A Differential Scanning Calorimetry Study of the Interaction of the Antimalarial Agent Halofanthrine with Dipalmitoyl Phosphatidyl Choline Bilayers

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The influence of the antimalarial agent halofanthrine on the phase transition temperature (T_m) of dipalmitoyl phosphatidyl choline (DPPC) liposomes was investigated by differential scanning calorimetry (DSC). The effects of increasing drug content and varying pH conditions (to give predominantly charged and uncharged states of the drug) on the interaction were considered and compared with those caused by structurally related antimalarials, mefloquine and quinine, under similar conditions. Increasing concentrations of halofanthrine resulted in the gradual disappearance of the original transition endotherm and the appearance of a new drug-induced endotherm at a lower temperature and with a decreased enthalpy of transition. Qualitatively similar observations were made at different pH conditions, suggesting little difference in the mode of interaction of charged and uncharged halofanthrine molecules with DPPC. Increasing concentrations of mefloquine and quinine also caused a decline in T_m but neither drug-induced endotherms was associated with a decreased enthalpy of transition. The results indicate that halofanthrine, unlike mefloquine and quinine, intercalates in the hydrophobic interior of the phospholipid. Its localization among the fatty acid side chains of the phospholipid is possibly related to its greater hydrophobic character compared to mefloquine and quinine. The disruption of phospholipid packing and the consequential effects on membrane permeability and integrity may be relevant to the greater *in vitro* antimalarial activity of halofanthrine and should be considered in any delibration of its pharmacodynamic action.

Key words calorimetric study; antimalarial; dipalmitoyl phosphatidyl choline bilayer; halofanthrine; mefloquine; quinine

Malaria is one of the most serious parasitic diseases confronting developing nations of the world. The problem is compounded by the declining efficiency of several classical antimalarial drugs due to growing parasite resistance. The antimalarial action of the quinolinemethanol antimalarials, such as mefloquine and quinine, has been shown to involve various membrane related processes such as penetration of erythrocyte membrane and accumulation within plasmodial infected erythrocytes.[1] Such processes would conceivably depend on membrane lipid composition and it has been shown that mefloquine and quinine do indeed intercalate into lipid bilayers.[2] Drug sensitivity among these antimalarials has also been correlated to membrane phospholipid composition of various strains of *Plasmodium falciparum*.[3]

In view of these findings, it would be of interest to investigate the interaction of halofanthrine, a newly introduced phenanthrenemethanol antimalarial agent,[4] with phospholipid bilayers. This has led to the present work which looks into the effects of varying drug concentration and pH conditions on the interaction of halofanthrine with dipalmitoyl phosphatidyl choline (DPPC) bilayers using differential scanning calorimetry (DSC). The interactions of mefloquine and quinine with DPPC bilayers under similar conditions are also considered in order to assess the effects of structural variation and hydrophobic character on the intercalation process among these three structurally related antimalarial agents (Fig. 1).

Experimental

Materials Halofanthrine hydrochloride and (±) *erythro*-mefloquine hydrochloride were gifts from Smith Kline Beechams Laboratories (Hertfordshire, UK) and Roche Pharmaceuticals (Basle, Switzerland) respectively. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC, approximately 99% purity) and quinine hydrochloride were purchased from Sigma Chemical Company (St. Louis, MO). Other reagents were of analytical grade.

Preparation of Liposomes An aliquot (5 ml) of chloroform containing 36.5 mg of DPPC was delivered into a Quickfit flask. Removal of chloroform by rotary evaporation resulted in the deposition of a thin lipid film on the inside wall of the flask. The latter was dried under

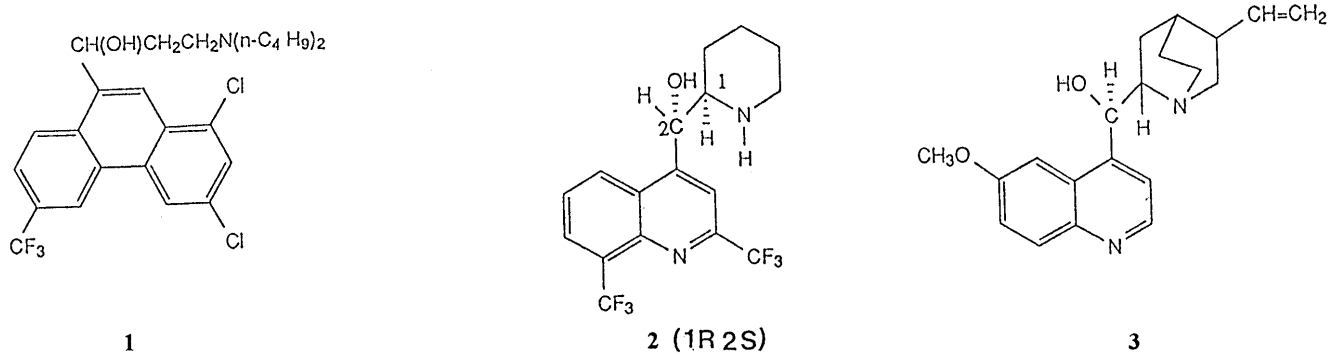

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Fig. 1. Structural Formulae of Halofanthrine (**1**), Mefloquine (**2**) and Quinine (**3**)

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vacuum overnight. Multilamellar liposomes were prepared by adding 1 ml of an appropriate buffer solution to the flask, vortexing (1 min) and rotating the flask and its contents on a rotary evaporator for 30 min at 50 °C. The milky suspension was then shaken for 2 h on a shaking water bath at 29 °C. For the preparation of drug liposomal dispersions, the drug was dissolved in methanol or chloroform and added at the stage involving phospholipid film formation. Removal of the organic solvent gave drug–lipid films which were dried and reconstituted with buffer solution (1 ml) to give liposomal dispersions as described earlier. The following buffer solutions were used: imidazole buffer (0.01 M pH 6.0), Tris buffer (0.05 M pH 7.4), glycine buffer (0.01 M pH 10) and phosphate buffer (0.01 M pH 11.0).

The quantity of drug used, expressed as the % molar ratio of the amount of drug added to the amount of phospholipid present, varied from 0.5% to 160%. Milky drug liposomal dispersions were obtained under these conditions and there was no evidence of a detergent effect normally associated with high drug concentrations.[5] The actual amount of drug and phospholipid present were quantified by UV spectroscopy for the drug and by the Stewart assay for the phospholipid.[6]

DSC Measurements Measurements were carried out on a Perkin Elmer DSC-4 differential scanning calorimeter. Aliquots (15 μl, containing approximately 0.55 mg of phospholipid) of the dispersion were encapsulated in hermatically sealed aluminium pans (Perkin Elmer) and heated from 20 °C to 50 °C at a rate of 2 °C per min, using empty aluminium pans as the reference. The sensitivity range was set at 1.5 mcal/s. The temperature scale and enthalpy measurement were calibrated using indium as standard. The enthalpies of the phase transition were evaluated by area measurements of the transition endotherms which were calculated by the Perkin Elmer System 4 Thermal Analysis Microprocessor Controller. The peak temperature (T_m) of the endotherm associated with the gel to liquid crystal phase transition of DPPC was noted.

Octanol–Buffer Partitioning The distribution coefficient D, defined as the ratio between the drug concentration in preequilibrated organic and aqueous phases, was determined in octanol and phosphate buffer (0.01 M pH 6.0) by a shake flask method for halofanthrine, mefloquine and quinine. A volume of 5 ml octanol and 5 ml of aqueous phase containing the drug were placed together in a flask and agitated for 15 h at 29 °C, after which the phases were separated, centrifuged (1000 rpm) and the concentration of the drug in the octanol phase determined by UV spectroscopy at appropriate wavelengths. The concentration of drug in the aqueous phase was determined by mass difference. D values were calculated from the mean of seven or more determinations and converted to partition coefficient (P) using the equation:

$$P = D(1 + 10^{pK_a - pH})$$

The pK_a is taken to be 8.6 (mefloquine),[7] 8.2 (quinine)[8] and 10.8 (halofanthrine).[9]

Results and Discussion

Mefloquine and quinine have two basic centers each. The pK_a values of quinine are 4.1 (quinolyl nitrogen) and 8.2 (quinuclidinyl nitrogen).[8] Only the pK_a (8.6) of the piperidinyl nitrogen of mefloquine has been reported.[7] The pK_a of its quinolyl nitrogen is assumed to be close to that of quinine. Therefore, mefloquine and quinine would exist mainly in the monocationic state at pH 7.4 (94%) and pH 6.0 (99.3%) respectively. In contrast, the uncharged forms of mefloquine (98.5%) and quinine (98.4%) would predominate at pH 10. The tertiary amino function is the only basic centre in halofanthrine. The pK_a is estimated to be 10.8, after related compounds listed in the literature.[9] Thus halofanthrine would be completely ionized at pH 7.4 but only one-third ionized at pH 11.

Although the pH of the liposomal suspension has been adjusted to give predominantly charged or uncharged forms of the drug, the actual species intercalating with the phospholipid is not known with certainty. Other investigators have assumed that the predominant form of the drug present is also the species involved in the intercalation process,[10,11] and a similar assumption is made here.

Under the prevailing pH conditions (pH 6—11) DPPC would exist in the neutral zwitterionic state, with an anionic

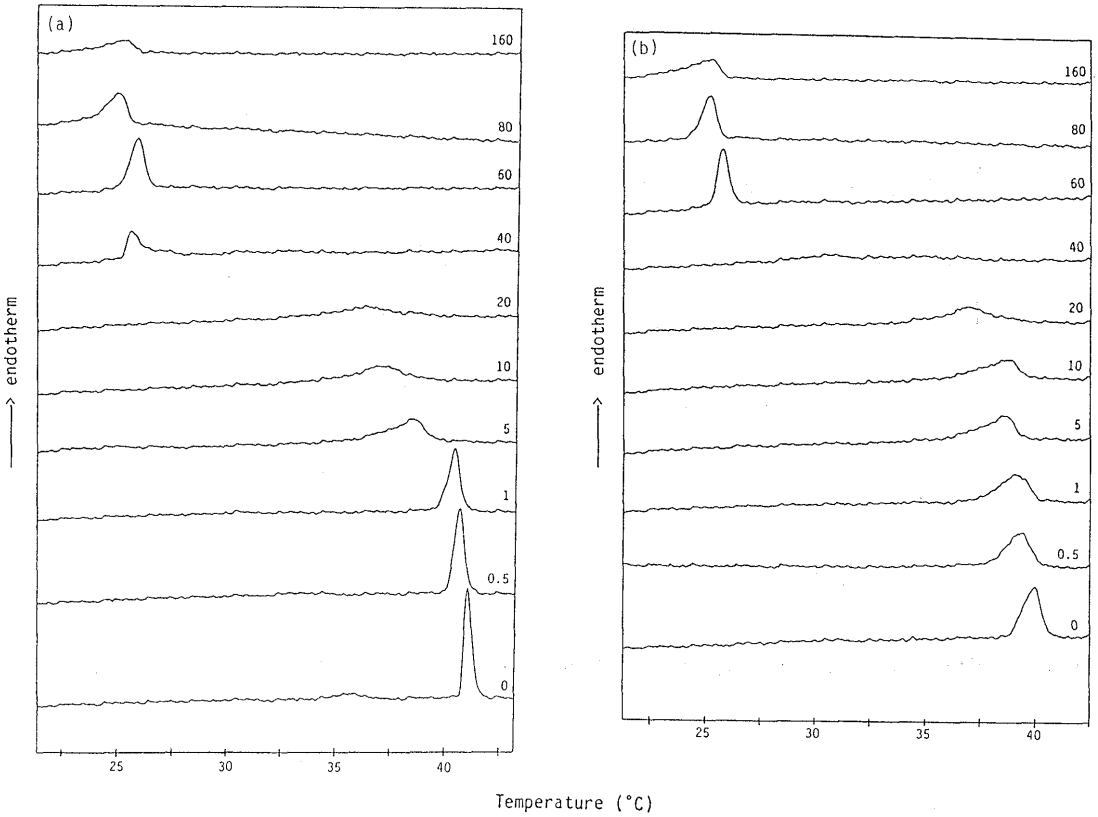

Fig. 2. DSC Curves of DPPC Liposomes Containing 0% to 160% Molar Ratio of Halofanthrine/DPPC Obtained at (a) pH 7.4 and (b) pH 11.0

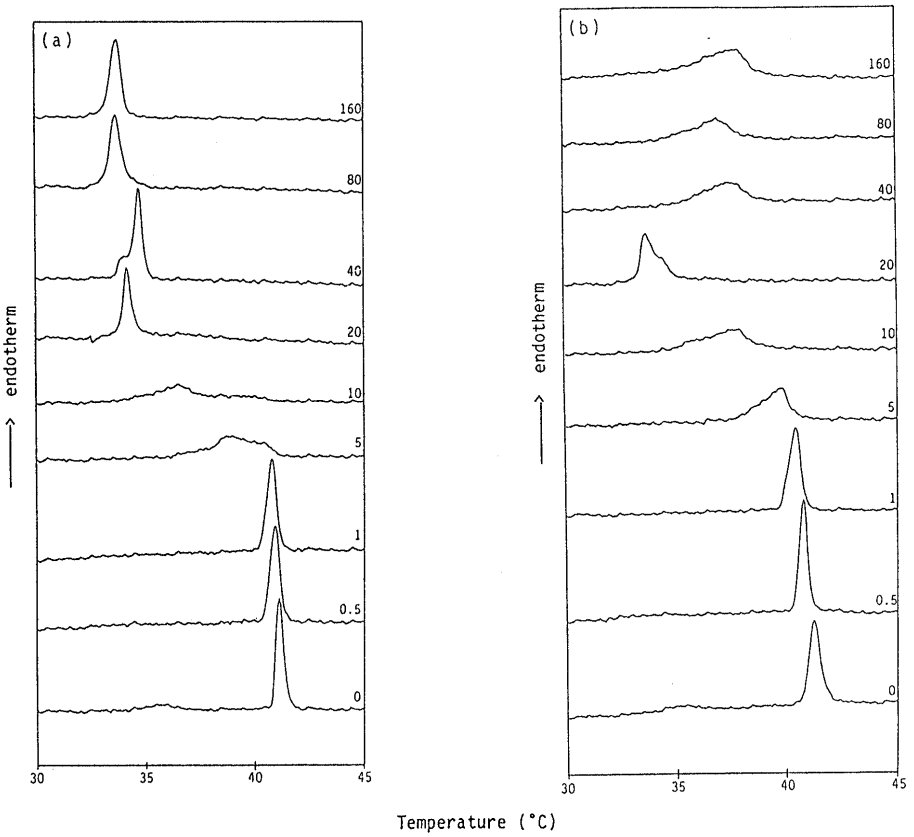

Fig. 3. DSC Curves of DPPC Liposomes Containing 0% to 160% Molar Ratio of Mefloquine/DPPC Obtained at (a) pH 7.4 and (b) pH 10.0

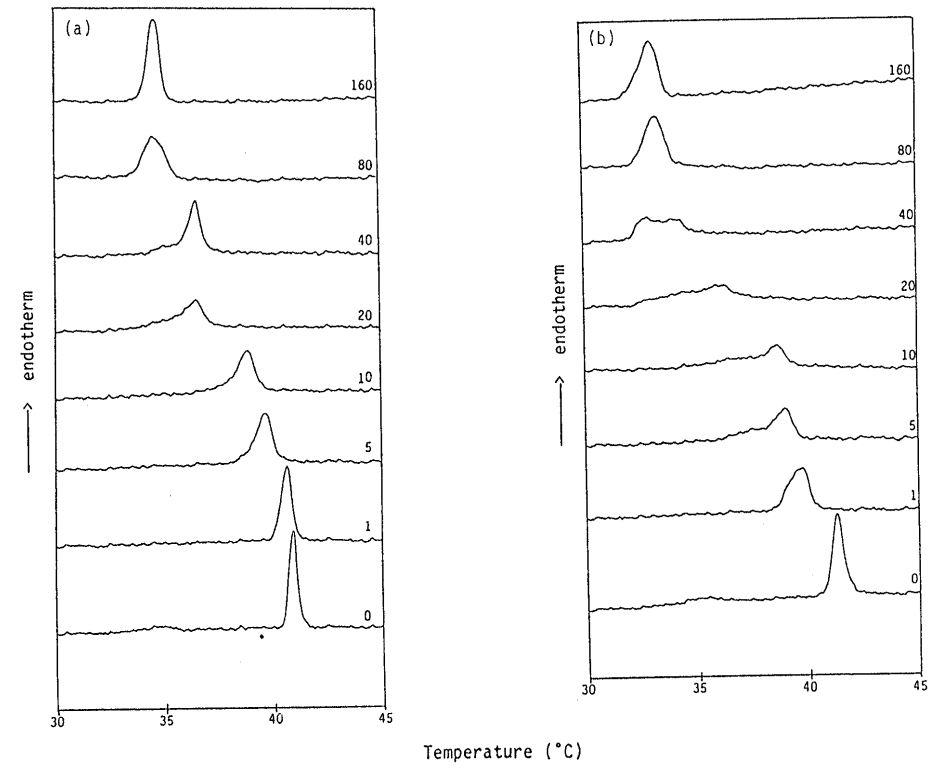

Fig. 4. DSC Curves of DPPC Liposomes Containing 0% to 160% Molar Ratio of Quinine/DPPC Obtained at (a) pH 6.0 and (b) pH 10.0

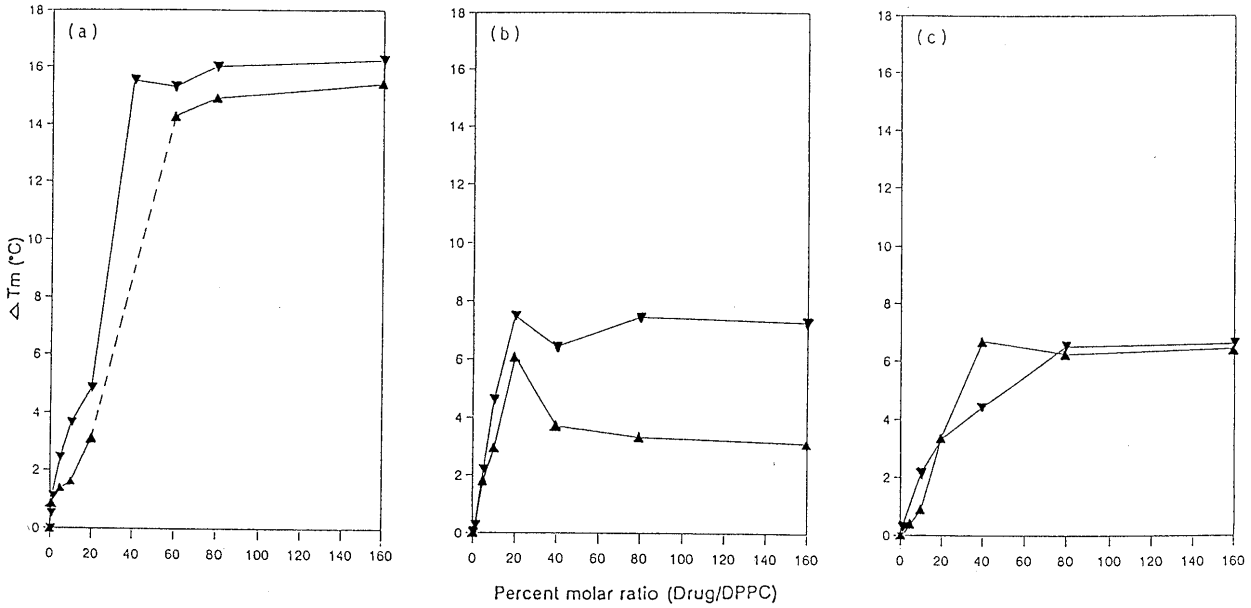

Fig. 5. Variation of the Change in Transition Temperature (ΔT_m) as a Function of (a) Halofanthrine/DPPC Molar Ratio (%) at pH 7.4 [▼] and pH 11.0 [▲]; (b) Mefloquine/DPPC Molar Ratio (%) at pH 7.4 [▼] and pH 10.0 [▲] and (c) Quinine/DPPC Molar Ratio (%) at pH 6.0 [▼] and pH 10.0 [▲]

ΔT_m is the difference between the control T_m and the drug-induced T_m value. Dashed lines in (a) are due to the absence of an endotherm at 40% molar ratio of halofanthrine/DPPC.

phosphate group (pK_a 3.7)[12] and a quaternary ammonium group. The T_m of DPPC has been reported to be independent of proton concentration at pH>4.[13] It has a value of 41°C (±0.5) over the pH range of 6 to 11.

When the pH of the liposomal suspension was adjusted to 6.0 or 7.4, the monocationic species of halofanthrine, mefloquine and quinine would predominate. Under these conditions, increasing the molar concentration of the drug caused the transition endotherm to become broad and flat, before narrowing down eventually to a sharp peak located at lower temperature than the control T_m. The appearance of a sharp peak suggests that the DPPC molecules have been homogenously affected by the incorporated drug molecules. Further broadening of this peak is evident with increasing molar ratios of halofanthrine (Fig. 2a), indicating disruption of this homogenous domain with increasing drug concentration.

Concurrent with the change in shape of the transition peak was a decline in the T_m value. Taking ΔT_m as the difference between the control T_m and the drug-induced T_m values, Fig. 5 shows that the drugs decreased T_m to different extents although a constant ΔT_m is eventually attained for each drug. The extent to which T_m is decreased may be correlated to the degree of penetration of the drug into the hydrophobic region of the phospholipid.

The presence of mefloquine and quinine did not cause significant changes to the enthalpy (ΔH) of the transition (Fig. 6). The small variations observed in ΔH were related to changes in the shape of the transition endotherm with concentration. In contrast, halofanthrine caused a large decrease in ΔH of transition, suggesting a different mode of interaction from the quinolinemethanols.

The effect of mefloquine and quinine on the transition endotherm of DPPC is consistent with their catamphiphilic character. Such molecules are characterized by a hydrophobic ring and a positively charged side chain and are known to cause shifts in T_m while causing little change to the ΔH of transition.[5,11] Mefloquine and quinine would then interact with DPPC molecules with their cationic groups placed between the anionic phosphate groups while their hydrophobic rings are directed towards the fatty acid interior of the phospholipid, as have been proposed for other catamphiphilic drugs.[14-16]

Despite its catamphiphilic character, the halofanthrine–DPPC interaction is characterized by an unexpected change in ΔH. This suggests that halofanthrine has a different mode of interaction with DPPC, which may be related to its greater hydrophobic character. Partition coefficient measurements in octanol/buffer showed that this is indeed so. Hydrophobicity (log P given in parentheses) increased in the order quinine (2.87) < mefloquine (3.42) < halofanthrine (3.73). The greater hydrophobic character of halofanthrine would favour more penetration and binding to the phospholipid and would also explain the large reduction in T_m observed in its presence (Fig. 5). This is in keeping with the relationship between hydrophobicity and ΔT_m observed by other investigators.[17,18] The localization of halofanthrine in this manner would disrupt lipid packing and reduce van der Waals interaction which is a major contributor to the phase transition enthalpy change.[19]

Under pH conditions where the uncharged forms of halofanthrine, mefloquine and quinine predominated, an increase in drug concentration also caused a decrease in T_m and a change in the shape of the transition endotherm. The transition endotherm became broad and flat, and except for halofanthrine, did not eventually form a sharp peak as observed at lower pH. There were no significant changes in ΔH for the quinine and mefloquine-induced transitions at pH 10, as noted at pH 6.0/7.4. In the case of halofanthrine, a decrease in ΔH was again observed.

It has been reported that the uncharged form of a drug

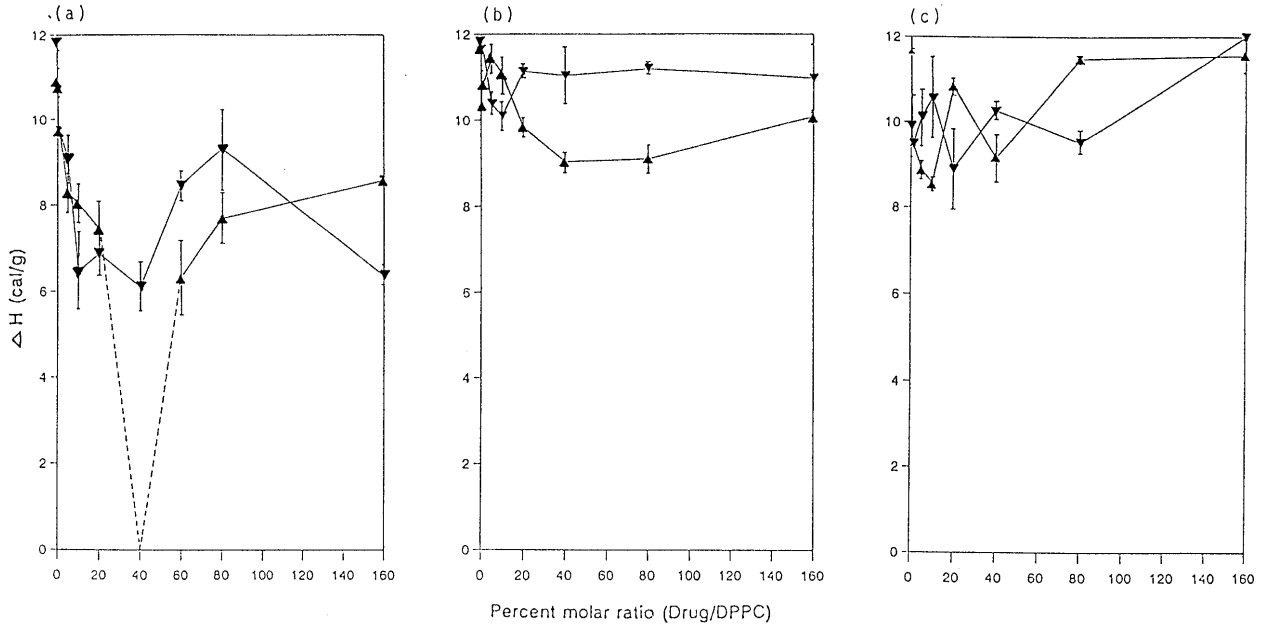

Fig. 6. Variation of the Enthalpy (ΔH) of the Main Transition Endotherm of DPPC as a Function of (a) Halofanthrine/DPPC Molar Ratio (%) at pH 7.4 [▼] and pH 11.0 [▲]; (b) Mefloquine/DPPC Molar Ratio (%) at pH 7.4 [▼] and pH 10.0 [▲] and (c) Quinine/DPPC Molar Ratio (%) at pH 6.0 [▼] and pH 10.0 [▲]

Vertical bar lines represent the standard deviations of the measurements.

is generally more extensively bound to the phospholipid bilayers and would be expected to cause a greater reduction in T_m than the charged species accordingly.[11] This has been demonstrated with acidic drugs.[10,11] In the case of some basic drugs,[11] it was shown that ΔT_m values for cationic and uncharged species were similar despite the greater proportion of uncharged drug bound to the phospholipid. In the case of mefloquine, however, it was observed that a larger reduction in T_m occurred at pH conditions where the charged species predominated. The difference in ΔT_m values for mefloquine at pH 7.4 and 10 is about 4 °C.

The greater reduction in T_m caused by monocationic mefloquine is somewhat unusual. It would imply that the monocationic species penetrated the phospholipid array to a greater extent than uncharged molecules. There is in fact some indication of this from the appearance of the mefloquine-induced transitions which remained broad and diffuse at high concentrations of the drug (>40%, pH 10), suggesting that DPPC molecules had not been homogenously affected by the incorporated drug molecules. In contrast, narrow endotherms were observed at comparable mefloquine concentrations at pH 7.4, which indicates homogenous mixing between drug and DPPC molecules.

These findings may be tentatively explained by considering the interaction of mefloquine with the phospholipid headgroup. The monocationic centre in mefloquine would be attracted to the anionic phosphate group but the lone pair of electrons on the uncharged mefloquine base would be repelled by the phosphate group and attracted instead to the cationic choline group of DPPC by dipole interactions. As the quaternary choline group is further from the hydrophobic interior of the phospholipid than the anionic phosphate group, the localization of uncharged mefloquine in this way would result in weaker hydrophobic interactions and a lesser ability to penetrate the phospholipid array. A related model has been proposed by Mohr and Struve[11] to explain the influence of charge on the ability of amphiphilic drugs to interact with DPPC bilayers.

Despite the structural resemblance of quinine to mefloquine, some differences are evident in the quinine–DPPC interaction, the most notable of which is the absence of pH effects on ΔT_m. If a similar mode of interaction is proposed for the monocationic species of mefloquine and quinine, it is difficult to explain why a change in pH to give the uncharged species did not cause a change in the quinine–DPPC interaction, as observed with mefloquine. A possible explanation may lie with the sterically bulky quinuclidine ring of quinine which could limit the penetration of both charged and uncharged quinine molecules into the phospholipid. Indeed, the smallest reduction in T_m is observed in the interaction of monocationic quinine with DPPC, which is in keeping with its low hydrophobic character.

Halofanthrine, mefloquine and quinine are structurally related arylmethanol antimalarial agents. They are also non-polar molecules with an approximate ten fold difference in hydrophobicity. Despite these broad similarities in structure and physicochemical properties, they seem to intercalate at different regions of the phospholipid. Of particular interest is the localization of halofanthrine (cationic and uncharged forms) within the hydrophobic interior of the phospholipid. Halofanthrine has also been shown to possess greater *in vitro* antimalarial activity than mefloquine.[20] Perhaps the localization of halofanthrine within the phospholipid interior may lead to changes in membrane permeability and lipid structure which could account for its greater antimalarial action.

The relevance of these findings to the pharmacodynamic action of halofanthrine warrants further investigation.

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