Convulsant agent pentylenetetrazol does not alter the structural and dynamical properties of dipalmitoylphosphatidylcholine model membranes

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ABSTRACT

Pentylenetetrazol (PTZ) is an epileptogenic agent, which is widely used in the determination of epilepsy-induced alterations and in the assessment of anticonvulsant agents in epileptic studies. Even though PTZ is suggested to induce repetitive firing of nerve fibers and shorten the refractory, its mechanism of action is only partially understood. In the literature there are discrepancies for its action mechanism. While some studies stated that primary sites of PTZ are membrane proteins, some reports indicated that PTZ acts on membrane lipids. In order to gain new insight for this we tested the possibility of interaction of PTZ with a simplified model system called dipalmitoylphosphatidylcholine (DPPC) multilamellar vesicles (MLVs) at agent concentrations (0–24 mol%) using differential scanning calorimetry (DSC), Fourier transform infrared (FTIR), electron spin resonance (ESR) and steady-state fluorescence spectroscopy. The results showed that PTZ at concentrations used (1–24 mol%), does not cause any significant change in lipid phase behavior, lipid dynamics (fluidity), lipid acyl chain flexibility (order), hydration state of the head group and/or the region near the head group of DPPC MLVs. These results clearly revealed that PTZ does not change the structural and dynamical parameters of neutral DPPC lipid vesicles and does not locate within the bilayer.

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1. Introduction

Epilepsy is one of the most common neurological disorders [1,2]. Although the condition is a well-known disorder affecting 1–2% of the population worldwide, epileptic seizures cannot be effectively controlled in at least 30% of all the cases due to the absence of definite cure for epilepsy [1].

In order to provide the improvement of therapeutic strategies with early and accurate diagnostic information, epilepsy-induced both biochemical and molecular alterations correlating with their pathology are needed to be determined. To achieve this, animal models have played important role to understand the basic physiological and behavioral changes due to epilepsy [3].

Pentylenetetrazol (PTZ)-induced epileptic animal models, which are widely used to produce PTZ threshold, kindling and acute convulsions, are accepted as good models in epilepsy research [4]. These models not only produce epileptic seizure activity, but also show seizure-induced changes similar to alterations observed in human epilepsy. Although PTZ, whose chemical structure is shown in Fig. 1, is extensively used [5–7] and some of its effects have been already described [5–8], the action mechanism of PTZ at the cellular level still is not fully understood [9–11]. The widely accepted mechanism is that PTZ displays its activity by binding to the picrotoxin binding site at GABA$_A$ receptor complex [12]. Accordingly, some binding studies revealed that PTZ also affects the function of benzodiazepine receptor [13], GABA$_A$ receptor [14], both ionotropic and metabotropic glutamate receptors [15]. Moreover, it was also shown that PTZ is not effective when injected into the neuron [7]. All these studies indicated that the epileptogenic agent PTZ acts at external site of cell membrane through binding on membrane channels and receptors. Despite these evidences there are also some reports stating that PTZ has the ability to penetrate and to interact with cell membrane through different mechanisms which may lead to the initiation of epileptic activity [5,16,17]. Previously, PTZ was suggested to be absorbed easily by membrane macromolecules [18]. Altrup et al. [17] recently reported that PTZ can incorporate with cell membrane and cause increased membrane pressure. Similarly, it was also shown that PTZ exerts its effect on potassium channel via interaction with cell membrane [16]. This corresponds to the fact that an increase of lipophilicity of PTZ elevates epileptogenic potency of the agent [19,20]. Moreover, PTZ
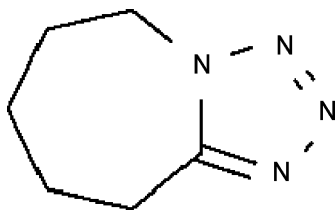
Fig. 1. Chemical structure of PTZ.

was also indicated to affect intracellularly GABA$_A$ receptor [6] and Na$^+$\K$^+$ pump [21] by penetrating into cell membrane. As a result, in spite of extensive use of PTZ for epileptogenic purposes, it is not clear that whether PTZ exerts its function through a direct interaction with membrane proteins or membrane lipids. PTZ has been previously characterized as an amphiphilic molecule, therefore; it has potential to modulate membrane lipids by altering the phase transition, fluidity and order/disorder state [17,18]. Since cell membrane is difficult to be characterized due to its complexity and constitutes of 35–60% phosphatidylcholine of all lipids [22], in the present study we used a simplified model membrane system comprised of dipalmitoylphosphatidylcholine (DPPC) multilamellar vesicles (MLVs) and widely used in biological studies [23–25]. We aimed to investigate the possibility of interaction of PTZ with PCs membrane lipids.

We investigated PTZ–DPPC MLVs interactions in terms of lipid phase behavior, order and dynamics and nature of hydrogen bonding around its polar part, using differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FTIR), electron spin resonance spectroscopy (ESR) and steady-state fluorescence spectroscopy.

2. Materials and methods

2.1. Chemicals

PTZ (1,8,9,10-tetrazabicyclo [5.3.0] deca-7,9-diene) was purchased from Sigma (St. Louis, MO, USA). Avanti Polar Lipids (Alabaster, AL) was the source of DPPC. Spin labels 5-and 16-doxyl stearic acid and the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) were obtained from Aldrich (St. Louis, MO) and Molecular Probes (Eugene, OR), respectively.

2.2. Methods

2.2.1. Solubility studies

Before starting spectroscopic experiments PTZ was dissolved (2 mg/ml) in different solvents, such as ethanol and methanol in order to determine the solvent which dissolves the PTZ with high-

est solubility. The samples were shaken for 2 h and the aliquots were filtered. The filtered samples were diluted with equal volume of same solvent and assayed spectrophotometrically at 220 nm. As seen from Fig. 2, PTZ dissolved in ethanol has higher absorbance values which indicates that ethanol is more suitable solvent to dissolve PTZ in our experiments.

2.2.2. DSC studies

A fully hydrated DPPC bilayer was used as our model membrane. MLVs were prepared in the absence and the presence of 1, 12, and 24 mol% PTZ. For the preparation of MLVs, PTZ dissolved in organic solvent mixed with DPPC dissolved in chloroform. The organic solvents were then removed under a stream of nitrogen followed by vacuum pumping overnight. The MLVs were formed by hydrating the lipids (10 mg of lipid/ml) in 10 mM sodium phosphate, pH 7.4 and vortex mixing at least 15 °C above the gel to liquid crystalline phase transition temperature of the phospholipids. The MLVs were frozen in liquid nitrogen and thawed three times in a water bath and then degassed for 30 min under vacuum. A 500 μl aliquot of the MLV suspensions was added to each of the three chamber in a Calorimetry Sciences MCDSC multi-cell differential scanning calorimeter (Calorimetry Sciences, Lindin, UT). The scans were made at 0.08 °C/min. Only heating curves are presented. Cooling curves were essentially identical.

The molar heat capacities and calorimetric enthalpies (ΔH_c) were computed using CpCalc software. The cooperativity unit (CU), which is a measure of the mean number of lipid molecules undergoing transition, was calculated as in Bhattacharya and Dileep [26] using the following approach:

$$CU = \frac{\Delta H_{vH}}{\Delta H_c}$$

where ΔH_{vH} is the van't Hoff enthalpy and ΔH_c is the calorimetric enthalpy. Van't Hoff enthalpy was calculated using the following equation:

$$\Delta H_{vH} = \frac{6.9 T_m^2}{\Delta T_{1/2}}$$

where $\Delta T_{1/2}$ is the full width at half-maximum of the calorimetric thermogram and T_m is the phase transition temperature.

2.2.3. FTIR studies

Phospholipid MLVs were prepared according to the procedure reported in our previous studies [23,27,28]. 5 mg DPPC was dissolved in chloroform and the solution was subjected to a stream of nitrogen to remove excess chloroform followed by the vacuum drying for 2 h. The thin films of lipid were then hydrated by adding 25 μl of phosphate buffer, pH 7.4. MLV were formed by vortexing
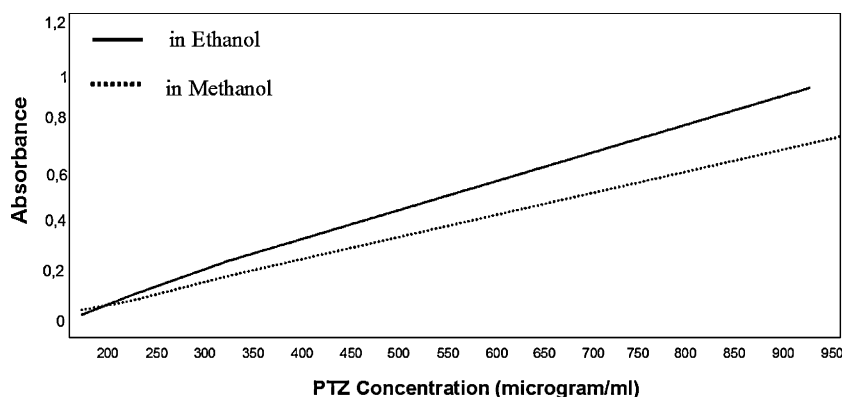

Fig. 2. UV absorbance values for different concentration of PTZ dissolved in methanol and ethanol.

the mixture for 30 min at least 15 °C above transition temperature of DPPC. In order to prepare PTZ containing MLV, the required amount of PTZ from stock solution was initially placed inside the sample tube. The excess of methanol was removed by a stream of nitrogen, DPPC in chloroform was added and the preparation of MLV was as described above. For FTIR measurement, 20 µl of liposomes were placed between CaF$_2$ windows with 12 µm sample thickness. The spectra were recorded using a PerkinElmer Spectrum One spectrometer equipped with a DTGS detector in the temperature range of 25–60 °C. The temperature was controlled digitally by Graseby Specac controller unit. The samples were incubated for 5 min at each temperature before acquisition of a spectrum. The interferograms were averaged for 100 scans at 2 cm^{-1} resolution. The spectral analysis was performed using Spectrum v5.0.1 software. To improve resolution of the infrared bands, the water bands were subtracted using same program. The band positions were measured from the center of weight.

2.2.4. ESR studies

MLV containing (1 mol%) 5- or 16-doxyl stearic acid in DPPC with 0, 1, 12 and 24 mol% PTZ were prepared by the same procedure outlined above except that 20 mM sodium phosphate (pH 7.5) was used to hydrate the lipids. Spectra were recorded on a Bruker ESP 300 X-band ESR spectrometer operating at 9.2 GHz. The following spectral parameters were used: microwave power, 200 mw; field strength, 3924 G; sweep width, 80–100 G; sweep time, 160–200 s; time constant, 500 ms; modulation amplitude, 1.0–2.0 G; and dataset, 1000–2000 points. The temperature samples (25 µl) placed in a capillary glass tube was regulated 50 °C, corresponding to the liquid crystalline phase, by a Love Controls 1600 Series temperature controller (Michigan City, IN).

The order parameters, S, were calculated from spectra in the upper part of the acyl chain (5-doxyl) according to the equation:

$$S = \frac{A_{\parallel} - A_{\perp} - C}{A_{\parallel} + 2A_{\perp} + 2C} 1.66$$

where $A_{\parallel}$ and $A_{\perp}$ are the apparent parallel and perpendicular hyperfine splittings, the constant $C = 1.4 - 0.053\,(A_{\parallel} - A_{\perp})$ is an empirical correction for the difference between the true and apparent values of $A_{\perp}$, and the factor 1.66 is a solvent polarity correction factor [29]. The correlation time τ_c was calculated from spectra in the lower part of the acyl chain (16-doxyl) by

$$\tau_c = 6.5 \times 10^{-10} W_0 \left[\left(\frac{h_0}{h_{-1}} \right)^{1/2} - 1 \right]$$

where $K = 6.5 \times 10^{-10}$ s G^{-1} is a constant depending on microwave frequency and the magnetic anisotropy of the spin label, W_0 is the peak-to-peak width of the central line, and h_0/h_{-1} is the ratio of the heights of the central and high field lines, respectively.

2.2.5. Steady-state fluorescence spectroscopy studies

For steady-state fluorescence spectroscopy, DPH was chosen as a probe. 2.5 mM MLV of DPPC with DPH at the ratio of 1:200 and containing different concentration of convulsants were formed employing the procedure mentioned above. The spectra were recorded on a PerkinElmer MPF-66 fluorescence spectrometer. The excitation and emission wavelength for DPH was 351 nm and 430 nm, respectively. The temperature range was varied in the range 10–60 °C and the samples were incubated for 5 min at each temperature before scanning.

The polarization parameter was calculated by

$$P = \frac{I_{vv} - G I_{vh}}{I_{vv} + G I_{vh}}$$

where I_{vv} and I_{vh} represent the intensity of excitation polarizer vertically oriented and emission polarizer vertically and horizontally oriented, respectively, components of emitted light when the excitation light is vertically polarized. The correction factor $G = I_{hv}/I_{hh}$ (horizontally polarized excitation beam) compensates for the differential in the monochromator's transmission efficiency for vertically and horizontally polarized light [30].

2.2.6. Statistical analysis

In the figures and tables the mean of at least three experiments was plotted and calculated together with the standard error of mean. Statistical significance was assessed using Mann–Whitney nonparametric test. Significant differences was statistically considered at the level of $p \leq 0.05$.

3. Results and discussion

In the literature, it has been suggested that most drugs exert their effects through incorporation or penetration into cell membranes by modulating lipid bilayer properties, which in turn, may also affect membrane proteins. This lipid mediated mechanism includes the change in lipid dynamics [31], localization at the membrane interface to modulate dipole potentials and modification of forces between headgroup and hydrocarbon domain. The detection of these kinds of molecular interactions is achieved by spectroscopic techniques since they are not only sensitive but also easy to use [32]. Since PTZ has been suggested to be amphiphilic and soluble in oil [33], it was expected to interact with membrane lipids and diffuse into the cell membrane [17,27,28]. Therefore, in this current study we tested the possibility of interaction of PTZ with DPPC MLVs by investigating structural and dynamical spectral parameters. Specifically, in the present study we examined, for the first time, the effects of different concentrations of PTZ on lipid dynamics (fluidity), lipid acyl chain flexibility (ordering), hydration state and phase behavior properties of DPPC MLVs using FTIR, DSC, ESR and steady-state fluorescence spectroscopy.

DSC was used to investigate the effect of PTZ on the phase behavior of DPPC MLV as used in the other study [34]. Fig. 3a shows the thermograms of DPPC MLVs in the absence and presence of different concentrations of PTZ. In the absence of PTZ, multilamellar liposome of DPPC demonstrates a pretransition from the gel phase to ripple phase at 35 °C and a main transition from the ripple phase to liquid crystalline phase at 41 °C. As seen from the figure, with the presence of the PTZ, the pretransition was broadened implying that there is perturbation of the ripple phase. This would suggest that there might be slight influence of PTZ at the surface of the phospholipid vesicles. As an amphiphilic molecule PTZ has both polar and non-polar moieties and may accumulate at the surface of the bilayer [35]. However, since the pretransition is highly sensitive to the presence of other molecules in the polar region of the phospholipids, the broadening of pretransition with the presence of PTZ cannot be attributed to any specific molecular changes as previously reported [36]. On the other hand, the presence of PTZ does not change the shape of main transition curve and also the main transition temperature as observed from the figure. In order to further elaborate the effects of PTZ on DPPC, the thermograms were analyzed in detail to yield enthalpy and cooperativity. The values of qualitative assessments are plotted against different PTZ concentrations in Fig. 3b and c, respectively. As can be observed from both figures, PTZ changes neither the enthalpy nor the cooperativity of the gel to liquid crystalline transition for DPPC MLVs. However, since DSC method detects the absorbed heat by the entire bilayer, it gives only bulk information on the thermotropic properties, but not the detailed dynamic behavior of different regions in the bilayer [37].
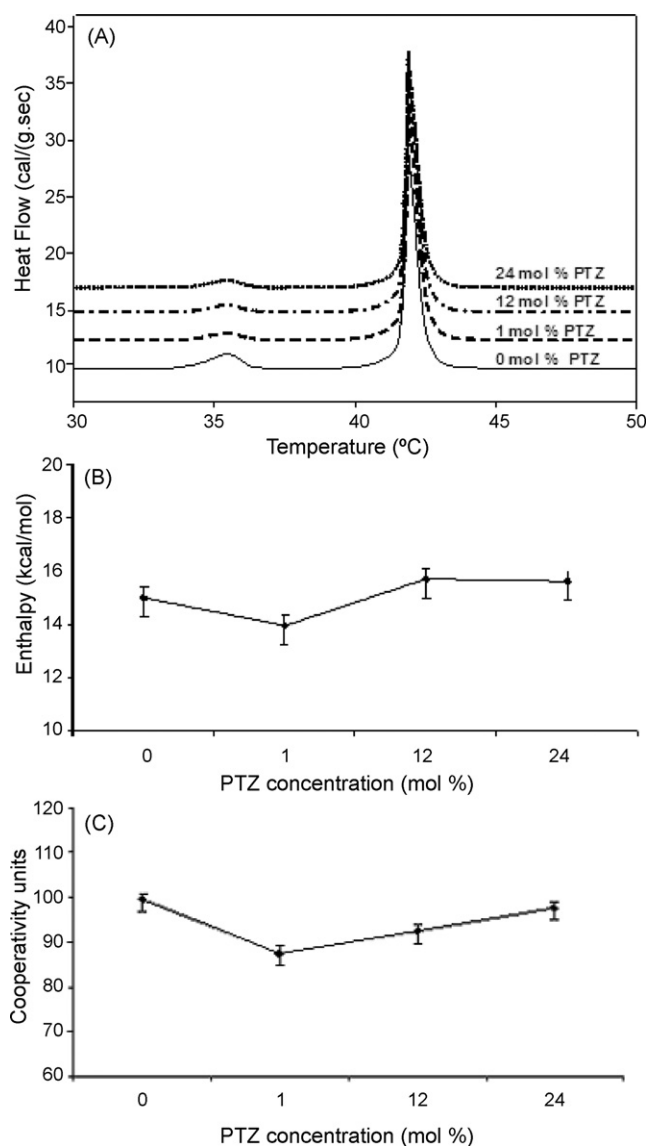
Fig. 4. Temperature dependence of the frequency changes of the CH_2 asymmetric stretching modes of DPPC MLVs in the absence and presence of PTZ in varying concentrations.

Fig. 3. (A) DSC thermograms of DPPC MLVs in the absence and presence of different concentrations of PTZ, (B) the variation of the enthalpy of the main transition as a function of PTZ concentration and (C) the variation of the cooperativity of the transition of DPPC MLVs as a function of PTZ concentration.

The information about the effect of PTZ on the phase transition behavior of DPPC MLVs was also obtained using FTIR spectroscopy by monitoring the frequency variations of the C–H stretching modes [34]. FTIR spectroscopy is a non-perturbing technique and directly monitors molecular vibrations belonging to different functional groups [34,38,39]. Before the data analysis, the water bands were subtracted from the sample spectrum since water absorption bands at 3050–2800 cm^{-1} and 1700–1500 cm^{-1} strongly overlap with the bands arising from different functional groups belonging to lipids. Fig. 4 shows the variation of the CH_2 asymmetric stretching frequency as a function of temperature. The abrupt increase in the frequency values at 41 °C corresponds to main phase transition. As seen from the figure with the addition of PTZ, at even high concentrations, neither the shape of the phase transition curve nor the main phase transition temperature did not change as in agreement with DSC results. Any possible PTZ-induced changes in the shape of main phase transition provide further information on the hydrophobic location of the agent in the bilayer matrix. Any alterations in the shape of the phase transition, such as broadening would be expected if the agent is localized in the outer hydrophobic
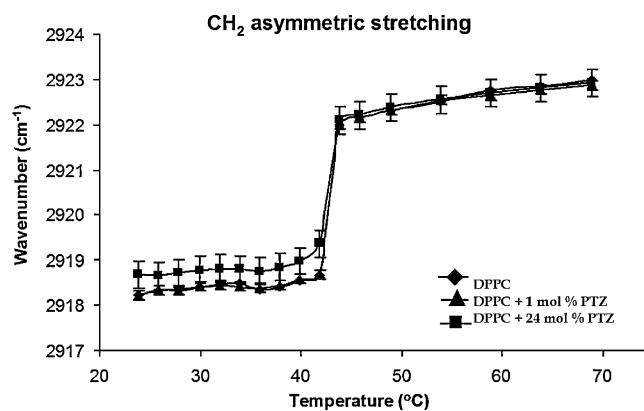
cooperative zone of the bilayer, i.e. in the region of C2–C8 carbon atoms of the acyl chain [40–42]. If the agent enters the hydrophobic part of membrane, it will disturb the van der Waals interactions between the hydrophobic acyl chains, e.g., it will disturb the tight packing between the hydrocarbon chains leading to the broadening in the phase transition curve [23,43,44]. In short, FTIR and DSC studies revealed that PTZ does not penetrate into the bilayer and does not localize in the bilayer structure of DPPC.

FTIR spectroscopy was further used to obtain information about lipid order, dynamics and hydration state of the head group and glycerol backbone close to aqueous interface. Fig. 5 shows representative FTIR spectra of DPPC MLVs in the absence and presence of PTZ at low and high concentrations in the liquid crystalline phase (50 °C), in the region of 3050-2800 cm^{-1}. The spectra were normalized with respect to the CH_2 asymmetric stretching mode to demonstrate visually the comparative difference in the frequency and bandwidth values of the specific bands of lipid. However the original spectra were used for the precise determination of variations in the frequency and bandwidth values. The infrared spectral parameters such as the frequencies and bandwidths are sensitive to the structural and dynamical properties of membrane lipid molecules [40,41,45]. The changes in acyl chain flexibility, e.g., order–disorder state of DPPC MLVs due to the presence of PTZ were examined by analyzing of the CH_2 asymmetric stretching mode frequency [23,46–48]. The wavenumber values below the main phase transition temperature for DPPC is characteristic of conformationally ordered acyl chains with a high content of trans isomers, whereas, the values at temperatures above the main phase transition is of conformationally disordered acyl chains with a high content of gauche conformers [49]. As seen from the figure, the frequency values of this band did not vary significantly with the addition of PTZ in the gel and liquid crystalline phases. This implies that the number of trans/gauche conformers did not change, which indicates that PTZ does not cause any alteration in order/disorder state of DPPC MLVs in both phases [27,41,47].

These results were further supported in the liquid crystalline phase by ESR spectroscopy using the spin label 5-doxyl stearic acid that gives information about membrane order in the upper part of the lipid chain. Fig. 6 shows the ESR spectra of DPPC MLVs labeled with 1 mol% 5-doxyl stearic acid at 50 °C which monitors to the liquid crystalline phase of DPPC MLVs in the absence and presence of PTZ. Order parameters were calculated from the ESR spectra and were listed in Table 1. As seen from the table, order parameters did not alter with the presence of PTZ.

Membrane fluidity is an important parameter for the proper functioning of cell membranes, which in turn, influences cellular
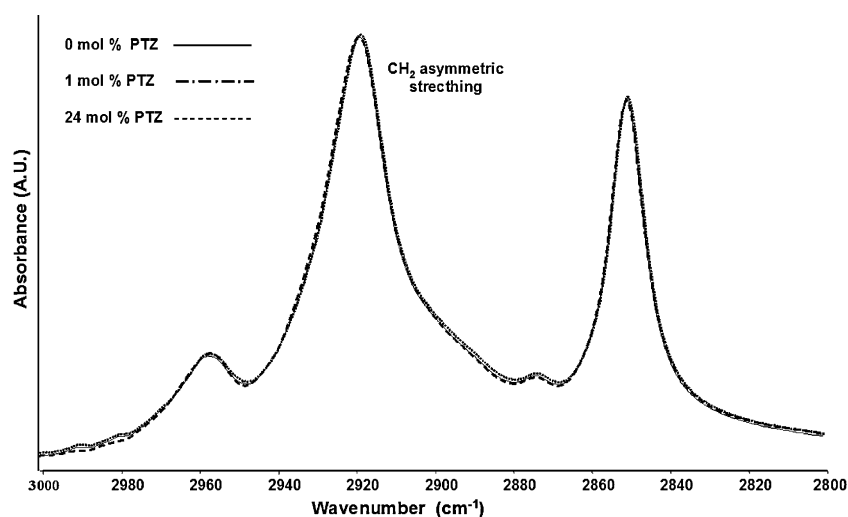
Fig. 5. Infrared spectra of DPPC liposomes in the absence and the presence of PTZ at 50 °C in the 3050–2800 cm^{-1} region (the spectra were normalized with respect to the CH$_2$ asymmetric stretching mode at 2925 cm^{-1}).

Table 1
Order parameter and correlation times for, respectively, 5- and 16-doxyl stearic acid intercalated into DPPC MLVs containing different concentrations of PTZ at a temperature corresponding to the liquid crystalline phase.

Sample	Membrane order parameter (S)		Correlation time (τ_c)	
	25 °C	50 °C	25 °C	50 °C
DPPC	0.525 ± 0.021	0.524 ± 0.033	4.90×10^{-10} s^{-1}	4.92×10^{-10} s^{-1}
1 mol% ptz	0.533 ± 0.002	0.532 ± 0.019	4.80×10^{-10} s^{-1}	4.81×10^{-10} s^{-1}
12 mol% ptz	0.528 ± 0.008	0.526 ± 0.021	4.74×10^{-10} s^{-1}	4.76×10^{-10} s^{-1}
24 mol% ptz	0.529 ± 0.013	0.532 ± 0.061	4.79×10^{-10} s^{-1}	4.81×10^{-10} s^{-1}

processes and disease states [50–52], therefore; we investigated the effect of PTZ on dynamics (fluidity) of DPPC MLVs by FTIR, ESR and steady-state fluorescence spectroscopy. In the FTIR experiments, the bandwidth values of the CH$_2$ asymmetric stretching band were studied since the variations in the bandwidth give information about dynamics of the system [23,46]. As seen from Fig. 5, the CH$_2$ asymmetric stretching mode was sufficiently separated after water subtraction; therefore, it was not necessary to used band deconvolution or fit routines to evaluate their bandwidths for relative measurements for this study as suggested by the others [51,52]. The bandwidth values were measured at 75% of height of the peak as reported in other studies [23,41]. Table 2 represents the changes in the bandwidth values with the presence of PTZ at two different temperatures which correspond to gel and liquid crystalline phases of DPPC MLVs. As observed from the table, PTZ did not exert significant effect on the fluidity of DPPC MLVs in both gel and crystalline phases.

In ESR spectroscopic studies we used the spin label 16-doxyl stearic acid that provides information on the rate of acyl chain motion in the lower portion of the chain towards the center of the membrane [53–55]. Fig. 6b displays the ESR spectra for DPPC MLVs labeled with 16-doxyl stearic acid at 50 °C with different concentrations of PTZ. The correlation time calculated from the spectra is listed in Table 1. As observed from the table, membrane dynamics

did not alter significantly in the presence of PTZ. This conclusion is in agreement with our FTIR results.

Polarization values for DPH incorporated into DPPC MLV were also measured by steady-state fluorescence spectroscopy to determine how PTZ affects fluidity in the membrane [50]. We applied DPH fluorescent probe which monitors the hydrophobic part of membrane [56]. The polarization parameter for DPPC MLVs in the absence and presence of PTZ is plotted as a function of temperature in Fig. 7. It has been suggested that DPH is positioned in the middle region of the lipid hydrocarbon chains, in a parallel alignment to the chains [57]. The aromatic structure (Fig. 1) of PTZ with less rotational freedom may not cause significant changes in the vicinity of the probe. For that reason, as can be observed from the figure, the polarization values of DPPC did not change significantly in the presence of PTZ which indicates that PTZ has no significant effect to alter fluidity (dynamics) of DPPC MLVs supporting ESR and FTIR results.

Although we have found that PTZ does not penetrate into the hydrophobic part of the DPPC membrane as an amphiphilic molecule it may have an interaction with the head group region without penetration in the hydrocarbon region [58]. For that reason, we studied the interaction of PTZ with the glycerol backbone near the head group of phospholipids in interfacial region and with the head group by FTIR spectroscopy by monitoring both the frequency changes of the C=O ester stretching (1730 cm^{-1}) and the PO$_2^-$ asymmetric double stretching modes (1230 cm^{-1}), respectively. According to the empirical rules, a decrease in these frequencies indicates an increase in the strength of hydrogen bonding whereas an increase in the frequency corresponds to dehydration [23,27,59]. The temperature dependent frequency change of these bands of DPPC MLVs containing different mole fractions of PTZ are shown in Figs. 8 and 9. As seen from the figures, both in the gel and liquid crystalline phases, the frequency values of these

Table 2
The bandwidth values of the CH$_2$ asymmetric stretching mode of DPPC in the absence and presence of PTZ at 35 °C and 50 °C.

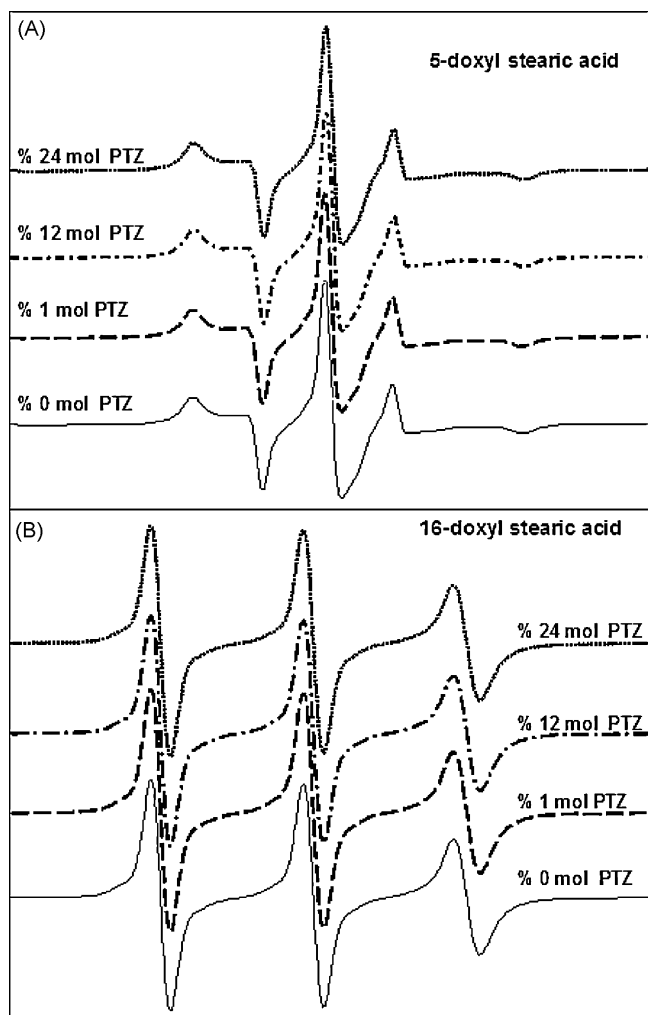| Sample	35 °C	50 °C
DPPC	22.43 ± 0.01 cm^{-1}	25.25 ± 1.02 cm^{-1}
1% mol PTZ + DPPC	22.72 ± 0.07 cm^{-1}	25.24 ± 0.04 cm^{-1}
24% mol PTZ + DPPC	23.19 ± 0.03 cm^{-1}	25.98 ± 2.05 cm^{-1}

Fig. 6. ESR spectra of stearic acid spin label in DPPC MLVs containing different concentrations of PTZ: (A) 5-doxyl stearic acid and (B) 16-doxyl stearic acid.

Fig. 8. Temperature dependence of the frequency changes of the C=O stretching mode of DPPC MLVs in the absence and presence of PTZ in varying concentrations.

bands have not altered with the presence of PTZ. The solubility of PTZ in water suggests that it locates along with carbonyl groups and first C atoms of the acyl chains of phosphatidylcholines, forming hydrogen bonds with two carbonyl esters [60]. However, both PTZ and PCs have only hydrogen bond acceptor groups not hydrogen bond donor groups, therefore; it does not seem to be possible to form hydrogen bond between these molecules, as revealed by FTIR results.

Besides the solubility of PTZ in water, its lipophilicity level is really important to interact with membrane lipids in order to show its action. The lipophilicity and effectiveness of PTZ on mem-
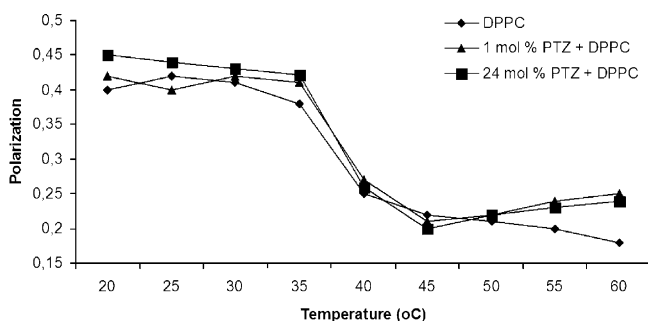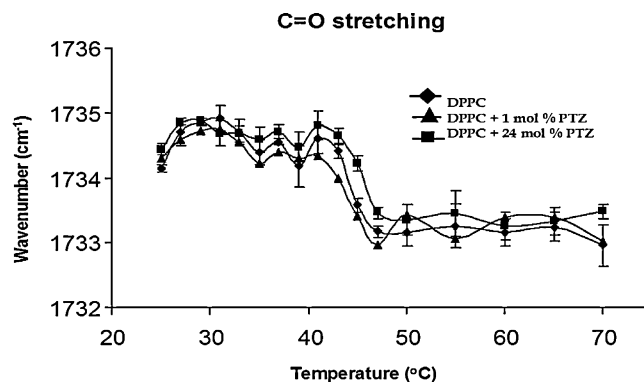

Fig. 7. Temperature dependent variations of polarization values for DPH in DPPC MLVs containing different concentrations of PTZ.

brane structure is expressed by its partition coefficient, which was measured by using isotropic two-phase solvent systems, such as octanol–water systems [61]. Since this system is independent of ester and amide groups present in phospholipids, it may not provide good models for PTZ partitioning into membranes. For that reason, the application of more structural-based biophysical methods is needed to understand the interaction of PTZ with membranes. In the current study, it is interesting to see that PTZ although it is a lipophilic molecule [33], does not change lipid phase transition profile, lipid dynamics and structural parameters such as lipid order, the strength of hydrogen bonding around glycerol backbone and lipid head group. We are aware of that several compounds, which are less lipophilic than PTZ, had strong interaction with neutral lipids both in the gel and the liquid crystalline phase [23]. On the other hand, estrogen having higher lipophilic character than PTZ did not alter the shape of the phase transition curve, which implies that it does not penetrate into hydrophobic zone of bilayer [62]. In addition, it has been demonstrated that two agents which have similar lipophilicity show different penetration and action profile on cell membranes [63,64]. Thus, lipophilic character does not truly reflect the behavior of agents interacting with membrane lipids. In other words, being lipid soluble like PTZ is not a prerequisite for an agent to interact with membranes and other factors are required for the interaction, as suggested by Goldstein [35]. Therefore, there is always possibility of interacting of PTZ with other types of lipids in the membrane. As we found in our preliminary studies (data not shown) PTZ may have different solubilities and effects on membranes with different lipid composition.

It should be kept in mind that electrostatic interactions have significant impacts on the accumulation and permeation of an
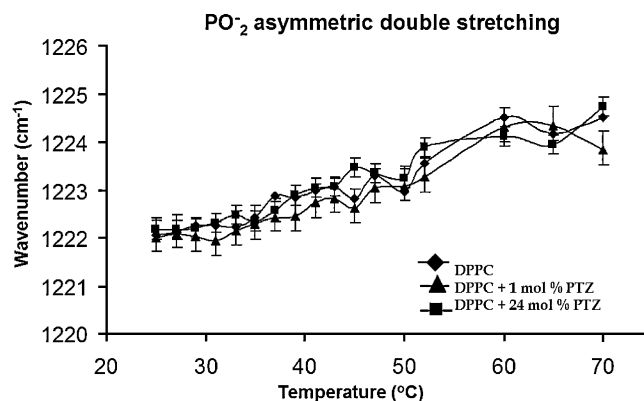

Fig. 9. Temperature dependence of the frequency of the PO_2^- asymmetric double bond stretching mode of DPPC MLVs in the absence and presence of PTZ in varying concentrations.

agent within the membranes [65]. In our case the contributions of electrostatic interactions between neutral molecules such as DPPC MLVs and PTZ might be very small and therefore, it can be neglected. Indeed, our preliminary studies on the interaction of PTZ with charged lipids indicated very strong effect on the spectral and calorimetric parameters of interest both in the gel and liquid crystalline phase (data not shown).

In some of the previous studies membrane lipids were assumed to be primary site of PTZ to develop epileptic activity [6,16,21]. In these studies, the effects of PTZ on the action of GABA$_A$ receptor [6], potassium channel [16] and Na$^+$\K$^+$ pump [21] were investigated using the *Xenopus leavis* oocytes, which include pool of different types of membrane lipids. Subsequently, PTZ was suggested to modify membrane lipids to alter membrane protein functions, indirectly. Contradictory to these studies, the current study clearly showed that the passage of PTZ through DPPC MLVs does not seem to be possible as it does not cause any perturbation in structural and dynamical properties of DPPC MLVs. Beside this, in the present study, PTZ was also found to be disable for the stabilization of this model membrane structure by ESR and FTIR results, which does not support the previous findings. The earlier findings mentioned above [6,16,21] have been performed not on artificial membrane composed of one or two type of lipid as in our system, instead on cell membranes. Due to absence of the typical cell membrane features in our system such as a variety of lipid composition and ionic current, the interactions of PTZ with model membrane might not exactly reflect all the aspects of cellular process [66]. For example, Wunderlich et al. [67] discussed that phase state of lipids and ionic current across the membrane are strongly affected by each other. The changes in the phase state of lipid by fluctuations in ion current cause alterations in membrane permeability to agents. Considering the existence of constant and regulated ionic transport across the cell membrane, PTZ may show different behavior such as partition into cell membranes which compose of different kind of lipids and proteins than artificial specific model membrane system composed of neutral DPPC as in the current study. Furthermore, it has been showed that paclitaxel penetrated differently in artificial monolayers than in monolayers composed of lipid extract from tissues [68]. These types of experiments have provided evidence that this discrepancy may be due to type of lipids present in neutral model membranes and in cell membranes. The previous studies, which reports that PTZ is able to interact with membrane lipids, did not differentiate which lipid is predominant in the interaction [6,16,21]. However, our current study clearly shows that PCs are not the effective lipids in the interaction. According to our unpublished data, PTZ can interact with sphingomyelin (SM) and phosphatidylethanolamine (PE), both of which have ability to form both intra and intermolecular hydrogen bonding capacity with PTZ due to having hydrogen bond donor groups [60]. As a result, it is obvious that the variation in chemical structure of lipid molecules may cause the differentiation binding capacity of PTZ to membrane lipids. Therefore, there might be a lack of interaction between PTZ and PC molecules as shown by our results. Accordingly, Noda et al. [69] found that PCs pretreatment did not show significant effect on the threshold dose of PTZ for inducing epileptic seizures in Mongolian gerbils. In the other study, Altrup et al. [17] suggested that PTZ can be incorporated into model membrane lipids which may lead to the disturbance of membrane fluidity. However, according to our ESR, FTIR and steady-state fluorescence data, PTZ does not affect fluidity of DPPC MLVs. The discrepancy between this finding and our results could be originating from the differences in experimental designs. Altrup and co-workers have performed their experiments on monolayers with saline sub phase, which may cause ionizable groups surround PTZ and/or lipid molecules. This alteration in the ionic environment of system may trigger the electrostatic interactions between PTZ and lipid molecules. Moreover, when the fatty acyl chains of lipid molecules exist in an ordered state, PCs form a more stable bilayer structure in comparison to monolayer structure. For this reason, more energy is needed for the partition of PTZ into PC's acyl chains in bilayer form, therefore; this would make it energetically unfavorable as pointed out in the current study.

4. Conclusion

There are some studies stating that PTZ has the ability to interact with membrane lipids without reporting which membrane lipid is predominant in this interaction. In order to gain insight, we have tested the possible interaction of PTZ with DPPC MLVs by studying its effect on phase behavior, lipid order, nature of hydrogen bonding and lipid dynamics. Our results revealed that PTZ does not alter the structural and dynamical properties of DPPC. This study indicated that PCs, which are found with a high percentage in cell membrane, do not play role in the interaction of PTZ with membrane lipids. We have proposed some explanations for the lack of interaction between and DPPC such as chemical and hydrogen bonding properties of both molecules.

According our preliminary data (DSC and FTIR studies) PTZ is able to interact with other types of membrane lipids such as SM and PE, Thus, all these findings showed that the composition and species of membrane lipids play crucial role in PTZ action on cell membrane. It is especially necessary to determine the effects of PTZ on structural and functional properties of different types of membrane lipids in order to explain the action mechanism of PTZ on membranes. These studies are under way.

Since it can be possible to alter membrane responsiveness and sensitivity of PTZ by changing membrane lipid composition, this type of biophysical investigations are further important in order to suggest promising approaches for the treatment of epilepsy.

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