ELSEVIER

Biophysical Chemistry 109 (2004) 399–412

Biophysical Chemistry

www.elsevier.com/locate/bpc

Presence of anionic phospholipids rules the membrane localization of phenothiazine type multidrug resistance modulator

Olga Wesołowska[a,*], Andrzej B. Hendrich[a], Noboru Motohashi[b], Masami Kawase[c], Piotr Dobryszycki[d], Andrzej Ożyhar[d], Krystyna Michalak[a]

[a]*Department of Biophysics, Wrocław Medical University, ul. Chałubinskiego 10, 50–368 Wrocław, Poland*
[b]*Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose-shi, Tokyo 204-8588, Japan*
[c]*Faculty of Pharmaceutical Sciences Josai University, Keyakidai, Sakado, Saitama 350-0295, Japan*
[d]*Division of Biochemistry, Institute of Organic Chemistry, Biochemistry & Biotechnology, Wrocław University of Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland*

Received 25 July 2003; received in revised form 17 November 2003; accepted 20 November 2003

Abstract

Substances able to modulate multidrug resistance (MDR), including antipsychotic phenothiazine derivatives, are mainly cationic amphiphiles. The molecular mechanism of their action can involve interactions with transporter proteins as well as with membrane lipids. The interactions between anionic phospholipids and MDR modulators can be crucial for their action. In present work we study interactions of 2-trifluoromethyl-10-(4-[methanesulfonylamid]buthyl)-phenothiazine (FPhMS) with neutral (PC) and anionic lipids (PG and PS). Using microcalorimetry, steady-state and time-resolved fluorescence spectroscopy we show that FPhMS interacts with all lipids studied and drug location in membrane depends on lipid type. The electrostatic attraction between drug and lipid headgroups presumably keeps phenothiazine derivative molecules closer to surface of negatively charged membranes with respect to neutral ones. FPhMS effects on bilayer properties are not proportional to phosphatidylserine content in lipid mixtures. Behavior of equimolar PC:PS mixtures is similar to pure PS bilayers, while 2:1 or 1:2 (mole:mole) PC:PS mixtures resemble pure PC ones.

Keywords: Phenothiazine derivative; Fluorescence spectroscopy; Differential scanning calorimetry; Anionic phospholipids; Drug–membrane interaction; Multidrug resistance modulation

Abbreviations: CPZ, chlorpromazine; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DPH, 1,6-diphenyl-1,3,5-hexatriene; DSC, differential scanning calorimetry; ΔH, enthalpy change during phase transition; FPhMS, 2-trifluoromethyl-10-(4-[methanesulfonylamid]buthyl)-phenothiazine; GP, Laurdan generalized polarization; MDR, multidrug resistance; NPN, *N*-phenyl-1-naphtylamine; PC, egg yolk phosphatidylcholine; PS, bovine brain phosphatidylserine; T_m, lipid phase transition temperature.

*Corresponding author. Tel.: +4871-7841402; fax: +4871-7840088.
E-mail address: olawes@biofiz.am.wroc.pl (O. Wesołowska).

0301-4622/04/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2003.11.004

1. Introduction

Biological membranes constitute the outer border of the cell and control the fluxes of all substances between the cell and its environment. The selective removal of xenobiotics and other toxic compounds from the cells is their basic line of defense. The outward transport of substances threatening the cell is a double-edged process, however, as the ability of cancer cells to pump out anticancer agents poses a major problem to a successful chemotherapy. This active outward efflux of the wide spectrum of cytotoxic compounds contributes to the phenomenon of multidrug resistance (MDR). The drug transport is performed by transmembrane proteins belonging to ABC proteins superfamily (ATP-binding cassette) such as *P*-glycoprotein (reviewed in [1]). The substrate specificity of *P*-glycoprotein is extremely wide. Apparently the vast majority of cationic amphiphiles can be bound and translocated by this transporter (see [2] for review), including some short chain lipid analogues [3,4]. Although ABC transporters are believed to play the main role in MDR there is more and more evidence gathered pointing to the important role of lipid phase of cell membranes in this process, too. Cell membrane constitutes the environment where MDR transporters recognize and bind their substrates. Its biophysical properties can also influence *P*-glycoprotein activity (reviewed in [5]). On the other hand, lipid phase of membranes constitutes the medium for the diffusional, passive, inward transport of the drugs.

Clinical importance of multidrug resistance that appears during a course of chemotherapy in vast number of malignancies has attracted a lot of scientists' attention to compounds able to reverse MDR. The reduction of MDR transporters' activity results in increased concentration of cytostatics inside the cancer cells and thereby restores drugs' ability to kill them. Compounds called MDR modulators share no common chemical structure. The majority of them, however, are relatively hydrophobic neutral or cationic molecules that partition easily into lipid bilayers [6,7]. As majority of P-glycoprotein substrates and MDR modulators are cationic amphiphiles it seems much probable that the presence of negatively charged phospholipids in membranes could rule their interactions with lipid bilayers. Recently, it has been shown that MDR modulator verapamil binds stronger to the membranes containing anionic lipids and competes with anticancer drug doxorubicin for membrane partitioning [8]. It also increases the rate of passive import of doxorubicin across the plasma membrane in this way reducing MDR.

Phenothiazines are clinically used as antipsychotic drugs. They also constitute the group of promising multidrug resistance modulators [9–11]. Although their potency to reverse MDR has been recognized for many years, little is known about the molecular mechanism of anti-MDR action of phenothiazine derivatives. Structural modifications that increase compound's hydrophobicity increase also multidrug resistance potency of phenothiazines [12,13]. Together with extremely wide scope of effects exerted by these compounds on membranes it suggests that interaction with lipid phase of cell membranes can be important for anti-MDR activity of phenothiazines. In physiological pH phenothiazine derivatives bear the positive charge [14]. It is thus probable that many of phenothiazine-associated effects in biological membranes are ruled by specific interactions of these cationic compounds with anionic phospholipids. Phenothiazine derivatives are known to cause erythrocyte hemolysis [15] and, in lower concentrations, to induce stomatocytosis of red blood cells [16,17]. According to bilayer couple hypothesis proposed by Sheetz and Singer in 1974 [18] invaginations of erythrocyte membrane are caused by compounds that specifically interact with phosphatidylserine and intercalate into inner monolayer of cell membrane. Chlorpromazine (CPZ) was also reported to cause partial phospholipid scrambling in erythrocyte membranes [19].

There is also a lot of data from model membrane experiments suggesting that phenothiazines interact differently with charged phospholipids than with neutral ones. We have shown previously by microcalorimetry that trifluoperazine induces phase separation in bilayers formed from zwitterionic DMPC and DPPC but not anionic DMPG [20]. Freeze-fracture electron microscopy of CPZ: phosphatidic acid bilayers has shown that the

addition of phenothiazine can result in hexagonal H_{II} phase formation [21]. Specific interaction of phenothiazine derivatives with phosphatidylserine was also observed. Pajeva et al. [22] have shown by DSC that some of these compounds interact with DPPC and DPPS in dissimilar way and that new kind of phospholipid organization is formed in charged bilayers in the presence of these drugs. The recent microcalorimetry and ^{13}C-NMR studies of Nerdal et al. [23] have demonstrated that CPZ interacts weakly with DPPC models systems and that strength of drug–lipid bilayer interaction raises dramatically after introducing bovine brain PS into membranes. The monolayer technique has been employed to show that the surface area of acidic phospholipids increases strongly after addition of chlorpromazine whereas the surface area of neutral phospholipids remains constant in spite of the presence of the drug [24]. Jutila et al. [25] have observed CPZ-induced changes in lateral organization of model membranes composed of DPPC:brain PS mixtures.

2-Trifluoromethyl-10-(4-[methanesulfonylamid]buthyl)-phenothiazine (FPhMS) was designed and synthesized with intention of its future use as a multidrug resistance modulator. Its anti-MDR activity was confirmed in drug-resistant cancer cells in culture [11,26]. The interactions of this compound with model membranes composed of zwitterionic DPPC were studied in details using DSC, EPR and fluorescence spectroscopy techniques [27]. We have also shown previously that FPhMS is able to induce phase separation in phosphatidylethanolamine bilayers [28].

The aim of the present work was to study the interactions of phenothiazine-type MDR modulator FPhMS with model membranes containing acidic phospholipids. We have employed DSC technique to compare the influence of this compound on neutral DMPC and charged DMPG bilayers. Fluorescence spectroscopy was used to study the binding of FPhMS to liposomes composed of PC:PS mixtures. We have shown that phenothiazine derivative probably occupies slightly different locations inside the lipid bilayer depending on the membrane charge. Additionally, fluorescence spectroscopy revealed that the extent of effect induced by phenothiazine derivative on membranes is not proportional to the molar ratio of neutral to anionic phospholipid in the bilayer.

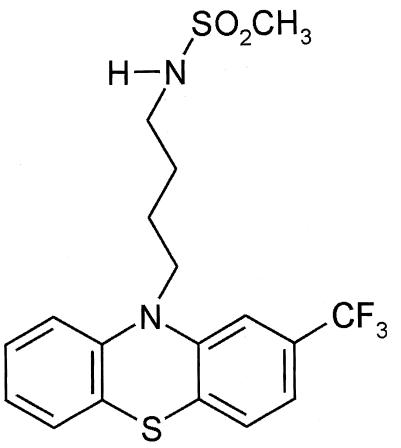

Fig. 1. Chemical structure of 2-trifluoromethyl-10-(4-[methanesulfonylamid]buthyl)-phenothiazine (FPhMS).

2. Materials and methods

1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylglycerol (DMPG) and 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Bovine brain L-α-phosphatidylserine (PS) and egg yolk L-α-phosphatidylcholine (PC) were from Sigma (St. Louis, MO, USA). Lipids were used without further purification. 1,6-Diphenyl-1,3,5-hexatrien (DPH) and *N*-phenyl-1-naphtylamine (NPN) were purchased from Sigma (St. Louis, MO, USA). 6-Lauroyl-2-(*N*,*N*-dimethylamino) naphthalene (Laurdan) was from Molecular Probes (Eugene, OR, USA).

2-Trifluoromethyl-10-(4-[methanesulfonylamid]buthyl)-phenothiazine (FPhMS) was synthesized as described in [29,30]. Its chemical structure is shown in Fig. 1. All other chemicals were of analytical grade.

2.1. Microcalorimetry

Stock solution (3.5 mM) of phenothiazine derivative was prepared in chloroform:methanol (1:1, v/v). For each sample 2 mg of appropriate lipid was dissolved in FPhMS stock solution. The

amount of FPhMS was chosen to obtain the desired drug:lipid molar ratio in the sample.

The samples were dried under the stream of nitrogen and the residual solvent was removed under vacuum for at least 3 h. Samples were hydrated by 20 μl of 20 mM Tris–HCl buffer (150 mM NaCl, 0.5 mM EDTA, pH = 7.4). Hydrated mixtures were heated to temperature ~10 °C higher than main phase transition temperature of a given lipid and vortexed until homogeneous dispersion was obtained. Samples were sealed in aluminum pans and scanned at the rate of 1.25 °C/min. Calorimetric measurements were performed using Rigaku calorimeter, which was partially rebuilt in our laboratory. Samples were scanned immediately after preparation. The temperature at which maximal deviation of transition peak from the base line was recorded was taken as a phase transition temperature. The area under the transition profiles was used to calculate the molar enthalpy change accompanying phase transition.

2.2. Steady-state fluorescence spectroscopy

To obtain PS, PC and PC:PS liposomes chloroform solutions of lipids were mixed in appropriate amounts. The mixtures of following PC:PS molar ratios were used: 5:1 (16.66 mol% of PS), 2:1 (33.33 mol% of PS), 1:1 (50 mol% of PS), 1:2 (66.66 mol% of PS) and 1:5 (83.33 mol% of PS). Organic solvent was then evaporated under stream of nitrogen and the samples were kept under vacuum for at least 2 h. The dry lipids were hydrated in 1/15 M Michaelis phosphate buffer (pH 7.4) in case of DPH and NPN experiments or in 20 mM Tris–HCl buffer (50 mM NaCl, 0.1 mM EDTA, pH = 7.4) in case of Laurdan experiments. Small unilamellar liposomes were obtained by sonication.

DPH stock solution (1 mM) was prepared in tetrahydrofuran. NPN (1 mM), Laurdan (1 mM) and FPhMS (5 mM) were dissolved in dimethyl sulfoxide. Liposomes (final phospholipid concentration 200 μM) were incubated with fluorescent probe (concentration 5 μM) in darkness for 30 min or 15 min (for DPH and NPN, respectively) at room temperature. FPhMS was then added (at concentration varying from 5–100 μM) and incubation was continued under the same conditions for the next 20 min (DPH) or 10 min (NPN). For Laurdan experiments liposomes (200 μM) were incubated with fluorescent probe (1 μM) for 30 min (darkness, room temperature).

Fluorescence measurements were performed with LS 50B spectrofluorimeter (Perkin–Elmer Ltd., Beaconsfield, UK) using emission and excitation slits of 5 nm. All measurements were done at 25 °C. Temperature was controlled by a water-circulating bath and the content of the cuvette was continuously mixed. DPH excitation and emission wavelengths were 380 nm and 450 nm, respectively. NPN fluorescence was excited at 350 nm and emission spectra were recorded in the range of 360–580 nm. Excitation wavelength for Laurdan was 320–400 nm and fluorescence emission spectra were recorded in the range of 410–540 nm. Data were collected and processed with FLDM Perkin–Elmer software. Laurdan generalized polarization was calculated according to the equation [31]:

$$\mathrm{GP} = \frac{I_B - I_R}{I_B + I_R} \quad (1)$$

where I_B and I_R are the fluorescence emission intensities at the blue and red edges of the emission spectrum, respectively. The GP values were calculated using emission intensities at 440 nm (I_B) and 490 nm (I_R).

DPH polarization degree (P) was calculated as:

$$P = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + GI_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the emission intensity with polarizer parallel ($\parallel$) and perpendicular ($\perp$) to the direction of polarization of the excitation light and G is the instrumental correction factor calculated by FLDM software.

2.3. Time-resolved fluorescence spectroscopy

The samples for fluorescence lifetimes measurements were prepared in the same way as for steady-

state fluorescence experiments. Concentration of FPhMS varied from 5–50 μM. In higher drug concentrations lifetime measurements were not performed due to very low fluorescence intensities. Measurements were performed with a SLM Aminco 48000S frequency–domain instrument using a 450 W xenon lamp as a light source and frequency range of 1–250 MHz. Samples were excited at 350 and 380 nm for NPN and DPH, respectively. The emitted light passed through a cut-off filter to eliminate the light below 370 nm for NPN and 410 nm for DPH experiments. All measurements were done at 25 °C unless stated otherwise. The lifetimes were calculated by multiexponential analysis from phase shifts and demodulation parameters using the fluorimeter software. When fluorescence decay was not a single exponential function the average lifetime $\langle\tau\rangle$ was calculated according to the equation:

$$\langle\tau\rangle = \sum_{i=1}^{n} f_i \tau_i$$

where τ_i is fluorescence lifetime and f_i is fractional intensity contribution.

3. Results

3.1. Microcalorimetry

The thermograms of pure DMPC and its mixtures with different amounts of newly synthesized phenothiazine derivative are presented in Fig. 2. DMPC is a zwitterionic lipid that thermotropic polymorphism is well known. In pure lipid (upper trace) we recorded the main phospholipid phase transition at 23 °C as well as pretransition centered at 13.8 °C. The addition of FPhMS to the lipid caused disappearance of pretransition and distinct symmetrical broadening of the gel–liquid crystalline transition peaks. The influence of studied compound on DMPC bilayer structure was so strong that main phospholipid phase transition disappeared completely at drug:lipid molar ratio 0.12. Also the parameters characterizing gel–liquid crystalline transition of DMPC: temperature (T_m) and enthalpy change (ΔH) during transition were strongly influenced by the presence of phenothiazine derivative. Both parameters monotonously decreased with increasing FPhMS concentration (Fig. 3a,b).

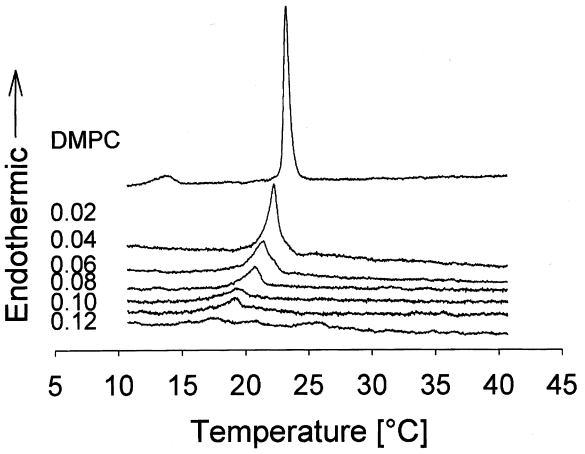

Fig. 2. Thermograms of DMPC (upper profile) and FPhMS:DMPC mixtures. Numbers on the figure represent drug/lipid molar ratios. Thermograms were normalised to equal amount of lipid for each profile.

Thermotropic behavior of mixtures of phenothiazine derivative with anionic lipid DMPG was also studied. For pure lipid two transitions were recorded: pretransition and main phospholipid phase transition at 14 °C and 24 °C, respectively (Fig. 4). The pretransition was abolished even at the smallest drug:lipid molar ratio studied. The addition of studied compound to DMPG model system resulted in symmetric broadening of transition peaks. This broadening was stronger than observed in FPhMS:DMPC mixtures. Addition of increasing concentrations of phenothiazine derivative to the lipid caused the transition temperature shift to lower values (Fig. 5a). The extent of T_m lowering induced by the drug in DMPG was approximately 4 °C, the same as in phosphatidylcholine. The effect of FPhMS on ΔH of anionic lipid was, however, different than in case of DMPC (Fig. 5b). Up to drug:lipid molar ratio 0.04 transition enthalpy remained constant. It dropped slightly only at higher phenothiazine derivative concentrations.

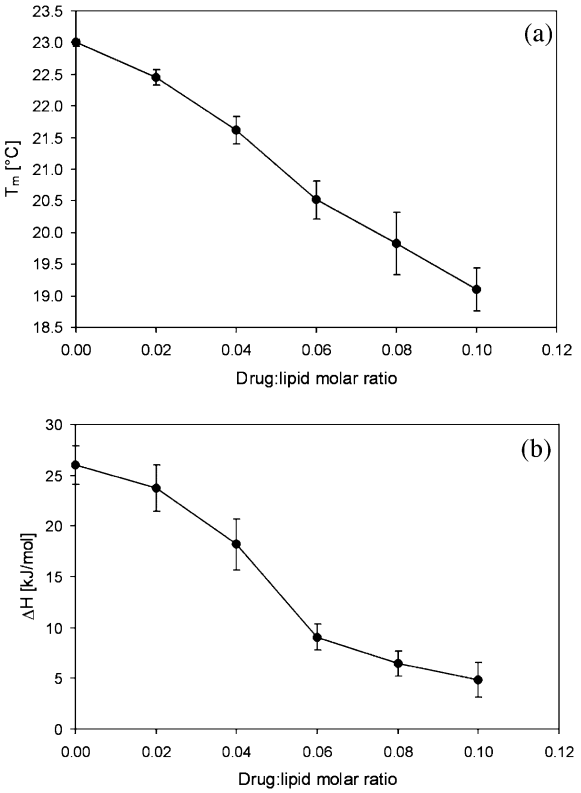

Fig. 3. Alterations of main phospholipid phase transition parameters: temperature (a) and enthalpy (b) induced by FPhMS in DMPC. Errors are given as standard deviation values of eight measurements.

3.2. Fluorescence spectroscopy

The interactions of FPhMS with model membranes composed of neutral phosphatidylcholine, negatively charged phosphatidylserine and their mixtures were studied by means of fluorescence spectroscopy. Addition of studied compound to the liposomes labeled with NPN resulted in strong quenching of its fluorescence. Stern–Volmer plots of quenching are presented in Fig. 6. In all types of lipid compositions studied NPN quenching increased with increasing of phenothiazine derivative concentration. The strongest quenching was observed in liposomes composed from pure PS and the weakest in pure PC. It is worth noticing that for concentrations of phenothiazine derivative higher than 50 μM the quenching of NPN fluorescence was slightly higher in PC:PS (1:1) mixtures than in two other PC:PS systems studied.

In all lipid systems studied Stern–Volmer plots of NPN quenching showed slight upward curvature with increasing phenothiazine derivative concentration. Only in case of pure PS significant curvature was observed. To investigate further this quenching phenomenon we measured NPN fluorescence lifetimes in PS liposomes in presence of FPhMS. NPN lifetime recorded in pure liposomes was 2.35 ns. The lifetime dropped to 1.49 ns after addition of 5 μM of the drug and remained constant up to FPhMS concentration of 50 μM.

Apart from reducing NPN fluorescence intensity FPhMS also caused the shift of fluorescence emission spectra maxima toward longer wavelengths as compared to the spectra recorded in the absence of this compound. Such a maximum red-shift was recorded in all types of PC:PS mixtures with the exception of PC:PS (1:2). The biggest red-shift was observed in PS liposomes (5 nm), almost twice smaller in equimolar PC:PS mixture (2.7 nm), even smaller in PC:PS (2:1) (2.4 nm) and the smallest one in pure PC (1.7 nm).

The presence of phenothiazine derivative influenced also the fluorescence of the probe DPH. Addition of studied compound to all types of liposomes resulted in concentration dependent

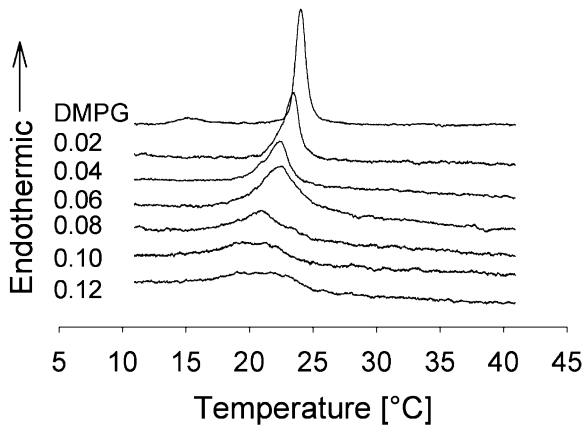

Fig. 4. Thermograms of DMPG (upper profile) and FPhMS:DMPG mixtures. Numbers on the figure represent drug:lipid molar ratios. Thermograms were normalized to equal amount of lipid for each profile.

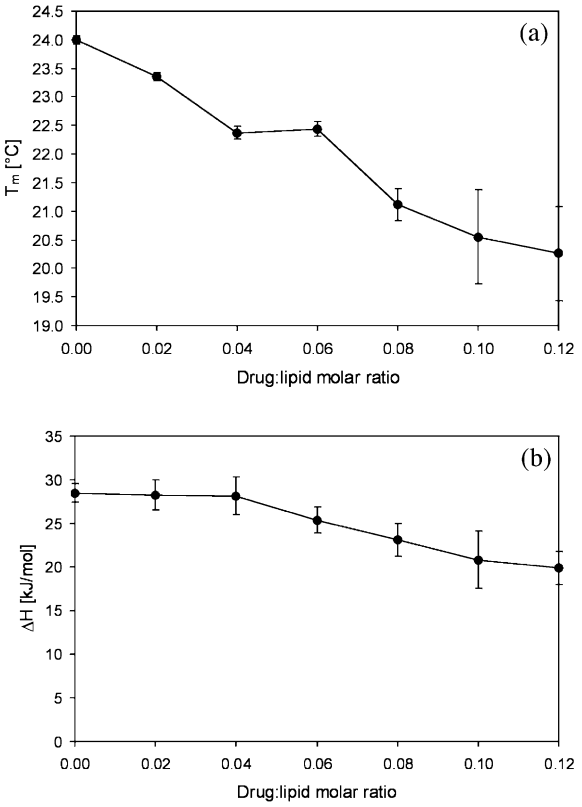

Fig. 5. Alterations of main phospholipid phase transition parameters: temperature (a) and enthalpy (b) induced by FPhMS in DMPG. Errors are given as standard deviation values of eight measurements.

DPH fluorescence polarization increase (Fig. 7). Only in PS model membranes at FPhMS concentrations above 50 μM polarization stopped increasing. The biggest drug induced changes in DPH polarization values were recorded in pure PC. The extent of polarization increase caused by FPhMS decreased in order: PC > PC:PS (2:1) > PC:PS (1:2) > PC:PS (1:1) > PS.

Apart from changes in DPH polarization phenothiazine derivative caused also fluorescence quenching of this probe (data not shown). The extent of quenching was similar for all lipids studied. DPH fluorescence lifetimes were also investigated (Fig. 8). DPH had the longest lifetime in membranes composed of pure phosphatidylserine. In pure lipid systems the fluorescence lifetime

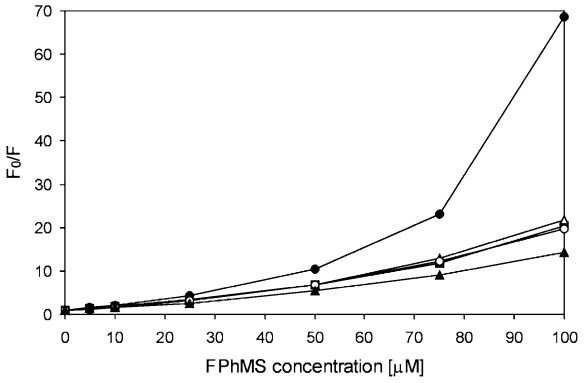

Fig. 6. Stern–Volmer plots of NPN fluorescence quenching caused by FPhMS in liposomes composed of PC:PS mixtures. Symbols represent: ▲ – PC, ○ – PC:PS (2:1), △ – PC:PS (1:1), ■ – PC:PS (1:2), ● – PS.

decreased together with the decrease of the amount of anionic lipid in the model membrane. The addition of phenothiazine derivative to liposomes caused DPH lifetime shortening. This change was the most pronounced in PS liposomes and almost negligible in PC liposomes. We decided also to study the influence of FPhMS on DPH fluorescence in DPPC model membranes in different phase states (Fig. 9). The addition of the drug caused DPH lifetime shortening in both gel-like and liquid–crystalline states, however the extent

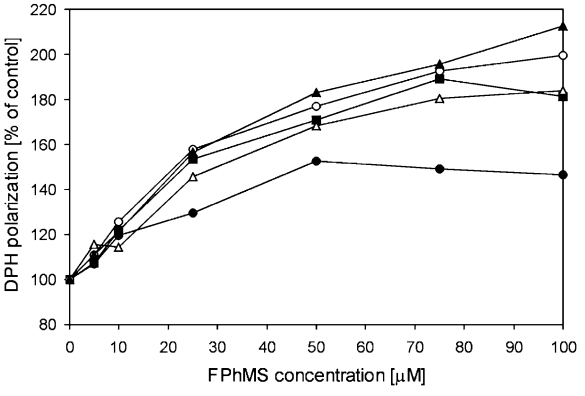

Fig. 7. DPH polarization as a function of FPhMS concentration in liposomes composed of PC:PS mixtures. Symbols represent: ▲ – PC, ○ – PC:PS (2:1), △ – PC:PS (1:1), ■ – PC:PS (1:2), ● – PS.

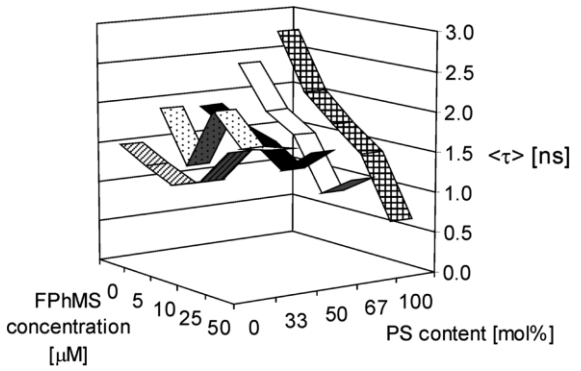

Fig. 8. DPH fluorescence lifetime as a function of FPhMS concentration and liposome composition: PC – stripped band, PC:PS (2:1) – dotted band, PC:PS (1:1) – filled band, PC:PS (1:2) – open band, PS – cross-hatched band.

of change due to increasing drug concentration was greater in lipid bilayers below T_m.

The study on FPhMS-induced NPN fluorescence quenching and DPH polarization changes revealed that phenothiazine derivative incorporated in the liposomes composed of PC:PS (1:1) mixture behaved more similarly to liposomes of pure PS than when incorporated in model membranes of PC:PS (1:2). This suggested that the range of model membrane perturbation induced by FPhMS was not proportional to anionic PS content in the system. Therefore we decided to characterize the properties of PC:PS membranes in absence of any drug more thoroughly. Table 1 presents DPH fluorescence polarization values obtained in liposomes composed of PC and PS mixed in different molar ratios. Again it could be noticed that DPH polarization value recorded in PC:PS (1:1) membranes was more similar to pure PS liposomes than polarization value in PC:PS (1:2) mixture.

Table 1
DPH polarization in liposomes composed of egg yolk PC, bovine brain PS and PC:PS mixtures. Errors are given as standard deviation values of six measurements

Lipid composition	DPH fluorescence polarization
PC	0.099±0.005
PC:PS (2:1)	0.102±0.003
PC:PS (1:1)	0.113±0.004
PC:PS (1:2)	0.107±0.004
PS	0.119±0.004

We have studied the properties of PC:PS model membranes also employing Laurdan as a fluorescent probe. We observed an increase in the intensity of the blue part of emission spectra with increase of PS content in the model system (data not shown). The Laurdan generalized polarization as a function of excitation wavelength (λ_{ex}) is presented in Fig. 10. All the GP vs. λ_{ex} plots had negative slopes i.e. they presented the typical features of the liquid–crystalline phase. The typical GP value recorded in this phase is approxi-

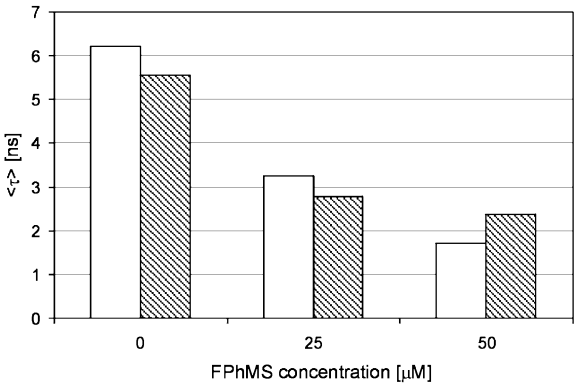

Fig. 9. Influence of FPhMS on DPH fluorescence lifetime in DPPC liposomes below (open bars; measured at 25 °C) and above (filled bars; measured at 45 °C) transition temperature.

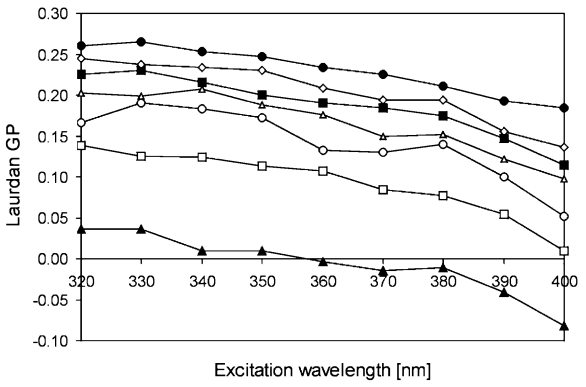

Fig. 10. Laurdan generalized polarization values in PC:PS liposomes as a function of excitation wavelength. Symbols represent: ▲ – PC, □ – PC:PS (5:1), ○ – PC:PS (2:1), △ – PC:PS (1:1), ■ – PC:PS (1:2), ◇ – PC:PS (1:5), ● – PS.

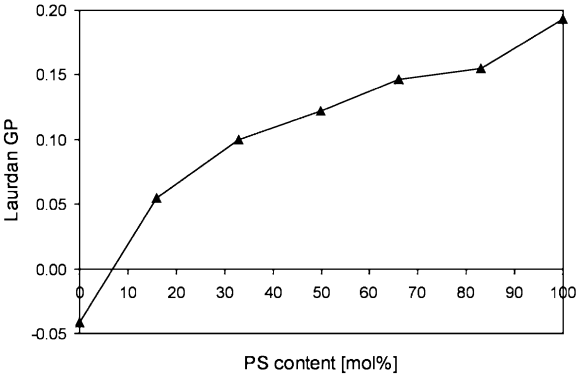

Fig. 11. Laurdan generalized polarization values in liposomes as a function of PS concentration. Excitation wavelength was 390 nm.

mately −0.2 if calculated for the same excitation and emission conditions as in Ref. [32]. As presented in Fig. 11 at 25 °C Laurdan in membranes composed of pure egg yolk PC possessed the most 'liquid-crystalline' GP values whereas in pure PS liposomes the highest Laurdan GP values were observed. It could be also noticed that the increase in generalized polarization was proportional to the anionic lipid content in the membranes. This dependence was by no means linear, however. Introducing even the smallest amount of PS to PC membrane (16.66 mol%) caused the most dramatic increase of GP value. Further raising phosphatidylserine content resulted in smaller changes in Laurdan generalized polarization.

4. Discussion

The results of calorimetric experiments has proved that FPhMS intercalates into membranes of both neutral DMPC and charged DMPG changing the parameters that characterize lipid bilayers' phase transitions. In both lipids phenothiazine derivative caused T_m lowering, gel–liquid crystalline transition peaks' broadening and decrease of transition enthalpy. Broadening of transition peaks together with lowering of transition temperature are typical changes induced by compounds that localize in C_1–C_8 methylene region of lipid bilayer [33]. Such a localization of FPhMS molecule is consistent with a model proposed by Nerdal et al. [23] for chlorpromazine. According to it the hydrophobic ring system of CPZ molecule penetrates the acyl chain region and is oriented along the chain direction. In the same time positively charged side chain group of chlorpromazine is positioned in the vicinity of the phospholipid headgroups what allows their mutual electrostatic interaction. Such a location of FPhMS molecule inside the membrane affects the interaction between phospholipid molecules both in polar and hydrophobic region of the bilayer that results in changes of all transition parameters induced by this drug.

Transition temperature of both lipids is affected to similar extent by phenothiazine derivative studied. Enthalpy change during main phospholipid phase transition is, however, much stronger affected by this compound in DMPC bilayers. Its influence is so strong that the transition is completely abolished at drug:lipid molar ratio 0.12. Main phase transition peaks appear on thermograms as the result of cooperative melting of phospholipid hydrocarbon chains. Peak's broadening and decreasing of its intensity are signs of perturbation of interactions between phospholipid acyl chains. At high drug:lipid molar ratios these interactions in DMPC model membrane are probably destroyed. Stronger influence of FPhMS on DMPC bilayers than on DMPG ones may seem a little surprising as strong specific interactions of phenothiazines with anionic phospholipids were reported previously [23,24]. We propose that the phenomenon that we observed could be explained by slightly different location of FPhMS molecule inside the bilayers composed of different lipids. Cationic side chain group of phenothiazine could interact strongly with negatively charged polar headgroups of DMPG. This interaction would anchor the drug molecule relatively close to membrane surface and thereby it could prevent the hydrophobic ring system from intercalating deeply between the acyl chains of phospholipid. On the other hand, in neutral DMPC interactions of FPhMS with polar region of the membrane would probably be weaker that could result in deeper compound's immersion inside the model membrane. Such a localization of phenothiazine derivative in zwitterionic lipid system would cause

a stronger perturbation of its transition enthalpy as compared with charged lipid system and that is what is observed.

The role of membrane charge in its interaction with phenothiazine-type MDR modulator was also studied by means of fluorescence spectroscopy. NPN is a fluorescent probe that locates at polar/apolar interface of lipid bilayer. Quenching of its fluorescence with concomitant red shift of its emission maximum is usually attributed to increased polarity in the fluorophore vicinity caused by the presence of the studied compound [34]. However, the upward curvature of Stern–Volmer plots (especially significant in case of PS liposomes) may suggest that more complex mechanism is responsible for NPN quenching caused by FPhMS. Such effect observed in homogenous solution could be explained by a mixture of dynamic and static quenching, however in liposomes also membrane effects must be taken under consideration. NPN fluorescence quenching depends on phenothiazine derivative concentration, whereas NPN fluorescence lifetime is lowered by even the lowest drug concentration tested and drug's amount increase does not change the lifetime any further, as if the interaction became saturated. In our opinion this point to some kind of direct interaction, i.e. complex formation between the drug and the probe inside the lipid bilayer. The nature of this putative complex is elusive; it could be formed by molecules in ground or in excited states. The red-shift of NPN emission maxima in the presence of FPhMS could point to exciplex formation. In our opinion, however, the observed shift is too small. If exciplex was formed we would rather expect the appearance of new band of fluorescence with distinctly different maximum.

We have observed that FPhMS caused the strongest NPN quenching in liposomes formed from anionic PS. Conversely the influence of this drug on fluorescence polarization of DPH was the most potent in model membranes composed of neutral PC. DPH is a hydrophobic probe locating deeply in the membrane core. Its fluorescence polarization changes can be due to the altered molecular order of hydrophobic region of membrane and/or probe's lifetime variations. As the FPhMS-induced polarization changes are accompanied by quenching of DPH fluorescence we decided to study the probe's fluorescence lifetimes, too. Our results show that DPH has the longest fluorescence lifetime in lipid bilayers composed of anionic phosphatidylserine and the shortest one in neutral PC membranes. PS model membranes are also characterized by the highest DPH polarization degree and the highest Laurdan GP values (see below). The addition of phenothiazine derivative to DPH labeled liposomes caused the shortening of fluorescence lifetime clearly visible in PS bilayers and almost negligible in membranes with high PC content. Such a behavior suggests that drug-induced DPH lifetime shortening is responsible for observed polarization increase in PS bilayers to much greater extent than in case of PC membranes. In the latter case the polarization degree increase is probably caused by membrane ordering effects, i.e. rigidifying of the model membrane. Therefore we can conclude that at least PC model membranes and PC:PS mixtures of high PC content (minimum 50%) are rigidified by FPhMS.

As fluorescent probe's lifetime variation can affect the results of fluorescence polarization measurements we decided to study the effect exerted by FPhMS on DPH fluorescence parameters in lipid systems in more detail. Previously, we have observed the opposite FPhMS-induced changes of DPH polarization depending on phase state of the lipid bilayer [27]. DPH polarization was reduced by phenothiazine derivative in membranes below transition temperature and increased above T_m, i.e. in liquid crystalline state. In the present study we measured DPH fluorescence lifetimes in both phase states of DPPC membranes in presence of the studied compound. We have demonstrated that DPH lifetime is shortened by FPhMS in both gel-like and liquid–crystalline lipid bilayers.

The picture generated by fluorescence spectroscopy is consistent with our hypothesis of slightly different localization of FPhMS molecules in membranes composed of neutral and charged lipids. According to our model phenothiazine derivative is localized closer to the bilayer surface in PS liposomes. That is why it affects the fluorescence of shallowly positioned probe NPN stronger in PS

than in PC model membranes. On the other hand, fluorescence polarization of deeply inserted DPH is affected by the studied compound to lesser extent in PS than in PC liposomes that is consistent with postulated deeper FPhMS insertion into PC bilayers.

Proposed different localization of phenothiazine derivative in model anionic and neutral membranes allows also to eliminate the apparent discrepancy between the results obtained by DPH fluorescence polarization and lifetime measurements. This discrepancy lies in the fact that FPhMS-induced change in polarization is the most pronounced in neutral PC systems whereas DPH lifetime is the most affected by the drug in anionic PS membranes. In PC liposomes molecules of fluorescent label and drug are presumably located much closer to each other than in PS bilayers. Thus in PC systems direct interactions between the drug and the probe molecules may lead to the observed lifetime effects. Since all of FPhMS concentrations used are much higher than the concentration of DPH their possible interaction may be saturated and that is why we observe no lifetime dependence on drug concentration. The deeper localization of phenothiazine derivative in PC model membrane would also cause the pronounced DPH polarization changes. On the other hand, in PS bilayers the effect exerted by phenothiazine derivative on DPH fluorescence lifetime is indirect and depicts the influence of the drug on membrane properties. As the lifetime shortening is often related to the increase of water content inside the membrane [35] we may assume that incorporation of FPhMS molecule closer to the polar/apolar membrane interface in PS liposomes may enhance water penetration into the membrane. DPH fluorescence polarization increase observed in PS liposomes is not contradictory to the postulate of increased water content in membrane as this polarization measurements series can be significantly biased by lifetime shortening. The above hypothesis seems to be supported by our NPN quenching results, which point to the increased polarity of probe surroundings in PS reach PC:PS mixtures.

There is one problem that still needs elucidation, however. We have observed that the changes of both NPN and DPH fluorescence parameters recorded in the presence of FPhMS are not proportional to the content of anionic PS in liposomes. Phenothiazine's behavior in liposomes composed of equimolar PC:PS mixture is more similar to its behavior in pure PS membranes, whereas in PC:PS (2:1) and PC:PS (1:2) mixtures it resembles more the pure PC system. To address this problem we decided to study the properties of PC:PS model membranes in the absence of the drug. DPH fluorescence polarization measurements have shown that molecular ordering in PC:PS (1:1) liposomes is more similar to pure PS than to pure PC membranes. Conversely the experiments with the use of Laurdan as a fluorescent probe have demonstrated that changes in its fluorescence parameters are proportional to the amount of PS in the model system studied. Laurdan reports on membrane region below the phospholipid ester groups and its spectral properties are highly sensitive to the polarity in the fluorophore vicinity [32]. The increase of hydration in membranes (e.g. caused by gel to liquid–crystalline phospholipid phase transition) results in decrease of intensity of Laurdan emission with concomitant red shift of its maximum (of approx. 40 nm). Due to Laurdan spectral properties its generalized polarization can be used to assess the coexistence of gel-like and fluid domains in membrane (for details see Ref. [32]). Briefly, GP values are approximately 0.6 in gel phase and approximately −0.2 in liquid–crystalline phase if calculated for the same excitation and emission conditions as in [32]. Also the slope of GP vs. λ_{ex} dependence is characteristic for a given phase. It is around zero in gel phase, negative in liquid–crystalline phase and positive when domains of both phases coexist in the system. Our results have shown that increasing amount of PS in liposomes results in the decrease of the polarity in membrane region where Laurdan molecules are localized. GP calculation has demonstrated that there is no typical gel–fluid phase separation in this system. Both lipids used: bovine brain PS and egg yolk PC are in liquid-crystalline phase under conditions of the experiment. It can be noticed both from Laurdan GP and DPH polarization studies that pure PC membranes are more fluid than pure PS liposomes.

Laurdan is able to detect phase coexistence in membranes only when the two phases present differ significantly in hydration. That is why, in spite of the results of Laurdan experiments, we suppose that higher similarity of equimolar PC:PS mixture to pure PS membranes may be caused by non-ideal mixing of these two lipids in the liquid-crystalline phase. PC- or PS-enriched regions could form in membrane as the result of different biophysical properties of the two phospholipids. However, such regions, if they existed, would be both fluid and they would not differ in lipid hydration enough to be detected by Laurdan fluorescence. We have also to bear in mind that electrostatic repulsion between negatively charged PS headgroups could hinder PS molecules from aggregating. It was demonstrated previously by fluorescence spectroscopic methods that in fluid mixtures of PC with such anionic lipids as phosphatidylserine and phosphatidic acid but not phosphatidylglicerol phase separation occurs [36]. The theoretical analysis of PC:PS mixing has led Huang and Feigenson [37] to the conclusion that the range of PS mole fraction where phase separation could be observed depends only on the strength of electrostatic repulsion between PS headgroups. Assuming that the nonelectrostatic excess mixing energy of a PC:PS pair is 2.22×10^{-21} J the authors calculated that in room temperature phase separation in PC:PS mixture existed only when the amount of PS in the system was between 25 and 65 mol%. Their expectations have been confirmed by experimental results by Hinderliter et al. [38]. Monitoring the binding of Ca^{2+} ions to PC:PS membranes and employing X-ray diffraction technique they have shown that phase separation occurs only in mixtures with 52–62 mol% of phosphatidylserine. The region of phase separation could be broadened up to 50–80 mol% of PS by increasing the ionic strength of the solution (the addition of 800 mM of KCl) that caused screening of electrostatic PS headgroups repulsion. The experiments presented in our work were performed in low ionic strength. It allows as to presume that in our system phase separation could occur only in PC:PS (1:1) mixture or occurred in this mixture to the highest extent. In the other PC:PS mixtures tested both kinds of lipids are probably mixed more homogenously. In equimolar PC:PS system the molecules of FPhMS could preferentially bind to PS-enriched regions of membrane or to PC:PS domain boundaries as proposed by Jutila [25] for CPZ. In our opinion such a behavior of phenothiazine derivative could explain the greater similarity of its influence on PC:PS (1:1) membranes than on pure PS liposomes.

Acknowledgments

This work was supported by the State Committee for Scientific Research (KBN) Grant No. 6 P05A 01221. Olga Wesolowska is grateful to the Foundation for Polish Science for the scholarship.

References

[1] I. Bosch, J. Croop, P-glycoprotein multidrug resistance and cancer, Biochim. Biophys. Acta 1288 (1996) F37–F54.
[2] S.V. Ambudkar, S. Dey, C.A. Hrycyna, M. Ramachandra, I. Pastan, M.M. Gottesman, Biochemical, cellular, and pharmacological aspects of the multidrug transporter, Annu. Rev. Pharmacol. Toxicol. 39 (1999) 361–398.
[3] I. Bosch, K. Dunussi-Joannopoulos, R.L. Wu, S.T. Furlong, J. Croop, Phosphatidylcholine and phosphatidylethanolamine behave as substrates of the human MDR1 P-glycoprotein, Biochemistry 36 (1997) 5685–5694.
[4] A. Pohl, H. Lage, P. Muller, T. Pomorski, A. Herrmann, Transport of phosphatidylserine via MDR1 (multidug resistance 1) P-glycoprotein in a human gastric carcinoma cell line, Biochem. J. 365 (2002) 259–268.
[5] J. Ferte, Analysis of the tangled relationships between P-glycoprotein-mediated multidrug resistance and the lipid phase of the cell membrane, Eur. J. Biochem. 267 (2000) 277–294.
[6] M. Wiese, I.K. Pajeva, Structure-activity relationships of multidrug resistance reversers, Curr. Med. Chem. 8 (2001) 685–713.
[7] C. Avendano, J.C. Menendez, Inhibitors of multidrug resistance to antitumor agents (MDR), Curr. Med. Chem. 9 (2002) 159–193.
[8] G. Speelmans, R.W.H.M. Staffhorst, F.A. de Wolf, B. de Kruijff, Verapamil competes with doxorubicin for binding to anionic phospholipids resulting in increased internal concentrations and rates of passive transport of doxorubicin, Biochim. Biophys. Acta 1238 (1995) 137–146.
[9] T. Tsuruo, H. Iida, S. Tsukagoshi, Y. Sakurai, Increased accumulation of vincristine and adriamycin in drug-resistant P388 tumor cells following incubation with

calcium antagonists and calmodulin inhibitors, Cancer Res. 42 (1982) 4730–4733.

[10] J. Molnar, A. Hever, I. Fakla, I. Ocsovski, A. Aszalos, Inhibition of the transport function of membrane proteins by some substituted phenothiazines in *E. coli* and multidrug resistant tumor cells, Anticancer Res. 17 (1997) 481–486.

[11] O. Wesolowska, J. Molnar, N. Motohashi, K. Michalak, Inhibition of P-glycoprotein transport function by *N*-acylphenothiazines, Anticancer Res. 22 (2002) 2863–2868.

[12] J.M. Ford, W.C. Prozialeck, W.N. Hait, Structural features determining activity of phenothiazines and related drugs for inhibition of cell growth and reversal of multidrug resistance, Mol. Pharmacol. 35 (1989) 105–115.

[13] A. Ramu, N. Ramu, Reversal of multidrug resistance by phenothiazines and structurally related compounds, Cancer Chemother. Pharmacol. 30 (1992) 165–173.

[14] W. Caetano, M. Tabak, Interaction of chloropromazine and trifluoperazine with ionic micelles: electronic absorption spectroscopy studies, Spectrochim. Acta A 55 (1999) 2513–2528.

[15] S.V. Malheiros, E. de Paula, N.C. Meirelles, Contribution of trifluoperazine/lipid ratio and drug ionization to hemolysis, Biochim. Biophys. Acta 1373 (1998) 332–340.

[16] B. Isomaa, H. Hagerstrand, G. Paatero, Shape transformations induced by amphiphiles in erythrocytes, Biochim. Biophys. Acta 899 (1987) 93–103.

[17] A.B. Hendrich, K. Lichacz, A. Burek, K. Michalak, Thioridazine induces erythrocyte stomatocytosis due to interactions with negatively charged lipids, Cell. Mol. Biol. Lett. 7 (2002) 1081–1086.

[18] M.P. Sheetz, S.J. Singer, Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions, Proc. Natl. Sci. USA 71 (1974) 4457–4461.

[19] J. Rosso, A. Zachowski, P.F. Devaux, Influence of chlorpromazine on the transverse mobility of phospholipids in human erythrocyte membrane: relation to shape changes, Biochim. Biophys. Acta 942 (1988) 271–279.

[20] A.B. Hendrich, O. Wesolowska, K. Michalak, Trifluoperazine induces domain formation in zwitterionic phosphatidylcholine but not in charged phosphatidylglycerol bilayers, Biochim. Biophys. Acta 1510 (2001) 414–425.

[21] A.J. Verkleij, R. de Maagd, J. Leunissen-Bijvelt, B. de Kruijff, Divalent cations and chlorpromazine can induce non-bilayer structures in phosphatidic acid-containing model membranes, Biochim. Biophys. Acta 684 (1982) 255–262.

[22] I.K. Pajeva, M. Wiese, H.P. Cordes, J.K. Seydel, Membrane interactions of some catamphilic drugs and relation to their multidug-resistance-reversing ability, J. Cancer Res. Clin. Oncol. 122 (1996) 27–40.

[23] W. Nerdal, S.A. Gundersen, V. Thorsen, H. Hoiland, H. Holmsen, Chlorpromazine interaction with glycerophospholipid liposomes studied by magic angle spinning solid state ^{13}C-NMR and differential scanning calorimetry, Biochim. Biophys. Acta 1464 (2000) 165–175.

[24] A. Varnier Agasoster, L.M. Tungodden, D. Cejka, E. Bakstad, L.K. Sydnes, H. Holmsen, Chlorpromazine-induced increase in dipalmitoylphosphatidylserine surface area in monolayers at room temperature, Biochem. Pharmacol. 61 (2001) 817–825.

[25] A. Jutila, T. Soderlund, A.L. Pakkanen, M. Huttunen, P.K.J. Kinnunen, Comparison of the effects of clozapine, chlorpromazine, and haloperidol on membrane lateral heterogeneity, Chem. Phys. Lipids 112 (2001) 151–163.

[26] A.B. Hendrich, O. Wesolowska, N. Motohashi, J. Molnar, K. Michalak, New phenothiazine-type multidrug resistance modifiers: anti-MDR activity versus membrane perturbing potency, Biochem. Biophys. Res. Commun. 304 (2003) 260–265.

[27] A.B. Hendrich, O. Wesolowska, M. Komorowska, N. Motohashi, K. Michalak, The alterations of lipid bilayer fluidity induced by newly synthesized phenothiazine derivative, Biophys. Chem. 98 (2002) 275–285.

[28] O. Wesolowska, A.B. Hendrich, N. Motohashi, K. Michalak, Phenothiazine derivative causes phase separation in phosphatidylethanolamine model membranes, Curr. Topics Biophys. 25 (2001) 71–73.

[29] N. Motohashi, M. Kawase, S. Saito, T. Kurihara, K. Satoh, H. Nakashima, M. Premanathan, R. Arakaki, H. Sakagami, J. Molnar, Synthesis and biological activity of *N*-acylphenothiazines, Int. J. Antimicrob. Agents 14 (2000) 203–207.

[30] N. Motohashi, M. Kawase, J. Molnar, L. Ferenczy, O. Wesolowska, A.B. Hendrich, M. Bobrowska-Hagerstrand, H. Hagerstrand, K. Michalak, Antimicrobial activity of *N*-acylphenothiazines and their influence on lipid model membranes and erythrocyte membranes, Arzneim–Forsch. 53 (2003) 590–599.

[31] T. Parasassi, G. de Stasio, A. d'Ubaldo, E. Gratton, Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence, Biophys. J. 57 (1990) 1179–1186.

[32] T. Parasassi, E. Gratton, Membrane lipid domains and dynamics as detected by Laurdan fluorescence, J. Fluorescence 5 (1995) 59–69.

[33] M.K. Jain, N.M. Wu, Effect of small molecules on the dipalmitoyl lecithin liposomal bilayer: III. Phase transition in lipid bilayer, J. Membrane Biol. 34 (1977) 157–201.

[34] G.K. Radda, in: E.D. Korn (Ed.), Methods in membrane biology, Fluorescent probes in membrane studies, 4, Plenum Press, New York, 1975, p. 97.

[35] C. Ho, C.D. Stubbs, Effect of *n*-alkanols on lipid bilayer hydration, Biochemistry 36 (1997) 10630–10637.

[36] T. Ahn, C.H. Yun, Phase separation in phosphatidylcholine/anionic phospholipid membranes in the liquid-crystalline state revealed with fluorescent probes, J. Biochem. 124 (1998) 622–627.
[37] J. Huang, G.W. Feigenson, Monte Carlo simulation of lipid mixtures: finding phase separation, Biophys. J. 65 (1993) 1788–1794.
[38] A.K. Hinderliter, J. Huang, G.W. Feigenson, Detection of phase separation in fluid phosphatidylserine/phosphatidylcholine mixtures, Biophys. J. 67 (1994) 1906–1911.