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Thermal behavior and elastic properties of dimyristoyl phosphatidylcholine bilayers under the effect of pentoxifylline

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Abstract

We have investigated the effect on dimyristoyl phosphatidylcholine bilayers of pentoxifylline, a derivative of xanthine by using two optical techniques, quasi-elastic light scattering (QLS) and Fourier transform infrared spectroscopy (FT-IR). The results show that in the presence of pentoxifylline, the bilayer phase transition point is lowered and that the elastic modulus is decreased. The FT-IR results indicate strong interactions in the aqueous interface regions of the bilayers. We discussed these results comparatively with those obtained from flavonoid derivatives whose effect was analogous and previously studied.

Key words: Pentoxifylline; Model membranes; Elasticity modulus; Quasi-elastic light scattering

1. Introduction

Pentoxifylline, a derivative of xanthine, has been known to possess many physiological and pharmacological activities [1–4]. It improves circulatory insufficiencies by decreasing the erythrocyte deformability, decreasing the blood viscosity and prevents the patelets aggregation. Among these properties, the amelioration of the deformability of red blood cells is noteworthy. This is comparable to the effect of flavonoid derivatives [5,6]. Up to nowadays, the molecular mechanism of action is still not well understood and such a study attracted much interest. By fluorescence and ESR techniques, Sato et al. [7–9] have shown that pentoxifylline interacts with ghost membranes in changing the fluidity of the outer side as well of the inner side. It is suggested that this enhancement of membrane fluidity may be related to some biological processes. By the filtration method, the improvement in rigidity index of red blood cells was observed [8]. On the other hand, it was demonstrated that pentoxifylline has an effect on polymorphonuclear leukocytes deformability [10]. Moreover, interactions of pentoxifylline molecules with phospholipid membranes were suggested [11]. From these studies, it may be that pentoxifylline interacts with proteins as well as with the lipid components of cell membranes. A study of the effect on one component, in the absence of the others, appears useful for the comprehension of these mechanisms.

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In this work, we are interested in the effect on the elastic and thermal behavior of pure phospholipid large unilamellar vesicles. The elastic property may be related to the cell membrane deformability which depends in a great part on the lipid bilayer [12].

For this purpose, we have chosen two 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 [13–17]. On one hand, QLS permits to determine the vesicle size and thus to monitor the thermal behavior and to measure the deformations under an osmotic pressure. The change in size allowed a determination of the elastic modulus. Such a determination of elastic modulus, successfully used by many workers for pure lipid vesicles, was suitable for a study of elastic properties of LUV under the effect of drugs. On the other hand, FT-IR spectroscopy has been used to locate the interaction sites between a drug and lipid bilayers [18]. We will compare these results to those observed with flavonoid derivatives.

2. Materials and methods

2.1. Materials

Pentoxifylline (Fig. 1) was furnished by Hoeschst. Dimyristoylphophatidylcholine (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 analytical grade and purchased from Merck or Aldrich.

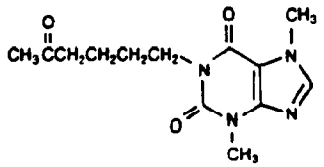

Fig. 1. Molecular formula of pentoxifylline.

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 [19], followed by successive centrifugations. This technique allowed us to obtain unilamellar vesicles in the range of 120–150 nm in diameter with a polydispersity factor of 0.15 to 0.20. The unimodal distribution of the dispersions was controlled by light scattering technique using the inverse Laplace Transform method (Malvern Software) [20].

Pentoxifylline is soluble in 10 mM Tris, pH 8 aqueous buffers used for the preparation of vesicles. In these buffers, pentoxifylline shows an absorption band at 233 nm which was used to titrate the drug concentration. Solutions of concentration 1 mg ml^{-1} were added to vesicle samples before measurements in order to obtain the desired drug concentrations and drug/lipid molar ratios.

2.2. Quasi-elastic light scattering

Quasi-elastic light scattering (QLS) has been performed on a photon self-beating spectrometer constructed by our Laboratory, operating with the green line of a Spectra Physics Ar^+ laser. Other experimental details were described previously (15). The autocorrelation functions of the scattered intensities were analyzed using the method of cumulants [21], which allows the determination of the average translational diffusion coefficient D. 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. From the measurements of R_h, the phase transition of the membranes and their elasticity can be monitored.

2.2.1. Phase transition

The phase transition was studied by following the hydrodynamic radius R_h versus the temperature. A change in R_h reflects a modification in the total surface area of the vesicles, which is a consequence of the change in the phospholipid

conformation occuring when phospholipid vesicles undergo the gel to liquid–crystalline phase transition.

2.2.2. Osmotic response and elastic modulus determination

The swelling of vesicles was provoked by a gradient of osmotic pressure between the inside and the outside of the vesicles then the vesicle sizes before and after the swelling were measured. This pressure gradient was obtained by diluting the external medium by free NaCl buffers and was estimated in accordance with the van't Hoff law. Once the swelling (expressed by the vesicle radius change $\Delta r/r$) was measured at time t, the Young elastic modulus E of the bilayers was calculated [15],

$$E = 2RT\frac{r_0(1-2e/r_0)}{4e}(C_{s0}-C_s) \times\left(\exp(-kt)\frac{r_0}{\Delta r}-\frac{3C_{s0}}{C_{s0}-C_s}\right), \quad (1)$$

where R is the ideal gas constant, T the absolute temperature, e the membrane thickness, r_0 the initial external radius and Δr the change of r. The corrective term $-3C_{s0}$ is due to the swelling and the exponential term describes the decay of the salt concentration gradient due to the leakage as a function of time. Although many authors considered that the permeability of small cations accross pure lipid bilayers is negligible, the importance of the leakage in the determination of E was recognized [15,22]. If there is no leakage ($k=0$) and in neglecting the term $2e/r_0$, the formula (1) becomes

$$E = 2RT\frac{r_0^2}{4e\Delta r}(C_{s0}-C_s)\left(1-\frac{3C_{s0}}{C_{s0}-C_s}\frac{\Delta r}{r_0}\right). \quad (2)$$

2.3. FT-IR spectroscopy

Infrared spectra with a spectral resolution of 1 cm^{-1} have been obtained using a Perkin Elmer 1760 Fourier Transform spectrophotometer with ZnSe as window material. The path width used was 50 μm and 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, pH8 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.

3. Results

3.1. Effect of pentoxifylline on the elastic modulus of DMPC LUV

For pure lipid LUV, when the external NaCl concentration was gradually diluted from the initial value 150 mM, the hydrodynamic radius R_h increased indicating a swelling of vesicles (Fig. 2a). The salt was moderately diluted in order to prevent the break up of vesicles. The polydispersity factor was slightly increased. The interval between two subsequent dilutions was 18 min. At each state of the swelling, the Young elastic modulus was calculated following relation (1). In this calculation, the corrective term due to leakage has been estimated from the constant k deduced from the loss of the isotope ^{22}Na measured by Singer [23]. We obtained the values of 1.6×10^8 dyn/cm^2 and of 2.7×10^8 dyn/cm^2 for the liquid crystalline phase and the gel phase respectively. In the liquid crystalline phase, the value of E, although smaller but in the same order as that in DMPC giant vesicles observed at 29°C by Evans and Needham [24], is very close to those obtained for DMPC vesicles with 106 nm in size [14] or for brush border vesicles in buffers containing 10 mM glucose [25]. If the leakage was not taken into account, the uncorrected value of E would be 2.9×10^8 dyn/cm^2. In the gel phase, the leakage is weak, the value of E, given in Table 1, differs only by 3.5% from that obtained without

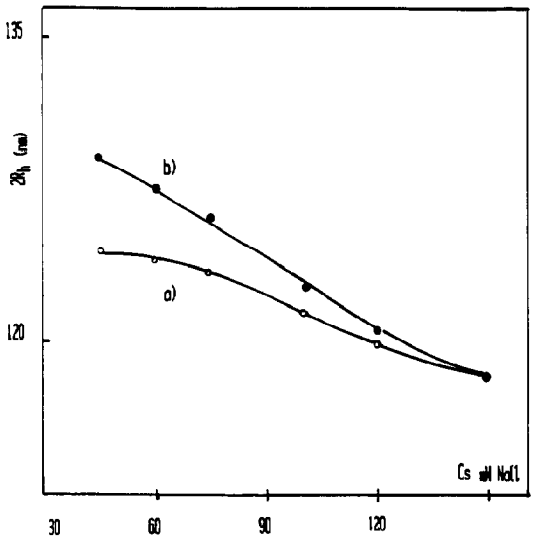

Fig. 2. Effect of the osmotic pressure gradient on DMPC LUV at 33°C. (a) in the absence and (b) in the presence of pentoxifylline (drug/lipid molar ratio $x = 0.3$). The plots represent the variation of the vesicle radius when the external NaCl concentration Cs is diluted from the initial value 150 mM in 10 mM Tris, pH 8 aqueous buffer. This dilution of the salinity implies also a dilution of the vesicle concentration C which, in the studied range, does not affect the diffusion coefficient and the measured vesicle radius.

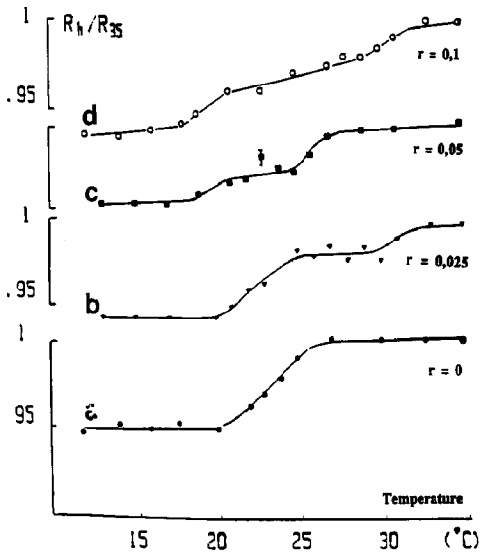

Fig. 3. Effect of pentoxifylline on the phase transition of DMPC LUV observed by quasielastic light scattering. The plots represent the temperature dependence of the vesicle hydrodynamic radius R_h (normalized to the value R_{35} of pure lipid LUV at 35°C) (a) LUV in the absence and (b)–(d) in the presence of pentoxifylline at various drug/lipid molar ratio x: (b) $x = 0.025$; (c) $x = 0.05$; (d) $x = 0.10$. Lipid concentration: 0.40 mM in 10 mM Tris, 150 mM NaCl, pH 8 aqueous buffer.

the corrective exponential term due to the leakage.

In the presence of pentoxifylline, the osmotic swelling was more important than that for pure lipid vesicles (Fig. 2b). This must correspond to a decrease of the elastic modulus or/and the ion leakage of DMPC model membranes. We have estimated the value of E in two extreme cases. In the first case we took the value of k corresponding to pure lipid vesicles (with an unchanged leakage), and in the second case, we took $k = 0$, corresponding to an impermeable bilayer (with a complete reduction of leakage). The values were given in Table 1. In both cases, it is obvious that there is a decrease of the Young elastic modulus in the presence of pentoxifylline. In other words, this drug diminishes their rigidity.

Table 1
Effect of pentoxifylline on the elastic modulus of DMPC bilayers (buffer: 10 mM Tris, 150 mM NaCl, pH 8)

DMPC LUV	Young elastic modulus E (10^8 dyn/cm^2)		
	15°C	33°C	
pure lipid	2.5–3 [a]	1.6 [a]	
in the presence of pentoxifylline			
drug/lipid molar ratio 1/10	1.2 [a]	0.9 [a]	
3/10		0.48 [a]	0.87 [b]

[a] Value obtained with leakage from pure lipid vesicles.
[b] Value obtained for completely impermeable bilayers.

3.2. Effect of pentoxifylline on the phase transition of LUV

3.2.1. Transition observed by QLS

The phase transition of DMPC LUV is shown by the variation of the vesicle area or the hydrodynamic radius R_h (normalized to its value at 35°C) depicted by Fig. 3. The plots correspond to various drug concentrations. In the absence of pentoxifylline (Fig. 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 [26]. At the transition, the change in the vesicle radius is about 6–7%. In the presence of pentoxifylline at various concentrations (Figs. 3b–3d), a complex transition was observed. At least, two steps were shown. For the low-temperature transition step, the concentration dependence of the transition point seems follow a linear law versus the drug concentration.

Besides this transition, at low pentoxifylline concentrations, the vesicles undergo a second transition at about 30–26°C, higher than the transition point of pure lipid vesicles. In this second transition step, the vesicle size change is small (1–2%). When the drug concentration is increased, the intermediate state between two step-transition points becomes complex and these step-transitions cannot be clearly described. In other words, one can say that there are many steps which spread in a broad temperature range.

3.2.2. Transition observed by FT-IR: effect of pentoxifylline in the spectral region 3000–2800 cm^{-1}

The phase transition was also observed by FTIR 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 Fig. 4. These shifts reflect the change of the *trans/gauche* ratio of the acyl chain conformers Wavenumbers 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 [17]. Figs. 4a and 4b depicted the transition curves of DMPC LUV in the absence and in the presence of pentoxifylline respectively. When the drug is added to the vesicle solution, the liquid crystalline to gel-like phase transition 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.

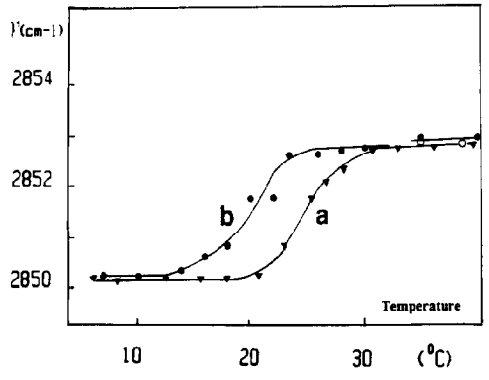

Fig. 4. Effect of pentoxifylline on the phase transition of DMPC LUV observed by FT-IR spectroscopy. The plots represent the temperature-dependence of the wavenumber of the symmetric CH_2 stretching mode (a) in the absence and (b) in the presence of pentoxifylline (drug/lipid molar ratio 0.2). Concentration of lipid: 50 mg ml^{-1} in 10 mM Tris 150 mM NaCl, pH 8 aqueous buffer.

3.3. Effect of pentoxifylline on the IR spectra of DMPC LUV

3.3.1. 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 is a broad band at about 1735 cm^{-1} (see Fig. 5). Two components at 1742 and 1727 cm^{-1} were shown to exist by a curve fitting procedure, corresponding to the vibrations of both C=O groups with different hydrations [27–29]. Their relative peak heights vary with temperature resulting in the shift of the peak wavenumber of the whole band profile [17,28].

In the used curve fitting procedure, we assumed a Gaussian–Lorentzian line shape for each of them (Fig. 5). For pure lipid vesicles, the best fit was obtained with a contribution of 40–70% for the Gaussian part. The heights and the widths of both components obtained after the curve fitting procedure are given in Table 2. When the drug is present, this table shows an increase of the peak height ratio and a change in the bandwidths. These changes are indicative of an effect

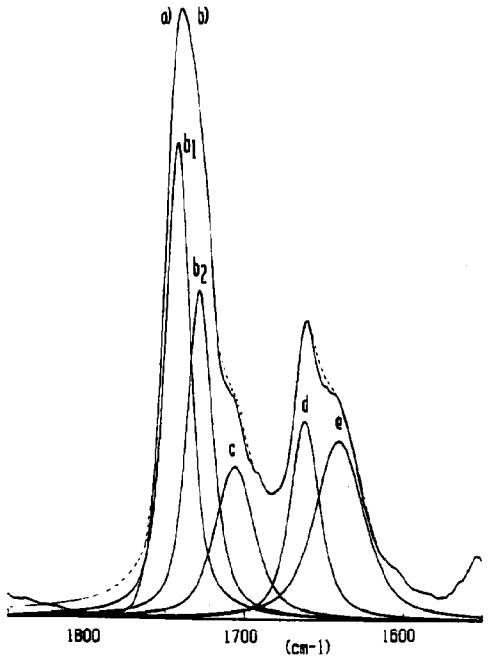

Fig. 5. FT-IR spectra in the spectral region corresponding to the stretching mode of the C=O groups of the DMPC LUV at 9°C (a) in the absence and (b) in the presence of pentoxifylline ($r = 0.5$) The curve fitting of each experimental spectrum (a) or (b) gives two components a_1 and a_2 or b_1 and b_2 whose sums are represented by dashed lines. Bands (c), (d) and (e) belong to pentoxifylline. Buffer: 10 mM Tris, 150 mM NaCl, pD 8 in D_2O.

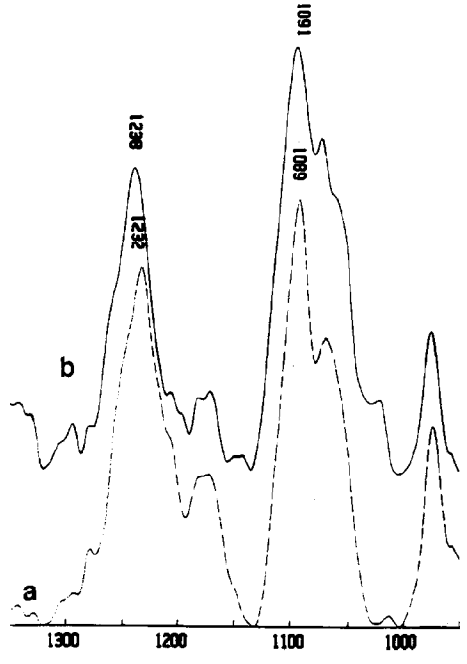

Fig. 6. FT-IR spectra in the region of the PO_2 stretching bands (a) pure DMPC LUV; (b) DMPC LUV in the presence of pentoxifylline (drug/lipid molar ratio 0.2). Buffers: H_2O, 10 mM Tris, 150 mM NaCl, pH 8. The spectrum (b) has been obtained by a subtraction of the absorption bands of the drug.

on the C=O groups by the presence of pentoxifylline.

3.3.2. Spectral region 1350–950 cm^{-1}

Infrared absorption spectra of DMPC LUV in the absence and in the presence of pentoxifylline in this spectral region were also recorded (Figs. 6a and 6b). In the plot 6b, the contribution of pure pentoxifylline has been subtracted. In the absence of pentoxifylline, 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 are shifted towards 1238 and 1091 cm^{-1}, respectively. By comparing the spectra, we also observed a change in the peak height ratio $I(1232)/I(1088)$ which is 8% smaller than that of pure lipid vesicles. These frequency shift and intensity change indicate an effect of pentoxifylline on the PO_2^- group region of the bilayers.

Table 2
Effect of Pentoxifylline on the relative peak height ratio I_1/I_2 and band widths Γ_1, Γ_2 (in cm^{-1}) of the components (1) at 1742 cm^{-1} and (2) at 1727 cm^{-1} of the band contour components corresponding to the C=O stretchings

	I_1/I_2		Γ_1/Γ_2			
	pure LUV	LUV + Ptox	pure LUV		LUV + Ptox	
	I_1/I_2	I_1/I_2	Γ_1	Γ_2	Γ_1	Γ_2
9°C	1.24	1.47	19.5	24	22	27
37°C	0.87	1.17	22	29	19	20

4. Discussion

Although pentoxifylline is very soluble in tris-buffer and the exact value of the partition coeffi-

cient between the lipid and the aqueous phases has not been known, the above results show interactions of pentoxifylline with DMPC bilayers and effects of this drug on their elastic properties as well as on their thermal behavior. The comparison of the results obtained here and those observed in the case of flavonoid derivatives shows that their interactions with the bilayers are qualitatively similar, i.e. they affect the aqueous interface and the inner hydrophobe region, decrease the bilayer rigidity and alter the phase transition.

First, at a molecular level, the presence of amphiphilic molecules, containing 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 [30]. In the outer region of the bilayers, IR data show a shift towards higher frequencies and a change in the peak height ratio $I(1232)/I(1089)$ of the IR absorption bands corresponding to the PO_2^- stretchings. This is indicative of an alteration of the polar head moieties. This result is consistent with the increase in the fluidity of the outer face of ghosts in the presence of pentoxifylline observed by Sato et al. [8]. The interaction with the phosphate groups would also be related to the change in surface charges [31] which, in turn, may result in a decrease of the patelets aggregation. This may also be in relation with the decrease of the bilayer stiffness [32]. In the interface region, the change of the band shape, of the relative peak height ratio $I(1742)/I(1727)$ of the components at 1742 and 1727 cm^{-1} and of the bandwidth (Table 2) indicate also an interaction between the drug molecules and the C=O groups. An analogous modification at the interface region of DPPC bilayers was observed in the presence of salicylic acid or phenol [18]. It may be that these alterations, relative to peak heights and bandwidths, corresponding to the double bond sn1-CO and sn2-CO stretchings, is due to a change in hydration and head group volume.

Second, at a more "macroscopic" level, one interesting result observed by QLS is that pentoxifylline decreases the Young modulus of DMPC membranes. This modulus E is two times, or more, smaller than that of pure lipid LUV indicating that the rigidity of the bilayers is decreased by the presence of the drug. An effect on the elastic modulus with the same order of magnitude was observed under the effect of flavonoid derivatives [13]. This effect may be correlated to the augmentation in the deformability of membranes in the presence of pentoxifylline.

On the other hand, QLS, as well as FT-IR, showed that pentoxifylline alters the liquid crystalline → gel-like phase transition. By comparison between the temperature-dependence plots of the vesicle hydrodynamic radius R_h in the presence and in the absence of pentoxifylline, one observes no 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 is more complex. The cooperativity of the transition was altered, indicating probably the existence of microdomains. At least two steps of the transition were shown. It might be that the low-temperature transition is related to the pretransition. However, in DMPC, this pretransition in pure lipid bilayers is weak and practically unobservable by QLS (plot 3a). Therefore it may be that the low-temperature step was the main transition shifted by the presence of the drug. Concerning the second transition step occurring at 26–30°C, it may be that it is related to a molecular rearrangement of the liquid crystalline phase before the real transition occurs. Note that this splitting is commonly observed when amphiphilic drugs such as loperamide, *d*-propranolol, gramicidin A, valinomycin or poly-lysine are added to DMPC LUV [34]. We think that the main effect of pentoxifylline is to shift the transition point but this lowering is accompanied by a rearrangement of the lipid molecules in the bilayers. This rearrangement may be related to the effects observed on the PO_2^- and C=O groups, probably due to a change in hydration at the aqueous interface.

It is interesting to note some analogy between the effects of pentoxifylline and that of the flavonoid derivative LEW-7/S1 on the elastic modulus as well on the main transition of the DMPC bilayers. In the presence of these drugs

the bilayer rigidity is decreased and the transition was altered. Thermodynamically, a shift in the transition point must be due to the dissolution of pentoxifylline and LEW-7/S1 in lipidic phases. However, compared to the effect of LEW-7/S1, the cooperativity of the transition seems to be more altered by the presence of pentoxifylline and the vesicle radius variation shows that intermediate states of the bilayer may exist (Figs. 3b, 3c and 3d).

In conclusion, with some differences in the effect on the phase transition, the interactions of pentoxifylline, as well as those of flavonoid derivatives with lipid bilayers, contribute to the modification of their phase transition and the decrease of their elastic modulus. This may be in relation with the decrease in deformabilty and the change in surface charge and therefore with some of its pharmacological properties.

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