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Tamoxifen perturbs lipid bilayer order and permeability: comparison of DSC, fluorescence anisotropy, Laurdan generalized polarization and carboxyfluorescein leakage studies

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Abstract

The perturbation of the lipid bilayer structure by tamoxifen may contribute to its multiple mechanisms of anticancer action not related to estrogen receptors. This study evaluates the effect of tamoxifen on structural characteristics of model membranes using differential scanning calorimetry (DSC), fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-[4-[trimethylammonium)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH), as well as 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) generalized polarization. The comparative measurements in multilammelar vesicles (MLV) prepared from dipalmitoylphosphatidylcholine (DPPC) revealed that tamoxifen decreases the phase transition temperature (T_m) paralleled by a broadening of the phase transition profile. In large unilamellar vesicles (LUV) prepared from egg yolk phosphatidylcholine (EPC), tamoxifen increased the lipid bilayer order predominantly in the outer bilayer region. From membrane permeability measurements, we conclude that the tamoxifen-induced release of entrapped carboxyfluorescein (CF) results from a permanent bilayer disruption and the formation of transient holes in the lipid bilayer.

Keywords: Tamoxifen; 1,6-Diphenyl-1,3,5-hexatriene (DPH); 1-[4-(Trimethylammonium)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH); Laurdan; Differential scanning calorimetry (DSC); Carboxyfluorescein leakage

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1. Introduction

Tamoxifen is a non-steroidal antiestrogen drug that is widely used in the treatment and prevention of breast cancer, as well as those of the liver, pancreas and brain [1]. The mechanism by which antiestrogens, such as tamoxifen, antagonize the growth of tumors indicates that its antitumor activity is due to a competition with endogenous estrogen-receptor binding sites. Thus, the tamoxifen–estrogen receptor complex interferes with the estrogen–estrogen receptor-mediated gene transcription, DNA synthesis, cancer cell growth and growth factors that may be involved in the cell proliferation [2]. However, a variety of other important actions of tamoxifen are now recognized. Tamoxifen interacts with membrane enzymes, particularly with protein kinase C, which has been implicated as a key enzyme in cellular growth regulation and apoptotic processes [3,4].

A subject of controversy is whether tamoxifen effects result from a direct interaction with relevant receptors or enzymes, or if they are a consequence of its interaction with the lipid phase of cellular membranes. As for most anticancer drugs, tamoxifen is an amphiphilic molecule of highly lipophilic character [5], likely to accumulate in membrane lipid and protein moieties. Experimental studies performed on artificial and biological membranes showed that tamoxifen enriches in lipid bilayers and affects both the physical properties [6–12] and the chemical composition [12] of the lipid bilayer. A fluidizing effect of the hydrophobic core in the gel phase and a small ordering effect of the hydrophilic region in the fluid phase in the presence of tamoxifen has been reported for liposomes prepared from pure phosphatidylcholine of different chain length using DPH fluorescence anisotropy and pyrene excimer measurements [6]. In contrast, Dicko et al. [10] observed a conformational disorder induced by tamoxifen in both the gel and fluid state of pure saturated lipid bilayers using Fourier-transform infrared spectroscopy. Wiseman and co-workers [7,8] observed an ordering effect of tamoxifen in both the hydrophobic and hydrophilic regions of the bilayer in artificial membranes prepared from ox-brain phospholipids using DPH fluorescence anisotropy measurements, whereas Dicko et al. [10] reported an increase in the membrane fluidity of striatum and frontal cortex membranes with the same method. The discrepancy between these results may be explained by the nature of the lipid used in the studies. On the other hand, the contrasting results may be due to the molecular interactions between tamoxifen and DPH which hamper the proper interpretation of the results revealed from fluorescence anisotropy data.

Given the controversies surrounding the mechanism of action of tamoxifen and the contradicting results reported so far, we were interested to carry out a study which compares the results obtained from different methodological approaches to investigate the effect of tamoxifen on artificial lipid bilayers. We have studied the capacity of tamoxifen to perturb the physical properties of lipid bilayers using three different methods:

1. non-invasive differential scanning calorimetry (DSC);
2. DPH and TMA-DPH fluorescence anisotropy. While DPH is supposed to distribute in the hydrophobic bilayer core, TMA-DPH is anchored at the hydrophobic–hydrophilic interface of the bilayer [13] and fixed in the outer region of the acyl chains. As the fluorescence anisotropy data in general are very sensitive to the interaction of drugs with the fluorescent probes, we additionally measured the absorption, fluorescence intensity and fluorescence lifetime of the fluorescent probes in the presence of tamoxifen in order to avoid misinterpretation of the obtained anisotropy data; and
3. generalized polarization of Laurdan fluorescence. This method is based on the bilayer fluidity-dependent fluorescence spectral shift of Laurdan which can be attributed to dipolar relaxation phenomena, originating from the sensitivity of the probe to the polarity of its environment [14]. Laurdan has been reported to be located at the glycerol backbone of the bilayer [15] with the lauric acid tail anchored in the phospholipid acyl chain region.

This paper describes the results of our studies on the effect of tamoxifen on the phase transition of model membranes constituted by multilayers (MLV) prepared from dipalmitoylphosphatidylcholine (DPPC) and on the physical state of large unilamellar vesicles (LUV) prepared from egg yolk phosphatidylcholine (EPC). These model systems have been used in our laboratory to investigate the interaction of volatile anesthetics [16] and neurotoxic compounds [17].

Another aim of this study is directed to the tamoxifen-induced increase in the permeability of model vesicles as a result of a disturbance of the structural integrity of the bilayer. One of the well-established methods to measure permeability of trapped fluorescent markers such as carboxyfluorescein (CF) through liposomal lipid bilayers is to monitor the increase of fluorescence intensity accompanying the dilution of the self-quenched fluorophore into the surrounding medium [19]. We have measured the increase of the CF fluorescence intensity at increasing tamoxifen concentrations in order to elucidate the relation between bilayer disruption and the perturbation of the physical state of the lipid bilayer.

Selected tamoxifen concentration varied in the range 1–20 μM. As the perturbing molar fraction of incorporated tamoxifen is to be evaluated, we calculated the molar drug/lipid ratio from the lipid–water partition [5] and from the ratio of lipid/aqueous volume.

2. Materials and methods

2.1. Chemicals

1,2-Dipalmitoyl-*sn*-glycero-3-phosporylcholine (DPPC) and egg yolk phosphatidylcholine (EPC) were purchased from Lipoid KG (Ludwigshafen, Germany). Lipid purity was greater than 99% and the lipids were used without further purification. Tamoxifen was from Orion Corporation Ltd. (Turku, Finland). The fluorescent probes 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-[4-[trimethylammonium)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH), 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) and carboxyfluorescein (CF) were purchased from Molecular Probes (Eugene, OR, USA). Cholesterol, α-tocopherol and ethanol were from Sigma (Deisenhofen, Germany). Dimethylformamide (DMF) was obtained from ACROS (Geel, Belgium) and Triton X from Serva (Heidelberg, Germany). All other chemicals were of research grade. Solutions were prepared in deionized ultra pure water.

2.2. Liposome preparations

For the phase transition and steady-state membrane fluidity measurements, EPC or DPPC dissolved in ethanol was evaporated to deposit a thin lipid film on the wall of a glass tube. The final traces of residual solvent were removed under vacuum at 50°C overnight. Lipids were dissolved in an appropriate amount of Tris buffer (100 mM Tris, pH 7.4) to give a lipid concentration of approximately 50 mg ml^{-1} for DSC or 2 mg ml^{-1} for fluorescence spectroscopic measurements and vigorously vortexed at temperatures above the phase transition. For the preparation of large unilamellar vesicles (LUV), the resulting multilamellar vesicle (MLV) dispersion was sonicated with a Bandelin sonoplus HD70 (Bandelin electronics, Berlin, Germany) for 15 min at maximal power (cycle 30%) under nitrogen and transferred to a thermostatted membrane extruder system (Lipex Biomembranes Inc., Vancouver, Canada), which allowed the extrusion of unilamellar vesicles of 200 nm final diameter. The final lipid concentration of both MLV and LUV suspensions was determined for each preparation [18]. The liposomal suspensions were stored under nitrogen in darkness at 4°C to avoid lipid peroxidation. All liposomal preparations were used within 2 weeks.

For the membrane permeability measurements, liposomes were prepared as described above from EPC/cholesterol/α-tocopherol (5:1:0.3 molar ratio). The liposomes were dissolved in 80 mM CF-buffer (5 mM MOPS, 50 mM NaCl, 1 mM EDTA, 0.02% NaN_3, pH 7.4) and extruded to obtain 200 nm LUV. The non-encapsulated CF was separated from the liposomes by chromatography on a Sephadex G50 column at 4°C. Liposomes were diluted in MOPS buffer (5 mM

MOPS, 150 mM NaCl, 1 mM EDTA, 0.02% NaN_3, pH 7.4) and stored in small glass tubes at 4°C under nitrogen in darkness.

2.3. Differential scanning calorimetry (DSC)

DSC measurements on MLV prepared from DPPC were performed in a DSC 20 scanning calorimeter (Mettler, Gießen, Germany) with the original data acquisition and analysis software. The temperature difference between sample and reference was measured. Samples were heated in the temperature range 25–50°C at a scan rate of 0.5°C min^{-1}. The appropriate amount of tamoxifen [see Eq. (4)] was added to 500 μl of the liposomal preparation in an eppendorf tube and vortexed to insure a homogeneous distribution of the drug in the bilayer. For the measurements, aliquots of 100 μl were transferred to the sample compartment of the DSC.

2.4. Fluorescence quenching measurements

Fluorescence quenching measurements of DPH and TMA-DPH by tamoxifen in LUV prepared from EPC were carried out in a computer-controlled Perkin-Elmer LS-50 luminescence spectrometer equipped with a thermostatted cuvette (Julabo Labortechnik, Seelbach, Germany). The final lipid concentration was 0.02 mg ml^{-1}. DPH or TMA-DPH was added from a 0.5×10^{-3} M stock solution in DMF to give a final lipid/probe ratio of 500. All samples were incubated for 1 h before the measurements. Excitation and emission wavelength were 359 and 429 nm, respectively; a 5-nm slit width was set for both excitation and emission. Tamoxifen was added in concentrations according to Eq. (4).

2.5. Fluorescence anisotropy measurements

Fluorescence anisotropy measurements were carried out on a computer-controlled SFM 25 Kontron spectrofluorimeter (Kontron Instruments, Eching, Germany) equipped with an L-format anisotropy inset and a Julabo HC thermostat (±0.5°C) (Julabo Labortechnik, Seelbach, Germany). Fixed wavelengths of 359 nm for the excitation and 429 nm for the emission of both DPH and TMA-DPH were used at a slit width of 5 nm for both the excitation and emission wavelengths. In the case of phase-transition measurements, the temperature of the 1-cm^2-cross-section quartz cuvettes were scanned in the same temperature range as the DSC measurements. All samples were thermostatted for 10 min before each measurement. Fluorescence intensities were measured every 1°C near the phase transition. All measurements were performed in triple and corrected for light scattering.

The degree of fluorescence anisotropy was calculated according to the equation [20,21]:

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp}) \quad (1)$$

where r is the steady-state fluorescence anisotropy and $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities at 429 nm parallel and perpendicular to the polarization plane of the excitation light, respectively.

Prior to the fluorescence anisotropy experiments, DPH or TMA-DPH from a 0.5×10^{-3} M stock solution in DMF were added to the LUV liposomal suspension prepared from EPC or MLV prepared from DPPC. Lipid concentrations were adjusted to 0.1 mg ml^{-1} (0.2 mg ml^{-1} in the case of TMA-DPH) to give a final lipid/fluorescent probe ratio of 500. All samples were incubated at room temperature for 1 h before the addition of tamoxifen to allow the fluorescent probe to incorporate into the lipid bilayer.

2.6. Laurdan generalized polarization

Laurdan emission spectra in MLV prepared from DPPC or LUV prepared from EPC were obtained using the thermostatted Perkin-Elmer LS 50 luminescence spectrometer at an excitation wavelength of 387 nm and a slit width of 5 nm for both excitation and emission. Emission spectra were scanned from 400 to 580 nm. The lipid concentration of both EPC unilamellar and DPPC multilamellar vesicle suspensions were adjusted to 0.02 mg ml^{-1} and Laurdan was added from a 5×10^{-3} M stock solution in DMF to give a lipid/probe ratio of 500. All samples were incubated at room temperature for 1 h before the

addition of tamoxifen to allow the fluorescent probe to incorporate into the lipid bilayer. The generalized polarization GP_{em} of Laurdan was calculated from the emission spectra as follows [14]:

$$GP_{em} = (I_{440} - I_{475})/(I_{440} + I_{475}) \quad (2)$$

where I_{440} and I_{475} are the intensities at the emission maxima of 440 and 475 nm, respectively, using a fixed excitation wavelength of 387 nm.

2.7. Fluorescence lifetime measurements

The fluorescence lifetime of DPH and TMA-DPH was measured in LUV prepared from EPC at a lipid concentration of 0.02 mg ml^{-1} and a lipid/probe ratio of 500 in the presence of increasing tamoxifen concentrations. Lifetimes were determined from fluorescence decays measured by the time-correlated single-photon counting method (IBH Scotland). Samples were excited at 359 nm and fluorescence was collected at 428 nm (a slit width of 10 nm for both excitation and emission was set for these measurements, due to the very low TMA-DPH fluorescence intensity). Data were calculated using single exponential analysis.

2.8. Carboxyfluorescein efflux

Experiments were performed with a Perkin-Elmer LS 50 luminescence spectrometer using a fixed wavelength of 492 nm for excitation and 518 nm for emission. The temperature was kept at 20°C. Both excitation and emission slits were set to 5 nm spectral bandwidth. The samples contained 3 ml of buffer and 1 μl of CF liposomes. Samples were stirred continuously during the measurements and tamoxifen was added to the cuvette from a 10^{-3} M stock solution in DMF. A 100% reference point was determined at the end of each measurement from the total release of CF after the addition of 2 μl of a 10% Triton X solution. The concentration gradient-driven release of CF is expressed by fluorescence intensities, and usually obeys first-order kinetics. In the presence of higher tamoxifen concentrations, the fluorescence intensity curves obtained follow second-order kinetics, and the velocity constants are calculated from the following equation:

$$F(t) = (F_t - F_o)/(F_{max} - F_o) = 1 - [A_1 * \exp(-k_1 t) + A_2 * \exp(-k_2 t)] \quad (3)$$

where F_o, F_t and F_{max} stand for the fluorescence intensities at time zero, time t and after total solubilization by Triton X, respectively; k_1 and k_2 (s^{-1}) are the velocity constants and A_1 and A_2 are their relative fractional contributions.

2.9. Drug exposure protocol

The appropriate amount of drug for a given molar tamoxifen/phospholipid ratio (mol.% tamoxifen with respect to phospholipid) was calculated according to following equation:

$$m_D = (1 + \nu_{B/L}/\gamma) x_{D/L} \cdot n_L \cdot M_D \quad (4)$$

m_D: amount of added tamoxifen (g)
$\nu_{B/L}$: buffer/lipid volume ratio
γ: lipid/water partition coefficient of tamoxifen [5]
$x_{D/L}$: molar tamoxifen/lipid ratio (mol.%)
n_L: molar lipid concentration in the assay (M)
M_D: molecular weight of the investigated tamoxifen (g)

Appropriate amounts of tamoxifen from a 10^{-3} M stock solution in DMF were added to the liposomal suspensions according to Eq. (4). The final tamoxifen concentration varied in the range 1–20 μM. In our studies, higher tamoxifen concentrations induced turbidity of the samples, which may interfere with the fluorescent measurement. Samples were incubated at room temperature for 30 min before each measurement.

3. Results

3.1. DSC

Fig. 1 shows original DSC transition curves of MLV prepared from DPPC with increasing tamoxifen concentrations. The presence of tamoxifen in the bilayer lipid leads to a broadening of the transition profile and a lowering of the main phase-transition temperature (T_m) with increasing tamoxifen concentration.

3.2. DPH and TMA-DPH fluorescence quenching

The fluorescence intensity of both DPH and TMA-DPH at temperatures from 10 to 40°C decreases with increasing tamoxifen concentration. The Stern–Volmer plots of the fluorescence quenching of DPH (Fig. 2a) and TMA-DPH (Fig. 2b) by tamoxifen show a saturation effect. The quenching of DPH by tamoxifen is small compared to TMA-DPH and is independent of temperature. TMA-DPH fluorescence is more sensitive to quenching by tamoxifen, but the quenching effect decreases with increasing temperature. From this temperature dependence, we conclude that the fluorescence quenching must be of a static nature. From absorption measurements of DPH in presence of tamoxifen in ethanol (data not shown), we conclude that reabsorption by tamoxifen is not responsible for the decrease in fluorescence intensity. We determined the fluorescence lifetime of DPH and TMA-DPH in the presence of tamoxifen. Table 1 shows that the fluorescence lifetime of both fluorophores is not decreased by tamoxifen. In the case of dynamic fluorescence quenching, which is an excited-state reaction, a decrease in fluorescence lifetime would be reasonable.

3.3. DPH and TMA-DPH fluorescence anisotropy

We have measured the fluorescence anisotropy using DPH and TMA-DPH in LUV and MLV prepared from EPC or DPPC, respectively. Temperature-dependent anisotropy values of DPH (Fig. 3a) and TMA-DPH (Fig. 3b) in MLV of DPPC, calculated according to Eq. (1), are plot-

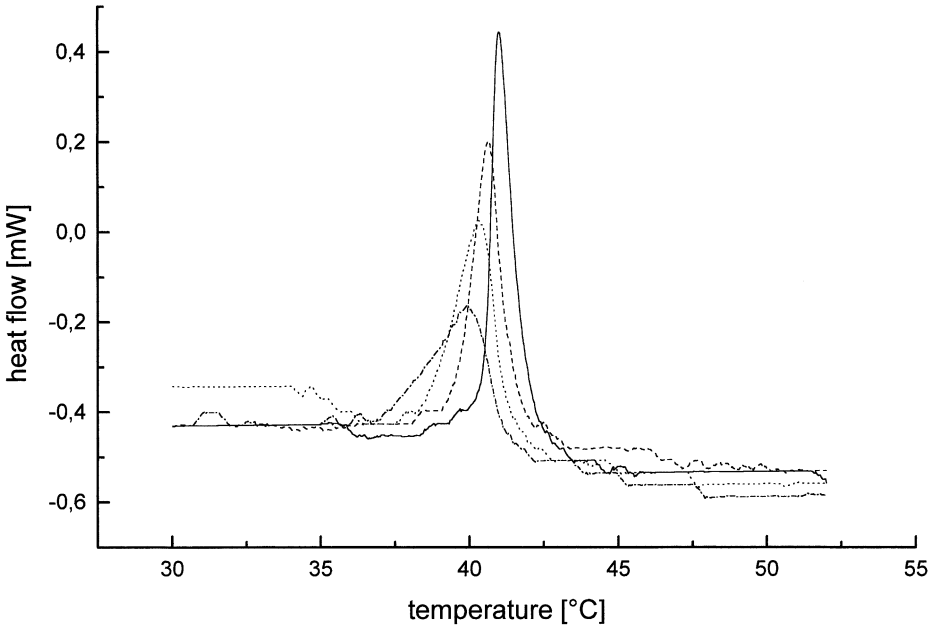

Fig. 1. Original DSC scans on MLV prepared from DPPC at increasing tamoxifen concentrations. Tamoxifen with respect to DPPC: ——— 0; – – – 3.5; · · · 5.25; and – · – · – · 7 mol.%.

Table 1
Estimated DPH and TMA-DPH lifetimes in LUV prepared from EPC in the presence of increasing tamoxifen concentrations[c]

Tamoxifen/lipid ratio [mol.%]	DPH t [ns][a]	TMA-DPH t [ns][b]
0	5.9	3.52
1.56	5.8	3.08
3.13	5.9	3.37
6.25	6.1	3.07
12.50	5.8	3.06

[a]Accuracy, 0.1 ns.
[b]Accuracy, 0.3 ns.
[c]χ^2 1.2–1.3.

ted for increasing tamoxifen concentrations. A decrease in T_m and a broadening of the phase transition with increasing tamoxifen concentration are detected by both DPH and TMA-DPH. A slight decrease of the anisotropy values was observed in the gel and fluid states for both DPH and TMA-DPH.

The effect of tamoxifen on LUV prepared from EPC was also evaluated using fluorescence anisotropy of DPH and TMA-DPH at 25°C (Fig. 4). No effect of tamoxifen on the anisotropy of DPH was observed, while the anisotropy of TMA-DPH exhibits a small, but significant, increase with increasing tamoxifen concentrations.

3.4. Laurdan generalized polarization

We studied the effect of tamoxifen on the phase transition of MLV prepared from DPPC using the Laurdan generalized polarization method. In contrast to DPH and TMA-DPH, Laurdan fluorescence intensity is not affected by the presence of tamoxifen. The method is based on the change in the dipolar relaxation of Laurdan [14]. The molecular origin of the dipolar relaxation has been attributed to the water molecules present at the hydrophobic–hydrophilic interface of the bilayer. The dynamics of the water molecules change in the different phases of the lipid bilayer [14]. The dynamic changes of penetrating water molecules are reflected by the spectral distribution of the Laurdan emission. The relaxation rate of the water molecules is of the same order of magnitude as the fluorescence lifetime of Laurdan in the liquid-crystalline phase of the bilayer and, in consequence, Laurdan emission is red-shifted. In the gel phase, water rotation is restricted and Laurdan emission is observed at shorter wavelengths.

In the gel phase of the phospholipid vesicles, the dipolar relaxation rate, with and without tamoxifen, is very small and the emission displays a maximum at approximately 440 nm (Fig. 5 35°C). In the liquid-crystalline phase, with and without tamoxifen, the rate of dipolar relaxation is much higher and the Laurdan emission displays a maximum at approximately 475 nm (Fig. 5 50°C). At temperatures near the phase transition the half-width of the emission spectra increases and both maxima are present due to the coexistence of the gel and liquid-crystalline phases (Fig. 5 40°C). In the presence of tamoxifen, the maximum at 475 nm already appears at lower temperatures (Fig. 5 40°C). This effect is dose-dependent and indicates an increased rate of dipolar relaxation with increasing tamoxifen concentration. From the emission spectra, we have calculated the emission GP_{em} according to Eq. (2). Fig. 6 shows the temperature-dependent plots of the calculated GP_{em}. The observed shift of the phase transition to lower temperatures with increasing tamoxifen is paralleled by a broadening of the phase transition profile. In contrast to the fluorescence anisotropy measurements, no effect was observed in the pure gel and fluid state of the bilayer.

In order to obtain detailed information on the effects of tamoxifen on LUV prepared from EPC, we studied the temperature-dependent Laurdan generalized polarization in the presence of increasing tamoxifen concentrations. The emission spectra of Laurdan in the EPC vesicles display maxima at 440 and 475 nm and their ratios depend on the temperature. In the presence of tamoxifen, the relative intensity of the 475-nm maximum decreases with respect to the maximum at 440 nm, indicating a decrease in dipolar relax-

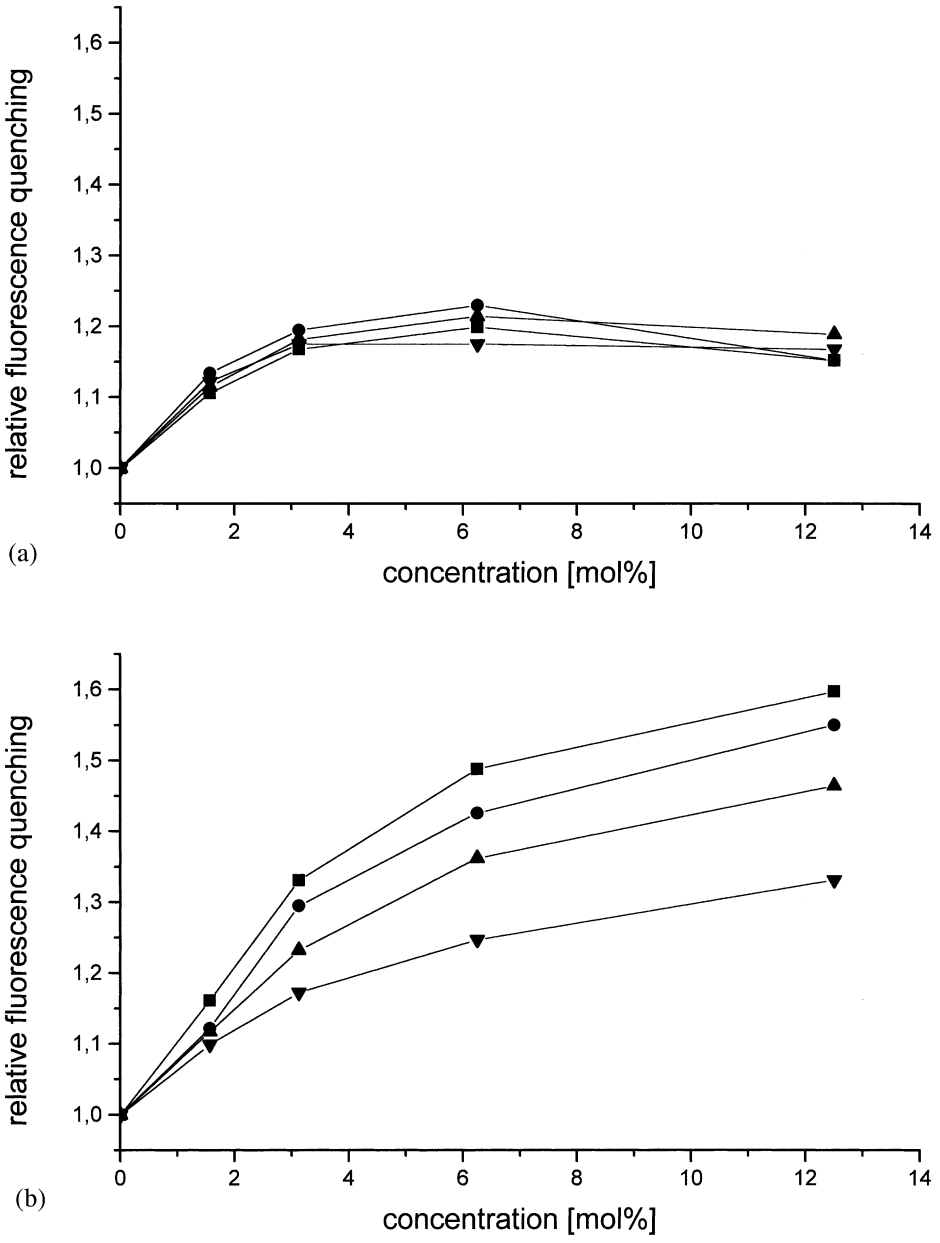

Fig. 2. Stern–Volmer plots of the relative (a) DPH and (b) TMA-DPH fluorescence in LUV prepared from EPC at increasing tamoxifen concentrations and temperatures: ■, 10; ●, 20; ▲, 30; and ▼, 40°C.

ation with increasing tamoxifen concentrations. Generalized emission GP_{em} were calculated according to Eq. (2) and plotted as function of temperature. Fig. 7 shows an almost linear decrease of the Laurdan emission GP_{em} with increasing temperature. Increasing tamoxifen con-

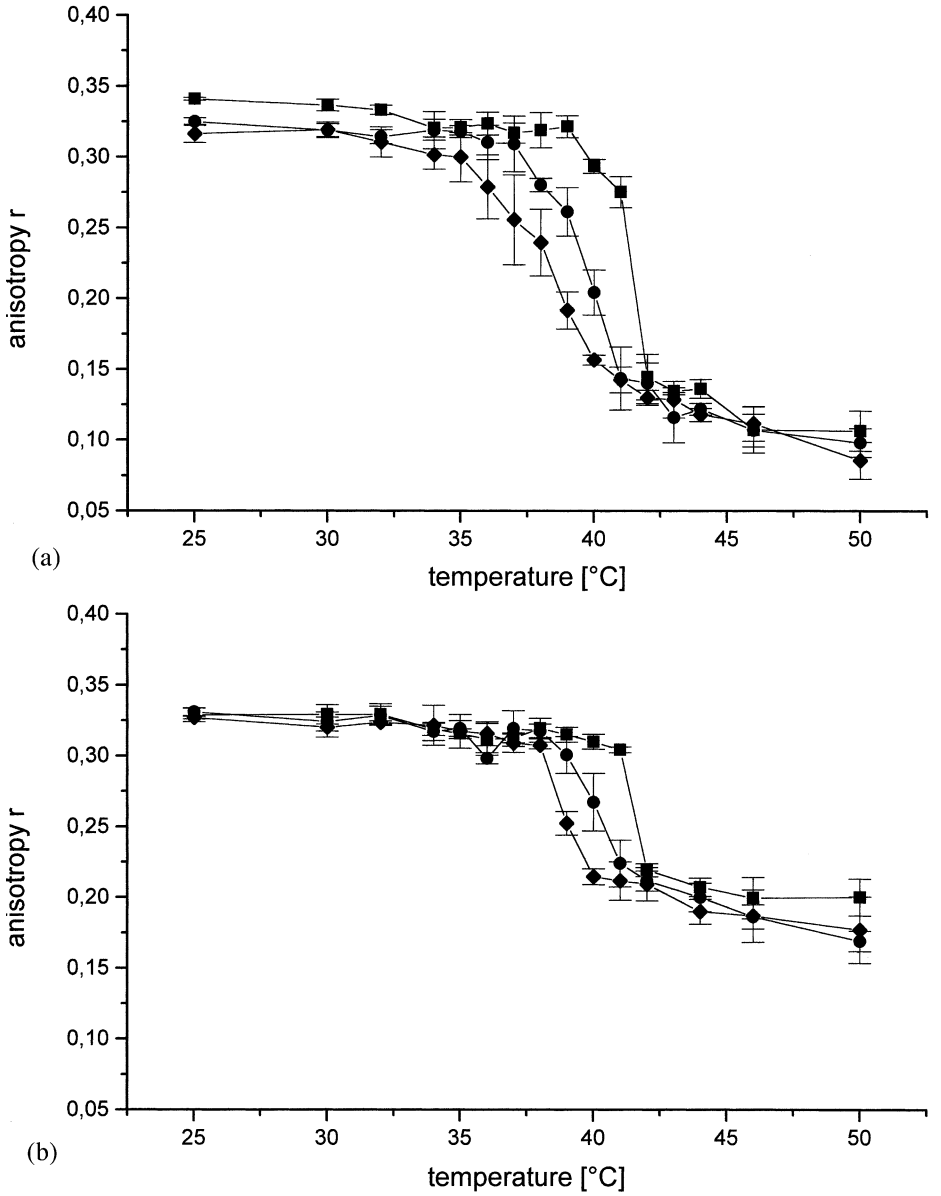

Fig. 3. Temperature-dependent fluorescence anisotropy of (a) DPH and (b) TMA-DPH in MLV prepared from DPPC at increasing tamoxifen concentrations: ■, 0; ●, 6.25; and ♦, 12.5 mol.% tamoxifen/lipid ratio. Vertical bars denote S.E. of the mean of three separate determinations.

centrations lead to a small, but significant, increase in the GP_{em}. This effect is higher at low temperatures where the membrane is less fluid, and it almost disappears at higher temperatures where the membrane is in a more fluid state.

3.5. Carboxyfluorescein leakage

The influence of tamoxifen on the permeability of the lipid bilayer of model vesicles was determined from the increase in fluorescence inten-

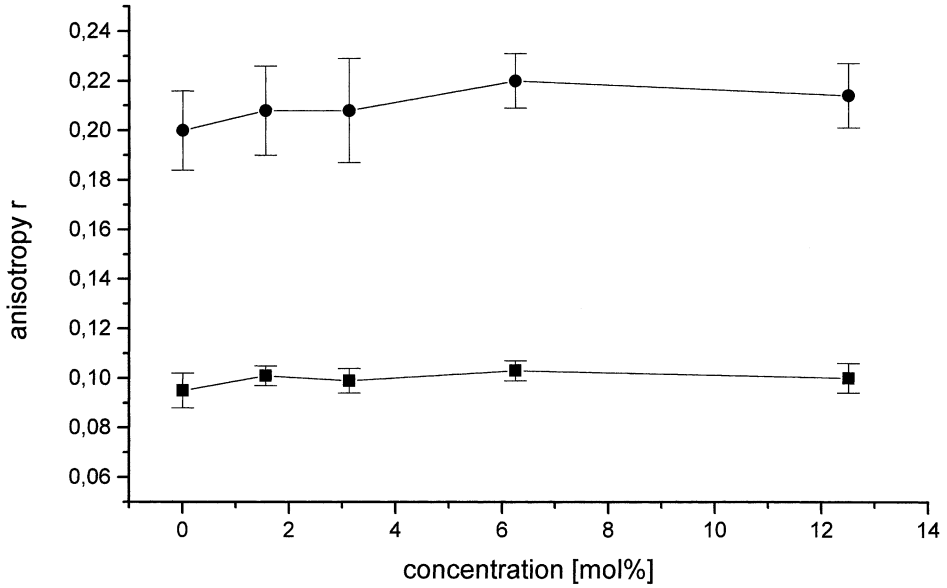

Fig. 4. Fluorescence anisotropy of DPH (■) and TMA-DPH (●) at 25°C in LUV prepared from EPC at increasing tamoxifen concentrations. Vertical bars denote SE of the mean of three separate determinations.

sity which results from the dilution of self-quenched CF. After 50 min, 10% Triton X was added to obtain 100% fluorescence intensity after total solubilization of the liposomes. As shown in Fig. 8, addition of tamoxifen after 300 s of measurement results in a concentration- and time-dependent increase in the fluorescence intensity. An almost complete CF release already occurs at a 5 mol.% tamoxifen/lipid ratio after 50 min. The increase in fluorescence intensity for the control sample and for the 1.25 mol.% tamoxifen/lipid concentration ratio obeys first-order kinetics, whereas fluorescence data from samples with higher tamoxifen concentration can only be simulated from double exponential analysis. Velocity constants for the fluorescence increase and their relative fractional contribution calculated according to Eq. (3) are summarized in Table 2. The rate constant k_1 increases dose-dependently and must be attributed to a concentration-driven increase in CF release as a result of a permanent destabilization of the lipid bilayer due to tamoxifen incorporation. At higher tamoxifen concentrations (>2.5 mol.%), the rapid release of CF immediately after the addition of tamoxifen hints to an additional time-dependent process which leads to the transient increase in CF release.

4. Discussion

In order to elucidate the controversy about the effect of tamoxifen on the lipid bilayer, we have applied three different methods to study tamoxifen-induced disorder in the lipid bilayer. The comparison of several methods is inevitable in studying drug effects on lipid bilayers, because: (a) of possible drug–probe interactions, which may hamper the interpretations of the obtained data; (b) the results are always related to the physical or chemical basis of the applied method; and (c) the order vertical to the plane of the lipid monolayer can be resolved using probes of different vertical location.

Experiments performed by DSC showed that tamoxifen interacts with DPPC multilamellar vesicles, which results in a shift of T_m to lower temperatures and the broadening of the phase transition profile. These results were confirmed by the fluorescence anisotropy measurements ob-

tained from DPH and TMA-DPH. T_m is shifted to lower temperatures and the phase transition profile is progressively broader with increasing tamoxifen concentration. The lower sensitivity of TMA-DPH anisotropy with respect to the tamoxifen-induced changes can be attributed to a steric

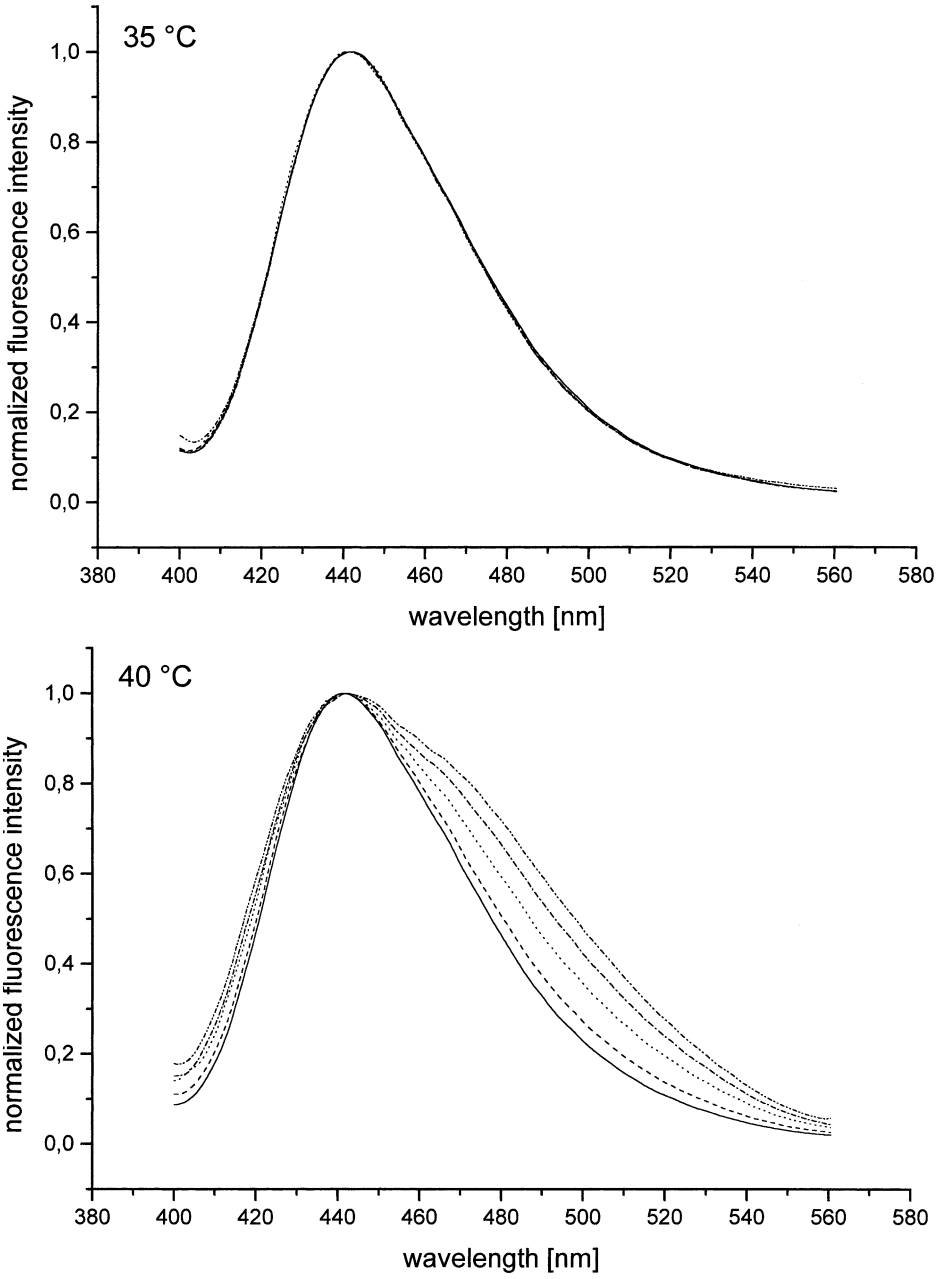

Fig. 5. Normalized Laurdan emission scans in multilamellar vesicles of DPPC with excitation at 387 nm at 35, 40 and 50°C and increasing tamoxifen concentrations. Tamoxifen with respect to DPPC: ——— 0; – – – 1.56; · · · 3.13; – · – · – · 6.25; and ·· – ·· – ·· – 12.5 mol.%.

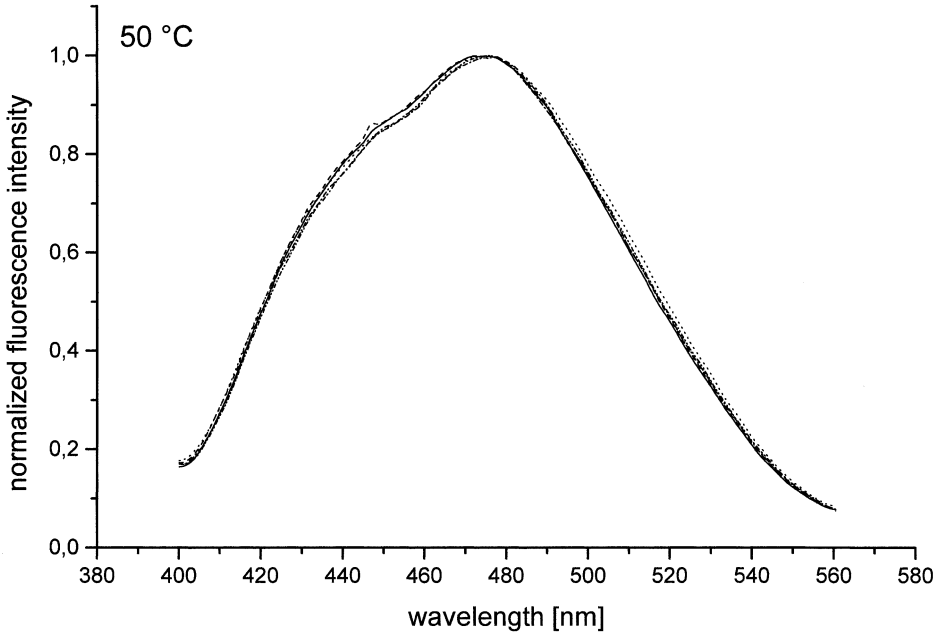

Fig. 5. (*Continued*).

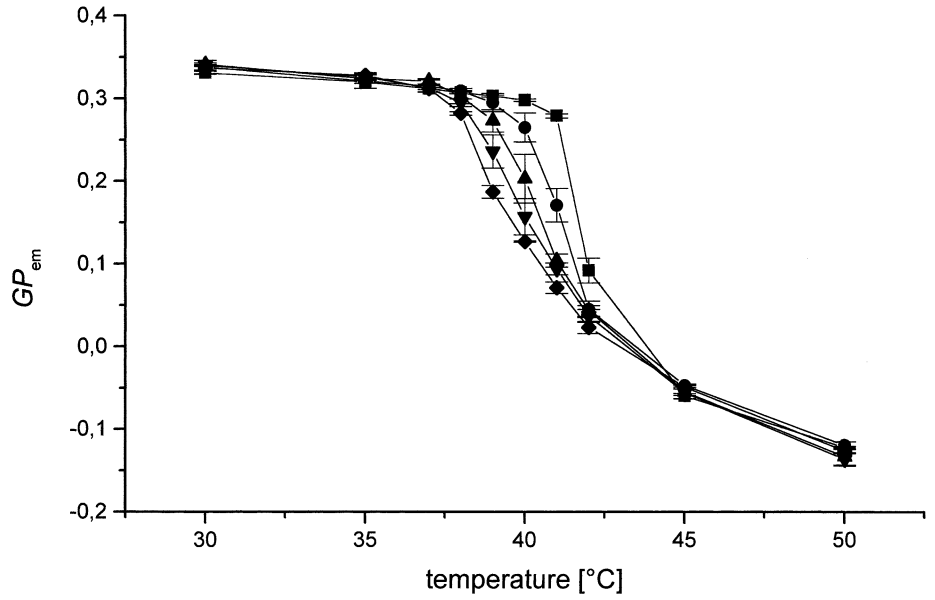

Fig. 6. Temperature-dependent generalized emission polarization of Laurdan in MLV prepared from DPPC at increasing tamoxifen concentrations: ■, 0; ●, 1.56; ▲, 3.13; ▼, 6.25; and ◆, 12.5 mol.% tamoxifen/lipid ratio. Vertical bars denote S.E. of the mean of three separate determinations.

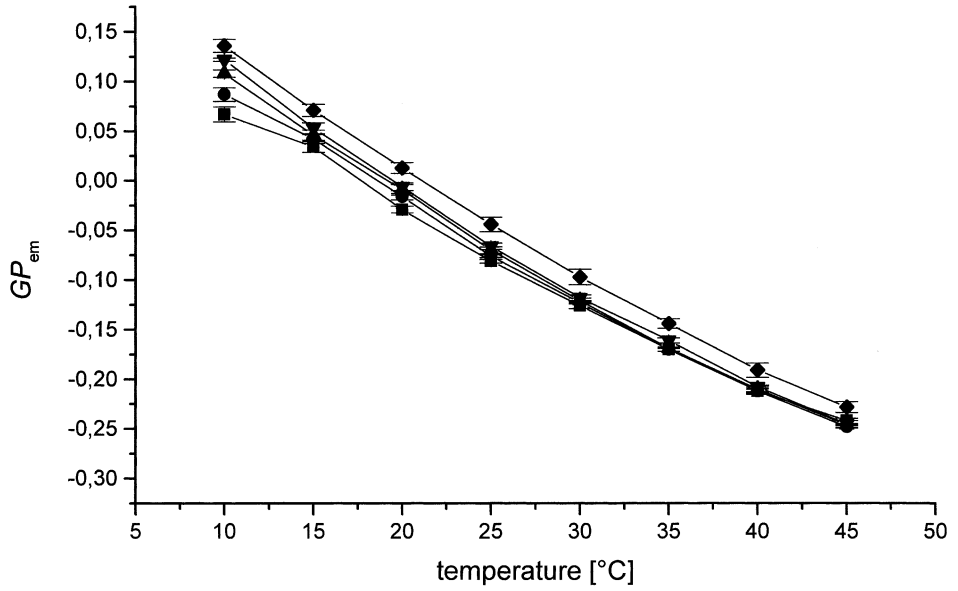

Fig. 7. Temperature-dependent generalized emission polarization of Laurdan in LUV prepared from EPC at increasing tamoxifen concentrations: ■, 0; ●, 1.56; ▲, 3.13; ▼, 6.25; and ◆, 12.5 mol.% tamoxifen/lipid ratio. Vertical bars denote S.E. of the mean of three separate determinations.

hindrance of the TMA-DPH mobility compared to DPH. Because contradicting results have been reported for the tamoxifen effects on the membrane fluidity determined by DPH fluorescence anisotropy, we also used the Laurdan generalized polarization method. Laurdan is a fluorescent membrane probe that has the advantage of displaying a spectral sensitivity to the phospholipid phase state, which is based on dipolar relaxation phenomena, originating from the sensitivity of the probe to the polarity of its environment [14]. In the temperature range of the phase transition of DPPC, we observed an increase in the emission maximum at 475 nm with increasing tamoxifen concentration, indicating an increased dipolar relaxation rate of penetrated water molecules. The calculated GP_{em} values revealed that the temperature interval of the phase transition is progressively broader and shifted to lower temperatures by increasing tamoxifen concentration.

In summary, all three methods, which are based on different physical mechanisms, reveal corresponding results: a comparable shift of T_m and the broadening of the transition profile. The influence of tamoxifen on the main phase transition can be explained by the 'excluded volume interac-

Table 2
Velocity constants (k_1 and k_2) and their relative fractional contributions (A_1 and A_2) of CF release from LUV determined according to Eq. (3) from Fig. 8

Tamoxifen/lipid ratio [mol.%]	k_1 [s^{-1}]	k_2 [s^{-1}]	A_1	A_2
0	$1.46 \times 10^{-5} \pm 2.2 \times 10^{-8}$	–	0.99	–
1.25	$9.68 \times 10^{-5} \pm 6.3 \times 10^{-8}$	–	0.99	–
2.50	$1.33 \times 10^{-4} \pm 1.7 \times 10^{-6}$	$1.01 \times 10^{-3} \pm 1.2 \times 10^{-5}$	0.70	0.27
5.00	$4.84 \times 10^{-4} \pm 2.0 \times 10^{-6}$	$2.77 \times 10^{-3} \pm 1.5 \times 10^{-5}$	0.48	0.43

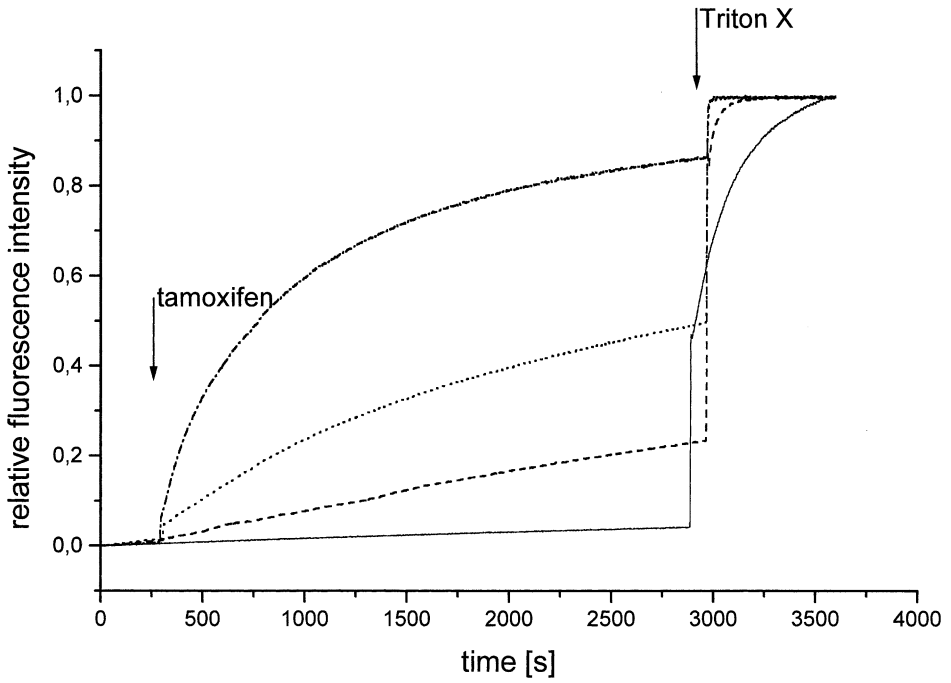

Fig. 8. Time courses of relative fluorescence intensity due to CF release from LUV prepared from EPC/cholesterol/α-tocopherol (5:1:0.3 molar ratio) at: ——— 0; – – – 1.25; · · · 2.5; and – · – · – · 5.0 mol.% tamoxifen/lipid ratio at 20°C. Maximum fluorescence was induced after total solubilization of liposomes by Triton X. The arrows mark the addition of tamoxifen and Triton X, respectively.

tion' theory [22]. The phase transition results from a 'melting' or a condition of disorder due to the *trans*-gauche-isomerization of the acyl chains of the lipids. Lipid molecules that are packed into a lipid bilayer are not free to disorder gradually, but due to the close packing, the temperature-dependent increase in chain rotation is a cooperative process, giving rise to a sharp anomaly, the phase transition. The insertion of foreign molecules in the acyl chain region disturbs this cooperative process of the acyl chains, and the lipids exhibit a more gradual disorder, which results in the broadening of the phase transition. In the case of an impact of foreign molecules in the headgroup region, T_m decreases as a result of a weaker interaction of the headgroup moieties, paralleled by a lateral expansion of the interface region. From the depression of T_m coincident with the broadening of the transition profile, we conclude that tamoxifen accumulates in the upper 10 carbons of the acyl chains, i.e. in the cooperativity region.

At temperatures corresponding to the DPPC gel phase, and at high temperatures corresponding to the pure liquid-crystalline phase, the addition of tamoxifen causes a small decrease in the fluorescence anisotropy values in the gel and fluid state, but no change in GP_{em}. The discrepancy of the results obtained from the different methodical approaches can be explained by the different location of the fluorescent probes. While Laurdan is situated in the glycerol backbone of the lipid layer [15], both DPH and TMA-DPH are located more towards the bilayer center [13]. DPH is free to move in the transverse region of the acyl chains up to the hydrophobic core of the bilayer, while TMA-DPH is fixed in the outer acyl chain region. Obviously tamoxifen only disorders the acyl chain region of DPPC in the gel and fluid state, but not the glycerol backbone region. In

addition, the sensitivity of TMA-DPH to the fluorescence quenching by tamoxifen indicates that tamoxifen accumulates in the acyl chain region, where TMA-DPH is located.

Custodio et al. [6] have observed an increase in the fluidity of the gel phase and a slight decrease in the liquid-crystalline phase of liposomes prepared from different pure phospholipids. Wiseman and Quinn [8] have reported that the DPH fluorescence anisotropy in ox-brain phospholipids is reduced in the presence of tamoxifen, but a disordering effect of tamoxifen in crude brain membrane preparations from rat frontal cortex and the striatum has been observed by Dicko et al. [10]. In a bacterial model system [12], tamoxifen has been shown to induce disorder in the gel and slight order in the fluid state. All reports have in common that the DPH fluorescence anisotropy method was used to determine the disordering/ordering effects of tamoxifen. The physical interpretation of the fluorescence anisotropy measurements using DPH or DPH-derivatives has some contentious aspects. According to our measurements, the fluorescence intensity of DPH and TMA-DPH is decreased in the presence of tamoxifen in liposomal preparations. This effect may result from reabsorption, or from dynamic or static fluorescence quenching of the DPH fluorescence by tamoxifen. As tamoxifen does not decrease DPH fluorescence in ethanol, even at very high concentrations (data not shown), reabsorption due to high optical density is not responsible. Obviously, a close approach of DPH and tamoxifen molecules, as in the case of a simultaneous incorporation in lipid bilayers, is a precondition for the quenching effect. From our temperature-dependent measurements of tamoxifen fluorescence quenching and fluorescence lifetime of DPH and TMA-DPH in multilamellar EPC liposomes in the presence of increasing tamoxifen concentration, we conclude that the fluorescence quenching is of a static nature. Dynamic quenching is an excited-state reaction which leads to a decrease in fluorescence lifetime of the DPH molecule. The fluorescence lifetimes of DPH and TMA-DPH are not affected by tamoxifen. More proof for the static nature of the quenching process is the temperature dependence: fluorescence quenching decreases with increasing temperature. The fluorescence intensity of TMA-DPH is more sensitive to the presence of tamoxifen than is DPH. This effect is in agreement with the location of TMA-DPH predominantly in the cooperativity region, where tamoxifen also accumulates. DPH, on the other hand, is free to distribute to the hydrophobic core in the central region of the bilayer, apart from the cooperativity region.

We determined the Laurdan generalized emission, as well as the DPH and TMA-DPH fluorescence anisotropy, in LUV prepared from EPC at increasing temperatures and tamoxifen concentrations. EPC is in a more or less fluid state, depending on the temperature. Therefore, Laurdan emission GP_{em} shows an almost linear decrease with increasing temperature, due to an increased dipolar relaxation rate. In the presence of tamoxifen, the calculated GP_{em} values are increased with respect to the control, because of a decrease in the dipolar relaxation rate, i.e. tamoxifen restricts the molecular motion of the Laurdan environment. This effect is small and almost disappears with increasing temperature. A small increase in fluorescence anisotropy in LUV from EPC was also found using TMA-DPH as the fluorescent probe, but not for DPH. In summary, our results on LUV of EPC suggest that the ordering effect of tamoxifen is predominant in the bilayer region where TMA-DPH and Laurdan are located, whereas the hydrophobic core is not affected.

The fact that such ordering effects are not observed for liposomes prepared from DPPC can be explained by the nature of the lipid used in both studies. While DPPC is composed of saturated lipids, EPC contains more than 50% of unsaturated lipids. The effect of a given compound on a membrane depends on the degree of unsaturation of the lipid acyl chains. For example, a considerable decrease in membrane fluidity of liposomes from ox-brain phospholipids with increasing tamoxifen concentration using fluorescence anisotropy measurements has been reported [7]. Ox-brain is rich in polyunsaturated chains, and thus is more fluid than saturated DPPC.

The effect of tamoxifen on the lipid bilayer permeability was evaluated from the increase in CF fluorescence intensity as a function of tamoxifen concentration in LUV. Our results reveal that CF efflux is concentration- and time-dependent and that maximum CF fluorescence already occurs at a 5 mol.% tamoxifen/lipid ratio after 50 min of measurement. As the level of fluorescence depends on the rate at which entrapped CF leaks out of the vesicles, we studied the changes of release kinetics. The results calculated according to Eq. (3) are summarized in Table 2. Most interestingly, the release kinetics at 2.5 and 5 mol.% tamoxifen/lipid ratio do not obey first-order kinetics, but can be simulated by double exponential analysis. One explanation for this phenomenon is that asymmetric absorption of tamoxifen by the outer monolayer of the membrane results in a difference of tamoxifen concentrations in the two lamellae, generating a membrane tension which, above a threshold concentration, causes membrane defects until the foreign molecules have equilibrated across the membrane [23]. Membrane holes that form during redistribution lead to a rapid release of entrapped CF due to the formation of short-lived transient holes. These transient holes result in a fast transient CF release with high rate constant (k_2 in Table 2), whereas the smaller rate constant (k_1) relates to a permanent perturbation of the lipid bilayer, which leads to a concentration-driven release of CF across the membrane.

These results on membrane permeability of tamoxifen support the hypothesis that tamoxifen locates in the outer bilayer region and not in the central core of the bilayer. The headgroup region is the main barrier for permeation, and permeability changes are determined by the degree of order in the headgroup region. Previous data from our investigations on the effect of volatile anesthetics [16] on membrane order, phase transition and permeability revealed that polar molecules, for example diethylether, accumulate near the headgroup region and facilitate the permeation of CF, whereas non-polar molecules, such as *n*-pentane, which distribute in the central core of the bilayer, do not affect the permeability.

In addition, our results are in line with the observed induction of hemolysis in red blood cells [24]. According to this study, the hemolytic action of tamoxifen putatively results from perturbation of erythrocyte membrane integrity and structure, by impairing lipid–lipid, lipid–protein and protein–protein interactions. Our results support the concept of tamoxifen-induced hemolysis occurring via direct membrane disruption.

In conclusion, the results obtained from three different methods for studying the effect of tamoxifen on phosphatidylcholine bilayer demonstrate that tamoxifen disturbs the cooperative process of the phase transition as a result of tamoxifen insertion in the outer acyl chain region of the lipid bilayer. One important outcome of this study is that the fluorescence intensity of DPH and TMA-DPH is decreased by tamoxifen as a result of a static quenching process. Therefore, results obtained from fluorescence anisotropy experiments using DPH, TMA-DPH, or any other DPH-derivative may be hampered due to tamoxifen–fluorescent probe interactions and lead to misinterpretations.

In spite of its moderate ordering effect in the outer bilayer region of EPC unilamellar vesicles, tamoxifen decreased the mechanical stability of these vesicles, resulting in the release of entrapped CF. The method of carboxyfluorescein release proved to be a very sensitive tool in studying the effect of tamoxifen on lipid bilayers. Since cell leakage is a final stage of cytotoxicity, the disruption of the lipid bilayer structure by tamoxifen may contribute to its multiple mechanisms of anticancer action not related to estrogen receptors.

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References

[1] H. Wiseman, Tamoxifen: new membrane mediated mechanisms of action and therapeutic advances, TiPS 15 (1994) 83–89.

[2] A. Jaiyesimi, U. Buzdar, D.A. Decker, G.N. Hortobagyi, Use of tamoxifen for breast cancer: twenty-eight years later, J. Clin. Oncol. 13 (1995) 513–529.

[3] K.M. Eyster, M.R. Clark, Non-estroidal antiestrogen inhibition of protein kinase C in human corpus luteum and placenta, Biochem. Pharmacol. 38 (1989) 3497–3503.

[4] T. Hayon, A. Dyilamsky, L. Oriev, I. Nathan, Non-steroidal antiestrogens induce apoptosis in HL60 and MOLT3 leucemic cells; involvement of reactive oxygen radicals and protein kinase C, Anticancer Res. 19 (3A) (1999) 2089–2093.

[5] J.B.A. Custodio, L.M. Almeida, V.M. Madeira, A reliable procedure to estimate drug partitioning in biomembranes, Biochim. Biophys. Acta 176 (1991) 1079–1085.

[6] J.B.A. Custodio, L.M. Almeida, V.M. Madeira, The anticancer drug tamoxifen induces changes in the physical properties of model and native membranes, Biochim. Biophys. Acta 1150 (1993) 123–129.

[7] H. Wiseman, P. Quinn, B. Halliwell, Tamoxifen and related compounds decrease membrane fluidity in liposomes. Mechanism for the antioxidant action of tamoxifen and relevance to its anticancer and cardioprotective actions? FEBS 330 (1993) 53–56.

[8] H. Wiseman, P. Quinn, The antioxidant action of synthetic estrogens involves decreased membrane fluidity: relevance to their potential use as anticancer and cardioprotective agents compared to tamoxifen? Free Radical Res. 21 (1994) 187–194.

[9] M. Suwalsky, P. Hernández, F. Villena, F. Aguilar, C.P. Sotomayor, Interaction of the anticancer drug tamoxifen with human erythrocyte membrane and molecular models, Z. Naturforsch. 53c (1998) 182–190.

[10] A. Dicko, M. Morisette, S.B. Ameur, M. Pésolet, T. Di Paolo, Effect of estradiol and tamoxifen on brain membranes: investigation by infrared and fluorescence spectroscopy, Brain Res. Bull. 49 (1999) 401–405.

[11] H. Boyer, F. Severcan, Tamoxifen–model membrane interactions: an FT-IR study, J. Mol. Struct. 408/409 (1997) 265–268.

[12] C. Luxo, A.S. Jurado, J.B.A. Custodio, V.M. Madeira, Use of *Bacillus stearothermophilus* as a model to study tamoxifen-membrane interactions, Toxicol. In Vitro 10 (1996) 463–471.

[13] W.H. Sawyer, Fluorescence spectroscopy in the study of membrane fluidity: model membrane systems, in: R.C. Aloia, C.C. Curtain (Eds.), Methods for Studying Membrane Fluidity, Alan R Liss, New York, 1988, pp. 161–191.

[14] T. Parasassi, G. De Stasio, G. Ravagnan, R.M. Rusch, E. Gratton, Quantification of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence, Biophys. J. 80 (1991) 179–189.

[15] P.L.-G. Chong, P.T.T. Wong, Interactions of Laurdan with phosphatidylcholine liposomes: a high-pressure FTIR study, Biochim. Biophys. Acta 1149 (1993) 260–266.

[16] M. Engelke, R. Jessel, A. Wiechmann, H.A. Diehl, Effect of inhalation anaesthetics on the phase behavior, permeability and order of phosphatidylcholine bilayers, Biophys. Chem. 67 (1997) 127–138.

[17] M. Engelke, H. Tähti, L. Vaalavirta, Perturbation of artificial and biological membranes by organic compounds of aliphatic, alicyclic and aromatic structure, Toxicol. In Vitro 10 (1996) 111–115.

[18] J.C.M. Stewart, Calorimetric determination of phospholipids with ammonium ferrothiocyanate, Anal. Biochem. 104 (1980) 10–14.

[19] T. Hernández-Caselles, J. Villalain, J.C. Gómez-Fernandez, Stability of liposomes on long-term storage, J. Pharm. Pharmacol. 42 (1990) 397–400.

[20] M. Shinitzky, Y. Barenholz, Fluidity parameters of lipid regions determined by fluorescence polarization, Biochim. Biophys. Acta 515 (1978) 367–394.

[21] M. Shinitzky, A.-C. Dianoux, C. Gitler, G. Weber, Microviscosity and order in the hydrocarbon region of micelles and membranes determined with fluorescent probes. I. Synthetic micelles, Biochemistry 10 (1971) 2106–3113.

[22] J.F. Nagle, Theory of the main lipid bilayer phase transition, Annu. Rev. Phys. Chem. 31 (1988) 157–195.

[23] J. Lasch, Interaction of detergents with lipid vesicles, Biochim. Biophys. Acta 1241 (1995) 269–292.

[24] M.M. Cruz Silva, V.M.C. Madeira, L.M. Almeida, J.B.A. Custódio, Hemolysis of human erythrocytes induced by tamoxifen is related to disruption of membrane structure, Biochim. Biophys. Acta 1464 (2000) 49–61.