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A study on the perturbation of model lipid membranes by phenoxazines

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Abstract—The interactions of six newly synthesized phenoxazine derivatives with lipid bilayers were studied by means of calorimetry, fluorescence spectroscopic methods and electron spin resonance. Depending on their structure studied compounds decreased membrane fluidity and increased lipid order in liquid-crystalline bilayers to different degrees. These studies showed also that phenoxazine molecules are located close to the polar/apolar interface of bilayer. The results allow to conclude that phenoxazines rather weakly interact with lipid bilayers.

1. Introduction

Phenoxazines are structurally similar to phenothiazines, both groups of compounds are well-known drugs widely used in treatment of psychiatric diseases. Phenoxazines, instead of sulfur atom present in phenothiazines, possess the oxygen atom in the tricyclic ring system (Fig. 1). Due to this structural similarity phenoxazines are supposed to exert biological effects comparable to phenothiazines. Among others, since many years phenoxazines are supposed to be the effective modulators of multidrug resistance of cancer cells.[1] Phenoxazines act as an antioxidant in cultured neuronal cells,[2] with the half-maximal effective concentrations of 20–75 nM. They are as active as phenothiazines and about two orders of magnitude more effective than common phenolic antioxidants. Phenoxazine derivative (2-amino-4,4α-dimethyl-4α,7-dimethyl-3*H*-phenoxazin-3-one) was found to down-regulate the expression of IgM and inhibit B cell antigen receptor-mediated signalling and IgM secretion. Such properties of this phenoxazine derivative may make it useful as an immunosuppressive agent for therapeutic purposes.[3] This compound exerts also anticancer effects—induction of apoptosis and necrosis in human B cell lymphoblastoid cell lines was recorded by Koshibu-Koizumi et al.[4] Additionally this phenoxazine derivative arrests the GM2 stage of cultured adeno-

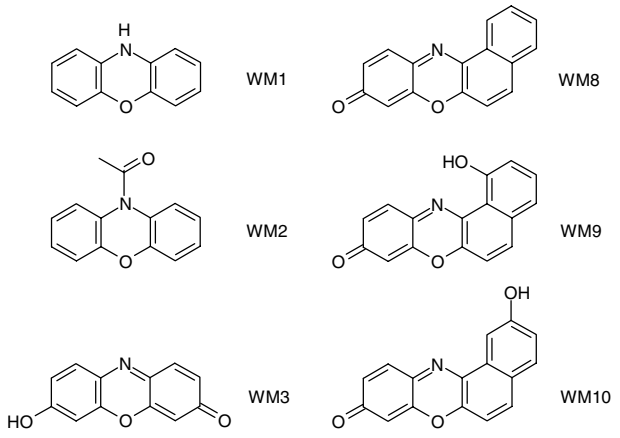

Figure 1. Chemical structures of phenoxazine and its derivatives: WM1, phenoxazine; WM2, 10-acetylphenoxazine; WM3, 7-hydroxy-3H-phenoxazin-3-one; WM8, 9*H*-benzo[*a*]phenoxazin-9-one; WM9, 1-hydroxybenzo[*a*]phenoxazin-9-one; WM10, 2-hydroxybenzo[*a*]phenoxazin-9-one.

Keywords: Phenoxazine; Lipid bilayer; Fluidity; Fluorescence spectroscopy; Microcalorimetry.
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carcinoma cell cycle and induces apoptosis of these cells.[5]

Unmodified phenoxazine was the most effective anticancerogenic agent among the group of 29 different tri-heterocyclic compounds.[6] Phenoxazine showed remarkable inhibitory effects against mouse skin tumour promotion. It was also found that in murine model the positive anticancer effects were not accompanied by any negative side effects. Recently Iwata et al.[7,8] presented the evidence that 2-amino-4,4α-dihydro-4α,7-dimethyl-3*H*-phenoxazin-3-one and 3-amino-1,4αl-dihydro-4α,8-dimethyl-2*H*-phenoxazin-2-one are effective antiviral agents.

The ability of phenoxazine derivatives to interact with albumin was tested by Channu et al.[9] It was found that binding of different phenoxazine derivatives to protein correlates with their hydrophobicity (expressed in terms of octanol/water partition coefficient—log *P*). Authors suggested that phenoxazine benzene rings and tertiary amines attached to the side chain of phenoxazine moiety bind to the hydrophobic fragments of albumin molecule. Interaction of phenoxazine with P-glycoprotein (P-gp) was proposed as a putative mechanism of increase of vinca alkaloid accumulation in multidrug resistant (MDR) human adenocarcinoma and HeLa cell lines[10] induced by the drug. This MDR modulating effect of phenoxazine was additive with the effects exerted on the studied cells by verapamil. The same research team tested also a group of 21 N-substituted phenoxazines for their ability to modulate MDR of cancer cells.[11] Some of these compounds potentiated the cytotoxicity of vinca alkaloids. Structure–activity relationship studies were also performed using a group of 2-chloro-N(10)-substituted phenoxazines.[12] It was found that lipophilicity of these compounds is important for their anti-MDR activity, however other structural features also determine the potency of these molecules. The same research group has also demonstrated that at least part of the N-substituted phenoxazines' activity could be mediated through the P-glycoprotein-independent mechanism.[13]

The lipophilic character of phenoxazine molecule provides the opportunity for the rapid uptake and efflux of phenoxazine into the cells and binding of this drug to the hydrophobic regions in the cells.[14] Since no correlation between the phenoxazine cellular transport and P-gp content was recorded, the authors concluded that direct interaction of the drug with transport protein is unlikely or at least incidental.

Despite the well-documented binding of phenoxazine and its derivatives to hydrophobic domains of proteins and other non-polar cellular regions not much is known about the possible drug–lipid interactions. To characterize the influence of phenoxazine derivatives on the model lipid bilayers we carried out fluorescence spectroscopic, microcalorimetric and electron spin resonance studies. Experimental results allowed us to conclude that apart from direct influence exerted on membrane proteins also the alteration of lipid phase properties induced by phenoxazine and its derivatives could be considered as a possible molecular mechanism underlying the biological activity of these compounds.

2. Results

2.1. Microcalorimetry

In microcalorimetric experiments we determined the influence of studied phenoxazines on the temperature and enthalpy change of DPPC main phase transition. Figure 2 presents the thermograms obtained for DPPC mixtures with WM2 at different molar ratios. Thermograms for other phenoxazines are not presented here since they were qualitatively similar to those shown in Figure 2. In most cases the shifting of transition temperatures and enthalpy changes of DPPC main phase transition were accompanied by broadening of transition peaks. The phenoxazine influence on pretransition of DPPC is not described here since even at lowest drug–lipid molar ratio phenoxazines caused disappearance of this transition. As it is shown in Figure 3 the most of phenoxazines exerted only moderate effects on DPPC main phase transition. The transition temperature was not altered by WM8. WM3 and WM2 decreased T_m only moderately, while WM10 and WM1 induced substantial decrease of DPPC transition temperature (Fig. 3A). The most pronounced transition temperature changes were recorded for WM1—at the highest of the studied phenoxazine–lipid molar ratios (0.12) T_m was decreased more than 3.5 °C. DPPC main phase transition enthalpy was changed by phenoxazines to even smaller extent than transition temperature (Fig. 3B). Only for WM1 and WM2 we observed the concentration-dependent decrease of transition enthalpy. For the highest of examined drug–lipid molar ratios (0.12) transition enthalpy was reduced to approximately 60% of the value obtained for pure lipid. In the case of WM8–DPPC mixtures a slight concentration-dependent increase of transition enthalpy was recorded. Other

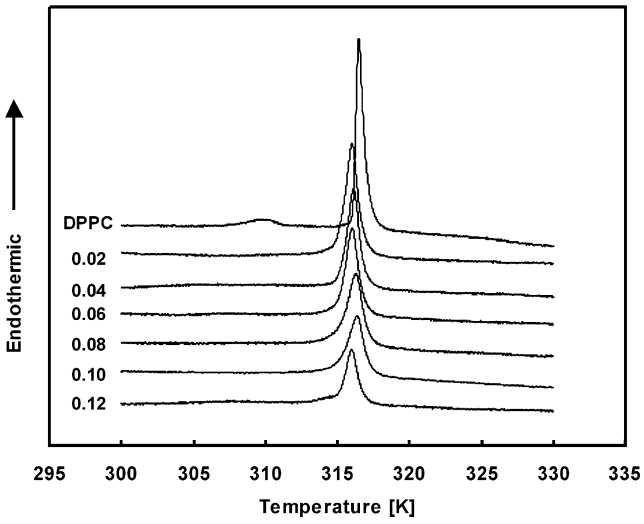

Figure 2. The microcalorimetric thermograms of DPPC mixed with WM2 at different phenoxazine–lipid molar ratios.

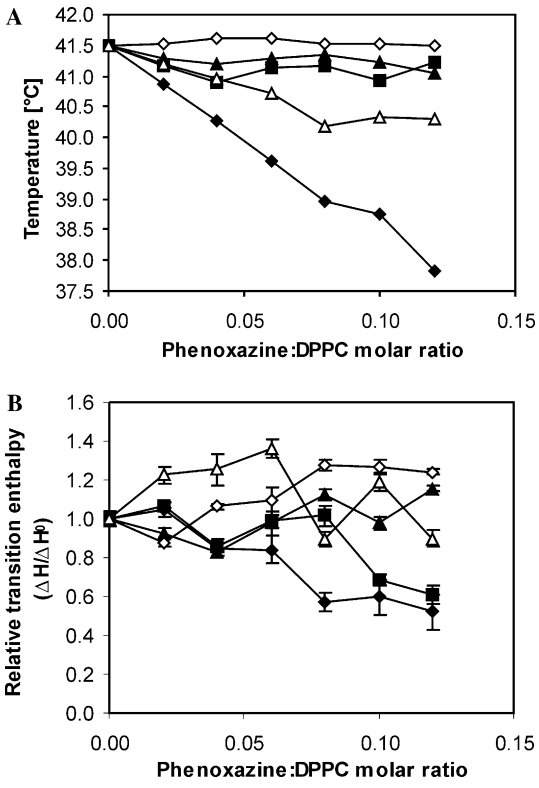

Figure 3. The influence of phenoxazines on the main phase transition of DPPC. (A) The effects exerted on phase transition temperature (T_m); (B) The effects exerted on phase transition enthalpy. In both panels: ♦—WM1; ■—WM2; ▲—WM3; ◊—WM8; Δ—WM10.

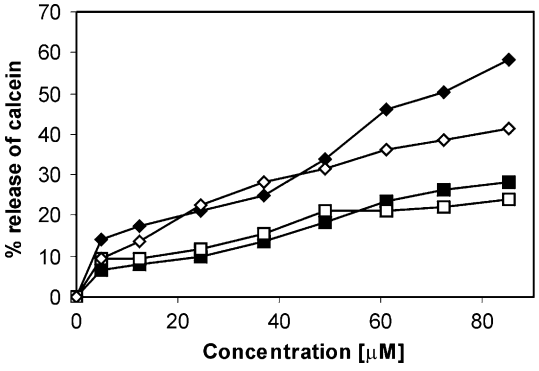

Figure 4. Calcein leakage from small unilamellar EYPC liposomes induced by phenoxazines. ♦—WM1; ■—WM2; ◊—WM8; □—WM9.

phenoxazines exerted no significant influence on the enthalpy of lipid phase transition.

2.2. Calcein leakage

In calcein leakage studies we used exclusively WM2, WM9, WM1 and WM8 phenoxazines (Fig. 4). WM10 and WM3 were not tested because their emission spectra overlapped with the emission spectrum of calcein. Regarding the ability to induce calcein release from liposomes studied phenoxazines could be divided into two groups. WM9 and WM2 showed only a moderate influence—we observed a concentration-dependent calcein release, however at the maximal concentration of the drugs (84 μM) only approximately 20% increase of calcein leakage was recorded. Two other compounds (WM8 and WM1) were much more effective. Their influence on the calcein release was also dose-dependent and at maximal concentrations used they induced much intensive dye efflux from liposomes: 35% and 57% for WM8 and WM1, respectively.

2.3. Fluorescence spectroscopy

The temperature dependence of Laurdan generalised polarization (GP) was used to characterize the influence of phenoxazines on the molecular order in gel and liquid-crystalline phases of DPPC bilayers. All plots representing GP-temperature dependency were composed of three different fragments (see Fig. 5). In temperatures below the lipid main phase transition a high (about 0.6) and almost constant GP values were obtained. In the temperature range corresponding to the main phase transition (T_m) the dramatic decrease of GP was observed. For temperatures above T_m further (but less dramatic) decrease of GP or constant GP values were obtained. GP values obtained for phenoxazine-DPPC mixtures at temperatures below the main phase transition were in most cases similar to the control values recorded for pure DPPC (Fig. 5). The exception was WM10, for which a slight increase of GP was recorded. More profound alteration of GP values was found for temperatures close and above the main phase transition of DPPC. For WM1 and WM2 a decrease of GP values started at 40 °C—unlike for other phenoxazine–DPPC mixtures, for which the GP values begun to decrease at approximately 42 °C. Except for WM2 and WM9, the decrease of GP values was not as pronounced as observed for pure DPPC. For WM1, WM8 and WM10 even in the last phase the GP-temperature dependence had not reached the negative values.

Similar temperature dependence was also observed for DPH fluorescence polarization anisotropy (Fig. 6). At temperatures below T_m for WM10 and WM8 values of DPH fluorescence polarization anisotropy were slightly

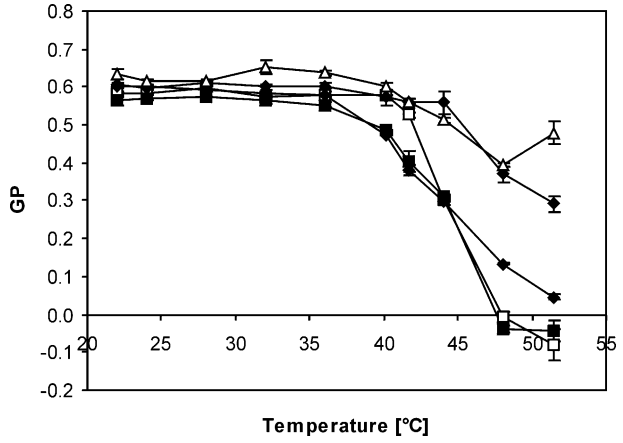

Figure 5. The dependence of Laurdan generalised polarization (GP) on temperature. Liposomes were formed of DPPC and the effects of the following phenoxazines were studied: ♦—WM1; ■—WM2; ◊—WM8; □—WM9; Δ—WM10; ●—pure DPPC.

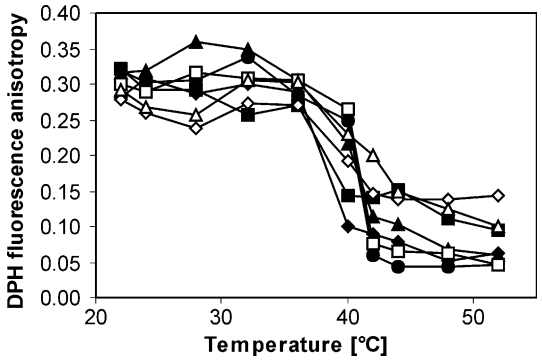

Figure 6. The dependence of DPH fluorescence polarization anisotropy on temperature. Liposomes were formed of DPPC and the effects of the following phenoxazines were studied: ♦—WM1; ■—WM2; ▲—WM3; ◊—WM8; □— WM9; Δ—WM10; ●—pure DPPC.

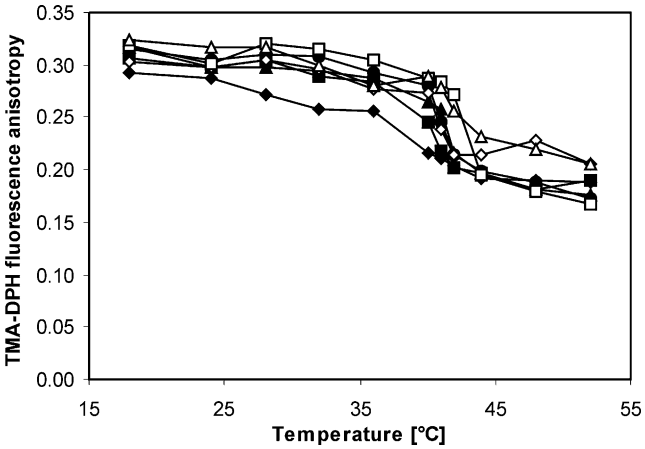

Figure 7. The dependence of TMA-DPH fluorescence polarization anisotropy on temperature. Liposomes were formed of DPPC and the effects of the following phenoxazines were studied: ♦—WM1; ■—WM2; ▲—WM3; ◊—WM8; □— WM9; Δ—WM10; ●—pure DPPC.

smaller than in case of pure lipid, values for WM1, WM2 and WM9 were close to those obtained for DPPC, while fluorescence polarization anisotropy of WM3 was slightly higher than that for pure lipid. The decrease of anisotropy started at temperatures lower than 41.5 °C for the most of phenoxazine–lipid mixtures. Only for WM9 the anisotropy decrease started at the same temperature as for pure DPPC. Well above the DPPC main phase transition temperature the DPH fluorescence polarization anisotropy for all of studied mixtures was higher than for pure lipid. There are, however, some differences between the phenoxazines used, because for WM1, WM2, WM3 and WM10 we observed further (but slower than in previous phase) decrease of anisotropy with temperature, while for WM8 and WM9 fluorescence polarization anisotropy remained almost constant in the liquid-crystalline phase.

The dependence of TMA-DPH fluorescence polarization anisotropy on temperature of DPPC liposome suspensions was also studied. Results obtained in these experiments were qualitatively similar to those recorded for DPH, however the extent of fluorescence polarization anisotropy changes during main phase transition of DPPC was much smaller than it was observed for DPH (see Fig. 7). In temperatures below T_m of DPPC only WM1 caused a significant decrease of TMA-DPH fluorescence polarization anisotropy with respect to values obtained for liposomes composed of pure lipid. In the transition region (i.e., near 41.5 °C) WM1 and WM2 decreased fluorescence anisotropy values. For temperatures higher than 43 °C only WM8 and WM10 caused increase of TMA-DPH fluorescence polarization anisotropy with respect to DPPC.

2.4. Electron spin resonance

The tumbling correlation time (τ_c) and order parameter (S) values obtained for spin probes present in pure DPPC bilayers and in its mixtures with WM1, WM2 and WM8 are given in Table 1. Regarding the influence on order parameter the effects of WM1 and WM8 were similar: both compounds increased the values of this parameter. The tumbling correlation time of 16DSA probe was decreased by WM1 and increased by WM8 (comparing to pure DPPC). WM2 mixed with DPPC caused effects opposite to WM1, it decreased the S and significantly increased τ_c values.

Table 1. Order parameter and tumbling correlation time for 5DSA and 16DSA spin probes located in the DPPC bilayers containing phenoxazines

Sample	5DSA order parameter ($S \times 10^{-1}$)	16DSA tumbling correlation time ($\tau_c \times 10^{-8}$) [s]
DPPC	6.50	3.34
WM1	6.91	2.87
WM2	6.34	3.61
WM8	6.61	5.38

Phenoxazine–lipid molar ratio were given in the above table.

3. Discussion

The structural similarity between phenothiazines and phenoxazines allows to expect that these compounds will also similarly interact with lipid bilayers. It was shown in our laboratory[15–17] as well as by other research groups[18–20] that phenothiazines generally intercalate into the lipid bilayers and are located in the vicinity of the polar/apolar membrane interface. The details of drug–lipid interactions depend, however, both on the lipid type as well as on the type of substitutions present at certain phenothiazine derivative molecules.

The results of microcalorimetric experiments showed that phenoxazines (WM3, WM2, WM10 and WM1) presumably interact with lipid bilayers, however in most cases this interaction seems to be weaker than that observed for phenothiazines. Only WM1 showed pronounced effects on both transition temperature and enthalpy what suggested that presumably the polar as well as hydrophobic regions of bilayer were affected by this phenoxazine. Moderate effects exerted by other studied phenoxazines on the DPPC transition temperature showed that these compounds weakly interacted with the polar region of lipid bilayer. The strongest

WM1-bilayer interactions were confirmed by the results of other experiments. WM1 affected the integrity of lipid bilayer and induced the biggest calcein efflux from liposomes. Membrane permeabilization induced by different factors is often related to the increase of its fluidity but not in all cases. Tomisato et al.[21] found that some non-steroidal anti-inflammatory drugs (mefenamic acid, flufenamic acid, celecoxib and nimesulide) decreased liposome membrane fluidity and simultaneously increased its permeability. Our EPR results seem to confirm that also for WM1 the increase of liposome permeability induced by this compound was coupled with the decrease of membrane fluidity. It is worth to notice, however, that regarding the results obtained for 5DSA and 16DSA spin probes the influence of WM1 on the level of 16th carbon atom was weaker than that recorded closer to the bilayer polar region what was coherent with calorimetric data.

Spectrofluorimetric measurements confirmed that phenoxazines affect the phase properties of lipids, however each of the fluorescence labels used gave slightly different results for the same phenoxazines. The decrease of transition temperature was detected by DPH for almost all of phenoxazines (except for WM9), while Laurdan and TMA-DPH showed T_m decrease only for WM1 and WM2. This discrepancy may be caused by the different position of fluorescence probe molecules in lipid bilayers. DPH is buried in the hydrophobic bilayer centre,[22] while Laurdan[30] and TMA-DPH[23] are positioned closer to the surface of bilayer. Additionally DPH and TMA-DPH fluorescence polarization anisotropy is related to the mobility of probe surrounding (in other words it reflexs the ordering of the acyl chains), while Laurdan generalised polarization depends on the polarity of this probe environment and thus the packing of lipid molecules in bilayer.[24] The courses of DPH, TMA-DPH polarization anisotropy as well as Laurdan generalised polarization dependencies on temperature allow also to conclude that phenoxazines: WM2, WM8 and WM10 alter the properties of the gel state of lipids to the lesser extent than the liquid-crystalline state. As a result of phenoxazine–lipid interaction bilayers in liquid-crystalline state become less fluid (DPH, TMA-DPH) and more ordered (Laurdan) than those of pure lipid.

Comparing the chemical structures of well-known and clinically used phenothiazine derivatives (like chlorpromazine or trifluoperazine) with phenoxazines studied in this work it is clear that despite general structural similarity of phenothiazine/phenoxazine moieties these two types of molecules differ in the presence/absence of the side-chain group. The type of the side-chain group and eventually the group substituting the phenothiazine ring system at position 2 is essential for the different effects exerted by these compounds.[25,26] The role of similar, hypothetical substitutions of phenoxazine moiety can be easily demonstrated by calculations of octanol/water partition coefficient (log P) for model molecules containing (or not) the required elements. Addition of side-chain group at the position 10 of WM1 phenoxazine moiety (Fig. 8) increases log P from 1.997 (for phenoxazine) to 3.225 for the chain substituted compound. Further substitution at the position 2 of ring system by CF_3 group increases log P of such molecule up to 4.146. This difference may explain why phenoxazines show weaker interaction with the lipid bilayers than phenothiazines.

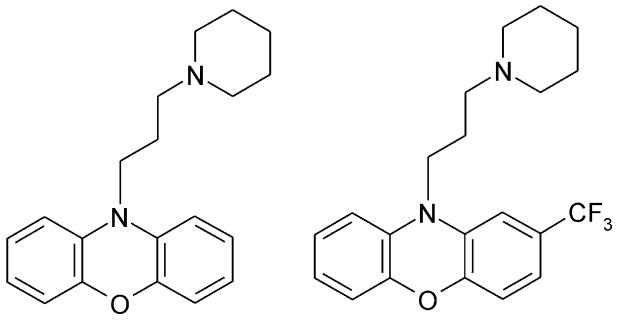

Figure 8. Chemical structures of hypothetical (phenothiazine-like) phenoxazine derivatives: left, WM1 substituted at position 10 by side chain; right, WM1 substituted at position 10 by side chain and CF3 group at position 2.

In last years the interest in phenoxazine derivatives' biological activities is increasing and it seems that these compounds are good candidates for future drugs. Some of phenoxazine activities suggest that they must cross the membranes and therefore their interactions with lipid phase of membranes have to be characterized. Present study has shown that phenoxazines interact with lipid bilayers and alter their properties (mainly in liquid-crystalline state). The strongest interaction was found for WM1, which is just a phenoxazine not substituted at any position of the rings. It seems therefore that any of the studied here molecule modifications: phenoxazine substitution by oxygen, hydroxyl and aldehyde groups or addition of the next benzene ring decreases the ability of such modified molecules to interact and perturb the bilayer structure.

4. Materials and methods

1,2-Dipalmitoyl-*n*-glycero-3-phosphatidylcholine (DPPC) and egg-yolk phosphatidylcholine (EYPC) were purchased from Sigma (St. Louis, MO, USA). Lipids were used without further purification. Fluorescent labels: calcein, 1,6-diphenyl-1,3,5-hexatriene (DPH), trimethylammonium-1,6-diphenyl-1,3,5-hexatriene (TMA-DPH) and spin probes: 5-doxylstearic acid (5DSA) and 16-doxylstearic acid (16 DSA) were purchased from Sigma (St. Louis, MO, USA). Calcein was used after purification (using column filled with Sephadex LH20). 6-Lauroyl-2-(*N*,*N*-dimethylamino)naphthalene (Laurdan) was purchased from Molecular Probes (Eugene OR, USA). Phenoxazine (WM1) was obtained from Aldrich Chemical Co. (Japan) and 7-hydroxy-3*H*-phenoxazin-3-one (WM3) was obtained from Tokyo Kasei Co. (Tokyo, Japan). The following compounds were prepared according to the previously published procedures: 10-acetylphenoxazine (WM2);[27] 9*H*-benzo[*a*]phenoxazin-9-one (WM8);[28] 1-hydroxybenzo[*a*]phenoxazin-9-one (WM9) and 2-hydroxybenzo[*a*]phenoxazin-9-one (WM10).[29]

The chemical structures and symbolic names of individual phenoxazines are shown in Figure 1.

4.1. Calcein leakage studies

Small unilamellar vesicles (SUVs) were prepared by extrusion of an aqueous suspension of EYPC multilamellar liposomes. Briefly, chloroform solution of lipid was placed in tubes and solvent was evaporated under a stream of nitrogen. Residues of the solvent were removed under vacuum for at least 2 h. The thin lipid film formed on the walls of the tubes was hydrated by vortexing with 1 ml of 36 mM calcein buffered in 10 mM Hepes buffer, pH 7.4. Liposome suspension was then manually extruded seven times through the polycarbonate filters (100 nm pore size). The calcein-containing vesicles were separated from the free calcein by molecular filtration on a Sepharose 4B column (1×20 cm) eluted with 10 mM Hepes buffer (150 mM NaCl, 1 mM EDTA) pH 7.4. NaCl (150 mM) was added to extravesiculer buffer to balance ionic strengths of intra- and extravesicular buffers. Final lipid concentration in unilamellar liposome suspension was 200 μM. Stock solutions of phenoxazines (5 mM) were prepared in DMSO. The liposomes containing calcein were incubated for 5 min with microliter amounts of phenoxazines (darkness, room temperature). The concentration of phenoxazines in samples was in the range between 4 μM and 84 μM. The degree of calcein release was determined spectrofluorimetrically. The fluorescence intensity at 520 nm was measured using a Perkin-Elmer spectrofluorimeter after excitation at 490 nm. Both the excitation and emission slit widths were set at 4 nm. Total (100%) amount of calcein in liposomes was assessed after lysing by addition of Triton X-100 of final concentration of 0.5%. The results of the leakage experiments are presented as the percentage of released calcein, calculated according to:

$$\%\ \text{release} = \frac{100 \cdot (F_t - F_0)}{F_\infty - F_0}\,[\%]$$

where: F_t is fluorescence of samples after incubation with phenoxazines, F_0 is initial fluorescence of samples (before incubation with phenoxazines) and F_∞ is maximal fluorescence of sample after lysis by Triton X-100.

Preliminary experiments have shown that calcein release from EYPC liposomes is not induced by 5 mM DMSO alone (without phenoxazines).

4.2. Microcalorimetry

For each calorimetric sample 1.5 mg DPPC was dissolved in the appropriate amount of stock solution of the studied phenoxazine. Stock solutions of phenoxazines (5 mM) were prepared in ethanol (WM1, WM2) or chloroform/methanol (1:1) (WM3, WM8, WM10). The amount of phenoxazine stock solution was chosen for each sample to obtain a drug to lipid molar ratio in the range between 0.02 and 0.2. The solvent was evaporated under stream of nitrogen to form a thin homogeneous film on walls of the tubes. Residuals of organic solvents were removed in vacuum for at least 2 h. 20 μl of 20 mM Tris–HCl buffer (150 mM NaCl, 0.5 EDTA), pH 7.5, was added to each tube containing a thin, dry film of DPPC–phenoxazine mixture. Hydrated mixtures were heated to 51 °C (a temperature about 10 °C above the gel–liquid crystalline phase transition temperature of DPPC) and shaken using a vortex. Aliquots of 20 μl of DPPC–phenoxazine mixtures were transferred to a DSC aluminium pan, sealed and submitted to DSC analysis. Calorimetric experiments were performed using scanning microcalorimeter type 600 (Unipan, Warsaw, Poland) operating at a 1 °C/min scanning rate. Thermograms were collected and analyzed off-line using software developed in our laboratory. Phase transition temperature was determined as a temperature at which the transition peak reaches the maximum. The data presented here are the average of eight measurements (two samples scanned four times each). Error bars on plots represent ±SD.

4.3. Fluorescence spectroscopy

Unilamellar DPPC liposomes for fluorescence spectroscopic measurements were obtained by sonication of 2 mM phospholipid suspension in 20 mM Tris–HCl buffer (50 mM NaCl), pH 7.4, using a UP 200s sonicator (Dr. Hilscher GmbH, Berlin, Germany). Stock solutions of phenoxazines (5 mM) were prepared in DMSO. Laurdan stock solution (1 mM) was also prepared in DMSO and tetrahydrofuran was used for DPH and TMA-DPH stock solutions' (1 mM) preparation. Liposomes (final phospholipid concentration 200 μM) were incubated with fluorescent probe (concentration 5 μM) in darkness for 30 min at room temperature. Phenoxazines were then added (concentration in the samples was 100 μM) and incubation was continued under the same conditions for next 15 min. In experiments with DPH and TMA-DPH, phenoxazines were added at varying concentrations ranging from 5 μM up to 100 μM. The degree of DPH and TMA-DPH fluorescence anisotropy was calculated according to the equation:

$$r = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)$$

where $I_\parallel$ and $I_\perp$ are the fluorescence intensities recorded in planes parallel (∥) and perpendicular (⊥) to the polarization plane of the excitation light. Fluorescence anisotropy measurements were performed with a LS 5013 spectrofluorimeter (Perkin-Elmer Ltd., Beaconsfiled, UK) equipped with a xenon lamp. Fluorescence of both DPH and TMA-DPH was excited at 360 nm. Emission of fluorescence was measured at 428 nm and 420 nm for DPH and TMA-DPH, respectively. Slit width was 5 nm for both the excitation and emission. Excitation wavelengths for Laurdan were in the range 320–400 nm (increasing in step of 10 nm) and fluorescence emission spectra were recorded in the range of 410–540 nm. Temperature-dependence measurements were carried out in temperatures varying between 22 °C and 52 °C and concentration-dependence measurements were performed at 25 °C. Temperature was controlled by a water-circulating bath and actual temperature was measured directly in the sample cuvette using platinum thermometer. The cuvette content was continuously stirred.

Generalised polarization (GP) was calculated from the Laurdan emission spectra using the following formula, proposed by Parasassi[30]:

$$GP = \frac{I_B - I_R}{I_R + I_R}$$

where I_B and I_R are the emission intensities at the blue (440 nm) and red (490 nm) edges of the emission spectrum and they correspond to the fluorescence emission maxima in the gel and the liquid-crystalline phases of a phospholipid bilayer, respectively. Laurdan fluorescence was excited at 390 nm.

In both cases: fluorescence polarization anisotropy and GP experiments the data presented here are the average of results of measurements performed using three separate liposome preparations. Error bars on plots represent ±SD.

4.4. Electron spin resonance

Stock solutions of spin probes—5-doxylstearic acid (5DSA), 16-doxylstearic acid (16DSA) (10 mM) were prepared in ethanol. The amount of stock solution of spin probe sufficient to obtain final probe–lipid molar ratio of 1:100 was dried on tube walls. Then the suspension of DPPC liposomes in Tris–HCl buffer (20 mM Tris, 150 mM NaCl and 0.5 mM EDTA, pH = 7.4) was added and the tube was mechanically shaken. After 20 min, spin probes were incorporated into liposome membranes. Phenoxazines were added to liposomes during their preparation. Final phenoxazine–lipid molar ratio in ESR samples was 0.05.

ESR spectra were recorded at room temperature using SE/X-28 electron spin resonance spectrometer (manufactured at Wrocław Technical University), operating in the X-band. From the obtained ESR spectra two parameters describing a motional freedom of spin probes in studied systems were calculated: the tumbling correlation time (τ_c—characterizing the rotational freedom of 16DSA probe) and order parameter (S—describing the 5DSA probe mobility). The details of the calculation methods were described previously in[15].

4.5. Log *P* calculations

Octanol/water partition coefficients of some studied experimentally and hypothetic phenoxazine molecules were performed using the Titan 1.0.8 software (Wavefunction Inc., Irvine USA and Schrodinger Inc., Portland USA). Calculations were done using AM1 semi-empirical molecular orbital model.

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