Interaction of the Anticancer Drug Tamoxifen with the Human Erythrocyte Membrane and Molecular Models

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Tamoxifen, Anticancer Drug, Erythrocyte Membrane, Phospholipid Bilayer

Tamoxifen is a non steroidal antiestrogen drug extensively used in the prevention and treatment of hormone-dependent breast cancer. To evaluate its perturbing effect upon cell membranes it was made to interact with human erythrocytes and molecular models. These consisted of bilayers of dimyristoylphosphatidylcholine (DMPC) and of dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipids classes located in the outer and inner leaflets of the erythrocyte membrane, respectively. Experiments by fluorescence spectroscopy showed that tamoxifen interacted with DMPC vesicles fluidizing both its polar head and acyl chain regions. These results were confirmed by X-ray diffraction which indicated that tamoxifen perturbed the same regions of the lipid. However, it did not cause any significant structural perturbation to DMPE bilayers. The examination by electron microscopy of human erythrocytes incubated with tamoxifen revealed that they changed their normal discoid shape to stomatocytes. According to the bilayer couple hypothesis, this result means that the drug is inserted in the inner leaflet of the erythrocyte membrane. Given the fact that tamoxifen did not interact with DMPE, it is concluded that it interacted with a protein located in the cytoplasmic moiety of the erythrocyte membrane.

Introduction

Tamoxifen, whose structural formula is shown in Fig. 1, is a non-steroidal antiestrogen drug extensively used in the prevention and treatment of hormone dependent breast cancer (Nayfield, 1995; King, 1995; Jordan, 1995) as well as those of the liver, brain and pancreas (Wiseman *et al.,* 1993). In approximately 30 years since its introduction tamoxifen has been established as the endocrine therapy of choice for estrogen- and progesteron-receptor-positive metastatic breast cancer because of its favorable adverse-effect profile (Higa, 1994; Jaiyesimi *et al.,* 1995). The mechanisms by which estrogens promote the growth of tumors remain unclear despite nearly half a century investigation. Similarly, are also unclear the mechanisms by which antiestrogens like tamoxifen antagonize the growth of the same tumors (Jaiyesimi *et al.,* 1995; Murphy, 1994; van den Koedijk *et al.,* 1994). In fact, several mechanisms of action have been proposed for tamoxifen (van den Koedijk *et al.,* 1994; Coletta *et al.,* 1994). The one that has found the highest acceptance indicates that its antitumor activity is due to a competition with endogenous estrogen for estrogen receptor (ER) binding sites (Jaiyesimi *et al.,* 1995; Murphy, 1994; van den Koedijk *et al.,* 1994). Thus, the tamoxifen-ER complex would interfere with estrogen-ER mediated gene

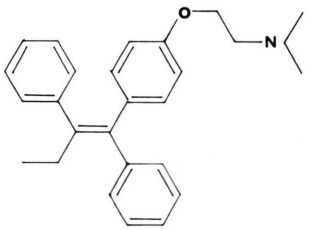

Fig. 1. Structural formula of tamoxifen (1-*p*-β-dimethylamino-ethoxyphenyl-*trans*-1,2-diphenylbut-1-ene).

Abbreviations: ER, estrogen receptor; AEBS, antiestrogen binding sites; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; LUV, large unilamellar vesicles; DPH, 1,6-diphenyl-1,3,5-hexatriene; Laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene; r, fluorescence anisotropy; GP, general polarization; SEM, scanning electron microscopy.

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transcription, DNA synthesis, cancer cell growth and growth factors that may be involved in cell proliferation (Jaiyesimi *et al.,* 1995). However, it has been suggested that besides ER there also high affinity membrane-associated binding sites identified in a variety of tissues called antiestrogen binding sites (AEBS), some of which bind tamoxifen but not estrogens (van den Koedijk *et al.,* 1994; Coletta *et al.,* 1994; Das *et al.,* 1993). Another hypothesis supposes the interaction of tamoxifen with membrane enzymes (Coletta *et al.,* 1994), particularly with protein kinase C which has been implicated as a key enzyme in cellular growth regulation (Eyster and Clark, 1989; O'Brien *et al.,* 1988; Horgan *et al.,* 1986).

An additional subject of controversy is whether tamoxifen effects result from a direct interaction with a relevant protein such as ER (Nayfield, 1995; Borgna, 1994), AEBS (van den Koedijk *et al.,* 1994; Das *et al.,* 1993) and enzymes (Eyster *et al.,* 1989; O'Brien *et al.,* 1988; Horgan *et al.,* 1986), or they are a consequence of its interaction with cell membranes (van den Koedijk *et al.,* 1994; O'Brien *et al.,* 1986; Custodio *et al.,* 1991, 1993; Luxo *et al.,* 1996). In fact, as most anticancer agents tamoxifen is a highly lipophilic molecule (Custodio *et al.,* 1991), likely to accumulate in membrane lipid and protein moieties in route to its target site. Experiments performed on native membranes, liposomes of their lipids or made of synthetic lipids showed that tamoxifen interacts with membranes and affects the physical properties of the lipid bilayers (Wiseman, 1993; Custodio *et al.,* 1993; Luxo *et al.,* 1996). Fluidizing and ordering effects were observed in hydrophobic and hydrophilic regions of the bilayers which, in general, depended on the nature, temperature and physical state of the specimens. Structural effects due to tamoxifen were also observed in membranes of human breast cancer cells (Clarke *et al.,* 1990). These effects on membranes have been related to antitumor and cytotoxic activities of anticancer drugs (Balasubramanian *et al.,* 1994).

Given the controversies surrounding the mechanism of action of tamoxifen we thought of interest to carry out a study concerning its interaction with cell membranes. This paper describes the results of our studies on the interaction of tamoxifen with human erythrocyte membranes and models constituted by phospholipid multilayers and large unilamellar vesicles. These systems have been used in our laboratories to determine the interaction and perturbing effects on membranes by several therapeutical drugs (Suwalsky *et al.,* 1988, 1991, 1993, 1994, 1995, 1996). The multilayers consisted in the phospholipids dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipid classes located in the outer and inner monolayers of the human erythrocyte membrane, respectively (Devaux and Zachowsky, 1994). Given the amphipatic character of tamoxifen and both phospholipids their interactions were assayed in hydrophobic and aqueous media in a wide range of concentrations. The capacity of tamoxifen to perturb the multilayer structure of DMPC and DMPE was determined by X-ray diffraction. The effect of tamoxifen in the physical properties of the bilayer of DMPC large unilamellar vesicles was studied evaluating DPH steady state fluorescence anisotropy and Laurdan fluorescence spectral shifts. Interaction of tamoxifen with human erythrocytes were observed by scanning electron microscopy to detect shape changes induced by the drug.

Materials and Methods

X-ray diffraction analysis of phospholipid multilayers

Synthetic DMPC (lot 80H-8371 A grade MW 677.9), DMPE (lot 13H-83681 A grade MW 635.9) and tamoxifen (lot 63H-0349 MW 371.5) from Sigma were used without further purification. About 3 mg of each phospholipid were mixed with the corresponding weight of tamoxifen in order to attain DMPC:tamoxifen and DMPE:tamoxifen powder mixtures in the molar ratios of 10:1, 5:1 and 1:1. Each mixture was dissolved in chloroform:methanol 3:1 v/v and left to dry. The recrystallized samples were placed in special glass capillaries 0.7 mm dia. They were diffracted in Debye-Scherrer cameras of 114.6 mm dia and flat-plate cameras with 0.25 mm dia glass collimators provided with rotating devices. The same procedure was also followed with samples of each phospholipid and tamoxifen. The aqueous specimens were prepared in 1.5 mm dia glass capillaries mixing each phospholipid and tamoxifen in the same proportions as described above. Each capillary was then filled with about 200 uL of distilled water.

These specimens were X-ray diffracted 2 days after preparation in flat-plate cameras. Specimen-to-film distances were 8 and 14 cm, standardized by sprinkling calcite powder on the capillary surface. Ni-filtered CuKα radiation from a Philips PW1140 X-ray generator was used. The relative reflection intensities were obtained from films by peak-integration in a microdensitometer (Joyce-Loebl MKIIICS) interfaced to a PC. No correction factors were applied. The experiments in water were performed at 17 ± 2 °C, which is below the main transition temperature of both DMPC and DMPE.

Fluorescence measurements on large unilamellar vesicles (LUV)

DMPC LUV suspended in water were prepared by extrusion of frozen and thawed multilamellar liposome suspension (final lipid concentration 0.5 mM) trough two stacked polycarbonate filters of 400 nm pore size (Nucleopore, Corning Costar Corp.) employing nitrogen pressure at 10 °C over the lipid transition temperature. DPH and Laurdan were incorporated into LUV by addition of small aliquots of concentrated solutions of the probe in tetrahydrofurane and ethanol respectively to LUV suspensions and gently shaken for about 30 min. Fluorescence spectra and anisotropy measurements were respectively performed in a Spex Fluorolog and in a phase shift and modulation Greg-200 steady-state and time-resolved spectrofluorometer (I. S. S.), both interfaced to computers. Software from I. S. S. were used for data collection and analysis. Measurements of LUV suspensions were made at 18 °C in 10 mm path-length square quartz cuvettes. Sample temperature was controlled by an external bath circulator (Cole Parmer) and measured prior and after each measurement using a digital thermometer (Omega). Anisotropy measurements were made in the "L" configuration using prism polarizers (Glan Thompson) in both exciting and emitting beams. The emission was measured across a high pass filter (Schott WG-420) with negligible fluorescence. Laurdan fluorescence spectral shifts were quantified through the General Polarization (GP) concept which was evaluated by GP=(Ib-Ir)/(Ib+Ir), where Ib and Ir are the intensities at the blue and red edges of the emission spectrum, respectively. These intensities have been measured at the emission wavelengths of 440 and 490 nm, which correspond to the emission maxima of Laurdan in the gel and liquid crystalline phases, respectively (Parasassi and Gratton, 1995). Tamoxifen was incorporated in LUV suspensions by addition of small aliquots of a concentrated ethanolic solution and incubated at 40 °C for ca. 15 min. Samples with probes but without tamoxifen showed no variation in the measured parameters during periods longer than those employed in the experiments. Blank subtraction was performed in all measurements using unlabelled samples without probes. The partition coefficient of tamoxifen in octanol/buffer pH 7.4, determined by spectrophotometry, was found to be 63.8. This value is close to the reported ratio of tamoxifen concentrations in human normal and malignant tissues and in serum during treatment (Lien *et al.*, 1991).

Scanning electron microscope (SEM) studies on human erythrocytes

The interaction of tamoxifen with human erythrocytes was achieved by incubating blood samples from clinically healthy male adult donors by puncture of the ear lobule disinfected with 70% ethanol. Two drops of blood were collected in a plastic tube containing 1 ml of saline solution (0.9% NaCl) at 5 °C. This solution was then used to prepare the following samples: a) control, by mixing 0.1 ml with 0.9 ml of saline solution, b) one that was 1.5 mM tamoxifen in the aqueous phase and 98.5 mM in the erythrocytes, and c) another tamoxifen solution which was 10-fold as concentrated as that of b), by mixing 0.1 ml of blood solution plus 0.9 ml of tamoxifen saline suspensions of adequate concentrations. These samples were incubated at 37 °C for 1 h. They were later fixed with glutaraldehyde adding one drop of each sample to a tube containing 1 ml of 2.5% glutaraldehyde in saline solution, reaching a final fixation concentration of about 2.4%. After resting overnight at 5 °C the fixed samples were directly placed on Al stubs, air dried at 37 °C for half to one h and gold coated for 3 min at 10^{-1} Torr in a sputter device (Edwards S150). The observations and photographic records were performed in an Etec Autoscan SEM. The concentrations of tamoxifen in the samples were calculated in base of its 63.8 partition coefficient octanol/buffer 7.4.

Results

X-ray studies on phospholipid multilayers

Given the amphipathic nature of tamoxifen and of the phospholipids DMPC and DMPE, their molecular interactions were assayed in hydrophobic and aqueous media. Table I shows the interplanar spacings and relative intensities of the reflections produced by DMPC, tamoxifen and their 10:1, 5:1 and 1:1 molar mixtures after their interaction and recrystallization from chloroform:methanol 3:1 v/v solutions. Their corresponding diffractograms are compared in Fig. 2a. The analysis of these results indicated that the X-ray pattern of DMPC was affected by tamoxifen. In fact, the reflection intensities of the phospholipid significatively decreased with increasing concentrations of the drug. On the other hand, several new reflections corresponding to tamoxifen showed up in the 5:1 and 1:1 specimens. It was also observed that the bilayer width of DMPC remained practically constant at 54.5 Å. These results clearly indicated that part of tamoxifen penetrated into the phospholipid bilayer structure perturbing its molecular arrangement.

Table II and Fig. 2b show the results obtained after DMPC, tamoxifen and their molar mixtures in the same ratios as above were immersed in an excess of distilled water. As it can be observed, water dramatically changed the X-ray pattern of DMPC. In fact, its bilayer width expanded from 54.5 Å when dry to 64.0 Å and its reflections were reduced to only the first three orders of the bilayer

Table I. Comparison of observed interplanar spacings (do) and relative intensities (Io rel) of dimyristoylphosphatidylcholine (DMPC), tamoxifen and of their 10:1, 5:1 and 1:1 molar mixtures [a-c].

DMPC		DMPC : Tamoxifen 10:1		DMPC : Tamoxifen 5:1		DMPC : Tamoxifen 1:1		Tamoxifen	
do(Å)	Io rel	do(Å)	Io rel	do(Å)	Io rel	do(Å)	Io rel	do(Å)	Io rel
54.5*	1660*	54.5*	1410*	54.5*	1185*	54.5*	1070*	–	–
27.2*	8*	27.2*	4*	27.2*	4*	27.2*	3*	–	–
18.3*	3*	18.3*	2*	18.3*	2*	–	–	–	–
–	–	–	–	–	–	15.2	5	15.2	33
13.7	12	13.7	9	13.7	7	13.7	5	–	–
–	–	–	–	–	–	–	–	12.7	2
–	–	–	–	–	–	10.2	3	10.2	20
9.30	7	9.30	6	9.30	5	9.30	4	9.30	3
8.30	6	8.30	5	–	–	–	–	–	–
6.28	17	6.29	11	6.28	10	6.28	10	6.28	13
–	–	–	–	5.62	4	5.63	6	5.62	30
–	–	5.17	5	5.17	10	5.17	15	5.16	82
–	–	–	–	–	–	–	–	4.87	6
4.76	4	4.75	3	4.74	2	4.74	5	4.74	7
4.54	4	4.57	3	–	–	4.58	4	4.57	43
–	–	–	–	–	–	4.45	10	4.44	20
4.30	33	4.31	34	4.30	25	4.31	22	–	–
–	–	–	–	–	–	–	–	4.23	6
4.14	100	4.13	99	4.13	47	4.13	40	4.14	52
–	–	–	–	–	–	3.92	9	3.91	27
3.87	28	3.88	18	–	–	–	–	–	–
–	–	–	–	3.82	7	3.81	9	3.80	13
–	–	–	–	–	–	3.73	5	3.72	12
3.51	4	–	–	–	–	–	–	3.57	8
–	–	–	–	–	–	–	–	3.49	7
–	–	–	–	–	–	3.38	3	3.37	14
–	–	–	–	–	–	3.28	2	3.29	9
3.16	2	–	–	–	–	–	–	3.14	6

a) All the specimens were recrystallized from $CHCl_3$:CH_3OH 3:1 (v/v).
b) The interplanar spacings and intensities of the reflections were measured in X-ray diagrams from Debye-Scherrer and flate-plate cameras with 8 and 14*cm specimen-to-film distance.
c) Only the main observed reflections are included.

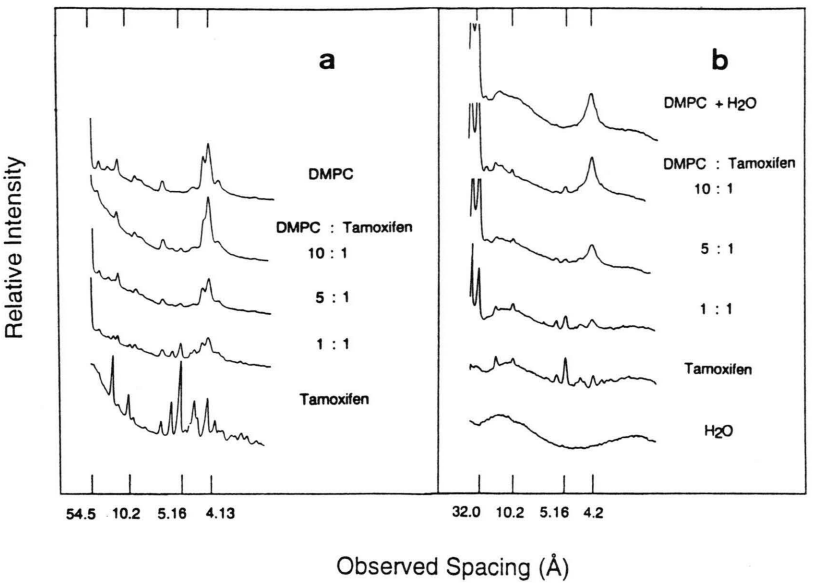

Fig. 2. Microdensitograms from X-ray diffraction diagrams of dimyristoylphosphatidylcholine (DMPC), tamoxifen and of their 10:1, 5:1 and 1:1 molar mixtures. Flat-plate cameras, specimen-to-film distance 8 cm. (a) Recrystallized from $CHCl_3$:CH_3OH (v/v); (b) immersed in water.

Table II. Comparison of observed interplanar spacings (do) and relative intensities (Io rel) of dimyristoylphosphatidylcholine (DMPC), tamoxifen and of their 10:1, 5:1 and 1:1 molar mixtures in water[a-c].

DMPC		DMPC : Tamoxifen						Tamoxifen	
		10:1		5:1		1:1			
do(Å)	Io rel	do(Å)	Io rel	do(Å)	Io rel	do(Å)	Io rel	do(Å)	Io rel
64.0*	470*	64.0*	270*	64.0*	225*	64.0*	127*	–	–
32.0*	167*	32.0*	110*	32.0*	98*	32.0*	50*	–	–
21.3	3	21.3	7	21.3	3	–	–	–	–
–	–	15.2	4	15.2	5	15.2	6	15.2	11
–	–	10.2	4	10.2	6	10.2	8	10.2	10
–	–	5.62	3	5.62	5	5.63	9	5.63	13
–	–	5.17	5	5.17	8	5.16	20	5.16	24
–	–	4.58	3	4.58	4	4.59	5	4.58	15
–	–	–	–	–	–	4.45	7	4.44	8
4.2	100	4.2	96	4.2	73	4.2	24	4.14	19
–	–	3.94	2	3.94	2	3.94	2	3.93	5
–	–	–	–	–	–	3.29	2	3.28	5
–	–	–	–	–	–	–	–	3.15	5

a) The samples were diffracted 2 days after preparation.
b) The interplanar spacings and intensities of the reflections were measured in X-ray diagrams from flat-plate cameras with 8 and 14*cm specimen-to-film distance.
c) Only the main observed reflections are included.

width and a relatively intense reflection of 4.2 Å. The latter arose from the stiff and fully extended DMPC hydrocarbon chains organized with rotational disorder in an hexagonal lattice. As before, increasing concentrations of tamoxifen produced a gradual decrease of the phospholipid reflection intensities without affecting its bilayer width. On the other hand, reflections arising from the drug were observed even in the 10:1 molar mixture. These results indicated that again part of tamoxifen, despite of its low solubility in water, was able to penetrate into DMPC bilayer perturbing its molecular structure. The results obtained after DMPE was made to interact with tamoxifen in the hydrophobic medium are presented in Table III and Fig. 3a, whereas those obtained in the aqueous one are shown in Table IV and Fig. 3b. As it can be appreciated, the effects of the drug upon the

Table III. Comparison of observed interplanar spacings (do) and relative intensities (Io rel) of dimyristoylphosphatidylethanolamine (DMPE), tamoxifen and of their 10:1, 5:1 and 1:1 molar mixtures[a-c].

DMPE		DMPE : Tamoxifen						Tamoxifen	
		10:1		5:1		1:1			
do(Å)	Io rel	do(Å)	Io rel	do(Å)	Io rel	do(Å)	Io rel	do(Å)	Io
51.4*	970*	51.0*	870*	51.0*	822*	51.0*	772*	–	–
25.5*	4*	25.5*	3*	25.5*	2*	–	–	–	–
16.9	4	16.9	3	16.9	2	–	–	–	–
–	–	–	–	15.1	8	15.1	7	15.2	41
12.7	6	12.7	6	12.7	6	12.7	3	12.7	4
–	–	–	–	–	–	10.2	4	10.2	24
–	–	–	–	–	–	–	–	9.30	4
–	–	–	–	6.29	2	6.29	3	6.28	16
5.94	12	5.94	9	5.94	7	5.94	3	–	–
5.69	9	5.69	6	–	–	–	–	–	–
–	–	–	–	5.62	8	5.62	10	5.62	37
5.47	5	5.48	4	–	–	–	–	–	–
5.22	4	5.20	4	5.18	14	5.16	24	5.16	100
5.06	6	5.06	5	–	–	–	–	–	–
–	–	–	–	–	–	–	–	4.87	7
4.79	19	4.78	12	4.77	12	4.76	12	4.74	8
4.63	11	4.62	9	4.63	7	–	–	–	–
								4.57	52
–	–	–	–	4.49	18	4.49	20		
								4.44	25
4.23	5	4.24	4	4.24	4	4.23	4	4.23	7
–	–	–	–	4.16	4	4.15	6	4.14	63
4.05	100	4.05	89	4.04	85	4.04	76	4.07	3
3.91	9	3.91	8	3.91	7	3.91	7	3.91	32
3.80	55	3.80	40	3.80	53	3.80	48	3.80	11
3.63	4	3.63	3	3.63	2	–	–	–	–
–	–	–	–	–	–	–	–	3.72	15
–	–	–	–	–	–	–	–	3.57	9
–	–	–	–	–	–	–	–	3.49	9
–	–	–	–	–	–	3.39	3	3.37	17
–	–	–	–	–	–	–	–	3.29	11
–	–	–	–	–	–	–	–	3.14	7

a) All the specimens were recrystallized from $CHCl_3$:CH_3OH 3:1 (v/v).
b) The interplanar spacings and intensities of the reflections were measured in X-ray diagrams from Debye-Scherrer and flate-plate cameras with 8 and 14*cm specimen-to-film distance.
c) Only the main observed reflections are included.

structure of DMPE were considerable milder than those observed in DMPC under the same physicochemical conditions. In fact, even at such a high molar ratio as 1:1 tamoxifen did not induce a significative perturbation to DMPE, particularly in the presence of water. The fact that the intensities of the drug reflections are practically the same when immersed in pure water and in the presence of DMPE indicated that tamoxifen failed to penetrate into the bilayer. It is also worth to note that besides a small decrease of the reflection intensities the X-ray pattern of DMPE was not significatively affected by water.

Fluorescence measurements on large unilamellar vesicles (LUV)

The influence of tamoxifen upon the bilayer of DMPC LUV was evaluated at the phospholipid acyl chain hydrophobic core and at the hydrophilic/hydrophobic interface, i.e., the phospholipid polar head level. This was achieved evaluating DPH steady state fluorescence anisotropy (r) and Laurdan fluorescence spectral shifts, respectively. The latter was quantified through the general polarization (GP) parameter (Parasassi and Gratton, 1995). The presence of increasing concentrations of tamoxifen produced a significative decrease in DPH fluorescence anisotropy and Laurdan GP as

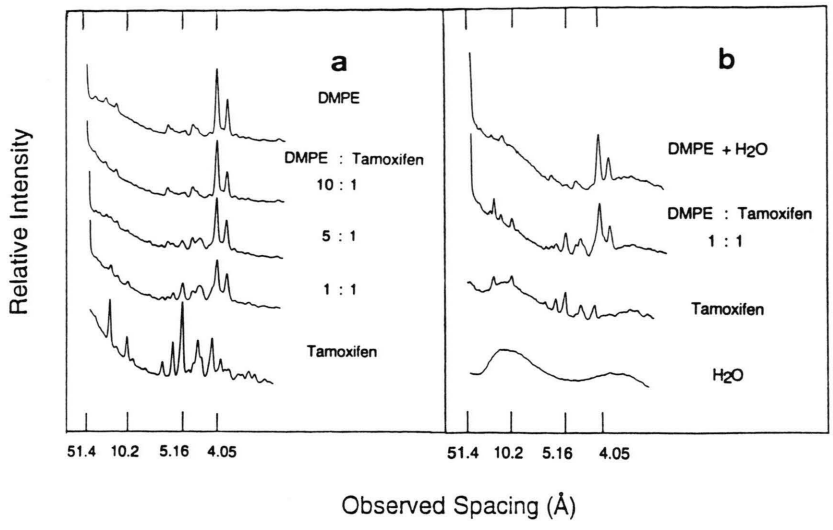

Fig. 3. Microdensitograms from X-ray diffraction diagrams of dimyristoylphosphatidylethanolamine (DMPE), tamoxifen and of their 10:1, 5:1 and 1:1 molar mixtures. Flat-plate cameras, specimen-to-film distance 8 cm. (a) Recrystallized from $CHCl_3$:CH_3OH (v/v); (b) immersed in water.

Table IV. Comparison of observed interplanar spacings (do) and relative intensities (Io rel) of dimyristoylphosphatidylethanolamine (DMPE), tamoxifen and of their 1:1 molar mixtures in water[a-c].

DMPE		DMPE:Tamoxifen 1:1		Tamoxifen	
do(Å)	Io rel	do(Å)	Io rel	do(Å)	Io rel
51.4*	1400*	51.0*	1270*	–	–
25.7*	2*	25.5*	2*	–	–
17.2	6	17.2	3	–	–
–	–	15.2	18	15.2	18
12.8	16	12.7	8	–	–
–	–	10.2	13	10.2	14
5.94	8	5.93	7	–	–
–	–	5.63	18	5.63	19
–	–	5.16	42	5.16	52
5.08	5	–	–	–	–
4.64	9	4.58	26	4.58	21
–	–	4.42	11	4.44	12
4.25	5	–	–	–	–
–	–	4.15	2	4.14	27
4.06	100	4.05	97	–	–
3.94	4	3.93	4	3.93	7
3.81	48	3.81	44	3.84	6
3.65	2	3.60	2	3.57	5
–	–	3.38	5	3.39	5
–	–	3.28	5	3.28	7
–	–	3.14	5	3.15	7

a) The samples were diffracted 2 days after preparation.
b) The interplanar spacings and intensities of the reflections were measured in X-ray diagrams from Debye-Scherrer and flat-plate cameras with 8 and 14*cm specimen-to-film distance.
c) Only the main observed reflections are included.

shown in Table V. The decrease of these parameters can be explained as a disorder effect induced by tamoxifen on the acyl chain packing and an enhancement of molecular dynamics or water penetration at the phospholipid polar head level, respectively. The latter effects could be a consequence of a mild structural disorganization at this level.

Scanning electron microscopy (SEM) studies on human erythrocytes

The examination by SEM of red cells incubated with tamoxifen revealed abnormalities in their shapes. In contrast to the normal discoid erythrocyte profile a great number of tamoxifen treated cells underwent stomatocytic shape changes, i.e., evagination of one surface and invagination of the opposite face. This effect was observed in all the assayed samples and the extent of the shape alteration was always the same.

Discussion

Several controversies surround the mechanism of action of the anticancer drug tamoxifen. One of them is whether it interacts directly with a relevant membrane protein such as ER, AEBS or an enzyme (Das *et al.*, 1993; Eyster *et al.*, 1989; O'Brien *et al.*, 1988; Horgan *et al.*, 1986) or indirectly through structural modification of the membrane where the protein is located (O'Brien *et al.*, 1986; Custodio *et al.*, 1991, 1993; Luxo *et al.*, 1996). In order to elucidate this controversy we studied the interaction of tamoxifen with the human erythrocyte membrane and bilayers built up of DMPC and DMPE.

Table V. Effect of Tamoxifen on the fluorescence anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) and the general polarization (GP) of Laurdan embedded in large unilamellar vesicles (LUV) of dimyristoylphosphatidylcholine (DMPC) (probe:lipid ratio 1:600).

Tamoxifen Conc. (μM)* in Water	LUV	DMPC: Tamoxifen molar ratio	r DPH	GP Laurdan
0	0	0	0.315	0.418
0.15	9.85	50:1	0.299	0.409
1.5	98.5	5:1	0.188	0.194
8.0	492.0	1:1	0.185	0.098

* The drug concentration in the the aqueous and LUV phases was calculated considering its 63.8 octanol/buffer pH 7.4 partition coefficient.

The experiments performed by fluorescence spectroscopy showed that tamoxifen interacted with DMPC under the form of large unilamellar vesicles (LUV) perturbing its polar head and hydrocarbon chain regions (Table V). These results were confirmed by the X-ray experiments. In fact, in molar ratios similar to those used in LUV, tamoxifen was able to perturb the same molecular regions of DMPC bilayers in both the hydrophobic and aqueous media where these interactions were assayed. However, tamoxifen did not cause any significative structural effect in DMPE bilayers. This difference can be related to their respective packing arrangements and the effects of water upon them. Chemically these phospholipids only differ in their terminal amino groups, being $+NH_3$ in DMPE and $+N(CH_3)_3$ in DMPC. Moreover, both molecular conformations are very similar in their dry crystalline phases (Suwalsky, 1988) with the hydrocarbon chains mostly parallel and extended with the polar groups lying perpendicularly to them. However, DMPE molecules pack tighter than those of DMPC due to its smaller polar group and higher effective charge, resulting in a very stable multilayer system which is not significatively affected by water (Suwalsky and Duk, 1987). On the other hand, the gradual hydration of DMPC results in water molecules occupying the highly polar interbilayer spaces. Therefore, its bilayer width increases from 54.5 Å when dried up to 64.0 Å when it was fully hydrated. This situation allowed the incorporation of tamoxifen into DMPC bilayers producing its structural perturbation, which was not the case of DMPE.

The examination by electron microscopy of the erythrocytes incubated with tamoxifen revealed that they changed their normal discoid shape to stomatocytes. Accordingly to the bilayer hypothesis, this means that the drug inserted into the inner monolayer of the erythrocyte membrane (Sheetz and Singer, 1974). This moiety is poor in the class of lipids represented by DMPC and rich in those similar to DMPE, which did not interact with tamoxifen. Therefore, it is likely that this drug preferentially interacted with proteins located in the inner leaflet of the membrane. This conclusion is supported by reports that 99% of medicated tamoxifen is protein-bound (Nayfield, 1995). Patients are normally treated with doses of 250 mg/kg (Jordan, 1995), reaching a concentration of about 1 mM in the serum (Jordan, 1990). On the other hand, the concentration of the drug and metabolites in humans are 10-to-60-fold higher in tissues than in serum (Lien *et al.*, 1991). Therefore, the concentrations used in the experiments with human erythrocytes, liposomes and phospholipid multilayers were within the range of the concentrations that are therapeutically attained.

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