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Tamoxifen and related compounds decrease membrane fluidity in liposomes

Mechanism for the antioxidant action of tamoxifen and relevance to its anticancer and cardioprotective actions?

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Tamoxifen and related compounds decrease membrane fluidity in ox-brain phospholipid liposomes: their order of effectiveness is, 4-hydroxytamoxifen > 17 β-oestradiol > tamoxifen > *cis*-tamoxifen > *N*-desmethyltamoxifen > cholesterol. A good positive correlation was demonstrated between the decrease in membrane fluidity by these compounds and their antioxidant ability as inhibitors of liposomal and microsomal lipid peroxidation (correlation coefficient, $r = 0.99$, $P < 0.001$, in both cases). The ability of tamoxifen to decrease membrane fluidity is suggested to be the mechanism of its antioxidant action and is discussed in relation to its anticancer and cardioprotective actions.

Membrane fluidity, Tamoxifen; Antioxidant action; Liposomal lipid peroxidation; Anticancer action; Cardioprotectant

1. INTRODUCTION

Tamoxifen is used widely and effectively in the treatment of breast cancer [1–4]. Indeed, it is now being assessed in clinical trials as a prophylactic agent against this disease [5–9]. It is also being used to treat cancers of the liver [10], brain [11,12] and pancreas [13] and may have general application in the prevention and treatment of cancer [14]. It is of considerable interest that tamoxifen and related compounds are membrane antioxidants; they inhibit metal ion-dependent lipid peroxidation in a range of membrane systems [15–17]. Furthermore, tamoxifen and 4-hydroxytamoxifen protect human LDL against oxidative damage [18]. It has been suggested that tamoxifen and related compounds may act as membrane antioxidants by stabilising the membrane against lipid peroxidation through a mechanism involving a decrease in membrane fluidity [16]. Indeed, it has been reported previously that tamoxifen and 17 β-oestradiol decrease membrane fluidity, as judged by the rotation of fluorescent probe molecules, in two breast cancer cell-lines [19].

In order to test our hypothesis, we determined the ability of tamoxifen, its *cis* isomer, its 4-hydroxy and *N*-desmethyl metabolites, 17 β-oestradiol and cholesterol (structures shown in Fig. 1) to decrease membrane fluidity. A model membrane system consisting of liposomes formed from ox-brain phospholipid dispersed in aqueous medium was the experimental system used. Fluidity was measured using the fluorescent probe diphenylhexatriene (DPH) [20,21] and its phosphatidylcholine derivative (DPH-PC) [21] and the results were correlated with the known membrane antioxidant abilities of these compounds in microsomes [15] and preformed liposomes.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Tamoxifen, cholesterol, 17 β-oestradiol, ox-brain phospholipids and DPH were from Sigma (Poole, Dorset, UK). 4-Hydroxytamoxifen, *cis*-tamoxifen and *N*-desmethyltamoxifen were from ICI Pharmaceuticals p.l.c. (Macclesfield, Cheshire, UK). DPH-PC was from Molecular Probes Inc (Eugene, OR, USA). All other compounds were of Analar quality from BDH.

2.2. *Preparation of liposomes*

Ox-brain phospholipid liposomes were prepared as follows. The phospholipid was added to phosphate-buffered saline (PBS) pH 7.4 (140 mM NaCl, 2.7 mM KCl, 16 mM Na_2HPO_4, 2.9 mM KH_2PO_4) at a final concentration of 5 mg/ml followed by vortexing in the presence of glass beads (2.5–3.5 mm in diameter) at 20°C. The resulting liposomes were left in sealed nitrogen-flushed bottles for 1 h before use.

2.3. *Fluorescence measurements*

Incubation mixtures contained (in a final volume of 3 ml) 1.5 ml of distilled water, 1.2 ml of PBS pH 7.4, and 0.3 ml of ox-brain phospholipid liposomes at a final concentration of phospholipid of 0.3 mM. Tamoxifen and related compounds, 17 β-oestradiol or cholesterol were each added in ethanol to give final concentrations in the range

0–45 μM, at a final concentration of ethanol of 0.5% (v/v). Controls using this concentration of ethanol were always included as control treatments for the experiments. DPH dissolved in ethanol was added to give a final concentration of 1 μM. Incubation prior to fluorescence measurements was for 1 h at 20°C to ensure distribution of compounds and probe in the phospholipid bilayers.

In experiments where DPH-PC was used as the probe a different approach to preparation was taken to ensure that the probe was distributed throughout the phospholipid bilayers because unlike DPH, DPH-PC becomes intercalated between the phospholipids in the bilayers [21]. DPH-PC was added in ethanol to the ox-brain phospholipid (dissolved in chloroform) to give a final concentration in the liposome suspension of 1 μM; the solvents were completely evaporated under a stream of oxygen-free dry nitrogen and liposomes were prepared as described above.

The excitation (8 nm slit) and emission (8 nm slit) wavelengths were 348 nm and 428 nm, respectively for DPH, and 362 nm and 433 nm for DPH-PC. All fluorescence measurements were performed at a temperature of 20 ± 1°C using a Perkin-Elmer MPF 44A spectrofluorimeter. Polarizers were mounted on the excitation and emission sides of the sample cuvette and measurements of fluorescence intensity were made with the polarizers in parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) configurations, respectively. Values for the fluorescence anisotropy ratio, A [20,21] were calculated.

2.4. *Inhibition of liposomal and microsomal lipid peroxidation by tamoxifen and related compounds*

The addition of tamoxifen and related compounds to ox-brain phospholipid liposomes (prepared as described above), subsequent peroxidation by Fe(III)-ascorbate and measurement of the extent of peroxidation by the thiobarbituric acid (TBA) test were as described in previously for rat liver microsomes [15], with the exception that the reactions were terminated by the addition of 100 μl of BHT (butylated hydroxytoluene: 0.2% w/v) dissolved in ethanol to suppress further peroxidation during the heating stage of the TBA reaction. Graphs plotted from these results were used to obtain the IC_{25} values for inhibition of liposomal and microsomal lipid peroxidation used for purposes of comparison (in Table I) and correlation with those for decreased membrane fluidity.

3. RESULTS

Tamoxifen, 4-hydroxytamoxifen, *N*-desmethyltamoxifen, *cis*-tamoxifen, 17 β-oestradiol and cholesterol were each added to the ox-brain phospholipid liposomes in ethanol (ethanol itself at the concentration used had no effect on membrane fluidity) to give final concentrations in the range 0–45 μM at a final concentration of phospholipid of 0.3 mM. Fig. 2 shows that these compounds all decreased membrane fluidity (increased the fluorescence anisotropy ratio, *A*) in a concentration-dependent manner and that 4-hydroxytamoxifen and 17 β-oestradiol were more effective at decreasing membrane fluidity than tamoxifen, whereas *N*- desmethyltamoxifen and *cis*-tamoxifen were less effective. In addition, cholesterol itself was found to display only a poor ability to decrease membrane fluidity; at 45 μM an increase in *A* of only 7 ± 4.1% (S.E.M., $n = 3$) was found with this approach. When DPH-PC (1 μM) was used as the probe tamoxifen and 4-hydroxytamoxifen (both at a concentration of 45 μM) increased *A* by 22 ± 2.4% (S.E.M., $n = 3$) and 36 ± 4.1% (S.E.M., $n = 3$), respectively compared to 25 ± 2.8% (S.E.M., $n = 3$) and 34 ± 4.9% (S.E.M., $n = 3$) when DPH (1 μM) was the probe used (see Fig. 2). The differences in the potency of these compounds to decrease membrane fluidity (increase the fluorescence anisotropy ratio, *A*) are reflected in their IC_{25} values (see Table I: IC_{25} rather than IC_{50} values were calculated because none of the compounds, over the concentration range used, increased *A* by as much as 50%).

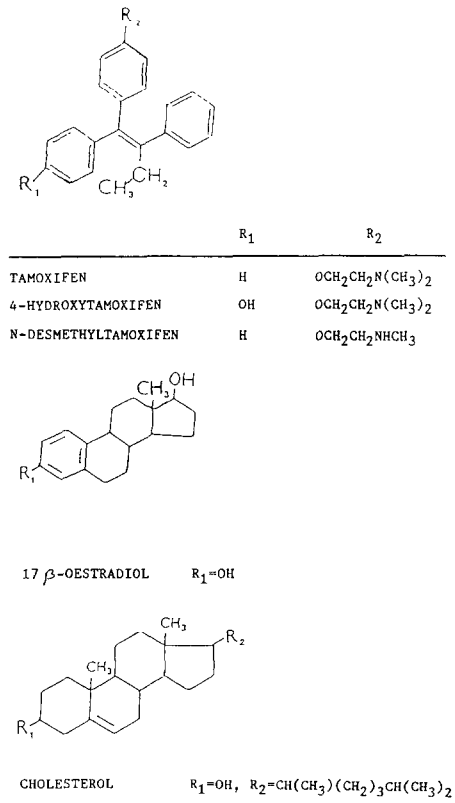

Fig. 1 Structures of tamoxifen, related compounds and 17 β-oestradiol and cholesterol. Structural similarity of the triphenylethylene compounds to cholesterol and 17 β-oestradiol.

Fig. 2 and Table I show that the order of effectiveness of these compounds in decreasing membrane fluidity is 4-hydroxytamoxifen > 17 β-oestradiol > tamoxifen > *cis*-tamoxifen > *N*-desmethyltamoxifen > cholesterol. Fig. 3 shows that a good positive correlation was found between the IC_{25} values for decreased membrane fluidity and the IC_{25} values for the inhibition of liposomal and microsomal lipid peroxidation (correlation coefficient, $r = 0.99$, $P < 0.001$, in both cases).

4. DISCUSSION

Our results show that tamoxifen, *cis*-tamoxifen, 4-hydroxytamoxifen, *N*-desmethyltamoxifen, 17 β-oestradiol and cholesterol all decreased membrane fluidity in ox-brain phospholipid liposomes and that their order of effectiveness was 4-hydroxytamoxifen > 17 β-oestradiol > tamoxifen > *cis*-tamoxifen > *N*-desmethyltamox-

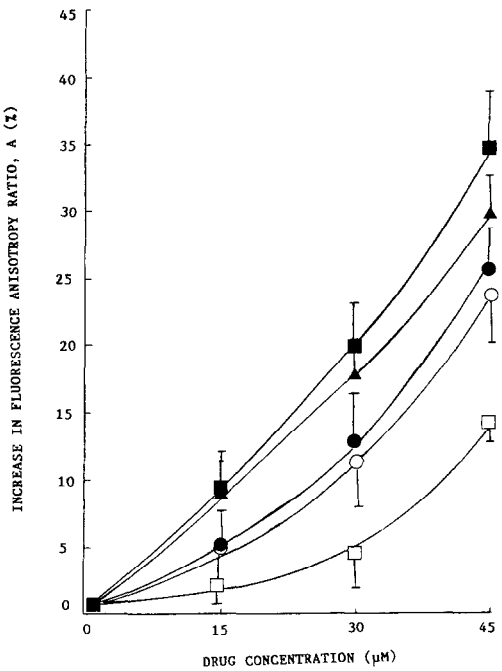

Fig. 2. Concentration-dependent increase in fluorescence anisotropy ratios, A, measured using DPH (1 μM) as the probe, by (●) tamoxifen, (■) 4-hydroxytamoxifen, (○) *cis*-tamoxifen, (□) *N*-desmethyltamoxifen, or (▲) 17 β-oestradiol, all in the range 0–45 μM. Results are shown as mean ± S.E.M., $n = 3$

ifen > cholesterol (see Fig. 2 and Table I). This order parallels their antioxidant ability as inhibitors of liposomal and microsomal lipid peroxidation and is reflected in the good positive correlation coefficients ($r = 0.99$, $P < 0.001$, in both cases: see Fig. 3) between the two parameters.

The relatively poor ability of cholesterol to decrease membrane fluidity reflected also in its weak antioxidant activity may reflect the use of a lipid substrate rich in polyunsaturated fatty acid side-chains. The well-recognized ability of cholesterol to stabilize membranes is thought to occur via an interaction between the rigid hydrophobic ring structure of cholesterol and the saturated, mono-unsaturated, and to a much lesser extent the poly-unsaturated, fatty acid side-chains of phospholipids, which decreases membrane fluidity [22]. Examination of the chemical structure of tamoxifen (see Fig. 1) indicates that it is unlikely to act chemically as a chain-breaking antioxidant (no potentially donatable hydrogen atom, such as a phenolic hydrogen) and indeed neither it nor its 4-hydroxy metabolite show the kinetics characteristic of such an antioxidant mechanism [16]. The present results clearly establish a membrane stabilizing action against lipid peroxidation via decreased membrane fluidity by tamoxifen and related compounds, which share a structural similarity with cholesterol [23] as the mechanism of their antioxidant action.

The close agreement between the results for tamoxifen and 4-hydroxytamoxifen with the two probes, DPH and DPH-PC, reflect a consistency of the effects on membrane fluidity in two different domains within the membrane lipid bilayer reported by the respective probes. Moreover, these findings are in agreement with a previous study using DPH as the fluorescent probe [19] that reported that although in the oestrogen receptor-positive MCF-7 cell-line tamoxifen (at 1 μM and 10 μM) decreased membrane fluidity more effectively than 17 β-oestradiol at the same concentrations, in the oestrogen receptor-negative MDA-MD-436 cell-line 17 β- oestradiol was more effective than tamoxifen (as found in our liposomal study). Perhaps in the oestrogen-receptor positive cells a decreased amount of 17 β-oestradiol was present in the membrane lipid bilayer because of its preferential binding to the oestrogen receptor.

It is noteworthy that some metastatic cancer cells have been reported to show increased membrane fluidity [24] that could thus be counteracted by this ability of tamoxifen to decrease membrane fluidity. This effect of tamoxifen could also contribute to its anticancer action by inhibiting the action of membrane enzymes, receptors and channels such as adenylate cyclase, the activity of which has been shown to decrease with decreased membrane fluidity [25]. This could lead to decreased levels of cellular cAMP levels thereby causing inhibition of the growth of some cancer cells [26].

We have recently reported that tamoxifen and in par-

Table I

IC_{25} values for the inhibition of microsomal and liposomal lipid peroxidation and for the increase in the fluorescence anisotropy ratio, A, indicating decreased membrane fluidity by tamoxifen and related compounds

Compound	IC_{25}		
	Membrane fluidity (μM)	Lipid peroxidation	
		Microsomes (μM)**	Liposomes (μM)***
Tamoxifen	45	8	7
4-Hydroxytamoxifen	36	2	2
17 β-Oestradiol	40	5	4
cis-Tamoxifen	48	11	9
N-Desmethyltamoxifen	53	14	11
Cholesterol	NR	NR	NR

*Values are deduced from the graphs shown in Fig. 2 in which each point represents the mean ± S.E.M. of three separate experiments.
**Values are deduced from the graphs shown in [15].
***Values are deduced from graphs, not shown (H. Wiseman, unpublished data).

NR, not reached. IC_{25} was the concentration (in μM) of each compound required to either inhibit microsomal or liposomal lipid peroxidation or to decrease membrane fluidity (increase the fluorescence anisotropy ratio, A) by 25%.

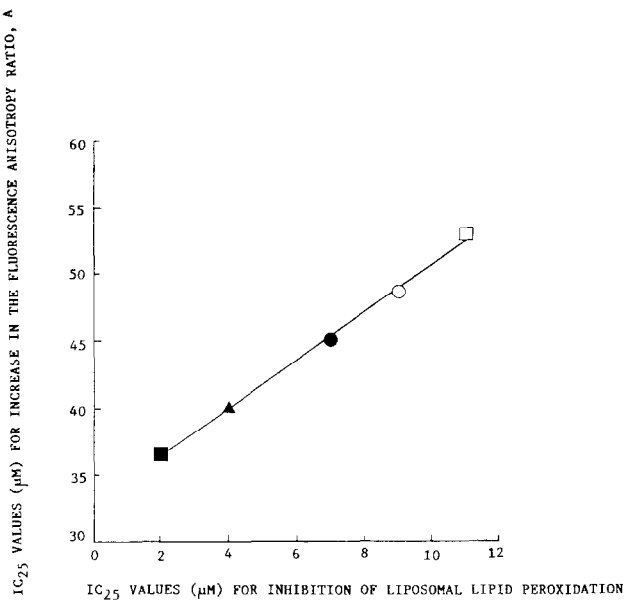

Fig. 3. Correlation between the IC_{25} values for decreased membrane fluidity (increase in the fluorescence anisotropy ratio, *A*) and inhibition of liposomal lipid peroxidation by (●) tamoxifen, (■) 4-hydroxytamoxifen, (○) *cis*-tamoxifen, (□) *N*-desmethyltamoxifen and (▲) 17 β-oestradiol.

ticular 4-hydroxytamoxifen protect human LDL against oxidative damage [18] and that this may contribute to the observed cardiovascular benefits of tamoxifen therapy [27–30]. We proposed that these compounds may stabilize LDL against peroxidation by interaction between their hydrophobic rings and the polyunsaturated residues of the phospholipid layer of LDL. This is supported by the decreased membrane fluidity arising from similar interactions in liposomal membranes, measured in the present study.

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