Interaction of Amiodarone and Desethylamiodarone with Dimyristoyl Phosphatidylcholine Bilayers

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The interaction between amiodarone or desethylamiodarone and dimyristoyl phosphatidylcholine bilayers was investigated by two optical techniques, quasi-elastic light scattering (QLS) and Fourier transform infrared spectroscopy (FT-IR). The results show that in the presence of the drugs, the bilayer phase transition point is lowered and at high drug concentration there is an incresase in the vesicle size. The FT-IR results indicate interactions in the hydrocarbon region and also in the carbonyl interface region of the bilayers. There may be an interdigitation of two leaflets of the bilayer. We discussed these results comparatively with those obtained from propranolol whose effect was analogous and previously studied.

Key words amiodarone; desethylamiodarone; model membrane; FT-IR spectroscopy; quasi-elastic light scattering

Amiodarone is known to possess multiple biological and pharmacological activities and is widely used clinically.[1-8] Among its therapeutic properties, are a powerful antiarrhythmic and antianginal effect; however, when chronically administered, several side effects were reported. Amiodarone was long considered to be a typical class III antiarrhythmic drug, but recent studies showed that it has actions of all four antiarrhythmic drug classes, in particular, it may act equally as a class I sodium channel blocker.[1,9,10] Its effect is also to some extent comparable with that of propranolol.[2,11,12,16] Although the molecular mechanism of action has been studied by many investigators, there is not yet concensus on a common mechanism and such a study always attracts interest. Fluorescence and X-ray techniques,[12,14,15] showed that amiodarone interacts with the hydrophobic inner region of phospholipid bilayers, while NMR investigation showed interactions localized, instead, in the polar head region.[18] Based on these studies, it has been believed that amiodarone interacts with the lipid components of cell membranes or mitochondrial membranes.[14-19] It was shown that amiodarone possesses a strong hydrophobic behavior and lipophilic properties with a very high partition coefficient.[15,16] The lipid component of membranes might therefore be implicated in its effects *via* a modification of functional properties of concerned enzymes or receptors embedded in biological membranes.[2]

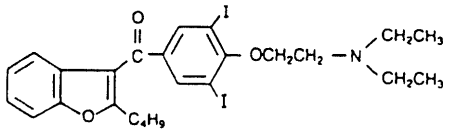
On the other hand, numerous recent works suggested that its active metabolite desethylamiodarone also interacts with membranes.[6] Assays *in vitro* as well as *in vivo* indicated similar effects as with amiodarone although there was some difference in the degree of these effects.[20-22]

Against this background, a study of the effect of amiodarone and desethylamiodarone on lipid component appears useful for the comprehension of these mechanisms. In this work, we were interested in the effect of the drugs on the thermal behavior of pure phospholipid large unilamellar vesicles which would reflect the drug–membrane interactions, and in spectroscopic data which would permit to locate the drug with respect to the bilayer. For this purpose, we chose two complementary optical techniques, quasi-elastic light scattering (QLS) and Fourier transform infrared spectroscopy (FT-IR). These techniques can provide information useful for the understanding of the interaction between drugs and lipid vesicles at molecular and macroscopic levels.[23-25] On one hand, QLS permits determination of the vesicle size and thus monitoring of the thermal behavior,[23,24] while, on the other hand, FT-IR spectroscopy has been used to clarify the location of the interaction sites between a drug and lipid bilayers.[25,26] We will compare these results to those observed with propranolol previously studied.[38]

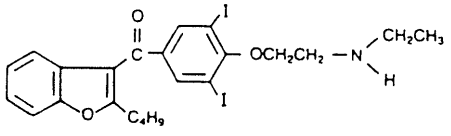
Experimental

Materials Amiodarone chloride was furnished by Sigma (Ref. A8423), and desethylamiodarone was a kind gift of the Laboratoire SANOFI (Ref. LB 33020). The chemical formulas are given in Fig. 1. Dimyristoylphosphatidylcholine (DMPC) was purchased from Sigma and used without further purification. The purity was checked by using thin layer chromatography. Other chemical agents used for the preparation of buffers were of analytical grade and purchased from Merck or Aldrich.

The bilayers used for this work were large unilamellar vesicles (LUV) of DMPC prepared in 150 mM NaCl, 10 mM Tris, pH 8 aqueous buffers by the reverse phase evaporation (REV) method of Szoka and Papahodjopoulos,[27] followed by successive centrifugations to eliminate aggregates and too large vesicles. This technique allowed us to obtain unilamellar vesicles of 120—150 nm in diameter with a polydispersity factor of 0.15 to 0.20. The unimodal distribution of the dispersions was



a)



b)

Fig. 1. Molecular Formulas of Amiodarone (a) and Desethylamiodarone (b)

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controlled by light scattering technique using the inverse Laplace transform method (Malvern software).[28,29]

Amiodarone (or desethylamiodarone) was initially dissolved in dimethyl formamide (DMF) to obtain solutions of concentration 1 mg·ml^{-1} and stocked in the dark. To obtain the desired drug concentrations and drug/lipid molar ratios, aliquots of these solutions and pure DMF were added to vesicle samples and incubated at 35 °C for one night before measurements. In all preparations we always used the same volume fraction of DMF in aqueous buffer and the effect of DMF in the results was controlled. In Tris, pH 8 aqueous buffers, amiodarone showed an absorption band at 310 nm which was used to titrate the drug concentration.

Quasi-Elastic Light Scattering QLS was performed on a photon self-beating spectrometer constructed by our laboratory, operating with the 514.5 nm line of a Spectra Physics Ar^+ laser. Other experimental details were described previously.[30] The autocorrelation functions of the scattered light were analysed following the cumulant method[31] which allows the determination of the average translational diffusion coefficient D and the polydispersity factor. The hydrodynamic radius R_h was then calculated *via* the Stokes–Einstein relation $R_h = k_B T/6\pi\eta D$ where η is the viscosity of the solvent at the temperature T. By following the hydrodynamic radius R_h *versus* the temperature, the phase transition of the membranes was monitored. A change in R_h reflected a modification in the total surface area of the vesicles, which was a consequence of the change in the phospholipid conformation occurring when phospholipid vesicles undergo the liquid-crystalline to gel phase transition.

FT-IR Spectroscopy Infrared spectra with a spectral resolution of 1 cm^{-1} were obtained using a Perkin Elmer 1760 Fourier Transform spectrophotometer with ZnSe as window material. Each spectrum was an average of 10—20 scans. DMPC unilamellar bilayers with concentrations of about 50 mg·ml^{-1} were prepared in 150 mM NaCl, 10 mM Tris–HCl, pH 8, H_2O or D_2O buffers depending on the spectral region investigated. The spectra were recorded at discrete temperatures with a waiting time of 15 min between two subsequent spectra. We explored three absorption spectral regions of major interest, corresponding respectively to the vibrational stretching modes of the CH_2 groups in the hydrophobic region, to those of the PO_2^- group of the polar heads and to those of the C=O groups in the interface of the bilayers.

Results

Effect of Amiodarone and Desethylamiodarone on the Phase Transition of DMPC LUV Transition Observed by QLS: The phase transition of DMPC LUV is shown by the variation in vesicle area or the hydrodynamic radius R_h (normalized to its value at 35 °C) depicted by Figs. 2 and 3. The plots correspond to various drug concentrations. In the absence of drug (Figs. 2a and 3a) the transition curve is a sigmoidal shape and the midpoint is shown at 24 °C. This is in agreement with the transition point observed by different techniques.[23,32] At the transition, the change in the vesicle radius is about 6—7%. In the presence of amiodarone (Fig. 2b—d) or desethylamiodarone (Fig. 3b—d) at various concentrations, a complex transition was observed. At temperatures higher than 30 °C, one observed practically the same value of R_h of pure lipid vesicles in the liquid crystalline phase while at low temperature, the behavior was different. With low drug/lipid molar ratios (Figs. 2b and 3b), the R_h value was as small as the value currently observed in the gel phase. When the drug/lipid molar ratio is increased, R_h is raised gradually, attains the value of Fig. 3c and then becomes higher (Figs. 2d and 3d) than that of the liquid crystalline phase. As indicated by Fig. 2c, it is clear that there was a two-step transition. At low drug concentration, the intermediate state between two step-transition points is not shown, but it becomes more and more clear when the drug concentration is increased. The results were reproducible after heating to 35 °C.

Transition Observed by FT-IR: Effect of Amiodarone and Desethylamiodarone in the Spectral Region 3000—2800 cm^{-1}: The phase transition was also observed by

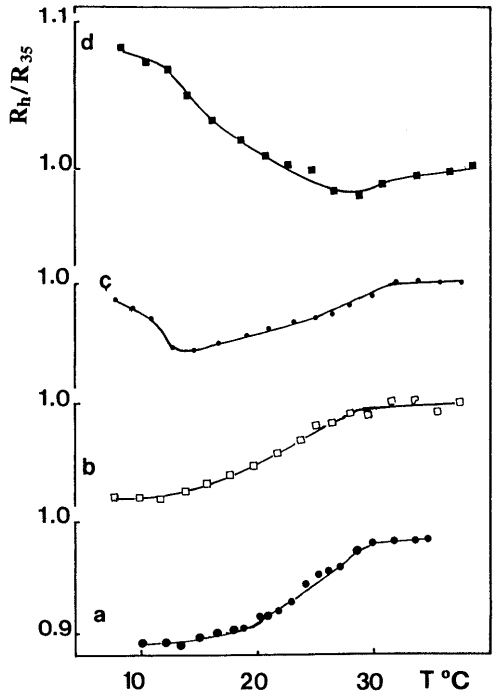

Fig. 2. Effect of Amiodarone on the Phase Transition of DMPC LUV Observed by Quasi-elastic Light Scattering

The plots represent the temperature dependence of the vesicle hydrodynamic radius R_h (normalized to the value R_{35} of pure lipid LUV at 35 °C). LUV in the absence (a) and in the presence (b—d) of amiodarone at various drug/lipid molar ratios (x): b) $x=0.05$; c) $x=0.10$; d) $x=0.40$. Lipid concentration: 0.40 mM in 10 mM Tris, 150 mM NaCl, pH 8 aqueous buffer.

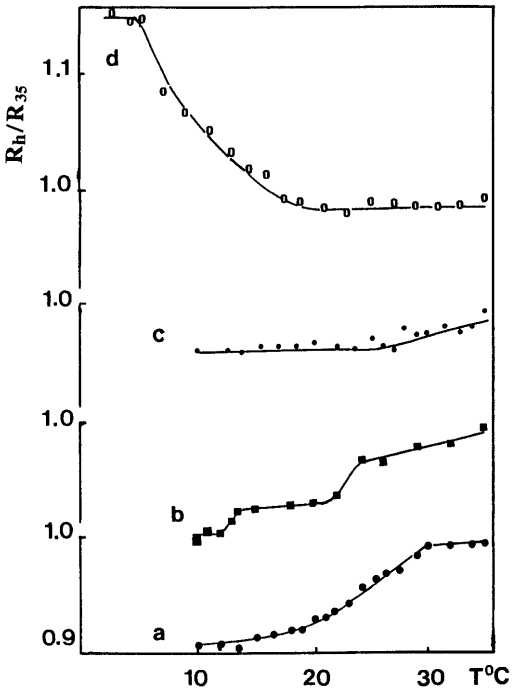

Fig. 3. Effect of Desethylamiodarone on the Phase Transition of DMPC LUV Observed by Quasi-elastic Light Scattering

The plots represent the temperature dependence of the vesicle hydrodynamic radius R_h (normalized to the value R_{35} of pure lipid LUV at 35 °C). LUV in the absence (a) and in the presence (b—d) of desethylamiodarone at various drug/lipid molar ratios (x): b) $x=0.10$; c) $x=0.20$; d) $x=0.40$. Lipid concentration: 0.40 mM in 10 mM Tris, 150 mM NaCl, pH 8 aqueous buffer.

FT-IR spectroscopy. In the spectral region 3000—2800 cm^{-1}, the strong absorption band at 2850 cm^{-1}, assigned to the symmetric stretching vibration of the methylene groups, shows temperature-induced frequency shifts as shown in Figs. 4 and 5. These shifts reflect the change of the *trans/gauche* ratio of the acyl chain conformers. Wave numbers near 2853 cm^{-1} are characteristic of conformationally disordered polymethylene chains with a high content of *gauche* conformers, while lower values at about 2850 cm^{-1} are characteristic of ordered methylene chains as found in the gel state.[25] Figures 4a and 5a depict the transition curves of DMPC LUV in the absence of drugs and Figs. 4b, c and 5b, c show the effect of amiodarone and desethylamiodarone, respectively. When the drug is added to the vesicle solution, the liquid crystalline to gel-like phase transition point is shifted. This indicates an effect of the drug on the fluidity of the hydrocarbon chains of the bilayers and is in agreement with the change in vesicle size observed by QLS.

Effect of Amiodarone and Desethylamiodarone on the IR Spectra of DMPC LUV Spectral Region 1800—1500 cm^{-1}: The interface region of the bilayers is constituted by the carbonyl groups sn-1 CO and sn-2 CO which link the polar head to the acyl tails of every phospholipid molecule. In pure lipid vesicles, the IR absorption band characteristic of these groups was a broad band, the wave number of its maximum was shifted when the temperature varied (Figs. 6a or 7a). This change was explained by the fact that it was composed of two components at 1742 and 1727 cm^{-1}, corresponding to the vibrations of both C=O groups with different hydrations.[33,34] These components can be shown by a curve fitting procedure. Their relative peak heights vary with temperature resulting in the shift of the peak wave number of the whole band profile from 1733 to 1737 cm^{-1} when the liquid crystalline–gel transition occurs. A lower value of this wavenumber reflects a more hydrated state of the C=O groups in the interface region of the bilayer.

When the drugs are present, Figs. 6b or 7b shows clearly a dramatic modification of the transition plots of ν(CO). For amiodarone, at high temperature the wavenumber of the whole band profile seems to be raised while at low

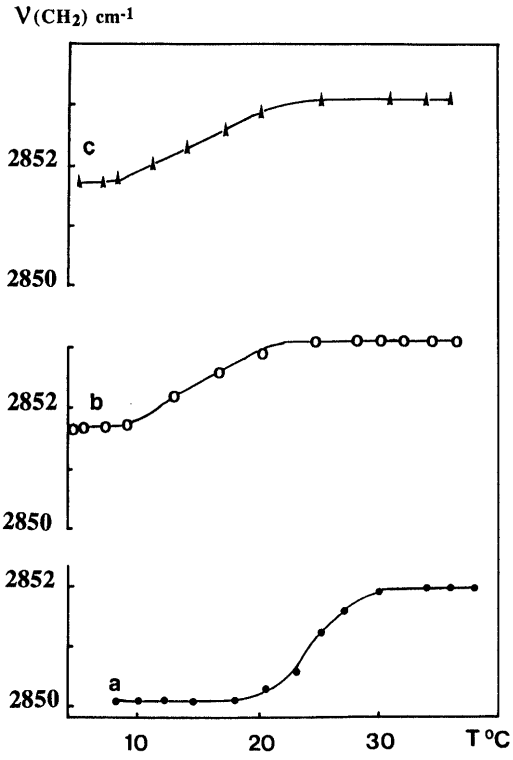

Fig. 4. Effect of Amiodarone on the Phase Transition of DMPC LUV Observed by FT-IR Spectroscopy

The plots represent the temperature-dependence of the wave number of the symmetric CH_2 stretching mode in the absence (a) and in the presence (b, c) of amiodarone (drug/lipid molar ratio 0.3 (b) and 0.53 (c)). Concentration of lipid: 50 mg/ml in 10 mM Tris, 150 mM NaCl, pH 8 aqueous buffer.

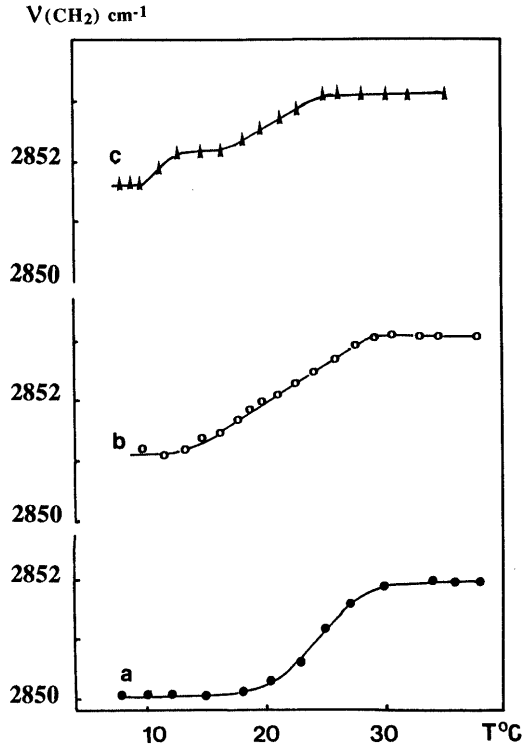

Fig. 5. Effect of Desethylamiodarone on the Phase Transition of DMPC LUV Observed by FT-IR Spectroscopy

The plots represent the temperature-dependence of the wave number of the symmetric CH_2 stretching mode in the absence (a) and in the presence (b, c) of desethylamiodarone (drug/lipid molar ratio 0.3 (b) and 0.53(c)). Concentration of lipid: 50 mg/ml in 10 mM Tris, 150 mM NaCl, pH 8 aqueous buffer.

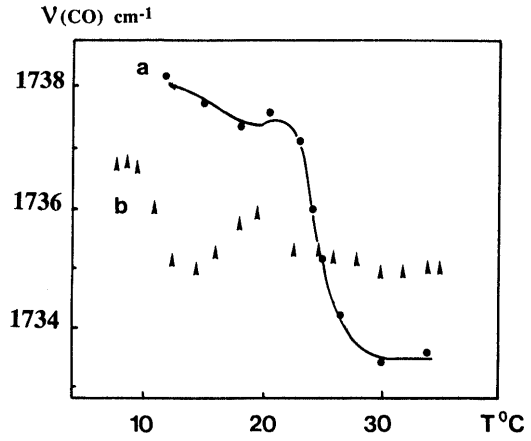

Fig. 6. Variation *versus* Temperature of the ν(CO) of the Whole Band Profile Corresponding to the Stretching Vibration Modes of C=O Groups of the DMPC Bilayer in the Absence (a) and in the Presence (b) of Amiodarone (Drug/Lipid Molar Ratio 0.53)

Buffer: 10 mM Tris, 150 mM NaCl, pD 8 in D_2O.

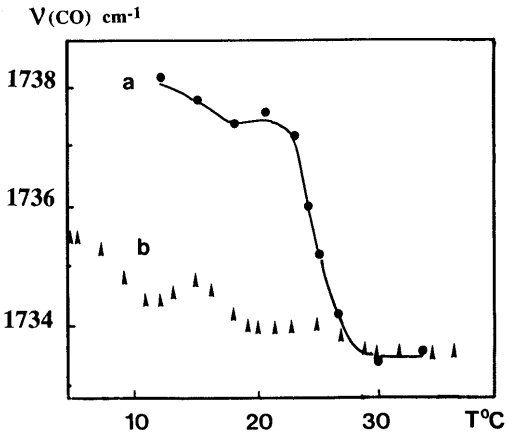

Fig. 7. Variation *versus* Temperature of the ν(CO) of the Whole Band Profile Corresponding to the Stretching Vibration Modes of C=O Groups of the Bilayer in the Absence (a) and in the Presence (b) of Desethylamiodarone (Drug/Lipid Molar Ratio 0.53)

Buffer: 10 mM Tris, 150 mM NaCl, pD 8 in D_2O.

temperature it was lowered, resulting in a very small wave number shift. For desethylamiodarone, the plot shows practically the value of ν(CO) as in the liquid crystalline phase. These modifications of the transition feature of ν(CO) are indicative of an effect on the C=O groups by the presence of amiodarone or desethylamiodarone.

Spectral Region 1350—950 cm^{-1}: Infrared absorption spectra of DMPC LUV in the absence and in the presence of amiodarone and desethylamiodarone in this spectral region were also recorded and the contribution of pure amiodarone or desethylamiodarone has been subtracted. In the absence of the drugs, the spectra are characterized by two strong bands at 1232 and 1088 cm^{-1} corresponding to the antisymmetric and symmetric stretching modes of the polar head PO_2^- groups. In the presence of the drug, these bands were not altered either in frequency or in peak height indicating that amiodarone or desethylamiodarone did not affect the PO_2^- group region of the bilayers.

Discussion

The above results show interactions of the drug with DMPC bilayers, in particular, the effect of the drugs on their thermal behavior. The comparison of the results obtained here and those observed in the case of propranolol shows that their interactions with the bilayers are, aside from some details, qualitatively similar. They affect the interface and the inner hydrophobe hydrocarbon region of the lipid bilayer.

Although neither amiodarone nor desethylamiodarone is very soluble in Tris-buffer and the exact value of the partition coefficient between the lipid and the studied aqueous phases has not been known, it is reasonably assumed that it is very high as suggested by Chatelain *et al.*[2,15] On the other hand, the amiodarone molecule, with a tertiary amine group,[3,5] possesses a $pK_a = 8.7$,[19,36] and desethylamiodarone, with a secondary amine group, displays a larger value of pK_a. This suggests that at pH = 8, these molecules would be ionized and both drugs would be positively charged. This cationic character confers some particular behaviors on molecules.

First, at a molecular level, the presence of amphiphilic molecules which contain polar and hydrophobic parts, may contribute to modify the stability of bilayers insured by an equilibrium between the electrostatic interaction of the polar heads and the hydrophobic interaction of the acyl tails. In the hydrophobic region, the shift of the transition observed in the $\nu(CH_2)$ plots *versus* temperature actually reflects an interaction with the hydrocarbon chains. In the interface region, the change of the band profile wavenumber ν(CO) indicates a strong interaction between the drug molecules and the C=O groups. A similar modification at the interface region of DPPC bilayers was observed in the presence of salicylic acid or phenol.[26] It may be that these alterations, relative to the change of two component peak heights corresponding to the double bond sn1-CO and sn2-CO stretchings, are due to a change in hydration and head group volume. These results suggested that the amiodarone molecules would be located in the intermediate region between the hydrocarbon tails and the polar heads. This is consistent with the ionized form of amiodarone molecules under the studied pH condition. This point was discussed by Paillous and Fery-Forgues.[36] From Figs. 6 and 7, it is interesting also to note the difference between the effects of amiodarone and desethylamiodarone on the C=O groups. Desethylamiodarone induced a more hydrated state of the C=O moiety. It may be that the desethylamiodarone molecules, which are more hydrophilic than amiodarone, are located closer to the C=O region than the amiodarone ones. In all cases, both drugs have no or little effect on the PO_2^- groups of the external face of the bilayer. These results are consistent with those observed by X-ray scattering and fluorescence[12,16] and with a relatively high value of the partition coefficient of amiodarone. This is in contrast to D-propranolol, the presence of which alters the PO_2^- group but not the C=O region, probably due to a deeper location of amiodarone or desethylamiodarone in the bilayer than propranolol.

At a more "macroscopic" level, QLS as well as FT-IR showed that either amiodarone or desethylamiodarone alters the liquid crystalline→gel-like phase transition and the membrane fluidity. Such a modification would result in a change of the response of the membrane. By comparison between the temperature-dependence plots of the vesicle hydrodynamic radius R_h in the presence and in the absence of the drugs, one observes a modification of the ratio between the vesicle radii in the liquid crystalline phase (at 35 °C) and in the gel phase (at 12 °C). Whereas in pure lipid vesicles practically only the main transition at 24 °C was observed, in the presence of the drug the transition was more complex. It seems that there were two steps in the transition as shown in plots c) and d) in Fig. 2 and plot b) in Fig. 3: a transition above 20 °C at which the vesicle size was decreased and another below 20 °C at which the vesicle size was increased at high drug content. These steps indicate three states of the bilayers when the temperature is lowered. The vesicle size in the intermediate and in the low-temperature state was decreased at low drug content but it was increased when the drug concentration was high. This increase is more important with

desethylamiodarone than with amiodarone. It is noteworthy that this splitting of the transition is commonly observed when amphiphilic drugs such as loperamide, D-propranolol, gramicidin A, valinomycin or poly-lysine are added to DMPC LUV.[37,38] In these cases, the low-temperature step was the main transition shifted by the presence of the drug while the high-temperature transition step was related to a molecular rearrangement of the liquid crystalline phase before the real transition occurs. The shift of the main transition towards a temperature below 20 °C (Fig. 2c, d) observed by QLS is consistent with the shift in $\nu(CH_2)$ observed by FT-IR (Fig. 4b, c). This rearrangement may be related to the effects observed on the C=O groups due to a change in hydration at the aqueous interface.[26,33,34]

At low drug/lipid molar ratio x, the R_h plots show a common feature of a transition from a disordered liquid crystalline state to a more ordered gel state. The intermediate state was not clear. When x was increased, the R_h value and thus the vesicle area at low temperature was raised which indicated that the mean molecular section area of the lipid molecule per polar head was increased. At $x=0.4$, this section was even higher than that in the liquid crystalline state. However, the low value of the $\nu(CH_2)$ wave number showed that the acyl chains were in the *trans*-conformation as in the gel phase. To explain this apparent discrepancy, we think that at this concentration of drugs, with amiodarone as well with desethylamiodarone, the bilayers were in an unusual gel state. There may be an interdigitation between the monolayers of the lipid membrane. This state is a particular gel phase observed when the bilayers are in the presence of amphiphilic agents such as chlorpromazine, tetracaine, ethanol.[39-42] In these cases, the drug molecules interact with both polar heads and acyl chains of the bilayer, but their length is short and leave vacant places in the interchain space which can be occupied by the chains of the opposite layer. Our hypothesis is based on the facts that: i) amiodarone and desethylamiodarone molecules are embedded between the aqueous interface and the hydrophobic region; ii) these drug molecules are not long enough to equal the length of the acyl tails (with 14 carbon atoms) of the lipid molecules, so that the consequence must be that there are vacancies induced by their presence; and iii) the minimum energy corresponds to the interdigitation when the other monolayer comes to occupy the vacant places as indicated by Simon *et al.*[42] The drug molecules are located between the C=O region and the hydrophobic acyl region. This is slightly different from the scheme proposed in ref. 16 where the amiodarone molecules are located deeper in the hydrophobic region; the difference may be due to the difference in length of the lipid molecules.

In comparison with propranolol, although its interaction is located closer to the polar heads than that of amiodarone, the transition of DMPC bilayers was altered in a similar way. At high concentration of propranolol, the results of various techniques showed a partial interdigitation state between two leaflets of the bilayers.[38,43] Pharmacologically, propranolol and amiodarone show a common behavior, the so-called "membrane stabilizing effect" or "quinidine-like effect" and are known as Na^+ blockers. This effect can be explained partially by the interdigitation which renders the bilayer more compact and may result in a compaction of the conformation of any protein or sodium channel embedded in the membrane. This compaction is also consistent with the decrease of deformability observed in erythrocytes when amiodarone was present.[44,45] We believe that this compaction effect can be implicated in modification of the geometry of the enzymes or receptors which results in the therapeutic ability of the drugs.

In conclusion, although differing in their effect on the phase transition, the interactions of amiodarone or desethylamiodarone with lipid bilayers contribute to the modification of their phase transition. Amiodarone seems to be located deeper in the bilayer than desethylamiodarone. This modification is somewhat similar to that of propranolol at high content of drug. This may be related to the compaction of the lipid bilayer and therefore to some of the pharmacological properties of amiodarone and desethylamiodarone.

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