concentration of halofantrine could be specially high. The formation of such domains would facilitate the activity of halofantrine even when its concentration is low and have an important bearing on the pharmacodynamic action of the drug.

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