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# The alterations of lipid bilayer fluidity induced by newly synthesized phenothiazine derivative

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**Abstract**

Using fluorescence spectroscopy, calorimetry and ESR the interactions of the phenothiazine derivative 2-trifluoromethyl-10-(4-[methylsulfonylamid]buthyl)-phenothiazine (FPhMS) with lipids were studied. Calorimetry showed biphasic effect of FPhMS on main phase transition of DPPC. At molar ratios up to 0.06 drug induced decrease of transition temperature and enthalpy, while at higher concentrations it caused subsequent increase of these parameters. For all concentrations studied we observed gradual broadening of transition peaks. Fluorescence polarization revealed that in FPhMS/lipid mixtures, order in bilayers is decreased in the gel state and increased in the liquid crystalline state. ESR experiment showed that at molar ratio of 0.06, FPhMS reduces the mobility of spin probes located in both polar and hydrophobic regions. Comparing observed effects with those reported for cholesterol/lipid mixtures, we conclude that at higher concentrations FPhMS presumably induces a new mode of bilayer packing. This structure is less co-operative than an unperturbed bilayer, but locally the mobility of lipid molecules is decreased.
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*Keywords:* Phenothiazine derivative; Lipid bilayer fluidity; MDR reversal; DSC; Fluorescence spectroscopy; ESR

*Abbreviations:* MDR, multidrug resistance; DSC, differential scanning calorimetry; ESR, electron spin resonance; *P*-gp*P*, glycoprotein; MRP1, multidrug resistance-related protein; CPZ, chlorpromazine; TFP, trifluoperazine; FPhMS, 2-trifluoromethyl-10-(4-[methylsulfonylamid]buthyl)-phenothiazine; EYPC, egg yolk phosphatidylcholine; DPPC, 1,2-dipalmitoyl-*n*-glycero-3-phosphatidylcholine; DMPC, 1,2-dimyristoyl-*n*-glycero-3-phosphatidylcholine; DMSO, dimethyl sulfoxide; DPH, 1,6-diphenyl-1,3,5-hexatriene; 5DSA, 5-doxylstearic acid; 7DSM, 7-doxylstearic-methyl ester; 16DSA, 16-doxylstearic acid.

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## 1. Introduction

Development of the mechanism of active, outward drug transport is a common strategy used by cancer cells to defeat themselves against chemotherapeutic agents. For this reason, the circumvention of multidrug resistance (MDR) seems to be one of the conditions necessary for successful chemotherapy. The active drug transport is performed by transport proteins, members of the ATP-binding-cassette (ABC) protein superfamily which are overexpressed in membranes of the resistant cancer cells (reviewed in [1]). P-glycoprotein (P-gp) and multidrug resistance-related protein (MRP1) are presumably the best known and extensively studied examples of such proteins. Most of the transport proteins share one common feature—the range of their substrates is usually very wide [2,3]. Taking into account the role played by these proteins in resistant cells, it is generally believed that the resistance depends on the balance between inward (passive) and outward (active) fluxes of cytotoxic substances [4]. In sensitive cells the efflux is smaller than influx and intracellular accumulation of drugs occurs. Drug accumulation cannot take place in resistant cells because the outward drug transport prevails over drug influx. To obtain the accumulation of anticancer drugs in resistant cells either the permeability of the cell membrane must be increased and/or the efficiency of efflux pumps must be reduced. The direct relation between membrane permeability and physical state of lipids is obvious, but also the activity of transporters should be modulated by the lipid composition [5] and/or biophysical properties (e.g. fluidity) of membrane [6]. For the above reasons, it seems that changes in membrane lipid phase induced by the presence of MDR modulators could be directly or indirectly involved in drug accumulation recovery.

Phenothiazine-related compounds are used as tranquilizers and recently are also considered as potent modulators of multidrug resistance [7]. The most commonly studied phenothiazines—chlorpromazine (CPZ) and trifluoperazine (TFP)—interact with cellular membranes and model lipid bilayers, and modify their biophysical properties. In erythrocytes chlorpromazine induces an increase in membrane thickness [8], transbilayer lipid redistribution [9,10] and shape changes [11,12]. Partition of the drug into the erythrocyte membrane causes its stabilization [13], presumably due to induction of direct binding of membrane skeleton proteins to the lipid bilayer. The experiments performed on erythrocytes allowed also to conclude that CPZ interacts with phosphatidylserine more strongly than with other membrane lipids [11]. This is in keeping with the results of experiments performed on model membranes [14], however, the ability of the drugs to interact with different lipid classes depends also on the type of phenothiazine. After incorporation into the model lipid bilayers, phenothiazine-related compounds are presumably located at polar/apolar interface of the single lipid leaflet [15–17]. Chlorpromazine induces also the dramatic surface area increase of the monolayers formed from acidic phospholipids [18], while the effects exerted on neutral phospholipid monolayers are weaker. The influence of a wide group of MDR modulators on the biophysical properties of liposomes was studied by Driori et al. [19]. Among these modulators both CPZ and TFP were found to increase membrane permeability, but unlike other chemosensitizers they reduced the fluidity of lipid bilayers.

In our previous work [17] we have shown that trifluoperazine partitions into lipid bilayers and in zwitterionic lipids induces phase separation in the gel state. In this present work, we used the newly synthesized trifluoperazine analog: 2-trifluoromethyl-10-(4-[methylsulfonylamid]buthyl)-phenothiazine (FPhMS) to further study the mechanism of interaction between phenothiazine-related compounds and bilayers composed of zwitterionic lipids. FPhMS possesses the structural features that are believed to be important for the phenothiazines' anti-MDR activity. It is lipid soluble [20] but amphipatic [21], its phenothiazine ring is substituted with $-CF_3$ [22], and its acyl chain connecting ring system with an $-NHSO_2CH_3$ group is four carbon atoms long [23]. To characterize the interaction between FPhMS and lipid bilayers and to describe the alteration of bilayer properties induced by this compound, we have employed several experimental techniques including fluorescence spectroscopy, differential scan-

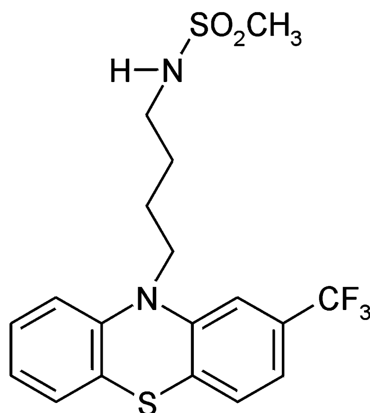


Fig. 1. Chemical structure of 2-trifluoromethyl-10-(4-[methylsulfonylamid]buthyl)-phenothiazine (FPhMS).

ning calorimetry and electron spin resonance.

## 2. Materials and methods

L-α-phosphatidylcholine (EYPC) from egg yolk was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). 1,2-Dipalmitoyl-*n*-glycero-3-phosphatidylcholine (DPPC) and 1,2-dimyristoyl-*n*-glycero-3-phosphatidylcholine (DMPC) were from Sigma (St. Louis, MO, USA). Lipids were used without further purification. 1,6-Diphenyl-1,3,5-hexatriene (DPH) and spin probes: 5-doxylstearic acid (5DSA), 7-doxylstearic-methyl ester (7DSM) and 16-doxylstearic acid (16DSA) were purchased from Sigma (St. Louis, MO, USA). TEMPO-palmitate (TP) was produced at the University of Łódź (Poland). 2-Trifluoromethyl-10-(4[methylsulfonylamid]buthyl)-phenothiazine (FPhMS) was synthesized as described by Motohashi et al. [7]. Its chemical structure is shown in Fig. 1. Since FPhMS was almost not soluble in water for experiments as DMSO solutions were used. All other chemicals used in experiments were of analytical grade.

### 2.1. Fluorescence polarization measurements

Unilamellar EYPC, DPPC or DMPC liposomes were obtained by sonication of 2 mM phospholipid suspensions in 1/15 M Michaelis phosphate buffer (pH 7.4) using UP 200s sonicator (Dr Hilscher, GmbH, Berlin, Germany). DPH stock solution (1 mM) was prepared in tetrahydrofuran. Stock solution of FPhMS (5 mM) was prepared in DMSO. Liposomes were incubated with fluorescent probe in darkness for 30 min at room temperature. Then, phenothiazine derivative was added and liposomes were incubated for 20 min (darkness, room temperature). In all experiments the final phospholipid concentration was 200 μM. The concentration of DPH was 5 μM. The FPhMS concentration in samples was 5–75 μM in concentration-dependence experiments and 100 μM for gel–liquid crystalline transition temperature ($T_m$) determination. The measurements below $T_m$ were carried out at 12 °C for DMPC ($T_m$=23 °C) and 23 °C for DPPC ($T_m$=41.5 °C). The experiments above $T_m$ were carried out at 40 °C for DMPC and 50 °C for DPPC.

Fluorescence measurements were carried out with LS 50B spectrofluorimeter (Perkin–Elmer Ltd., Beaconsfield, UK) equipped with a Xenon lamp using emission and excitation slits of 5 nm. Temperature was controlled by water-circulating bath and the actual temperature was measured directly in the sample cuvette using a platinum thermometer. The excitation wavelength was 380 nm and emission wavelength was 450 nm. Data were processed with FLDM Perkin–Elmer software.

The apparent gel to liquid-crystalline phase transition temperature was determined in DPPC liposomes. The $T_m$-value was calculated from the midpoint of the breaks related to the temperature-dependent DPH polarization degree values.

### 2.2. Calorimetric measurements

For each calorimetric sample 2 mg of DPPC were dissolved in 3.5 mM stock solution of FPhMS in organic solvents (methanol/chloroform, 1:1 v/v). The amount of FPhMS stock solution was chosen for each sample to obtain the desired drug/lipid molar ratio. The solvent was removed by a stream of nitrogen and the residual solvent was removed under vacuum for at least 4 h. Samples were hydrated by 20 ml of 20 mM Tris–HCl buffer (150 mM NaCl, 0.5 mM EDTA, pH=7.4).

Hydrated mixtures were heated to 51 °C (approx. 10 °C higher than the main phase transition temperature of DPPC) and vortexed until homogeneous dispersion was obtained. Completed samples were sealed in aluminum pans and scanned at a rate of 1.25 °C/min. Calorimetric measurements were performed using a Rigaku calorimeter, which was partially rebuilt in our laboratory. The thermograms were stored on hard disk and analyzed off-line using software developed in our laboratory.

### 2.3. Electron spin resonance spectroscopy

Stock solutions of all spin probes (5DSA, 7DSM, 16DSA, and TP) were prepared in ethanol (10 mM). The spin label/lipid molar ratio was 1:100. An appropriate amount of stock solution was dried on the tube walls. Afterwards, the suspension of DPPC liposomes in 20 mM Tris–HCl buffer (150 mM NaCl, 0.5 mM EDTA, pH= 7.4) was added to the tube. The mixture was mechanically shaken. After 20 min the spin probes were incorporated into lipid bilayers and the liposomes were sufficiently labeled for the ESR experiment.

All spectra were recorded at room temperature using a standard SE/X-28 electron spin resonance spectrometer (manufactured by the Technical University of Wrocław) operating in the X-band. In order to estimate the mobility of the spin probe, during its isotropic weakly restricted rotational motion, the tumbling correlation time ($\tau_c$) was calculated using Kivelson's method [24]:

$$\tau_c = 6.5\times 10^{-10}\, w_o[(h_o/h_{-1})^{0.5}-1]$$

where $w_o$, $h_o$, $h_{-1}$, are parameters taken from the ESR spectrum; $w_o$ is the midfield line width and $h_o$, $h_{-1}$ are mid- and high-field line amplitudes. The above method is suitable for 16DSA and TP spin probes incorporated into DPPC liposomes.

Doxyl-stearate spin probes (5DSA and 7DSM) under experimental conditions have strongly restricted motions in the membrane system. In this case the degree of restriction of their motion is expressed by the order parameter ($S$), which is a measure of the relative fluidity in the membranes. The order parameter was calculated from the equation:

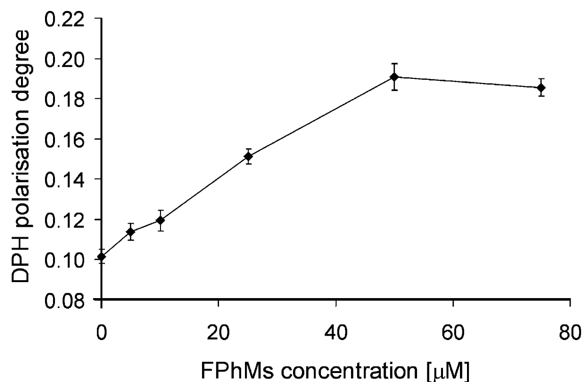


Fig. 2. DPH polarization degree in EYPC liposomes as a function of FPhMS concentration. Excitation and emission wavelengths were 380 and 450 nm, respectively. Buffer was 1/15 M Michaelis phosphate (pH 7.4), PC concentration = 200 μM, DPH concentration = 5 μM. Errors are given as standard deviation values.

$$S=\left[(A_{\parallel}-A_{\perp})/(A_{zz}-A_{xx})\right](a/a')$$

where $A_{\parallel}$, $A_{\perp}$ are maximal and minimal hyperfine splitting constants measured, respectively. $A_{zz}$, $A_{xx}$ are the hyperfine splitting tensors measured for probes in a crystal matrix and $a$ and $a'$ are the isotropic hyperfine splitting constants for nitroxides in the crystal matrix:

$$a=1/3\ (A_{zz}+2A_{xx})$$

and in membranes:

$$a'=1/3\ (A_{\parallel}+2A_{\perp})$$

## 3. Results

### 3.1. Fluorescence spectroscopy

The incorporation of FPhMS into EYPC liposomes caused the concentration-dependent increase in DPH polarization degree values (Fig. 2). The increase was monotonous up to 50 μM concentration of phenothiazine derivative. At this concentration the DPH polarization degree value was almost doubled in comparison with the control value (measured without FPhMS). When the phenothiazine derivative concentration was raised above 50 μM the DPH polarization degree stopped increasing. At the highest concentration used (75 μM) the DPH polarization degree value was slightly

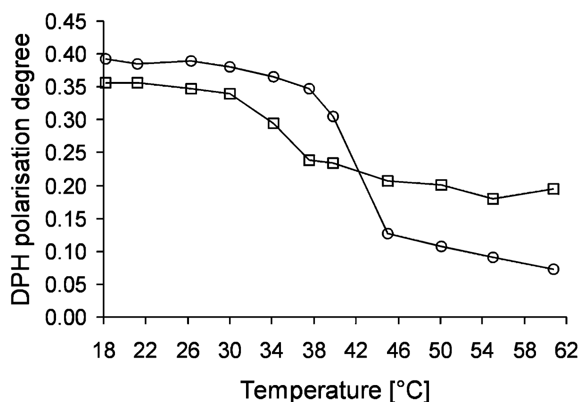


Fig. 3. DPH polarization degree in DPPC liposomes as a function of temperature: pure DPPC (○); DPPC with addition of 100 μM FPhMS (□). Excitation and emission wavelengths were 380 and 450 nm, respectively. Buffer was 1/15 M Michaelis phosphate (pH 7.4), DPPC concentration = 200 μM, DPH concentration = 5 μM. Errors are given as standard deviation values.

lower than the value at the preceding concentration.

The influence of FPhMS on the phase behavior of DPPC was followed by monitoring the dependence of DPH polarization degree on temperature. As compared with pure DPPC liposomes the DPH polarization degree values in the presence of phenothiazine derivative were lower in temperatures below phase transition, and higher in temperatures above phase transition (Fig. 3). The value of phase transition temperature determined by fluorescence spectroscopy was 41.5 °C for pure DPPC. For DPPC liposomes with addition of FPhMS the determination of $T_m$ was impossible due to the fact that the transition region of the fluorescence polarization vs. temperature plot was very broad and poorly defined. Nevertheless, it could be noticed that the onset of phase transition occurred at a much lower temperature in the DPPC/FPhMS systems than in pure DPPC. It has also been checked that DMSO alone has no influence on phase transition of DPPC (data not shown).

The dependence of DPH polarization degree on FPhMS concentration was also studied in DMPC and DPPC liposomes as well in gel as liquid crystalline states of lipid phase. For both systems in temperatures above $T_m$ DPH polarization degree values increased when the FPhMS concentration was raised (Fig. 4a). Already at a concentration of 25 μM DPH, the degree of polarization reached a value almost twice as big as the control value, in higher concentrations of FPhMS the increase of DPH polarization degree was weaker. The situation was different in DMPC and DPPC systems in temperatures below $T_m$ (Fig. 4b). At the lowest of the studied FPhMS concentrations (25 μM) for both lipids we observed a slight increase in the degree of DPH polarization. For FPhMS concentrations as high as 50 μM for DMPC and 75 μM for DPPC, a slight decrease in DPH polarization degree values was noticed. All changes in the

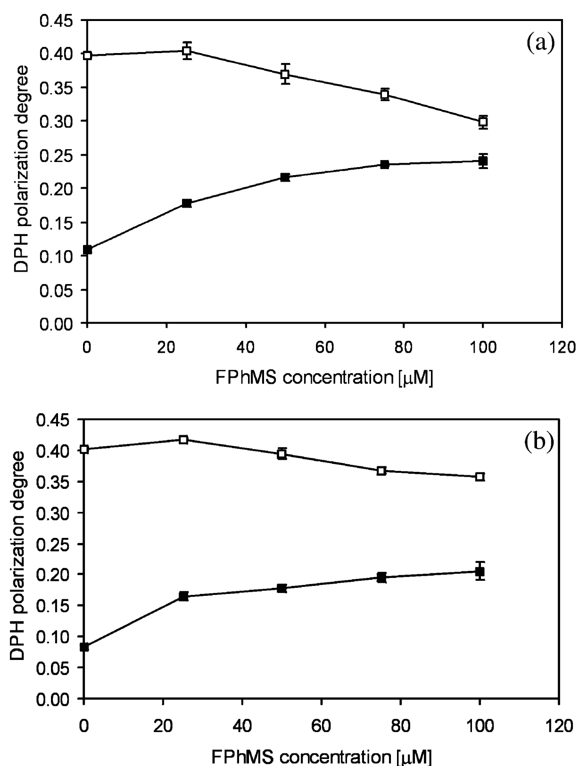


Fig. 4. DPH polarization degree values in DMPC (a) and DPPC (b) liposomes as a function of FPhMS concentration. Open symbols represent DPH polarization degree values recorded at temperature below $T_m$, filled symbols at temperature above $T_m$ of the appropriate lipid. Excitation and emission wavelengths were 380 and 450 nm, respectively. Buffer was 1/15 M Michaelis phosphate (pH 7.4), DPPC concentration = 200 μM, DPH concentration = 5 μM. Errors are given as standard deviation values.

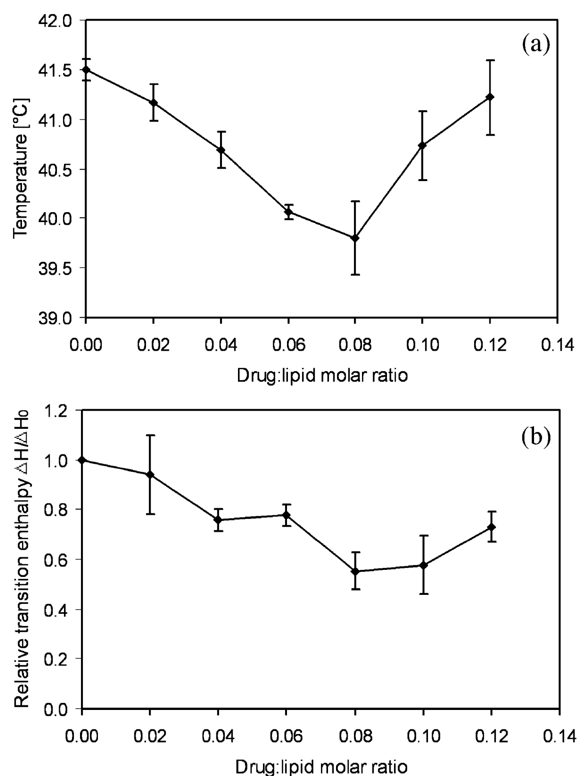


Fig. 5. The dependence of gel–liquid crystalline phase transition parameters: temperature (a); and relative transition enthalpy (b) on the FPhMS/DPPC molar ratio. Buffer was 20 mM Tris–HCl with 150 mM NaCl and 0.5 mM EDTA, pH=7.4.

degree of DPH polarization induced by FPhMS were more pronounced in DMPC than in DPPC.

### 3.2. Calorimetry

In the microcalorimetric experiments we used 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine —a zwitterionic phospholipid which thermotropic polymorphism is well known. Pretransition (occurring in pure DPPC in excess water at 35 °C) was abolished by the presence of FPhMS in the whole range of the molar ratios studied. The parameters characterizing gel–liquid crystalline phase transition of DPPC: temperature ($T_m$) and enthalpy change during transition ($\Delta H$) were altered by phenothiazine derivative under study in a concentration-dependent manner. The effects on transition temperature as well as on transition enthalpy were biphasic (see Fig. 5a)—for the FPhMS/DPPC molar ratios lying in the range 0.02–0.08, a gradual decrease in $T_m$ was observed, while for higher molar ratios (up to 0.12) $T_m$ increased. Transition enthalpy was decreased for FPhMS/DPPC molar ratios up to 0.08, while for 0.10 and 0.12 we observed a slight enthalpy increase (Fig. 5b). Increasing the amount of phenothiazine derivative in mixtures with DPPC we observed subsequent broadening of transition peaks. For molar ratios equal to 0.08 and higher the transition peaks were so broad that determination of transition temperature was inaccurate—this is reflected in Fig. 5a by the quite high values of standard deviation. Despite this uncertainty the increase in transition temperature for molar ratios higher than 0.06 was unquestionable.

Table 1
Tumbling correlation times and order parameters of four spin probes; TEMPO-palmitate TP, 16-doxylstearic acid (16DSA), 5-doxylstearic acid (5DSA) and 7-doxylstearic-methyl ester (7DSM) used as spin labels in FPhMS-modified DPPC liposomes

| Spin label | Tumbling correlation time ($\tau_c \times 10^{-9}$) | |
|---|---|---|
| | Control liposomes | Modified liposomes |
| TP | 2.60±0.11 | 3.77±0.30 |
| 16DSA | 2.22±0.12 | 2.60±0.19 |
| | Order parameter | |
| 5DSA | 0.640±0.002 | 0.684±0.022 |
| 7DSM | 0.616±0.038 | 0.675±0.020 |

Drug/lipid molar ratio was 0.06.

### 3.3. Electron spin resonance

From the calorimetric data it was obvious that for molar ratios equal to 0.08 and higher the DPPC bilayer structure was perturbed by FPhMS so strongly that precise determination of parameters describing the thermal behavior of this system was difficult. Therefore, the electron spin resonance experiments were performed using a phenothiazine derivative/lipid molar ratio of 0.06. The spectral parameters calculated from the experimental data according to the method described in Section 2 are given in Table 1. These parameters clearly

show the immobilization of all spin probes incorporated into modified liposomes. Four types of spin probes used in experiments enabled us to follow the mobility changes induced by FPhMS in different regions of DPPC bilayer. The results obtained using TEMPO-palmitate indicated probe immobilization in the lipid polar heads region while 5DSA, 7DSM and 16DSA showed that FPhMS reduced the spin probe mobility also in the hydrophobic region, starting from the polar/apolar interface down to the bilayer center.

## 4. Discussion

To characterize the interaction of newly synthesized model phenothiazine derivative with lipid bilayers composed of zwitterionic lipids we employed the following techniques: fluorescence polarization measurements, calorimetry and electron spin resonance. The results obtained by these three experimental approaches clearly show that FPhMS partitions into lipid bilayers and alters their biophysical properties.

Calorimetric measurements showed that FPhMS interacts with multilamellar DPPC structures and changes the lipid phase behavior in a concentration-dependent manner. Vanishing of the pretransition from the thermograms, observed even at the lowest of FPhMS/DPPC molar ratios examined, suggests that the presence of small amounts of drug affects the packing of lipid molecules strong enough to prevent formation of the ripple phase [25]. The effect of FPhMS on DPPC pretransition is stronger than observed by Jutila et al. [26] for chlorpromazine/DPPC/brainPS mixtures.

The biphasic effect exerted by FPhMS on DPPC gel–liquid crystalline phase transition temperature and enthalpy shows that different mechanisms are presumably involved in the drug–lipid interactions at low and high molar ratios. The decrease of $T_m$ and $\Delta H$ followed by broadening of transition peaks recorded for phenothiazine derivative/lipid molar ratios smaller than 0.06 are the effects of membrane structure perturbation induced by intruder molecules located near the hydrophilic/hydrophobic interface of the lipid leaflet. Such a location of drug molecules enables them to affect the interactions between the lipid molecules in both headgroup and hydrocarbon chain regions and thus after all phase-transition parameters [27]. Intercalation of the drug molecules into the polar/apolar lipid bilayer interface was confirmed by the ESR results. Immobilization of spin probes was observed not exclusively for TEMPO-palmitate (located in the lipid polar heads region), but also for 5DSA, 7DSM and 16DSA (located in different regions of the hydrophobic membrane core). It is thus obvious that FPhMS could not be bound to the bilayer surface or located exclusively in the polar region, since in such a case 7DSM and 16DSA probes should show no immobilization effect. Analysis of a phenothiazine derivative molecule structure together with the immobilization of the TEMPO-palmitate spin probe clearly show that FPhMS could not be buried in the hydrophobic region of the bilayer. The putative localization of FPhMS molecules at the polar/apolar interface of lipid bilayers is in keeping with the model deduced from $^{13}$C-NMR and microcalorimetric experiments, proposed by Nerdal et al. [16] for chlorpromazine. Assuming the above positioning of studied compound molecules in the lipid bilayer we may conclude that phenothiazine rings presumably affect the C1–C7 region of lipid acyl chains while sulfonylamide groups presumably interact with phosphate region of polar headgroups.

Insertion of increasing amounts of FPhMS into bilayer perturbs to some extent packing of lipid molecules, but does not destroy the bilayer structure. In calorimetric experiments we observed an increase of the transition temperature and enthalpy followed by further broadening of transition peaks for drug/lipid molar ratios higher than 0.06. The latter effect distinguish our results from those obtained previously by Frenzel et al. [15] for chlorpromazine/DPPC mixtures. These authors also observed the biphasic behavior of transition parameters, however, for CPZ/DPPC molar ratios higher then 0.1 the transition peaks sharpened. Sharpening of the transition peaks was also recorded by Hanpft and Mohr [28] in mixtures of chlorpromazine with dipalmitoylphosphatidylglycerol (DPPG) at molar ratios higher then 0.2. In both cases this concentration-dependent decrease of the peak half-width was attributed to the formation of the new mode of molecular packing

characterized by high co-operativity of phase transition. It is worth to emphasize here, that the nature of the new phase was differently described by these authors: Frenzel et al. [15] postulated formation of interdigitated phase while Hanpft and Mohr [28] suggested that in the putative new phase drug and lipid molecules are homogeneously mixed. Since our calorimetric data did not match the results obtained by other authors we performed the experiments in which the influence of FPhMS on DPPC gel–liquid crystalline phase transition was followed by DPH fluorescence polarization measurements (Fig. 3). This experiment fully confirmed that the phase transition temperature range in phenothiazine derivative/lipid mixtures is much broader than in pure lipid. So we conclude, on the basis of calorimetric and fluorescence polarization experiments, that it is likely also that at FPhMS/DPPC molar ratios higher than 0.06 a new phase or mode of packing appears, but this structure is much less co-operative than that formed by pure lipid.

Even if the phase transition temperature of FPhMS/DPPC mixtures at high molar ratios was not strongly modified with respect to the $T_m$-value of pure lipid (see Fig. 5a), peak broadening shifts the onset of transition towards lower temperatures and the transition completion temperature towards higher values (see Fig. 3). These results suggest that both gel and liquid–crystalline phases of studied mixtures are affected by the presence of FPhMS.

Alteration of the gel phase of lipid bilayers by phenothiazine derivative gives an ambiguous picture when observed by electron spin resonance and fluorescence. Our ESR data show the decrease of spin probes motional freedom in the gel DPPC bilayer caused by the moderate phenothiazine derivative concentration (drug/lipid molar ratio = 0.06). A slight increase in bilayer rigidity was observed also by fluorescence polarization but only for the lowest of the FPhMS concentrations studied (25 μM). On the other hand, DPH fluorescence polarization studies, performed at higher FPhMS concentrations, point to the concentration-dependent decrease in bilayer order induced by the studied compound in the gel state of lipid bilayers. An increase of the order of the gel-state lipid bilayers has been recorded in chlorpromazine/DMPC mixtures using either $^{31}$P NMR and $^{13}$C NMR [15] or by ESR [29] techniques. Recent solid-state $^{13}$C NMR investigations, however, gave different results: an increase in acyl chains mobility caused by the interaction of CPZ with phosphatidylserine polar headgroups was found [16]. Simultaneously, these authors reported only minute effects of CPZ exerted on DPPC/DMPC liposomes.

Influence of FPhMS on the liquid-crystalline phase of lipid bilayers gave a more coherent picture. In both natural extract (EYPC) and synthetic lipids (DMPC, DPPC) we observed a concentration-dependent increase of DPH polarization degree what suggests that the liquid-crystalline phase becomes more rigid (ordered) in the presence of FPhMS. These results are in general agreement with the majority of data obtained for interaction of CPZ either with liquid-crystalline bilayers or with cell membranes (which are considered rather to be in a fluid than a gel state); some papers, however, report effects different than those observed by us. In ESR experiments, the increase in membrane order upon the interaction with CPZ was found in phosphatidylcholine/phosphatidic acid liposomes [30] as well as in membranes composed of ox brain white matter lipids [31]. Fluorescence polarization experiments have shown, however, that the character of changes may depend on the type of fluorescence probe used. In the study performed on the brush border membrane vesicles Iseki et al. [32] have found that CPZ induces an increase in the degree of DPH fluorescence polarization while 1-anilino-8-naphthalene sulfonate (ANS) fluorescence polarization is reduced by this drug. Loosening of the phosphatidylcholine packing was deduced by Wadkins et al. [6] from the changes induced by CPZ in NPN fluorescence spectra.

In general, the influence of FPhMS on the properties of lipid bilayers as observed by experimental techniques used in this work resembles to some extent the effect of cholesterol. First of all, cholesterol also decreases DPH polarization anisotropy in the gel state of DPPC bilayers and increases it in the liquid-crystalline state [33]. It decreases the temperature and broadens the lipid phase transition peaks as observed by calorimetry

[34,35]. These similarities between FPhMS and cholesterol influence on lipid bilayers may arise from the similar (to some extent) structure of their molecules. They are both rigid and flat (aromatic rings' system) and after the incorporation into membrane they are able to affect the packing of lipid molecules. Since the cholesterol molecule is bigger and more hydrophobic than FPhMS, and it also possesses the ability to form hydrogen bonds between sterol 3β-hydroxyl and lipid-*sn*-2 carbonyl groups [36] (for review see also Ohvo-Rekilä [37]), its influence on lipids is probably stronger than that of phenothiazine derivative. Nevertheless, we postulate that FPhMS induces a new, concentration-dependent mode of lipid packing in model bilayers. At low molar ratios, phenothiazine derivative molecules presumably form complexes with lipid molecules and thus induce structural defects in bilayer. These defects decrease phase transition temperature, enthalpy and co-operativity of lipid bilayers. The packing disorder may also result in the appearance of holes in the plane of the bilayer as was reported for CPZ acting on erythrocytes [8]. Simultaneously, the presence of rigid molecules inside the bilayer may result in a decrease of the lipid molecules' mobility. This effect should be more striking in the liquid crystalline state than what was observed in our fluorescence polarization experiments. At higher molar ratios the FPhMS molecules become more homogeneously mixed with lipids and instead of local defects, a new bilayer packing mode is presumably established. Like the liquid-ordered phase induced by cholesterol concentrations above 7–10 mol% [36] FPhMS/lipid mixtures are characterized by low co-operativity and reduced molecule mobility—as compared with pure lipid.

In this work we attempt to elucidate the mechanism of interaction between the newly synthesized phenothiazine derivative and lipid bilayers. Our results show that FPhMS (possessing structural features that are believed to be of some importance for phenothiazine derivatives' biological activity) changes the biophysical properties of lipid bilayers. Structural defects in membranes caused by this compound are likely to increase membrane permeability. Such an increase could probably enhance the rate of passive anticancer drugs' influx, thus contributing to their accumulation in cancer cells. The direct influence on accumulation of chemotherapeutic agents could explain, at least in part, the mechanism of anti-MDR action of phenothiazines. However, an indirect mechanism of action—such as changes in activity of MDR transporters induced by membrane's altered fluidity—cannot be excluded at the moment. Further studies are required to elucidate the complex pattern of the biological activities of phenothiazine derivatives.

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