Effects of the antimalarial drug primaquine on the dynamic structure of lipid model membranes

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ABSTRACT

Primaquine (PQ) is a potent therapeutic agent used in the treatment of malaria and its mechanism of action still lacks a more detailed understanding at a molecular level. In this context, we used differential scanning calorimetry (DSC), pressure perturbation calorimetry (PPC), and electron spin resonance (ESR) to investigate the effects of PQ on the lipid phase transition, acyl chain dynamics, and on volumetric properties of lipid model membranes. DSC thermograms revealed that PQ stabilizes the fluid phase of the lipid model membranes and interacts mainly with the lipid headgroups. This result was revealed by the great effect on the pretransition of phosphatidylcholines and the destabilization of the inverted hexagonal phase of a phosphatidylethanolamine bilayer. Spin probes located at different positions along the lipid chain were used to monitor different membrane regions. ESR results indicated that PQ is effective in changing the acyl chain ordering and dynamics of the whole chain of dimyristoylphosphatidylcholine (DMPC) phospholipid in the rippled gel phase. The combined ESR and PPC results revealed that the slight DMPC volume changes at the main phase transition induced by the presence of PQ is probably due to a less dense lipid gel phase. At physiological pH, the cationic amphiphilic PQ strongly interacts with the lipid headgroup region of the bilayers, causing considerable disorganization in the hydrophobic core. These results shed light on the molecular mechanism of primaquine–lipid interaction, which may be useful in the understanding of the complex mechanism of action and/or the adverse effects of this antimalarial drug.

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1. Introduction

Malaria is one of the most common diseases in the tropical and subtropical world, being responsible for ca. one million deaths each year. At the end of 2006, over 200 million new malaria cases were reported among an estimated 3.3 billion people living in areas at risk [1]. Primaquine (PQ), an 8-aminoquinoline, has been playing a central role in malaria treatment especially because of its large spectrum of activities against all four species of plasmodia that are pathogenic to human being: *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* [2]. This drug is also an important prophylactic agent because it is the only compound currently available that prevents the relapsing malaria [3], and has also been used as gametocidal and sporonticidal agents [4]. Despite its clinical importance, the utility of this drug has been limited by its side effects such as gastrointestinal disturbances, methaemoglobinaemia and hemolytic anemia, particularly in patients with inborn erythrocytic glucose-6-phosphate dehydrogenase deficiency [5].

The mechanism of action of primaquine has not been completely understood so far, although it is believed that its antimalarial activity is probably attributable to interference with the parasite's DNA structure and the disruption of parasite mitochondrial membranes [6]. Another potential mechanism of action is through the oxidative stress [7] inside the erythrocytes caused by the generation of reactive oxygen species that suppress the cellular defenses and attack cell macromolecules [8]. However, there is no conclusive information showing whether the overall primaquine antimalarial activity is due to the direct action of this drug or only mediated by its metabolites [9,10]. The molecular targets of this drug, whether lipids and/or proteins, are still unknown. Thus, studies of primaquine–membrane interaction gain importance in understanding the overall mechanism of action of this drug.

The growing resistance of parasites to the traditional antimalarial drugs has increased the number of studies where the development of alternative drugs as well as the understanding of the mechanism of action at a molecular level has taken a paramount role. These mechanisms usually involve several distinct processes that may

include binding to specific sites in the tissues and interactions with biological membranes. In the latter, interaction with membrane proteins and/or modifications in the lipid structure can happen [11–14]. It is therefore evident the relevance of investigating drug–membrane interactions to gain detailed information about the molecular events taking place during that process. In this view, it is common to choose an approach in which different experimental techniques are employed. Among the methods usually used in those cases, differential scanning calorimetry (DSC), pressure perturbation calorimetry (PPC), and electron spin resonance (ESR) have given significant contributions [15–19].

In this work, we investigated the interactions of the antimalarial drug primaquine with zwitterionic lipid model membranes by means of DSC, PPC, and ESR experiments. The modifications in the macroscopic behavior of phospholipids caused by interaction with PQ and the local changes in the acyl chain structure and dynamics were assessed by combining these techniques. The significance of the strong membrane perturbation caused by PQ as an additional contribution to the PQ mechanism of action is discussed.

2. Materials and methods

2.1. Reagents

The phospholipids 1,2-dimyristoyl-sn-glycero-phosphatidylcholine (DMPC), 1,2-dipalmitoyl-sn-glycero-phosphatidylcholine (DPPC), 1,2-distearoyl-sn-glycero-phosphatidylcholine (DSPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine (POPE) and the spin labels 1-palmitoyl-2-stearoyl(n-doxyl)-sn-glycero-3-phosphocholine (n-PCSL, where n = 5, 7, 10, 12, 14, and 16) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The antimalarial drug primaquine diphosphate (PQ) was purchased from Sigma Chem. Co. (St. Louis). All reagents were used without further purification.

2.2. Sample preparation

Phospholipid and spin labels from chloroform stock solutions were mixed in a glass tube, dried under N_2 flow to form a lipid film, and ultracentrifuged under vacuum for 2 h to ensure complete removal of the solvent. After addition of appropriate buffer, the film was scraped off the wall, sonicated in a bath type sonicator for 15 min, and maintained at 22 °C during at least 2 h for hydration. A measured volume of a drug buffered solution was added to the vesicles, vortexed for a few minutes, and maintained at 22 °C during at least 5 h prior to the measurements. For ESR, 0.5 mol% of the spin labels was added to the chloroform lipid solution.

2.3. Calorimetric measurements

Measurements were carried out in 10 mM phosphate, borate, and acetate, pH 7.4 with 2 mg/mL of the final lipid concentration. Differential scanning calorimetry (DSC) experiments were performed in a VP-DSC MicroCal MicroCalorimeter (Microcal, Northampton, MA, USA). A heating rate of 30 °C/h was used to sweep the appropriate temperature range for each of the phospholipids. The thermograms were recorded only during the heating scans after 20 min of sample equilibration at the starting temperature. Reversibility of all transitions and reproducibility of the thermograms were observed in repeated heating scans. All experiments were done at least twice. Buffer subtraction, baseline correction, and integration of the calorimetric endotherms were performed using Microcal Origin software.

Pressure perturbation calorimetry (PPC) experiments were carried out on the same VP-DSC Calorimeter mentioned above equipped with a pressure perturbation accessory (Microcal, Northampton, MA, USA).

The same samples used for the DSC experiments were used without reloading for the PPC experiments. The isothermal mode of the calorimeter was used during the pressure jumps from 5 bar (~73 psi) excess pressure to ambient pressure or vice versa. Acquisition conditions: high gain, low noise, and 90 s of pulse duration. The results were analyzed in terms of the relative volume change (ΔV/V) of the lipid main phase transition obtained from the area underneath the thermal expansion coefficient (α_V) versus temperature curve. The DMPC specific volume used for data evaluation was 0.978 mL/g [20]. Basic thermodynamic theory of the PPC experiments and how the α_V curve was obtained are described elsewhere [20].

2.4. Electron spin resonance (ESR)

For ESR experiments, the samples were transferred to glass capillaries (1.5 mm I.D.) and centrifuged for 20 min. The capillaries were set into a quartz tube containing a mineral oil bath to help stabilize the sample temperature. ESR measurements were carried out in 50 mM Tris/HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4 with 10 mg/mL of the final lipid concentration. The amount of phospholipid and drugs used provided a 5:1 DMPC:PQ molar ratio. The temperature was controlled using an E257-X Varian temperature control unit coupled to an X-band E109 Varian spectrometer, and its uncertainty was about 0.2 °C. Acquisition conditions: field modulation frequency, 100 kHz; field modulation amplitude, 0.5 G; sweep width, 100 G; microwave power, 15 mW.

2.5. Non-linear least-squares ESR simulations

Non-linear least-squares simulations were performed using the NLSL software package developed by Freed and co-workers [21–23], which calculates an ESR spectrum by solving the stochastic Liouville equation. The parameters involved in the simulations were: hyperfine (A_{xx}, A_{yy}, and A_{zz}) and g-tensor (g_{xx}, g_{yy}, and g_{zz}) components, orienting potential coefficients (c_{20}, c_{22}, and c_{40}), rotational diffusion rates ($R_\perp$, $R_{//}$), and an anisotropic Lorentzian linewidth. It is worth mentioning that not all parameters are varied during the fitting procedure.

The dynamics of the spin label molecule is described through rates of rotational diffusion of the nitroxide moiety around axes parallel ($R_{//}$) and perpendicular ($R_\perp$) to the mean symmetry axis of the molecule. For n-PCSL chain labels, these rotational diffusion rates consist of approximations to trans-gauche isomerizations, represented by $R_{//}$ values, and the wagging motion of the long axis of the carbon chain, represented by $R_\perp$ values [24]. It is well known that $R_{//}$ values for spin labels in membranes are larger than $R_\perp$ values. Thus, due to the insensitivity of ESR spectra to variations in $R_{//}$ values, in the simulation process reported here, the ratio $R_{//}/R_\perp$ was kept fixed at 10 and the parameter $\overline{R} = \left(R_{//} R_\perp^2 \right)^{1/3}$ was varied instead [25,26].

Lipid and spin probe molecules in membrane bilayers sense a restoring potential that restricts the amplitude of the rotational motion. This potential also produces an orientational distribution of molecules with respect to the local ordering axis of the membrane bilayer. The parameters related to the membrane orienting potential used in the fitting process were c_{20}, c_{22} and c_{40}, the first three coefficients of the potential expansion in generalized spherical harmonics series [21]. Besides this microscopic molecular ordering, lipid bilayer fragments can overall be distributed randomly. In this particular case, the final theoretical spectrum is regarded as a superposition of the spectra from all those fragments. This microscopic order with macroscopic disorder (MOMD) model was introduced by Meirovitch et al. [27].

Seed values for the magnetic parameters were obtained from Earle et al. [28]. The strategy of the simulation was the following: for each spin label in pure DMPC vesicles, the magnetic parameters were initially kept fixed and the orienting potential coefficients and

dynamic parameters were varied. This was done until the best theoretical spectrum was achieved. After that, the magnetic tensor values were allowed to be slightly varied. However, when these parameters are varied together, high correlation values between them can come up. To avoid that least-squares minimization of those parameters were carried out separately. The simulation parameters obtained from the control sample (DMPC/n-PCSL vesicles) spectra were used as starting values for the fits of PQ-containing samples. In these samples and for the whole temperature range used, the magnetic parameters were always kept fixed. Consequently, spectral changes obtained with the incorporation of PQ should reflect changes of the ordering potential coefficients and dynamic parameters. Finally, in order to avoid local minima, different sets of seed values for $R_\perp$ and c_{20}, c_{22} and c_{40} coefficients were restarted in the simulation process.

3. Results

3.1. Calorimetric results

To investigate the interaction of primaquine with zwitterionic lipid bilayers, the thermotropic phase behavior of DMPC vesicles in the presence and absence of the drug was determined. Fig. 1 shows the DSC thermograms of multilamelar DMPC and DMPC/PQ mixtures at different molar ratios. In excess water, DMPC exhibited two calorimetric peaks upon heating: a broad and less energetic pretransition centered at $T_P = 13.8$ °C and a sharp and more enthalpic main transition centered at $T_M = 23.8$ °C [29]. The pretransition is characterized by the conversion of the lamellar tilted gel phase, $L_{\beta'}$, to the lamellar rippled gel phase, $P_{\beta'}$, whereas main phase transition corresponds to the conversion of $P_{\beta'}$ to the liquid-crystalline phase, L_α [29].

Incorporation of different concentrations of PQ affects the thermotropic behavior of DMPC vesicles in three remarkable ways: (1) decreasing the enthalpy of the pretransition endothermic peak (inset in Fig. 1), (2) a notable linear decrease of both T_P and T_M, and (3) a broadening of the main phase transition. The calorimetric parameters were summarized in Table 1S of the Supplementary material. Taken together, these results indicate that PQ destabilizes the rippled gel phase of DMPC liposomes. At high PQ concentration (33 mol%), the pretransition of the DMPC bilayers was completely abolished.
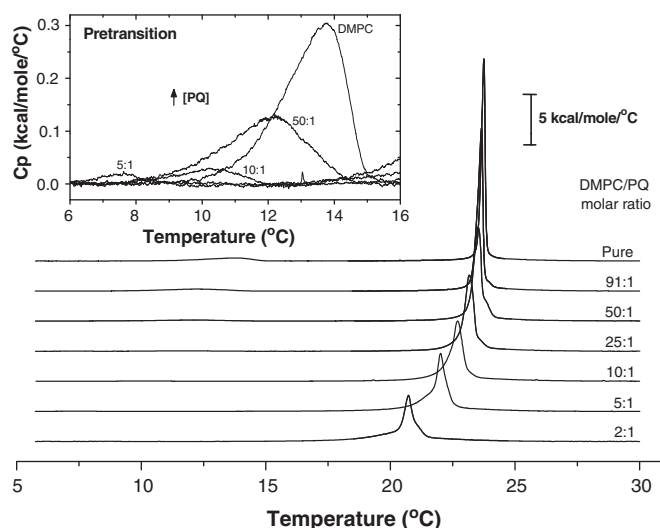
PPC experiments allow the determination of the thermal expansion coefficient (α_V) as function of temperature as well as the relative volume changes ($\Delta V/V$) accompanying lipid melting. From the measured heat changes for both compression and decompression experiments (Fig. 5S, Supplementary material) and the fits of the controls (buffer–buffer, buffer–water, and water–water), the temperature dependence of α_V for DMPC and DMPC/PQ mixtures was determined and yielded identical curves for both samples in the region far away from the phase transition (inset in Fig. 5S). From the area under the $\alpha_V(T)$ curve, a $\Delta V/V$ of 1.8% was obtained for pure DMPC and this value is a little lower than that reported (~3.0%) in the literature [20]. Despite the discrepancy, the absolute values were reproducible for repeated experiments and trends could thus be obtained regarding the PQ-induced DMPC volume changes. Incorporation of PQ into DMPC bilayers caused a reduction of $\Delta V/V$ to ~1.5% and seems to be PQ concentration-independent (Table 1S).

DSC was also used to monitor the perturbation of PQ on the membrane properties of other phospholipids. To do so we chose a phospholipid/PQ molar ratio that showed significant changes in the DMPC/PQ experiments (Fig. 1). Fig. 2 shows the effects of 20 mol% of PQ on excess heat capacities (C_P) of DPPC, DSPC and POPE. In all cases PQ decreases T_M, thus destabilizing the rippled gel phase. It was observed that the addition of PQ does not strongly affect the calorimetric enthalpy change of the observed main phase transition for all phospholipids (Table 2S, Supplementary material). Furthermore, the perturbation caused by PQ on phosphatidylcholine (PC) calorimetric parameters for both transitions was reduced by increasing the number of methylene groups on the lipid acyl chain (Table 2S).

The cooperative behavior observed in the lipid phase transitions has been explained as the coexistence of clusters of lipids with properties that are characteristic of the specific phases [30,31]. This means that the lipids do not melt independently of each other but rather transition as a cooperative domain. The width at half height ($\Delta T_{1/2}$) of the main phase transition peak is a calorimetric parameter whose value is inversely proportional to the size of this cooperative unit. Broadening of the main transition peak by the presence of PQ (Figs. 1 and 2; Tables 1S and 2S) indicates a destabilization of the phospholipid assemblies, suggesting an intercalation of this drug within the lipid bilayer. This fact could be related to a reduction of the average number of lipid molecules which undergo the transition as a cooperative unit. A similar effect was observed when the anesthetic procaine was added to DMPC bilayers [32].
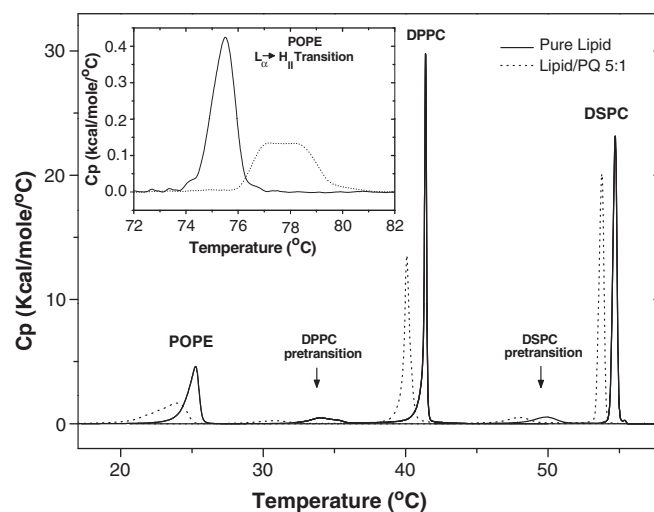

Fig. 1. Temperature dependence of the molar heat capacity of DMPC and DMPC/PQ vesicles at different DMPC/PQ molar ratios. The lipid main phase transition temperature decreases with increasing concentration of PQ. *Inset*: Representative thermograms illustrating the effects of the antimalarial drug on the pretransition of DMPC vesicles. Both T_P and pretransition enthalpy decrease with increasing PQ concentration.

Fig. 2. Temperature dependence of the molar heat capacity of POPE, DPPC and DSPC without and with addition of 20 mol% of PQ. Both DPPC and DSPC pretransitions are affected by PQ. *Inset*: Thermogram representing the effect of 20 mol% of PQ on the liquid-crystalline to inverted hexagonal phase transition temperature of POPE lipids.

In order to monitor the effects of PQ on membrane curvature, the phase transition temperature (T_H) of the L_α to the inverted hexagonal (H_{II}) phase transition of POPE vesicles was studied [33]. DSC experiments on POPE vesicles (inset in Fig. 2) showed that the L_α to H_{II} phase transition is characterized by a transition enthalpy of 470 cal/mol and $T_H = 75.5$ °C (Table 2S).

It is well known that T_H is markedly sensitive to the presence of foreign molecules [34]. An additive that stabilizes the typical negative (concave) curvature of the H_{II} phase will favor this phase by lowering T_H. Conversely, promotion of a positive membrane curvature stabilizes the L_α phase by increasing T_H. Addition of 20 mol% of PQ increased T_H to 78.1 °C and reduced the transition enthalpy to 370 cal/mol. This finding implies that PQ promotes positive membrane curvature strain and stabilizes the fluid phase of POPE vesicles.

3.2. ESR and NLSL simulations

To further investigate the effects, observed in our DSC experiments, caused by PQ on the lipid membrane properties, we performed a detailed ESR study using one of the DMPC/PQ molar ratios that showed significant changes (Fig. 1). The use of ESR and spectral simulation routines allowed us to gain information about local alterations in the lipid dynamic structure. To achieve this goal, we first discuss our ESR results obtained at 20 °C and then move on to analyze the temperature dependence of a set of spectra.

3.2.1. Rippled gel phase (20 °C)

Since PQ caused a destabilization of the DMPC rippled gel phase (Fig. 1), changes in local structure and dynamics of the lipid acyl chains after incorporation of this drug were investigated by means of spin labeling ESR technique. A complete study was made by using phospholipid derivatives, where the label is located at different positions along the acyl chain, thus monitoring the hydrophobic region of the bilayers. Fig. 3 shows the n-PCSL (n = 5, 7, 10, 12, and 14) ESR spectra and the best fits obtained from NLSL simulations in the

Fig. 3. n-PCSL (n = 5, 7, 10, 12, and 14) ESR spectra at 20 °C in DMPC vesicles in the absence (left) and in the presence (right) of PQ. Dotted lines represent the best theoretical fits obtained from NLSL simulations. In pure DMPC vesicles, 14-PCSL yielded two-component ESR spectrum. The two dashed lines in 14-PCSL ESR spectra represent the maximum hyperfine splitting of the 'rigid' component. The populations of both components are also presented. Total width: 100 G.

absence and in the presence of 20 mol% of PQ. Spectra were acquired at 20 °C, i.e., in the DMPC rippled gel phase.

Considerable changes can be seen in the ESR spectra of all spin probes upon incorporation of PQ. The order parameter (S_0) and the perpendicular rotational diffusion rate ($R_\perp$) obtained from non-linear least-squares simulations are presented in Fig. 4 (see also Table 3S, Supplementary material). The changes, in general, reflect a more fluid environment of the spin probes in DMPC vesicles in the presence of PQ. For pure DMPC vesicles, a decrease of S_0 (from 0.58 for 5-PCSL down to 0.22 for 16-PCSL) and an increase of $R_\perp$ (from 0.35×10^8 s^{-1} for 5-PCSL up to 0.76×10^8 s^{-1} for 16-PCSL) of the spin labels along the DMPC acyl chains towards the bilayer center were observed [35,36]. Spectral fittings showed that the effect of PQ on the dynamics ($R_\perp$) and ordering (S_0) of the lipid chains extends along the whole hydrophobic region of the bilayer. After incorporation of PQ, it is noticed a significant increase in the mobility of the spin labels (from 0.41 to 0.64×10^8 s^{-1} for 7-PCSL, for example) and a remarkable reduction of the order parameter for all positions (from 0.42 to 0.32 for 10-PCSL, for example) along the lipid chain as monitored by n-PCSL probes (Fig. 4).

As can be observed in Fig. 3, the n-PCSL probes present single-component ESR spectra except for 14-PCSL in pure DMPC vesicles. In this case, the shape of the low field peak is not characteristic of a spin label residing in only one microenvironment. Therefore, the resultant spectrum can be interpreted as a superposition of two spectra corresponding to the presence of spin labels in two structurally distinct micro-domains. One of the spectral components represents spin labels in a more fluid environment and the other arises from spin labels with restricted chain motion. As can be seen in Fig. 4, the fluid component (approximately half of the total spin population) has the usual behavior observed along the bilayer acyl chains for both S_0 (0.27) and $R_\perp$ (0.67×10^8 s^{-1}). On the other hand, the 'rigid' component has the same packing of the region monitored by 12-PCSL ($S_0 = 0.36$), but the rotational mobility of the spin labels is very restricted ($R_\perp = 0.36 \times 10^8$ s^{-1}) comparable to the 5-PCSL rotational motion ($R_\perp = 0.35 \times 10^8$ s^{-1}). Upon PQ addition, the two-component ESR spectrum of 14-PCSL is modified to a one-component spectrum (Fig. 3) with very low S_0 (0.12) and very high $R_\perp$ values (1.01×10^8 s^{-1}) as compared to the fluid component of 14-PCSL in pure DMPC (Table 3S, Supplementary material). The strong perturbation promoted by this drug on the rippled phase of DMPC bilayers is thus clearly seen from the spectra in Fig. 3 and the behavior of both S_0 and $R_\perp$ along the lipid acyl chains in Fig. 4.

3.2.2. Temperature variation — ESR analysis

The reduction in the DMPC melting temperature and the destabilization of the rippled phase after incorporation of PQ led us to monitor, also by ESR, the thermotropic phase behavior of DMPC and DMPC/PQ vesicles at 5:1 molar ratio. Due to their high resolution to spectral changes with temperature, phospholipids labeled at the end of the lipid acyl chains are the most appropriate spin probes in monitoring lipid phase transitions. Thus, we used 14-PCSL to monitor the main phase transition and investigate the effect of PQ on the thermotropic behavior of DMPC liposomes. It is worth mentioning that we also carefully characterized the dynamic structure of DMPC vesicles by using other spin labels. Temperature dependence of n-PCSL (n = 5, 10 and 12) ESR spectra were obtained and non-linear least-squares simulations showed an analogous thermotropic behavior for both S_0 and $R_\perp$ parameters as presented by 14-PCSL (Figs. 6S–8S, Supplementary material). Addition of PQ into lipid bilayers monitored by these other spin labels led to the same overall results obtained for 14-PCSL. Furthermore, since a two-component feature of ESR spectra was obtained only for 14-PCSL in the rippled gel phase, we chose that label for the temperature variation ESR studies.

Before analyzing the interactions of PQ with phospholipids, the dynamic structure of DMPC bilayers was characterized from non-
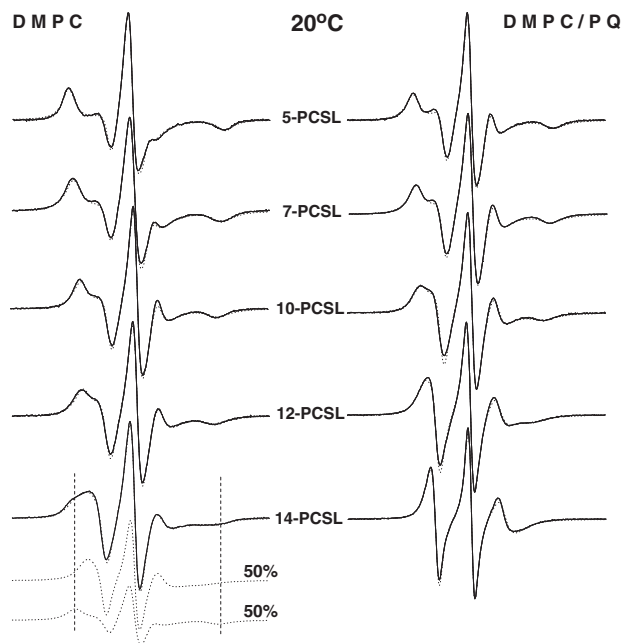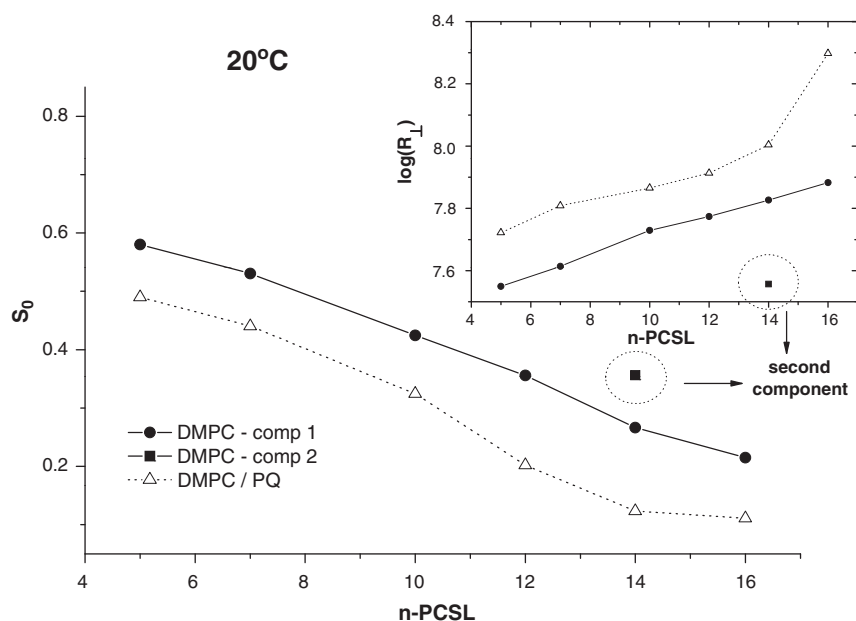
Fig. 4. Acyl chain ordering (S_0) and dynamics ($\log(R_\perp)$ — inset) profile obtained from non-linear least-squares simulations of DMPC/n-PCSL (n = 5, 7, 10, 12, 14, and 16) ESR spectra acquired at 20 °C before (solid circles) and after (open triangle) incorporation of PQ. The $R_\perp$ and S_0 uncertainties were estimated in 5% and 2%, respectively.

linear least-squares fits of 14-PCSL ESR spectra. Fig. 5 shows the temperature dependence of 14-PCSL ESR spectra in DMPC vesicles before and after mixture of PQ molecules. In that figure, it is also shown the best calculated spectra obtained from NLSL simulations. In the temperature range 15.0–23.0 °C, i.e. in the DMPC rippled gel phase, we were unable to obtain a reasonable simulation for DMPC/ 14-PCSL using only one component. Hence, a model including two ESR signals was used to fit 14-PCSL ESR spectra in the DMPC rippled phase.
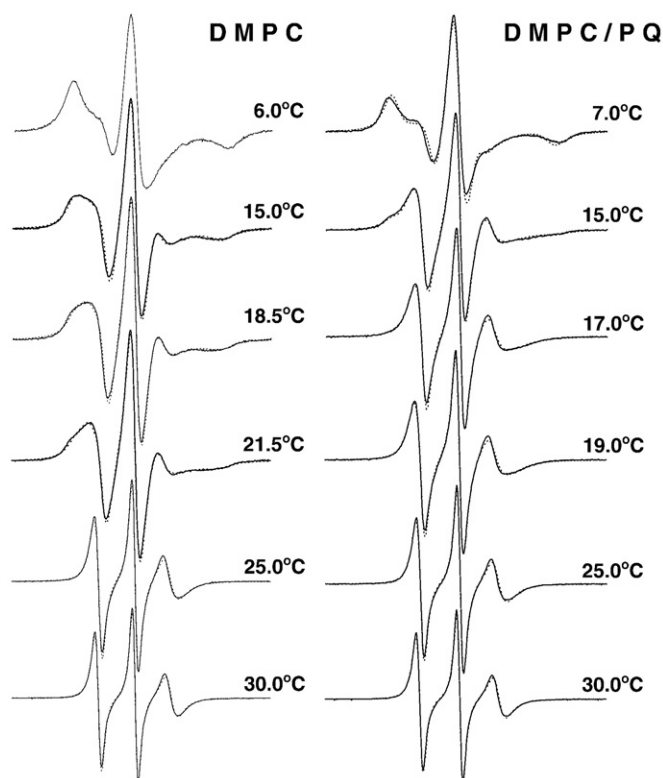
Fig. 6 shows ESR spectra that were best-fit with a two-component theoretical spectrum. For temperatures below 15.0 °C and above 24 °C, ESR spectra are characteristic of only one component. The thermotropic behaviors of S_0 and $R_\perp$ for DMPC/14-PCSL vesicles are presented in Fig. 7, and overall they show the usual trends observed for mobility and ordering of phospholipid acyl chains upon heating.

At 15.0 °C, S_0 value of 0.42 was found for the less mobile component and this value is comparable to that in the DMPC gel phase ($S_0 = 0.41$ at 10.0 °C), i.e. the packing of these domains is analogous to the tilted gel phase. On the other hand, the other component presents a less packed structure ($S_0 = 0.33$ at 15.0 °C) and a higher rotational dynamics of the spin labels ($R_\perp = 0.54 \times 10^8 \text{ s}^{-1}$) as compared to the less mobile sites ($R_\perp = 0.29 \times 10^8 \text{ s}^{-1}$).

Fig. 5. Temperature dependence of 14-PCSL ESR spectra in DMPC (left) and DMPC/PQ (right) vesicles. Dotted lines represent the best theoretical fits obtained from NLSL simulations. Total width: 100 G.
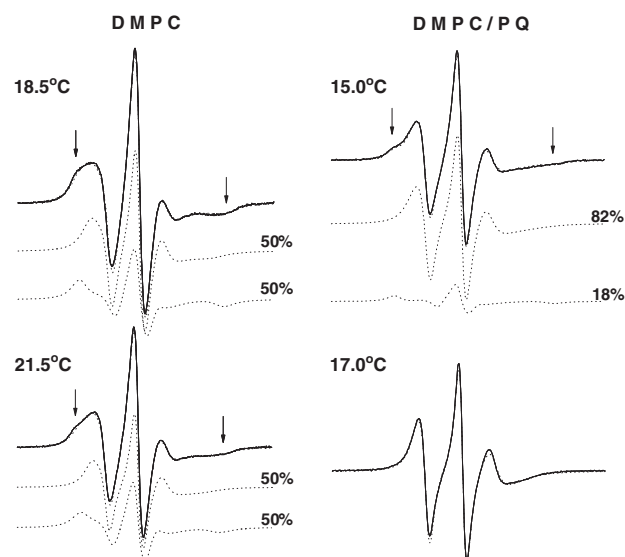

Fig. 6. 14-PCSL ESR spectra in DMPC vesicles in the absence (left) and in the presence (right) of PQ. Dotted lines represent the best theoretical fits obtained from NLSL. The arrows indicate the ESR spectrum of spin labels residing in a more 'rigid' lipid domain. The populations of the two components are also shown. At 17.0 °C, 14-PCSL ESR spectrum in DMPC/PQ vesicles was simulated with only one component. Total width: 100 G.
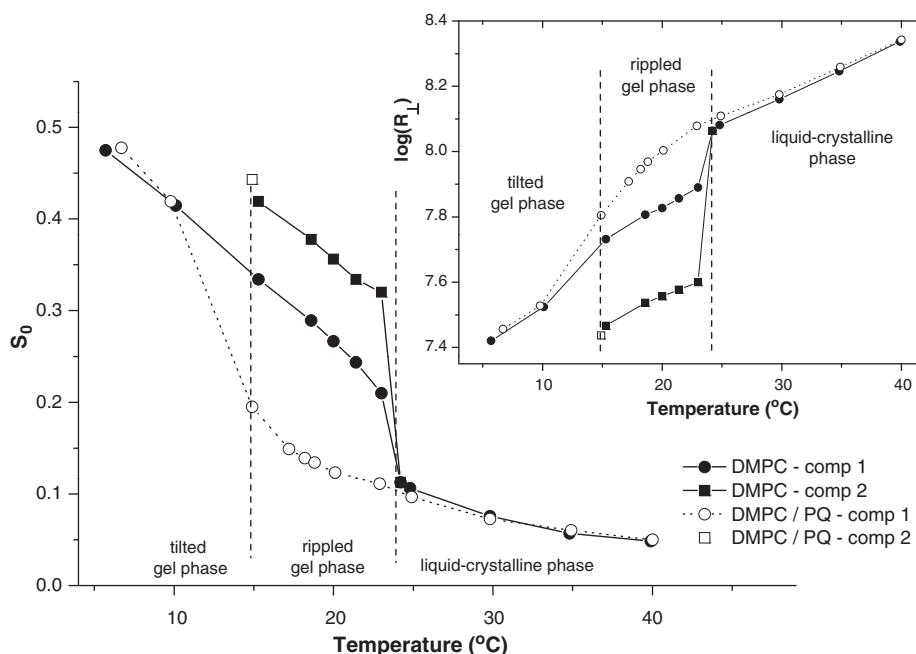
Fig. 7. Temperature dependence of the order parameter S_0 and $\log(R_\perp)$ (inset) of 14-PCSL in DMPC and DMPC/PQ vesicles. Parameters for the second component observed in the rippled gel phase of pure DMPC vesicles are also shown. The $R_\perp$ and S_0 uncertainties were estimated in 5% and 2%, respectively.

S_0 and $R_\perp$ values showed abrupt changes in the range 23.0–24.2 °C which are typical of lipids undergoing a phase transition. The DMPC transition temperature estimated by ESR spectroscopy was taken as the middle point of those two temperatures, i.e., 23.6 °C, which is in very good agreement with our DSC data (Fig. 1 − T_M = 23.8 °C).

Also in Fig. 5 are the ESR spectra of DMPC/PQ/14-PCSL in the temperature range 7.0–30 °C. For temperatures below 15.0 °C and above 24.0 °C, no spectral changes were observed after addition of PQ. However, in the temperature range corresponding to the pure DMPC rippled phase (from 15.0 °C to 23.0 °C), dramatic changes in 14-PCSL ESR spectra can be observed. This drug is capable of modifying the spectral lineshape yielding ESR spectra that reflect a more fluid environment of the spin probes.

Except for the spectrum obtained at 15.0 °C (Fig. 6), all other spectra obtained in the temperature range of 15–23 °C after addition of PQ presented only one component. PQ is able to modify the two-component feature of the spectrum, evidencing the strong perturbation caused by this drug on ordering and mobility of the acyl chains. Nevertheless, at 15.0 °C, it was found that 82% of the total spin population resides in a fluid domain (S_0 = 0.20 and $R_\perp$ = 0.64×10^8 s^{-1}) and 18% resides in a more packed microenvironment (S_0 = 0.44 and $R_\perp$ = 0.27×10^8 s^{-1}). This latter domain has the same packing and rotational mobility of 14-PCSL at 15.0 °C in pure DMPC (S_0 = 0.42 and $R_\perp$ = 0.29×10^8 s^{-1}). The great difference is related to the alteration of the fluid-like spin populations (from 57% in pure DMPC to 82% in DMPC/PQ vesicles), to its local ordering (from 0.33 to 0.20) and to the rotational dynamics of the acyl chains (from 0.54×10^8 s^{-1} to 0.64×10^8 s^{-1}). These results indicate a 'fluidizing' effect of PQ on DMPC bilayers. The second component is probably due to the remaining rippled structure of the lipid since PQ is not able to completely abolish the DMPC pretransition at this drug concentration (20 mol%). Fig. 7 summarizes the notable changes of the DMPC membrane properties with the temperature and the effects caused after addition of PQ.

4. Discussions

A great variety of molecular events can be involved in the mechanisms of drug action. Most drugs can strongly bind to specific sites in biological tissues. Nevertheless the nonspecific association of drugs with membranes is also an important and fundamental event at a molecular level. This association may induce modifications on lipid structure and may modulate protein conformational changes [37,38]. Thus, studies of drug–membrane interactions are pivotal for the elucidation of the mechanism of action.

The aim of this work was to investigate the interactions of the antimalarial drug primaquine with phospholipid model membranes. In order to achieve a detailed analysis, this study was performed using ESR, DSC and PPC techniques. The combination of these techniques is a valuable biophysical approach to assess the local changes in the lipid matrix and modifications on the macroscopic behavior of the phospholipids caused by the presence of small molecules such as drugs, peptides and hormones.

4.1. Effects of PQ on phospholipid membrane properties

DSC studies showed that the pretransition of phosphatidylcholine (PC) bilayers was strongly affected by the presence of PQ (Fig. 1). It has been suggested that the pretransition peak observed for pure PC liposomes in DSC experiments is due to structural changes in the lamellar lattice [39]. Ladbrooke and Chapman [40] also suggested that this transition is due to the rotation of the polar headgroups of the phospholipid molecules. Furthermore, headgroup hydration plays an important role in the rippled phase formation [41,42]. Hence, the pretransition is strongly influenced by the presence of foreign molecules on the bilayer surface [43,44]. The reduction of both pretransition temperature and enthalpy (Table 1S) with increasing PQ concentrations until the complete pretransition disappearance (at high molar concentration − 33 mol%) is a suggestion of a direct interaction of primaquine with the PC headgroup.

Usually the cooperativity of the phase transition and the lipid melting temperature are strongly affected by the presence of other molecules in the lipid matrix. The broadening of the main endothermic peak in DSC thermograms and the decrease of the chain-melting temperature (Fig. 2 and Table 2S) by incorporation of PQ are probably related to a reduction of the lipid–lipid interactions, yielding an alteration of the lipid chain packing. These results are consistent with the intercalation of PQ into the lipid structure. However, no changes in the associated calorimetric enthalpy mean that the interaction seems

to take place at the bilayer surface without deeply involving the acyl chains.

Despite the dependence of the DMPC calorimetric parameters on PQ concentration, the volumetric properties of the lipid bilayers were not perturbed by increasing the drug concentration (Table 1S). Even at the highest lipid:drug molar ratio (2:1), the relative lipid volume changes at the phase transition were very similar to those at the low PQ concentration. This slight difference in the DMPC volumetric properties in the presence of PQ ($\sim -0.3\%$) gives support to the hypothesis of a more superficial interaction between DMPC and PQ. A different kind of result was obtained by Okoro and Winter [45] in their studies of the volumetric properties of DPPC–sterol mixtures. They found that 5 mol% of cholesterol was able to induce a 0.8% negative change (from 4.0% to 3.2%) on $\Delta V/V$ for DPPC and these changes followed essentially a linear behavior as a function of sterol concentration. Additionally, the authors found comparable saturation behaviors for $\Delta V/V$ and the order parameter (S_0) in DPPC/stigmasterol mixtures as function of sterol concentration [46]. As indicated by our ESR experiments, primaquine does not cause any change on membrane packing (S_0) and on rotational dynamics ($R_\perp$) of the DMPC acyl chains at temperatures above T_M (fluid phase; see Fig 7). Hence, the combined ESR and PPC results suggest that the 0.3% negative volume changes of DMPC bilayers at the melting transition in the presence of PQ are probably due to a less dense lipid gel phase.

It is well known that PE phospholipids have strong tendencies to form H_{II} phases. It is believed that this phase plays significant role in membrane fusion and modulates several functions of membrane proteins [47–49]. Furthermore, it has also been shown that the effects of additives on membrane curvature play important biological roles in membrane function [50–52]. The stabilization of POPE L_α phase observed in our experiments (Fig. 2) is a strong evidence for an induction of positive membrane curvature strain by PQ and this is consistent with drug binding to the headgroup region of the lipid molecule. A similar finding has been observed for an analogue of the magainin antimicrobial peptide [53]. On the contrary, stabilization of the H_{II} phase is related to an increase of the negative curvature strain, promoted by ligands that insert deeply into the bilayer as observed for the antioxidant curcumin [54].

From our ESR experiments, nitroxide labels positioned at different bilayer depths showed that PQ induces a local disorder on the membrane packing of the DMPC rippled phase (Fig. 4). The increase of the rotational dynamics ($R_\perp$) and the reduction of the order parameter (S_0) along the lipid acyl chains in the temperature range corresponding to the pure DMPC rippled gel phase after addition of PQ (Fig. 7 and Figs. 6S–8S) could explain the decrease of the melting temperature and the reduction of the cooperativity of the main phase transition seen in our DSC results (Fig. 1). These changes can be related to an increase of the membrane fluidity for temperatures below T_M and above the pretransition temperature. Such effects on bilayer ordering caused by introduction of drugs have been explained by other authors as a fluidizing effect [55,56].

4.2. PQ binds to both gel-like and fluid-like domains

Another interesting result obtained from our ESR experiments was the two-component feature of 14-PCSL spectra in the rippled gel phase (Fig. 6). The existence of these two different micro-domains with distinct organization and dynamics may be due to the coexistence of 'gel-like' and 'fluid-like' domains in this phase. This assumption was proposed by various authors with both experimental and theoretical evidences [57–60]. Wittebort et al. [61] found that the carbon-13 nuclear magnetic resonance spectra in the temperature range corresponding to the DMPC rippled phase were a superposition of typical $L_{\beta'}$ and L_α spectra, thus exhibiting properties of both phases. Furthermore, Riske et al. [62] noted the existence of a second component on 16-PCSL ESR spectra in the temperature range

corresponding to the rippled gel phase for DPPG and DPPC model membranes. Using a statistical model, which took into account competing interactions between nearest and next-nearest lipid neighbors, the authors suggested that the lipid pretransition is the beginning of the whole acyl chain-melting transition, as proposed before by Heimburg [63]. Both papers support the idea that the pretransition is part of the full acyl chain-melting process.

As for the effects of PQ on the two-component feature of 14-PCSL spectra in pure DMPC, it was found that this drug dramatically modifies the dynamic and structural properties of the region monitored by this spin probe, leading to an almost complete suppression of the 'rigid' component (Fig. 6). The structural and dynamic parameters obtained for 14-PCSL in DMPC/PQ vesicles exhibit a more mobile and less packed lipid environment compared to the 'fluid' component of the pure lipid (Fig. 7). This is consistent with an increase in the fluidity of the rippled gel phase of the DMPC bilayers as discussed previously.

Our ESR results have also shown a change of the spin populations of both micro-domains after addition of PQ (Fig. 6), pointing out to a change in the equilibrium constant of the gel-like and fluid-like phases. In order to explain these findings we analyzed the effects of PQ binding to DMPC bilayers through the DSC data by using a quite simple binding model derived in details in Section 1S of the Supplementary material. This model allows for different drug affinities to both gel (K_G) and fluid (K_F) domains. With this model, a binding constant ratio (K_F/K_G) of 1.6 ± 0.4 between the fluid and gel membrane domains was determined. This result shows that PQ binds more effectively to the fluid domains. The absolute values of the binding constants (K_b), however, were only assessed by fluorescence experiments (Section 2S of the Supplementary material). PQ binding constants of 160 ± 20 M^{-1} and 110 ± 20 M^{-1} to DMPC liposomes at 30 °C (fluid phase) and 20 °C (rippled gel phase), respectively, were determined. A little higher value $\left(240 \pm 20\ M^{-1}\right)$ was obtained spectrophotometrically by Perussi et al. [64] for PQ binding to micelles of the zwitterionic N-hexadecyl-N,N-dimethyl-3-ammonio-1-propa-nesulfonate surfactants at pH 7.0. This difference is probably related to the denser lipid packing in the DMPC bilayer as compared to the micellar system. The same type of behavior was observed for the vasodilator and antitumor drug coactivator dipyridamole binding to DMPC and DPPC phospholipid vesicles and lysophosphatidylcholine micelles [65]. Additionally, a binding constant of 270 ± 40 M^{-1} for PQ to DMPC/cholesterol (90:10 molar ratio) unilamellar vesicles at 37 °C was also obtained spectrophotometrically [66]. Since the lipid packing of unilamellar and multilamellar liposomes are different, this parameter should have a great contribution to the observed discrepancy. Considering that, at 30 °C, DMPC is in the fluid phase so that the K_b measured by fluorescence reflects binding to the fluid domains (K_F) and using the K_b value at 20 °C as a weighted estimate of K_G, a ratio of 1.5 ± 0.5 of the binding constants determined by fluorescence is quite similar to the K_F/K_G value obtained from our DSC data analysis. From the values of the binding constants, the partition coefficients at both temperatures were calculated (Section 2S): $P(20°C) = 160 \pm 30$ and $P(30°C) = 240 \pm 30$. These values are in the same order of magnitude than those of the uncharged forms of several local anesthetics (procaine, 228, lidocaine, 223, mepivocaine, 245, and prilocaine, 305) in egg phosphatidylcholine bilayers at 22 °C [67]. However, there is a quite important difference: at physiological pH, monoprotonated species of PQ are the predominant form, since the pKa values of its protonated groups in an aqueous solvent are 4.1 for the quinoline ring nitrogen and 11.2 for the primary amine [68]. Thus, since the partition coefficients of charged drugs into zwitter-ionic membranes are lower than those of the uncharged species [69] probably due to electrostatic interactions with the phospholipid headgroups [70], our results gain importance inasmuch as the monoprotonated PQ partitions well in DMPC bilayers compared to various uncharged local anesthetics.

4.3. Insights on drug location

To gain insight on the exact PQ location into DMPC bilayers, we performed ESR experiments with the headgroup spin label dipalmitoylphosphatidyl-tempocholine (DPPTC). Representative ESR spectra in the absence and in the presence of PQ are presented in Fig. 3S and the temperature dependence of an empirical parameter reflecting ordering and dynamics of that spin probe is presented in Fig. 4S. The spectra showed that PQ binds to the DMPC headgroups in the whole temperature range studied (from 8 °C to 35 °C) and increases the headgroup packing and/or decreases the rotational diffusion rate of the spin probe. Additionally, a second and more ordered component was observed in DPPTC ESR spectra for temperatures below 18 °C, indicating a direct PQ adsorption in the DMPC headgroup (Section 3S). These results suggest that the dynamic structure of the PC headgroup region was strongly affected by the presence of PQ.

Taken together, our findings suggest an interaction of the zwitterionic headgroup region of the lipids with the positively charged PQ. Probably electrostatic interaction between the negatively phosphate group of the lipid polar region and the charged nitrogen of the drug takes place. Moreover, hydrophobic interactions can also play an important role by localizing the PQ quinoline ring into the DMPC hydrocarbon core. In this sense, primaquine might act as a spacer in the lipid structure with consequent perturbation of the acyl chain organization. This could lead to a weakening of van der Waals attractions among DMPC hydrocarbon chains and hence, increase the fluidity of the liposomes as indicated by our ESR measurements. However, this strong perturbation promoted by PQ is most likely due to a nonspecific interaction with phospholipids, which is supported by a previous work on the binding of this and another antimalarial drug, chloroquine, to micelles of surfactants with different charges of the headgroups [64]. Further detailed studies to exactly determine drug location and to gain more insights on the nature of the PQ–phospholipid headgroup interactions are needed.

4.4. Relevance of the results and correlation with other studies

Interaction of cationic amphiphilic drugs like primaquine with human erythrocytes was shown to alter the topological phospholipid asymmetry distribution of the two halves of the bilayer [71,72]. Asymmetries with respect to the charge of the lipid headgroups and to the degree of unsaturation of the acyl chains in the inner and outer leaflets of human erythrocyte membranes are well known [73,74]. The outer monolayer predominantly contains neutral and saturated phospholipids and thus presents a more packed structure. Since about 75% of the total PC is present in the outer layer, the results obtained in this work gain importance, and may be relevant from a physiological point of view. Furthermore, the choice of PC and PE as model membranes is appropriate because both lipids are the major classes of phospholipids present in the infected erythrocyte plasma membrane (about 40% of the total lipid composition for PC and 25% for PE) and the parasite membrane (about 55% and 27%, respectively) [75].

Primaquine was also shown to induce morphological changes in membranes of all exoerythrocytic stages of *P. fallax* [76]. The formation of cytoplasmatic vacuoles caused by incubation of PQ was identified as swollen parasite mitochondria. In addition, Ginn and co-workers [77] reported that the primaquine-induced hemolysis in normal red cells *in vitro* was probably related to the reduction in the surface area of the cell membrane. This morphologic change caused membrane invaginations which led to the internalization of the plasmalemma. Also, using a cell-free assay that mimics glycoprotein transport through the Golgi apparatus, Hiebsch et al. [78] found that PQ selectively inhibits the formation of functional transport vesicles. The authors suggested that the incorporation of PQ into the luminal leaflet of Golgi membranes may affect the vesicle budding and/or pinching off of the buds to form transport vesicles. Changes in PC headgroup ordering (Section 3S) and PE membrane curvature (Fig. 2) induced by PQ may contribute to vesiculation and erythrocyte shape changes and thus PQ could also be regarded as a curvature-inducing additive [79–81].

Phospholipid–drug interactions may also play an important role as an alternative additional route in the mechanism of drug action. Due to the high-affinity binding to phospholipids, Chevli and Fitch [82] proposed that mefloquine, an 4-aminoquinole antimalarial drug, may have a second mode of action involving strong association with membranes in addition to its interaction with ferriprotoporphyrin IX, a hemoglobin degradation product. Like mefloquine, the antimalarial activity of other structurally related arylmethanol agents such as quinine and halofanthrine has also been associated with strong interactions with phospholipid membranes [83–85]. Secondly, membrane phospholipid composition has been shown to play crucial role in drug sensitivity among quinoline-containing drugs [86]. Both cases, nonspecific membrane interactions and dependence on membrane composition, are supported by our results that point to these two features as part of a more general mechanism of drug action.

5. Conclusions

Our results indicate that the effects of primaquine on zwitterionic lipid membrane properties may be due to interactions of the charged form of this molecule with the polar region of the lipids. The drug may act as a spacer intercalating in the phospholipid headgroups, from where it perturbs the ordering and the rotational dynamics of the acyl chains, making the bilayer more fluid in the temperature range corresponding to the rippled gel phase of the PC phospholipids. In addition, PC headgroup increased ordering and PE positive membrane curvature changes were induced by PQ.

These findings can be of particular importance because the local disorder in the basic structure of the lipids promoted by primaquine might influence the function of one or more proteins which are embedded into the lipid environment and/or may start a biological signal, mediating another cellular event. Thus, despite the wide spread of biological effects promoted by PQ [87], the exact understanding of its mechanism of action is still unknown. Our results indicate that the nonspecific interaction of primaquine with biological membranes may play an important role and may represent an additional route in the overall mode of action of this drug and/or could be involved in its adverse effects. However, further detailed studies with distinct model membranes are needed to elucidate the complete mechanism of interactions of PQ with phospholipids and their relevance to drug action.

To the best of our knowledge this is the first detailed report concerning the effects of primaquine on the dynamic structure of phospholipid model membranes at a molecular level. Our results can provide a useful rationale for the elucidation of the complex mechanisms involved in the interaction of this and other nitrogen heterocyclic compounds with biomembranes, and thus help in understanding the more general way of action of antimalarial drugs at a molecular level.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbamem.2010.08.009.

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