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Mechanism of inhibition of lipid peroxidation by tamoxifen and 4-hydroxytamoxifen introduced into liposomes

Similarity to cholesterol and ergosterol

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The anticancer drug tamoxifen when introduced into phospholipid liposomes during their preparation inhibited Fe(III)-ascorbate induced lipid peroxidation to a greater extent than similarly introduced cholesterol. Ergosterol was equipotent with tamoxifen, but much less effective than 4-hydroxytamoxifen. Possible mechanisms underlying these effects are discussed in relation to structural mimicry of the sterols by these triphenylethylene drugs as membrane stabilizers against lipid peroxidation.

Tamoxifen; 4-Hydroxytamoxifen; Cholesterol; Lipid peroxidation; Liposome; Membrane stabilization

1. INTRODUCTION

Tamoxifen is an antioestrogen drug (one of a series of triphenylethylene compounds), which is widely used in the chemotherapy of breast cancer [1]. 4-Hydroxytamoxifen is produced by the metabolism of tamoxifen *via* cytochrome P-450 action in humans [2-6]. We have recently reported that tamoxifen and 4-hydroxytamoxifen are antioxidants in that they are potent inhibitors of lipid peroxidation in microsomal and preformed liposomal membrane systems when added in the carrier solvent ethanol [7]. However, the chemical structure of tamoxifen (Fig. 1) indicates that it is unlikely to act as a chain-breaking antioxidant because the compound contains no easily donatable hydrogen atom. Furthermore, chain-breaking antioxidants usually introduce a lag period into the peroxidation time-course and this was not observed for tamoxifen or 4-hydroxytamoxifen, even though the latter compound possesses a potentially donatable hydrogen atom [7].

Cholesterol when introduced into phospholipid liposomes [8-11] during their preparation has been reported to inhibit membrane lipid peroxidation apparently by a membrane stabilization effect [12]. Inhibition of lipid peroxidation by 17β-oestradiol introduced into liposomes has also been reported [13]. Using this same experimental procedure we now report that not only cholesterol and 17β-oestradiol but also tamoxifen, 4-hydroxytamoxifen and ergosterol, which replaces cholesterol in plant and fungal membranes, can be individually introduced into ox-brain phospholipid liposomes, where each decreases the susceptibility of the liposomes to lipid peroxidation by a characteristic amount.

We therefore suggest that tamoxifen and 4-hydroxytamoxifen may act as structural mimics of cholesterol and ergosterol (see Fig. 1) and thus may exert their reported antioxidant action [7] by a similar membrane stabilizing effect, which we observe as an inhibition of lipid peroxidation.

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2. MATERIALS AND METHODS

2.1. *Preparation of Liposomes*

Liposomes were prepared with and without the introduction of the compounds shown in Fig. 1, as described previously for cholesterol [12]. Ox-brain phospholipid was dissolved in chloroform to give a final concentration of 5 mg/ml, and a 0.8 ml aliquot was taken and mixed with an equal volume of cholesterol, ergosterol, tamoxifen or 4-hydroxytamoxifen taken from stock solutions in chloroform or, in the case of 17β-oestradiol in methanol-chloroform 1:2 (v/v), to provide the concentration range required for each of these compounds. The organic solvents were then completely evaporated in a stream of nitrogen. The residue was suspended in 0.8 ml of phosphate buffered saline (140 mM NaCl, 2.7 mM KCl, 16 mM Na_2HPO_4 and 2.9 mM KH_2PO_4) at pH 7.4 and after the addition of small glass beads was sonicated in a water-bath sonicator (containing ice) and vortexed. The resulting liposomes were left to stand in sealed nitrogen-flushed bottles at 4°C for 1 h prior to use.

Ox-brain phospholipid, cholesterol, ergosterol, tamoxifen and 17β-oestradiol were purchased from Sigma. 4-Hydroxytamoxifen was kindly supplied by ICI Pharmaceuticals plc. Purity was at least 99%.

2.2. *Peroxidation of Liposomes*

Reaction mixtures (final volume 1.0 ml) contained liposomes (0.5 mg in 0.1 ml of phosphate buffered saline), phosphate buffered saline

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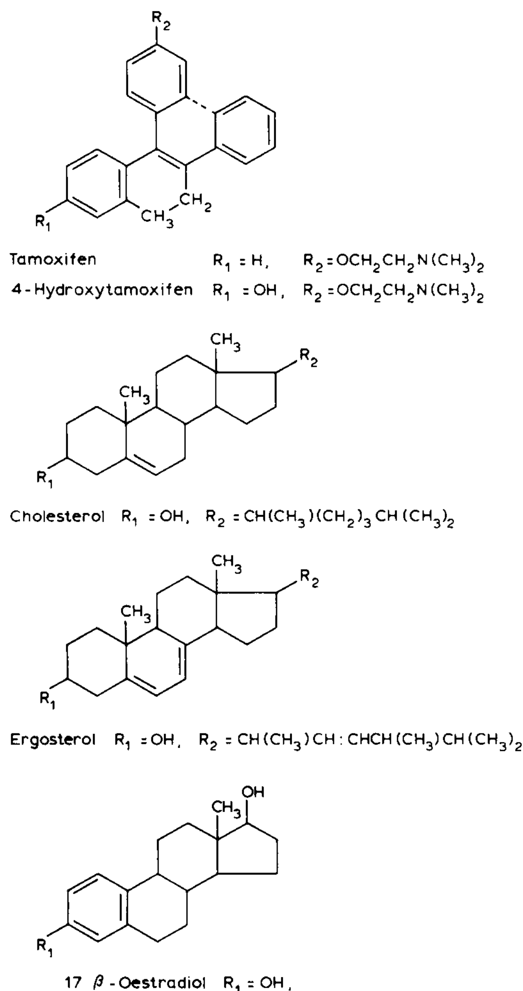

Fig. 1. Structural mimicry by tamoxifen and 4-hydroxytamoxifen of the sterols cholesterol, ergosterol and 17β-oestradiol.

pH 7.4 (0.5 ml) and water (0.2 ml). Peroxidation was started by adding freshly prepared aqueous solutions of $FeCl_3$ (0.1 ml) and ascorbate (0.1 ml) to give a final concentration of 100 μM of each. Incubations were carried out at 37°C for 20 min. The extent of lipid peroxidation was measured by the formation of thiobarbituric acid-reactive substances (TBARS) as described in [14]. HCl (0.5 ml of 25% v/v) was added to each reaction mixture, followed by 0.5 ml of thiobarbituric acid solution (1% w/v in 50 mM sodium hydroxide). After heating at 80°C for 30 min the chromagen was extracted with 2 ml of butan-1-ol and the A_{532} of the upper (organic) layer was measured.

3. RESULTS

Liposomes formed from ox-brain phospholipids act as a model membrane (lipid bilayer) system, which in the presence of Fe(III) and ascorbate at pH 7.4 is rapidly peroxidized [11] as measured by the TBA test. The compound/phospholipid ratios were expressed in molar form using an average molecular weight for the phospholipid based on the reported phospholipid composition [12]. The data in Fig. 2, expressed therefore as molar ratios (phospholipid at 5 mg/ml), show that tamoxifen, introduced into liposomes over the concentration range 0.01–8 mg/ml, inhibited lipid peroxidation to approximately the same extent as did ergosterol (0.01–4 mg/ml). Cholesterol (0.01–8 mg/ml) was a much less potent inhibitor than tamoxifen, whereas 4-hydroxytamoxifen (0.005–2 mg/ml) and 17β-oestradiol (0.005–4 mg/ml) were approximately equipotent. The latter two compounds were both more potent inhibitors of peroxidation than tamoxifen (see Fig. 2). This inhibitory ability can be expressed as the IC_{50} values derived for the compounds tested (see Table I). The results show that the concentration (or molar ratio) of tamoxifen or ergosterol that had to be introduced into the membrane to achieve 50% inhibition of lipid peroxidation was approximately 15-fold less than for cholesterol. Moreover the IC_{50} values of 4-hydroxytamoxifen and 17β-oestradiol were approximately 200-fold less than for cholesterol.

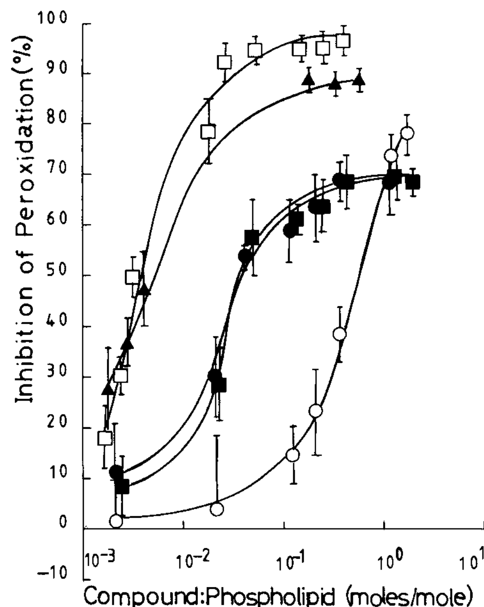

Fig. 2. Inhibition of lipid peroxidation by the compounds, (○) cholesterol, (■) tamoxifen, (•) ergosterol, (□) 4-hydroxytamoxifen and (▲) 17β-oestradiol each introduced into liposomes during their preparation. Results are mean ± SD, n = 6–9

Table I

IC_{50} values for the inhibition of lipid peroxidation by cholesterol, tamoxifen, ergosterol, 4-hydroxytamoxifen and 17β-oestradiol, each introduced into phospholipid liposomes during their preparation

Compound	IC_{50}		
	(mg/ml)	(mM)	mol/mol phospholipid
Cholesterol	2.8	7.2	7.5×10^{-1}
Tamoxifen	0.19	0.50	5.3×10^{-2}
Ergosterol	0.21	0.53	5.4×10^{-2}
4-Hydroxytamoxifen	0.014	0.036	3.8×10^{-3}
17β-Oestradiol	0.011	0.040	4.4×10^{-3}

Values are deduced from the graphs shown in Fig. 2, in which each point represents the mean ± SD of 6–9 separate assays.

For maximum stabilization against lipid peroxidation, cholesterol had to be introduced into the membrane at a molar ratio of approximately 1.0 (see Fig. 2), which is in agreement with earlier work [12]. Maximum stabilization against lipid peroxidation of membranes containing introduced tamoxifen or ergosterol was observed at the lower molar ratio of approximately 0.5. However, introduced 4-hydroxytamoxifen or 17β-oestradiol exhibited maximum stabilization against peroxidation at the greatly lower molar ratio of 0.05. Nevertheless, all of these compounds still retained this maximum inhibition of lipid peroxidation (*via* membrane stabilization) at a molar ratio of 1.0 in agreement with the original findings for cholesterol itself [12].

4. DISCUSSION

The stabilization by cholesterol (and its derivatives [15]) of membranes against lipid peroxidation is thought to occur *via* an interaction between the hydrophobic rings of cholesterol and the saturated, monounsaturated and polyunsaturated residues of phospholipid fatty acids [12,16], which reduces the fluidity of the membrane bilayer [8,9]. We have now shown that ergosterol, tamoxifen, 4-hydroxytamoxifen and 17β-oestradiol also exert membrane stabilizing effects against lipid peroxidation, possibly by a similar mechanism to that for cholesterol. Molecular models of the chemical structure of tamoxifen and 4-hydroxytamoxifen suggest that these compounds can take up conformations that structurally mimic the conformations of cholesterol, ergosterol and possibly 17β-oestradiol.

The molar ratio required for maximum stabilization against lipid peroxidation is important in identifying the structural relationship between the added compound and the membrane phospholipid of the liposome. The molar ratio of cholesterol to phospholipid in most animal membranes is approximately 1.0 and this ratio in ox-brain phospholipid liposomes offers maximum inhibition against lipid peroxidation [12] and thus maximum membrane stabilization, a result confirmed in our study. For ergosterol and tamoxifen, maximum membrane stabilization was reached at a molar ratio of approximately 0.5 (2-fold less than for cholesterol) and for 4-hydroxytamoxifen and 17β-oestradiol only 0.05 (20-fold less than for cholesterol). However, the IC_{50} values for these compounds were found to be more divergent from the cholesterol value and were approximately 15-fold less for tamoxifen and ergosterol and approximately 200-fold less for 4-hydroxytamoxifen and 17β-oestradiol than for cholesterol. The IC_{50} values indicate the relationships at the inflexion points of the curves in Fig. 2, rather than those near the maximum of these curves. It is the latter data that best reflects the similarity between cholesterol and the other compounds introduced into the membrane that mimic the structural role of cholesterol, in their ability to achieve maximum membrane stabilization against lipid peroxidation. The increased efficiency of these compounds as shown by the lower molar ratios in relation to cholesterol, may result from their interaction across more than one lipid bilayer in the multilayer liposome structure.

The potent inhibition of lipid peroxidation by 4-hydroxytamoxifen may reflect the presence of a phenolic hydroxyl group in this compound. Indeed the acetyl derivative of cholesterol is less effective than cholesterol as an inhibitor of lipid peroxidation suggesting that the hydroxyl group may be important for the correct orientation of cholesterol in the phospholipid bilayer [12].

Tamoxifen, 4-hydroxytamoxifen and 17β-oestradiol also show an inhibitory effect on lipid peroxidation when added in ethanol externally to preformed liposomes [7], whereas cholesterol and ergosterol under similar conditions have no activity (H.W., unpublished data). This difference may reflect the ease with which the drugs and hormones used are able to enter liposomes and also natural membranes [7].

It is particularly interesting that introduced ergosterol is a more powerful stabilizer against lipid peroxidation in the liposomal model membrane than introduced cholesterol. This may suggest an antioxidant action against lipid peroxidation that has been retained by plant and fungal cells during evolution.

Tamoxifen and in particular 4-hydroxytamoxifen, which has a higher affinity for the oestrogen receptor than tamoxifen, can also be considered to be structural mimics of 17β-oestradiol on the basis of their overall conformation (see Fig. 1). It is thought that it is the composition and positioning of the side chains of tamoxifen and 4-hydroxytamoxifen that give these compounds anti-oestrogenic rather than oestrogenic activity in breast cancer cells, which is mediated through the oestrogen receptor [17]. Similarly, membrane stabilization effects would be expected to reflect structural mimicry of 17β-oestradiol by tamoxifen and in particular 4-hydroxytamoxifen.

The ability of tamoxifen and 4-hydroxytamoxifen to stabilize membranes against lipid peroxidation beyond that achieved by cholesterol may reflect a further reduction in membrane fluidity, which is thought to be responsible for this stabilizing ability of cholesterol [8,9,12,16]. In the presence of these drugs therefore cell division may be slower as a result of reduced membrane fluidity, in agreement with the growth inhibitory properties of tamoxifen and 4-hydroxytamoxifen on oestrogen-dependent and oestrogen-independent breast tumours in vivo [5,18] and tumour cell lines in vitro [5,19,20]. Similarly in the oestrogen-independent yeast tumour cell model growth system [21–23] an explanation of the potent inhibition of growth by tamoxifen [22], which may be useful in the treatment of systemic

Candida infections (see [23]), may reside in the deleterious further reduction of membrane fluidity beyond that achieved even by ergosterol.

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