Biochimica et Biophysica Acta, 1150 (1993) 123–129

BBAMEM 76039

The anticancer drug tamoxifen induces changes in the physical properties of model and native membranes

José B.A. Custódio [a,b], Leonor M. Almeida [a,b] and Vítor M.C. Madeira [b]

[a] *Laboratório de Bioquímica, Faculdade de Farmácia, Universidade de Coimbra, Coimbra (Portugal) and* [b] *Centro de Neurociências, Universidade de Coimbra, Coimbra (Portugal)*

(Received 21 December 1992)
(Revised version received 1 February 1993)

Key words: Tamoxifen; Anti-cancer drug; Breast cancer; Membrane–lipid interaction; Membrane fluidity; Fluorescent probe

The interactions of tamoxifen with lipid bilayers of model and native membranes were investigated by fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) and by intramolecular excimer formation of 1,3-di(1-pyrenyl)propane (Py(3)Py). The effects of TAM on liposomes of DMPC, DPPC and DSPC are temperature dependent. In the fluid phase, TAM reduces dynamics of the upper bilayer region as observed by Py(3)Py and has no effect on the hydrophobic region as detected by DPH. In the gel phase, the effects of TAM evaluated by Py(3)Py are not discernible for DMPC and DPPC bilayers, whereas DSPC bilayers become more fluid. However, DPH detects a strong fluidizing effect of TAM in the hydrophobic region of the above membrane systems, where DPH distributes, as compared with the small effects detected by Py(3)Py. TAM decreases the main phase transition temperature but does not extensively broaden the transition thermotropic profile of pure lipids, except for bilayers of DMPC where TAM induces a significant broadening detected with the two probes. In fluid liposomes of sarcoplasmic reticulum lipids and native membranes, TAM induces an ordering effect, as evidenced by Py(3)Py, failing DPH to detect any apparent effect as observed for the fluid phase of liposomes of pure lipid bilayers. These findings confirm the hydrophobic nature of tamoxifen and suggest that the localization and effects of TAM are modulated by the order and fluidity of the bilayer. These changes in the dynamic properties of lipids and the non-specific interactions with membrane lipids, depending on the order or fluidity of the biomembrane, may be important for the multiple cellular effects and action mechanisms of tamoxifen.

Introduction

Tamoxifen (TAM, Fig. 1) is a non-steroidal antiestrogen drug widely used in the chemotherapy and chemoprevention of breast cancer [1]. However, the multiple cellular effects and action mechanisms are not sufficiently accounted for by the classical estrogen-receptor (ER) model, suggesting that antiestrogen actions may involve other mechanisms unclear at present [2].

Some cytostatic drugs interact strongly with the phospholipid components of several cellular membranes, changing the bilayer organization [3–5]. It is believed that these drug-membrane interactions play a key role in the cytotoxicity of anticancer agents [6].

Recently, we reported that tamoxifen strongly incorporates into biomembranes [7], explaining the high

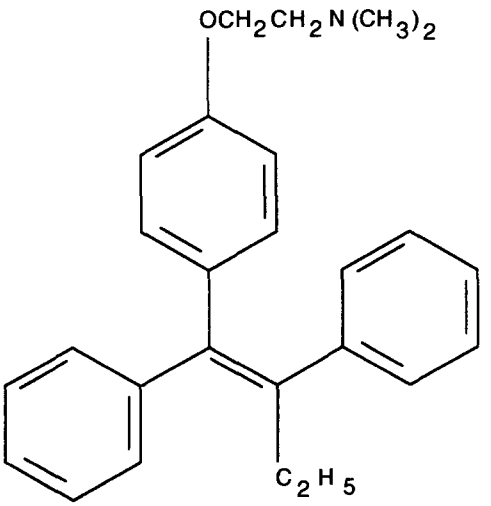

Fig. 1. Chemical structure of tamoxifen.

Correspondence to: J.B.A. Custódio, Laboratório de Bioquímica, Fac. Farmácia, Couraça dos Apóstolos, 51, r/c 3000 Coimbra, Portugal.

Abbreviations: TAM, tamoxifen; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; ER, estrogen receptor; SR, sarcoplasmic reticulum; DPH, 1,6-diphenyl-1,3,5-hexatriene; Py(3)Py, 1,3-di(1-pyrenyl) propane.

concentrations of this drug found by others in most tissues [8]. On the other hand, TAM inhibits enzymes involved in cellular proliferation by putative interactions with phospholipids [9]. Therefore, studies of the interactions between this drug with biomembranes are important to understand the multiple action mechanisms and cellular effects.

Previous work indicates that TAM decreases membrane fluidity in ER-positive and ER-negative human breast cancer cell lines, as evaluated from fluorescence polarization of DPH [10]. In the present study, experiments were designed to investigate the molecular interactions of TAM on lipid organization of pure phospholipids, SR membranes (taken as a native membrane model) and liposomes of SR lipids, by means of fluorescence polarization of DPH, a probe located in the bilayer core [11], and by intramolecular excimer formation of 1,3-di(1-pyrenyl)propane (Py(3)Py), a probe displaced to the outer regions of the bilayer [11].

Materials and Methods

Tamoxifen, dimyristoyl-, dipalmitoyl- and distearoylphosphatidylcholine, Tris-maleate, cholesterol and DPH were obtained from Sigma. The probe Py(3)Py was a gift of Dr. Zachariasse from the Max-Planck-Institut für Biophysikalische Chemie (Göttingen, Germany).

Membranes of sarcoplasmic reticulum (SR) were prepared from rabbit white muscles and SR lipids were extracted as previously described [12]. The lipid contents were determined by measuring the amount of inorganic phosphate [13] after hydrolysis at 180°C in 70% $HClO_4$ [14]. Liposomes were prepared, after solvent evaporation to dryness, by dispersion in 50 mM KCl, 10 mM Tris-maleate (pH 7) and the lipid suspensions were obtained as described elsewhere [15].

Py(3)Py and DPH were incorporated into membrane suspensions (345 μM in phospholipid) and mixtures

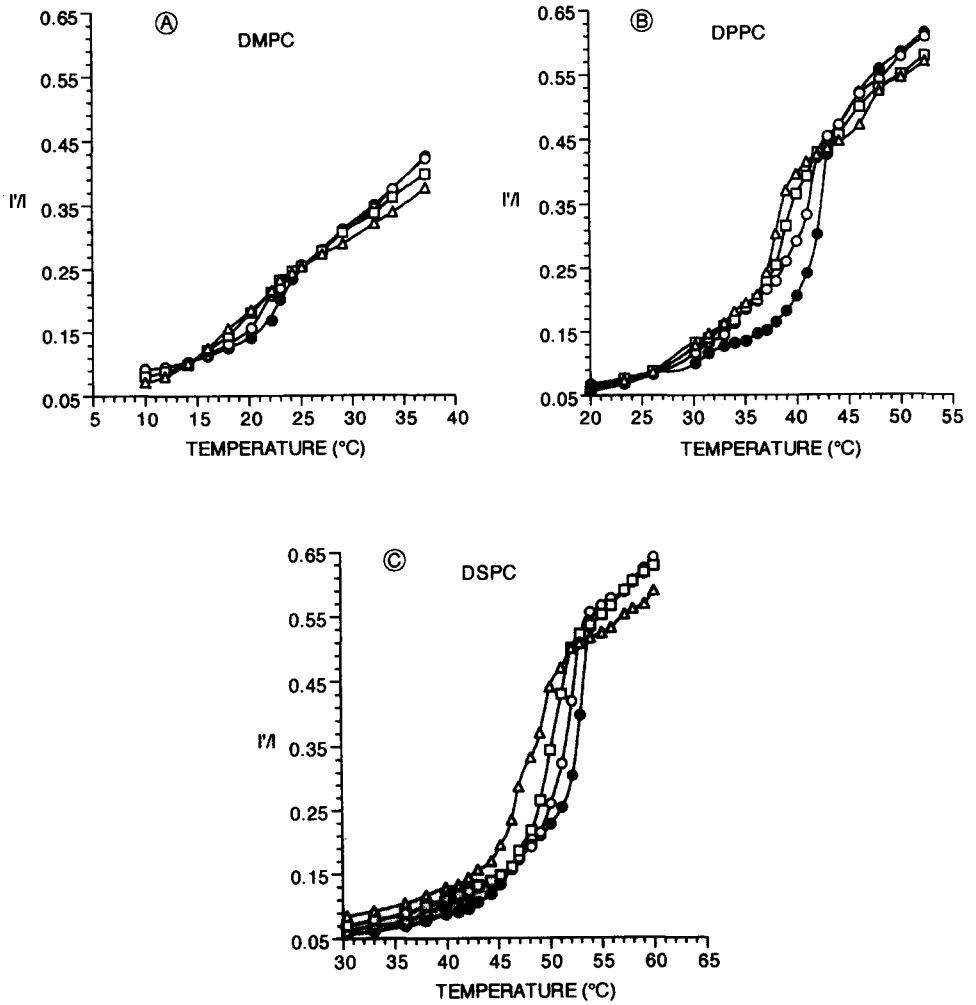

Fig. 2. Thermotropic phase transition profiles of DMPC, DPPC and DSPC bilayers determined by intramolecular excimerization of Py(3)Py as excimer/monomer fluorescence intensity ratio (I'/I), in the absence (●) and presence of 10 (○), 25 (□) and 50 μM (△) of TAM.

incubated as previously described [16,17], to give a lipid/probe molar ratio of about 900 and 400, respectively. After incubation with the probes, TAM was added to membrane systems and the mixtures were allowed to equilibrate for 20 min at the phase-transition temperature of the synthetic phospholipids and at 37°C for liposomes and native membranes of SR, before fluorescence measurements.

The fluorometric measurements were carried out with a Perkin-Elmer LS 50 computer controlled spectrofluorometer. In studies with DPH, the degree of fluorescence polarization (P) was determined as reported elsewhere [18], except that the excitation and emission slits were 3 nm and 4 nm, respectively. The intramolecular excimer formation of Py(3)Py was evaluated as the excimer to monomer fluorescence intensity ratio (I'/I), as previously described [16], using 3 nm excitation and 4 nm emission slits. Appropriate control experiments were carried out without added probes to correct for the contribution of light scattering. Other control assays with increasing concentrations of tamoxifen in solvents, at a constant temperature (25°C) have shown that the fluorescence quenching induced by the drug is of the dynamic or diffusional type, meaning that TAM interacts with the fluorophore in the excited state. However, tamoxifen does not affect either excimer/monomer ratio (I'/I) or fluorescence polarization of DPH (P) (unpublished data).

Results and Discussion

Liposomes of pure phospholipids

The effect of TAM on lipid organization of model membranes was studied by monitoring the thermotropic phase transition profiles of DMPC, DPPC and DSPC, using intramolecular excimer formation of Py(3)Py as reflected by the excimer/monomer ratio of fluorescence intensity (Fig. 2), and the fluorescence polarization of DPH (Fig. 3).

Py(3)Py was chosen to probe fluidity of the outer regions, whereas DPH was used to report the structural order of the lipid bilayer core. These two probes

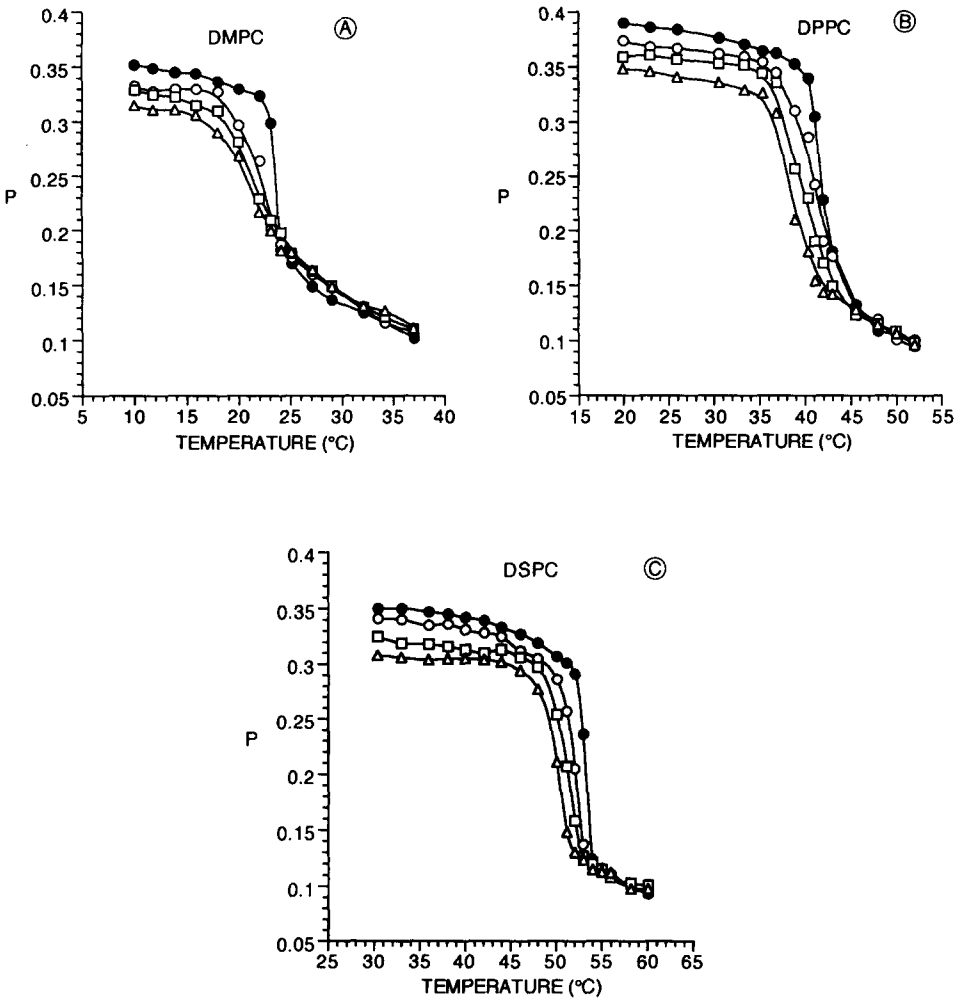

Fig. 3. Fluorescence polarization of DPH (P) on the thermotropic profile of DMPC, DPPC and DSPC bilayers as a function of temperature, in the absence (●) and presence of various TAM concentrations: 10 (○), 25 (□) and 50 μM (△).

were selected, since they report similar physical parameters and their locations across the membrane have been well-characterized [16,19–22]. Thus, DPH polarization reports the rotational diffusion of the probe and strongly depends on fluidity [23,24]. On the other hand, the intramolecular excimer formation of Py(3)Py is determined by the rate of motion about the σ bonds of propane linking the two pyrene rings and depends on the fluidity of probe environment [16,21,22]. Therefore, the term fluidity is used here in an operational sense and defined as being directly proportional to the fluorescence depolarization of DPH or to the rate of excimer formation by Py(3)Py. This fluidity is related but not identical with the physical definition of fluidity.

As detected by Py(3)Py (Fig. 2), TAM interacts with DMPC liposomes inducing condensing and fluidizing effects which depend on temperature, i.e., a fluidizing effect is noticed in the temperature range of the cooperative phase transition and a moderate condensing effect is detected in the fluid phase. As the fatty acyl chain length increases (DSPC liposomes), the intrinsic bilayer fluidity decreases and Py(3)Py detects a significant fluidizing effect also in the gel phase of this more ordered bilayer (Fig. 2).

On the other hand, a stronger fluidizing effect of the antiestrogen in the gel phase of the three membrane systems is observed by fluorescence polarization of DPH (Fig. 3), as compared with the small effects for the gel phase detected by Py(3)Py. In fact, in the gel phase, TAM significantly decreases the polarization in a concentration dependent way in the range of 10 to 50 μM, without affecting the fluid phase of lipid bilayers. These differential effects below and above phase transition may be explained by preferential accommodation of TAM in the hydrophobic core of ordered membrane systems, as observed for other drugs [17,18,25].

The stronger effects of TAM are detected by both probes at the temperature of the main phase transition, at which the drug incorporation is maximal [7]. TAM induces a shift of the main phase transition

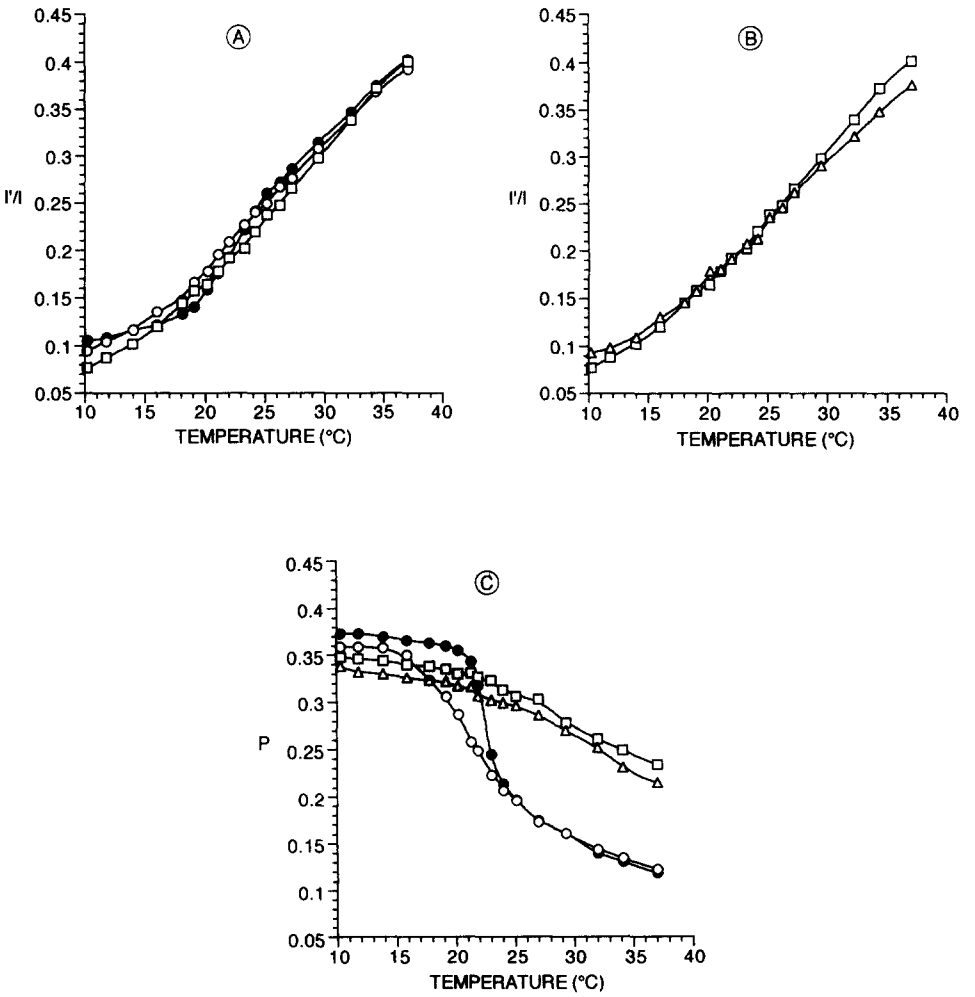

Fig. 4. Effect of TAM and cholesterol on the DMPC bilayer organization as a function of temperature, evaluated by intramolecular excimerization of Py(3)Py (I'/I) and fluorescence polarization of DPH (P). (●) DMPC alone, (○) 25 μM TAM, (□) 20 mol% cholesterol and (△) 20 mol% cholesterol plus 25 μM TAM. For the sake of clarity, data of I'/I was split into parts A and B.

temperature (3–5°C) with a relatively small broadening of the transition thermotropic profile for DPPC and DSPC, as compared with the strong broadening of the phase transition observed for other drugs [26–30]. In bilayers of higher intrinsic fluidity, e.g., DMPC (Figs. 2 and 3), these effects are, however, more pronounced.

According to Jain and Wu [31], the shifting and broadening of the transition profile indicates a localization of the perturber molecules in the cooperativity region of the bilayer, i.e., aligning with the segment corresponding to the first eight carbons of the acyl chains. TAM does not extensively perturb the cooperativity region of the above lipids, since a relatively small broadening in the temperature range of the transition profile was observed, suggesting that most TAM molecules are located in the hydrophobic interior region of the ordered bilayer and do not affect the cooperativity region (C_2–C_8) of the fatty acyl chains. However, since bilayers of DMPC are intrinsically more fluid, the drug may partially extend its distribution into the outer hydrophobic region of the bilayer, producing a more pronounced broadening of the transition profile of DMPC liposomes as compared to those in DPPC and DSPC liposomes.

In principle, changes in polarization could also result from changes in lifetime of DPH excited state. However, effects of TAM on lifetimes are probably negligible, due to the fact that polarization is practically insensitive to TAM in the fluid phase of artificial bilayers (Fig. 3).

In conclusion, data of Figs. 2 and 3 clearly indicate that, in the gel phase, TAM essentially affects the hydrophobic core of the bilayer, where DPH is located, whereas the outer region of the bilayer, where Py(3)Py distributes, is less perturbed by TAM. In the fluid phase only Py(3)Py detects some perturbation effects related with a fluidity decrease. Consequently, the localization and effects of TAM are apparently modulated by the order and the fluidity gradient of the bilayer.

DMPC-cholesterol bilayers

Considering that membrane physical properties influence membrane partition of TAM [7], and that plasma membranes of tumor cells have been shown to possess altered lipid fluidity [32], the effects of TAM on the fluidity of phospholipid bilayers as a function of temperature and cholesterol content were studied. As expected, cholesterol strongly broadens the transition profiles evaluated either by excimer formation of Py(3)Py or by DPH polarization (Fig. 4). The effect of tamoxifen in bilayers of DMPC containing cholesterol is generally small as compared with the effects observed in the absence of the sterol. Thus, a small fluidity increase is observed with DPH and either small increases or decreases are detected by Py(3)Py depending on temperature (Fig. 4).

Different locations are in principle expected for cholesterol and tamoxifen, owing to the different chemical structures. Whereas the cholesterol molecule contains a small polar hydroxyl group, a rigid steroid ring and a isooctyl side chain, the molecule of TAM contains a hydrophobic region of the three aromatic rings and a tertiary deprotonated amine side chain (Fig. 1) [33]. Thus, the cholesterol molecule located in the outer hydrofobic cooperativity region of the bilayer, with its rigid steroid ring extending to the depth of 7–10 carbon atoms of lipid alkyl chains [34], affects the cooperativity region of the bilayer; TAM putatively displaced to the central bilayer region induces a less broadening on the transition thermotropic profile as compared with the effect of cholesterol.

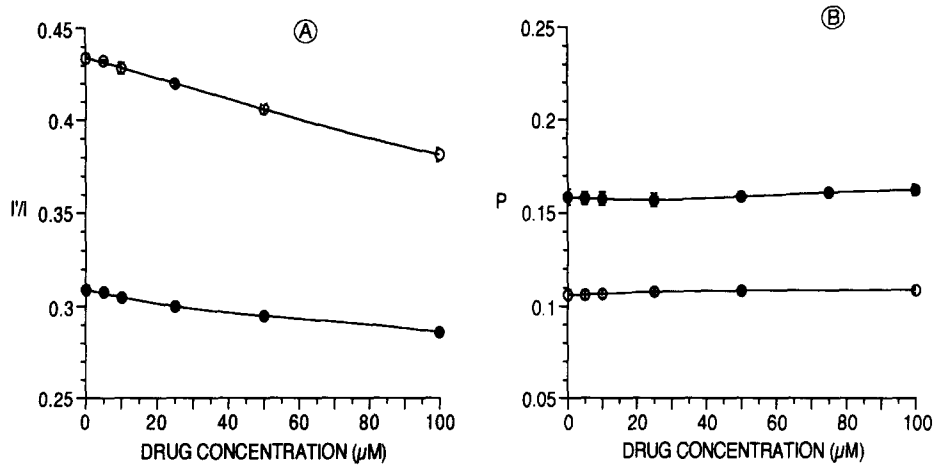

Fig. 5. Intramolecular excimerization of Py(3)Py (I'/I) and fluorescence polarization of DPH (P) in native SR membranes (full symbols) and liposomes of SR lipids (open symbols), as a function of TAM concentration, at 37°C. Normalized data are means ± S.D. for three experiments. Standard error bars are contained within the size of the symbols.

Liposomes and native membranes of SR

The concentrations of TAM used in our study reasonably compare with the concentrations used in other reported works [9,35–37]. However, it is difficult to precisely calculate the concentrations of TAM resulting from the pharmacological doses, since the recommended daily dose, depending upon the country, is 10 to 20 mg and the range of TAM serum levels is relatively large (50–300 ng/ml) [38]. On the other hand, more than 98–99% of TAM is bound to serum albumin [1,8] and, therefore, TAM has a long plasma half-life (7 days) and at least 4 weeks of administration are required to reach steady-state drug concentration [1,38]. The apparent volume of distribution for TAM is high, suggesting extensive distribution into peripheral tissues and the presence of a minor portion (0.1%) in serum, with levels 10–60-fold higher in tissues than in serum [8]. Therefore, estimated concentrations in peripheral tissues may reach values within the range of our studies.

The effects of TAM at 37°C in SR native membranes and in liposomes of SR lipids were also evaluated by means of the Py(3)Py I'/I ratio and DPH polarization (Fig. 5).

Similarly to the observed effects in the liquid-crystalline phase of pure phospholipids, TAM induces a concentration-dependent ordering effect in the lipid bilayer of fluid native SR membranes and liposomes of SR, as indicated by a decrease of Py(3)Py I'/I ratio (Fig. 5). This effect is more apparent in liposomes of SR lipids, suggesting that the drug in native membranes does not extensively disturb interactions between the fatty acyl chains of the bulk bilayer phospholipids. Since the SR membrane is more ordered than the lipid membrane, the additional effect of TAM is expected to be smaller. Moreover, in native SR membranes, the drug may strongly bind to membrane proteins, in agreement with the high efficiency of TAM to bind to proteins [1,8,39] with less perturbation of the bulk lipid structure.

The studies with DPH (Fig. 5) do not detect any significant effect of TAM in the organization of fluid native SR membranes or liposomes of SR lipids. These findings agree with studies in liposomes of pure phospholipids, which indicate that TAM, in the fluid phase, does not affect DPH polarization (Fig. 3). In the fluid bilayer, TAM tends to spread across the bilayer, extending to outer regions where Py(3)Py distributes. As indicated above, this probe detects limited ordering effects promoted by the drug.

In contrast to earlier reports [40–42] new experimental evidence suggests that only a minority of membrane enzymes is regulated by the fluidity of the lipid bilayer [43]. However, TAM binding to membrane proteins with consequent perturbation of lipid-protein interactions may also be involved in the molecular mechanisms by which the drug inhibits membrane enzymes involved in cell regulation, e.g., protein kinase C (PKC), a crucial enzyme in tumor promotion (35,44] and calmodulin-regulated enzymes, i.e., cAMP phosphodiesterase, a key enzyme for the metabolism of cyclic nucleotides [45] and (Ca^{2+}–Mg^{2+})-ATPase of red blood cell membranes [37]. A putative distribution of TAM at lipid-protein interfaces of native membranes may severely perturb the biochemical activities of membrane bound enzymes, viz., the Ca^{2+}-ATPase of SR. This Ca^{2+}-pump, very sensitive to changes at the interfacial lipids, is strongly inhibited by TAM at low added concentrations (unpublished data).

In conclusion, TAM interacts with membranes and affects the physical properties of the lipid bilayer. TAM induces a fluidizing effect in the gel phase of pure lipid bilayers and a rigidifying effect in fluid phase; however, the fluidity perturbations in the fluid phase are less pronounced in highly-fluid native membranes where the drug putatively interacts also with proteins modifying lipid-protein interactions. These lipid-mediated physical effects of TAM, as suggested for other drugs [46], are certainly important for the control of membrane functions and for an understanding of the cytotoxicity mechanisms of TAM. Furthermore, they may contribute for the multiple cellular effects and action mechanisms of TAM involved in the inhibition of cell proliferation.

Acknowledgement

This work was supported by Centro de Estudos Farmacêuticos of Instituto Nacional de Investigação Científica (INIC).

References

1 Nayfield, S.G., Karp, J.E., Ford, L.G., Dorr, F.A. and Karmer, B.S. (1991) J. Natl. Cancer Inst. 83, 1450–1459.
2 Kon, O.L. (1989) BioEssays 10, 210–213.
3 Wright, S.E. and White, J.C. (1986) Biochim. Biophys. Acta 863, 297–304.
4 Deliconstantinos, G., Kopeikina-Tsiboukidou, L. and Villiotou, V. (1987) Biochem. Pharmacol. 36, 1153–1161.
5 Canaves, J.M., Ferragut, J.A. and Gonzalez-Ros, J.M. (1991) Biochem. J. 279, 413–418.
6 Tritton, T.R. and Posada, J.A. (1989) in Resistance to Antineoplastic Drugs (Kessel, D., ed.), pp. 127–140, CRC Press, Boca Raton.
7 Custódio, J.B.A., Almeida, L.M. and Madeira, V.M.C. (1991) Biochem. Biophys. Res. Commun. 176, 1079–1085.
8 Lien, E.A., Solheim, E. and Ueland, P.M. (1991) Cancer Res. 51, 4837–4844.
9 O'Brian, C.A., Liskamp, R.M., Solomon, D.H. and Weinstein, I.B. (1986) J. Natl. Cancer Inst. 76, 1243–1246.
10 Clarke, R., Van den Berg, H.W. and Murphy, R.F. (1990) J. Natl. Cancer Inst. 82, 1702–1705.
11 Antunes-Madeira, M.C., Almeida, L.M. and Madeira, V.M.C. (1990) Biochim. Biophys. Acta 1022, 110–114.

12 Madeira, V.M.C. and Antunes-Madeira, M.C. (1976) Ciênc. Biol. 2, 265–291. (Portugese)
13 Bartlett, G.R. (1958) J. Biol. Chem. 234, 466–468.
14 Böttcher, C.J.F., Van Gent, C.M. and Pries, C. (1961) Anal. Chim. Acta 24, 203–204.
15 Jurado, A.S., Almeida, L.M. and Madeira, V.M.C. (1991) Biochem. Biophys. Res. Commun. 176, 356–363.
16 Almeida, L.M., Vaz, W.L.C., Zachariasse, K.A. and Madeira, V.M.C. (1982) Biochemistry 21, 5972–5977.
17 Antunes-Madeira, M.C. and Madeira, V.M.C. (1990) Biochim. Biophys. Acta 1023, 469–474.
18 Antunes-Madeira, M.C., Almeida, L.M. and Madeira, V.M.C. (1991) Pestic. Sci. 33, 347–357.
19 Andrich, M.P. and Vanderkooi, J.M. (1976) Biochemistry 15, 1257–1261.
20 Mulders, F., Van Langen, H., Van Ginkel, G. and Levine, Y.K. (1986) Biochim. Biophys. Acta 859, 209–218.
21 Almeida, L.M., Vaz, W.L.C., Zachariasse, K.A. and Madeira, V.M.C. (1984) Biochemistry 23, 4714–4720.
22 Zachariasse, K.A., Vaz, W.L.C., Sotomayor, C. and Kühnle, W. (1982) Biochim. Biophys. Acta 688, 323–332.
23 Shinitzky, M. and Barenholz, Y. (1977) J. Biol. Chem. 249, 2652–2657.
24 Litman, B.J. and Barenholz, Y. (1982) Methods Enzymol. 81, 678–685.
25 Sautereau, A.-M., Tournaire, C., Suare, M., Tocanne, J.-F. and Paillous, N. (1992) Biochem. Pharmacol. 43, 2559–2566.
26 O'Leary, T.J., Ross, P. and Levin, I.W. (1984) Biochemistry 23, 4636–4641.
27 Hanpft, R. and Mohr, K. (1985) Biochim. Biophys. Acta 814, 156–162.
28 Borchardt, K., Heber, D., Klingmüller, M., Mohr, K. and Müller, B. (1991) Biochem. Pharmacol. 42, 561–565.
29 Almeida, L.M., Vaz, W.L.C., Stümpel, J. and Madeira, V.M.C. (1986) Biochemistry 25, 4832–4839.
30 Subczynski, W., Markowka, E., Gruszecki, W.I. and Sielewiesiuk, J. (1992) Biochim. Biophys. Acta 1105, 97–108.
31 Jain, M.K. and Wu, N.M. (1977) J. Membr. Biol. 34, 157–201.
32 van Blitterwijh, W.J. (1984) in Physiology of Membrane Fluidity, Vol. II (Shinitzky, M., ed.), pp. 53–83, CRC Press, Boca Raton.
33 Bottega, R. and Epand, R.M. (1992) Biochemistry 31, 9025–9030.
34 McIntosh, T.J. (1978) Biochim. Biophys. Acta 513, 43–58.
35 O'Brian, C.A., Liskamp, R., Solomon, D.H. and Weinstein, I.B. (1985) Cancer Res. 45, 2462–2465.
36 Eyster, K. and Clark, M.R. (1989) Biochem. Pharmacol. 38, 3497–3503.
37 Lopes, M.C.F., Vale, M.G. and Carvalho, A.P. (1990) Cancer Res. 50, 2753–2758.
38 Jordan, V.G. (1990) Breast Cancer Res. Treat. 15, 125–136.
39 Oosbree, T.R.V., Olsen, M.R., Tate, A.C., Jordan, V.C. and Mueller, G.C. (1984) Mol. Cell. Endocrinol. 35, 143–149.
40 Dornmair, K., Overath, P. and Jahnig, F. (1989) J. Biol. Chem. 264, 342–346.
41 Gordon, L.M., Sauerheber, R.D., Esgate, J.A., Dipple, I., Marchmont, R.J. and Houslay, M.D. (1980) J. Biol. Chem. 255, 4519–4527.
42 Zakim, D., Vessey, D.A. and Hochman, Y. (1985) Biochem. Pharmacol. Toxicol. 1, 161–228.
43 Zakim, D., Kavecansky, J. and Scarlata, S. (1992) Biochemistry 31, 11589–11594.
44 Korgan, K., Cooke, E., Hallett, M.B. and Mansel, R.E. (1986) Biochem. Pharmacol. 35, 4463–4465.
45 Law, H.-Y. (1984) Biochem. Biophys. Res. Commun. 118, 27–32.
46 Jørgensen, K., Ipsen, J.H., Mouritsen, O.G., Bennett, D. and Zuckermann, M.J. (1991) Biochim. Biophys. Acta 1067, 241–253.