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TAMOXIFEN INHIBITS LIPID PEROXIDATION IN CARDIAC MICROSOMES

COMPARISON WITH LIVER MICROSOMES AND POTENTIAL RELEVANCE TO THE CARDIOVASCULAR BENEFITS ASSOCIATED WITH CANCER PREVENTION AND TREATMENT BY TAMOXIFEN

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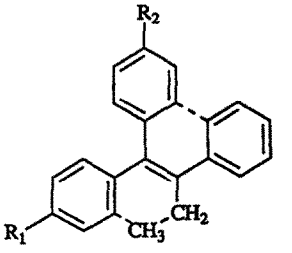
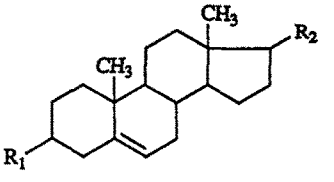
Abstract—Tamoxifen and 4-hydroxytamoxifen were both good inhibitors of iron-dependent lipid peroxidation in rat cardiac microsomes. Tamoxifen was also a good inhibitor of lipid peroxidation in liposomes prepared from the phospholipid obtained from rat liver microsomes. In a modified rat liver microsomal system containing a sufficiently low amount of peroxidizable phospholipid to make it comparable with the rat cardiac microsomal system, tamoxifen and 4-hydroxytamoxifen were of similar effectiveness as in the cardiac system. Tamoxifen is known to lower serum cholesterol levels, and the findings reported here indicate that the drug might also protect heart cell membranes against peroxidative damage. Potential cardioprotective and antiatherosclerotic benefits of tamoxifen are discussed in relation to the drug's use in cancer prevention and treatment.

Tamoxifen is now used in the prevention, as well as the treatment of, breast cancer [1–9]. It is also starting to be used to treat liver [10] and brain cancers [11]. Recent findings on the ability of tamoxifen to cause the production of transforming growth factor β in normal cells, which inhibits the growth of adjacent cancer cells [12], suggest that tamoxifen might find general application in the prevention and treatment of cancer. Cardiovascular benefits have also been reported in studies on the treatment of breast cancer in women with tamoxifen [13–16]. The incidence of fatal myocardial infarction was significantly less in the tamoxifen-treated group [13]. Furthermore, tamoxifen lowers serum cholesterol levels significantly [14, 15], and favourably affects lipid and lipoprotein profiles [15, 16]. In addition, tamoxifen decreases fibrinogen levels and this is associated with a decreased risk of arterial thrombosis [17]. Both tamoxifen and its metabolite 4-hydroxytamoxifen inhibit lipid peroxidation in rat liver microsomes and ox-brain phospholipid liposomes [18–22], and the role of lipid peroxidation in cardiovascular injury [23, 24] and the development of atherosclerosis is well documented [25–29].

We therefore investigated the ability of tamoxifen and 4-hydroxytamoxifen (structures shown in Fig. 1) to inhibit lipid peroxidation in a relevant rat cardiac microsomal system, as this system cannot be assumed to display the same features as the rat liver microsomal system and moreover is of special relevance to the proposed cardioprotective action of tamoxifen. Similar studies were carried out in a modified rat liver microsomal preparation that was comparable with the rat cardiac microsomal system. In addition, a liposomal system prepared from phospholipid extracted from rat liver microsomes, as the yield of rat cardiac microsomes was too low



Tamoxifen $R_1 = H$, $R_2 = OCH_2CH_2N(CH_3)_2$
4-Hydroxytamoxifen $R_1 = OH$, $R_2 = OCH_2CH_2N(CH_3)_2$



Cholesterol $R_1 = OH$, $R_2 = CH(CH_3)(CH_2)_3CH(CH_3)_2$

Fig. 1. Structural similarity of tamoxifen and 4-hydroxytamoxifen to the membrane sterol cholesterol.

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Table 1. Effect of tamoxifen and 4-hydroxytamoxifen on lipid peroxidation in rat cardiac microsomes, rat liver microsomes and rat liver phospholipid liposomes

Addition to reaction mixture	Extent of peroxidation A_{532}	Inhibition of peroxidation (%)
Cardiac microsomes		
None (control)	0.01	—
Fe(III)-ascorbate	0.25	—
30 μM Tamoxifen	0.033	90
30 μM 4-Hydroxytamoxifen	0.015	98
Liver microsomes		
None (control)	0.01	—
Fe(III)-ascorbate	0.27	—
30 μM Tamoxifen	0.02	96
30 μM 4-Hydroxytamoxifen	0.02	96
Liver phospholipid liposomes		
None (control)	0.01	—
Fe(III)-ascorbate	0.22	—
30 μM Tamoxifen	0.073	70

Results are the means of duplicate determinations taken from a representative experiment.
Percentage inhibition due to the triphenylethylene compounds is calculated after deducting the "control" level of peroxidation from the relevant values.

to make this practical, was used as another test system.

MATERIALS AND METHODS

Tamoxifen was from the Sigma Chemical Co. (Poole, U.K.). 4-Hydroxytamoxifen was kindly donated by ICI Pharmaceuticals (Macclesfield, U.K.). All other reagents were of the highest quality from the Sigma Chemical Co. or from BDH Ltd (Dagenham, U.K.).

Preparation of rat liver and cardiac microsomes. Rat liver microsomes were prepared from the livers of adult male rats by standard differential-centrifugation techniques as described earlier [30]; rat cardiac microsomes were prepared from the hearts of these rats by the same method despite the difficulties encountered in the homogenization of heart because of its tough muscular properties.

Extraction of phospholipid from rat liver microsomes. A lipid fraction was obtained from rat liver microsomes by extraction with chloroform–methanol [31]. The microsomes were mixed with an equal volume of chloroform–methanol 2:1 (v/v) and then centrifuged at speed number 7 for 10 min in a bench centrifuge. The lower organic layer was removed and 1 mL of water was added. The tubes were vortexed and centrifuged as before and the lower organic solvent layer was collected. This purification procedure was repeated twice. The organic solvent was evaporated under a nitrogen stream and the residue redissolved in chloroform and stored at −70°.

The lipid fraction was then separated into phospholipid and cholesterol components by silicic acid column chromatography [32]. The rat liver lipid fraction dissolved in chloroform was loaded on to the column (prewashed with petroleum ether). The column was washed several times with chloroform and the cholesterol present in the lipid fraction was collected (and discarded). The phospholipid was eluted with methanol and the column was again washed several times. The methanol was evaporated and the phospholipid residue was redissolved in chloroform. The residues dissolved in chloroform were stored at −70°.

Preparation of rat liver phospholipid liposomes. A 0.8 mL aliquot of rat liver phospholipid, derived from rat liver microsomes containing 11 mg of microsomal protein, dissolved in chloroform was evaporated in a stream of nitrogen. Liposomes were prepared from the residue by addition of 0.8 mL of phosphate buffered saline (140 mM NaCl, 2.7 mM KCl, 16 mM Na_2HPO_4, 2.9 mM KH_2PO_4) at pH 7.4, followed by sonication in a bath type sonicator at 0° and vortexing, both in the presence of small glass beads (2.5–3.5 mm in diameter). The resulting liposomes were allowed to stand in sealed nitrogen-flushed bottles at 4° for 1 hr before use [33, 34].

Peroxidation of rat liver phospholipid liposomes. The reaction mixture contained in a final volume of 1 mL, liposomes (0.1 mL), phosphate-buffered saline pH 7.4 (0.5 mL), water (0.2 mL) and 5 μL of ethanol or drug dissolved in ethanol. Freshly prepared aqueous solutions of $FeCl_3$ (0.1 mL) and ascorbate (0.1 mL) were added to the reaction mixture to give a final concentration of 100 μM of each. Ascorbate was added to start the peroxidation reaction and incubations were carried out at 37° for 20 min (unless stated otherwise).

Peroxidation of rat liver and rat cardiac microsomes. The reaction mixture contained in a final volume of 1 mL, rat liver microsomes (82 μg of microsomal protein) or rat cardiac microsomes (250 μg of microsomal protein), 10 mM KH_2PO_4–KOH buffer pH 7.4 (0.5 mL), water and 5 μL of ethanol or drug dissolved in ethanol. Ascorbate and $FeCl_3$ (0.1 mL) were added to the reaction mixture to give a final concentration of both of 100 μM. Although greater levels of peroxidation can be achieved in cardiac microsomes by using concentrations of $FeCl_3$ of up

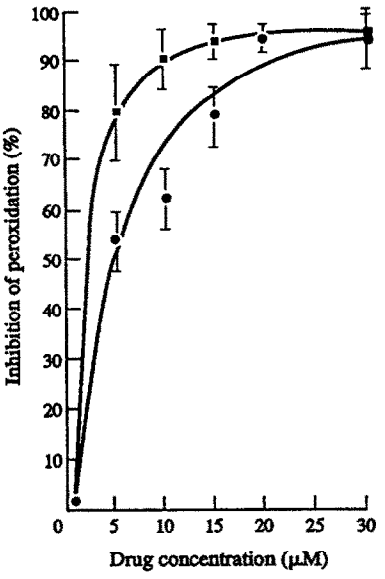

Fig. 2. Concentration-dependent inhibition of iron-dependent lipid peroxidation in rat cardiac microsomes. (●) Tamoxifen, (■) 4-hydroxytamoxifen. Results are mean ± SD, N = three to four tests.

to 2 mM, in this study 100 μM $FeCl_3$ was used to retain the standard parameters used with the liver microsomes. Ascorbate was added to start the peroxidation reaction and reaction mixtures were incubated as above.

Measurement of lipid peroxidation by the thiobarbituric acid (TBA) test. The amount of lipid peroxidation was measured by the formation of thiobarbituric acid-reactive substances (TBARS). The peroxidation reactions were stopped by the addition of HCl [0.5 mL of 25% (v/v)] followed by 0.5 mL of thiobarbituric acid solution [1% (w/v) in 50 mM (NaOH)]. After heating at 80° for 30 min the pink chromogen was extracted with an equal volume (2 mL) of butan-1-ol and the A_{532} of the upper (organic) layer was measured [30].

RESULTS

Inhibition of iron-dependent lipid peroxidation by tamoxifen and 4-hydroxytamoxifen in rat cardiac microsomes

Tamoxifen and 4-hydroxytamoxifen were added to the system dissolved in ethanol (ethanol itself had no effect on peroxidation). Table 1 shows experimental data for both of these triphenylethylene drugs at 30 μM. Tamoxifen and 4-hydroxytamoxifen were both good inhibitors of iron-dependent lipid peroxidation in the rat cardiac microsomal system. Figure 2 shows that 4-hydroxytamoxifen was more effective than tamoxifen as an inhibitor of lipid peroxidation in this system. This difference in potency is reflected in their IC_{50} values (see Table 2). Neither of these drugs interfered with the TBA test: no inhibition was observed when the compounds were added to peroxidizing microsomes at the same time as the TBA reagents instead of at the beginning of the incubation.

Inhibition of iron-dependent lipid peroxidation by tamoxifen and 4-hydroxytamoxifen in rat liver microsomes

Lipid peroxidation occurs rapidly when rat liver microsomes are incubated at pH 7.4 with Fe(III)-ascorbate [35]. Tamoxifen and 4-hydroxytamoxifen were both of similar effectiveness in this system (see Fig. 3) and this is also reflected in their IC_{50} values (see Table 2). Experimental data for tamoxifen and 4-hydroxytamoxifen are shown in Table 1.

Inhibition of iron-dependent lipid peroxidation by tamoxifen in rat liver phospholipid liposomes

Liposomes formed from phospholipids (including those from rat liver) act as a lipid bilayer system, which in the presence of Fe(III)-ascorbate at pH 7.4 is rapidly peroxidized [36] as measured by the TBA assay. Tamoxifen inhibited lipid peroxidation in this system (see Fig. 3) and representative experimental data at 30 μM are shown in Table 1. However, tamoxifen was a less effective inhibitor of lipid peroxidation in this system in comparison with the two microsomal systems (see Table 2).

DISCUSSION

Tamoxifen and its metabolite 4-hydroxytamoxifen inhibit lipid peroxidation in rat cardiac microsomes, although the IC_{50} value of 4-hydroxytamoxifen is 2-fold lower than that for tamoxifen (see Table 2). 4-Hydroxytamoxifen is also a more effective inhibitor of lipid peroxidation than tamoxifen in rat liver microsomes and ox brain phospholipid liposomes (4-fold and 7-fold lower IC_{50} values [18–20]). Comparison of the results for tamoxifen and 4-hydroxytamoxifen in the rat cardiac microsomal system with our previous work on these compounds in a standard rat liver microsomal system indicates that tamoxifen, and to a much lesser extent 4-hydroxytamoxifen, are more potent inhibitors of lipid peroxidation in the cardiac microsomal system (see Table 2). However, the positive control (i.e. microsomes plus Fe(III)-ascorbate) for the standard rat liver microsomal system had a much higher extent of peroxidation (A_{532} around 0.8) than for the rat cardiac microsomal system (A_{532} around 0.2), when 0.25 mg of microsomal protein was used for each. This indicates either an inherently lower content of peroxidizable phospholipid fatty acid side-chains in the cardiac microsomal system or possibly a loss of these components during preparation. Therefore, in order to make a more valid comparison of the protective effects of tamoxifen and its 4-hydroxy metabolite on the membranes of the two different tissues we used a modified rat liver microsomal system, which had a more comparable extent of peroxidation in terms of the positive control (see Table 1) against which all the inhibition levels are measured. In our modified rat liver microsomal system, tamoxifen was found to be just as effective as in the rat cardiac microsomal

Table 2. IC_{50} values for the inhibition of microsomal and liposomal lipid peroxidation by tamoxifen and 4-hydroxytamoxifen

System	IC_{50} values	
	Tamoxifen (μM)	4-Hydroxytamoxifen (μM)
Rat cardiac microsomes	5	2.5
Comparable rat liver microsomes	3.8	2.5
Rat liver phospholipid liposomes	9.4	NT
Standard rat liver microsomes	11*	3*
Ox brain phospholipid liposomes	63*	9*

NT, not tested.
* Data quoted from Ref. 20.
Values are deduced from the graphs shown in Figs 2 and 3 in which each point represents the mean ± SD of three to four separate assays.

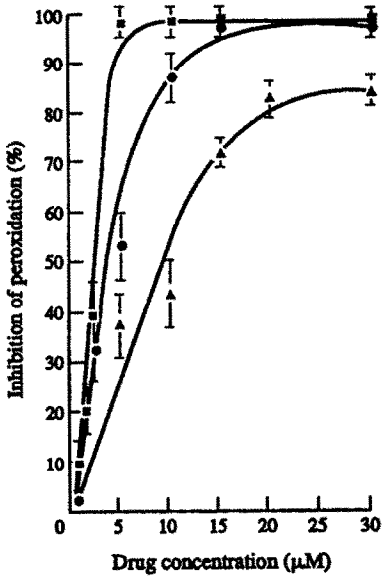

Fig. 3. Concentration-dependent inhibition of iron-dependent lipid peroxidation in rat liver microsomes. (●) Tamoxifen, (■) 4-hydroxytamoxifen and in rat liver phospholipid liposomes (▲) tamoxifen. Results are mean ± SD, N = three to four tests.

system. Caution is necessary, therefore, in making comparisons of the inhibitory effects of drugs on lipid peroxidation in microsomes prepared from tissues with different peroxidative abilities on a milligram of microsomal protein basis. When phospholipid was extracted from rat liver microsomes and liposomes prepared (again with a comparable positive control) tamoxifen was found to have an IC_{50} value of 9.4 μM showing it to be less effective than in the microsomal systems (see Table 2). Tamoxifen is known to be a weaker inhibitor of lipid peroxidation in liposomes compared to microsomes generally [18–20], and this may indicate an interaction of lipid and drug with membrane cholesterol or protein components found only in the microsomal systems. Furthermore, the membrane antioxidant action of tamoxifen (and 4-hydroxytamoxifen) is thought to be related to its structural similarity to cholesterol (see Fig. 1), which enables it to act like cholesterol in membranes, resulting in membrane stabilization against lipid peroxidation [19–22]. Additionally, the IC_{50} of tamoxifen in ox brain phospholipid liposomes has been found to be much greater (63 μM [20]) than in the standard rat liver microsomal system, and again this value is only directly comparable with data obtained from experiments where the A_{532} of the positive control is around 0.8, as with the standard rat liver microsomal system (see above).

The benefit of using tamoxifen to prevent cancer could be enhanced by the cardioprotective benefits of tamoxifen in both lowering serum cholesterol levels [14, 15] and, as found here, by actually protecting cardiac membranes against the damage caused by lipid peroxidation. This has important implications also for cancer treatments that use tamoxifen in combination with cardiotoxic anticancer drugs such as Adriamycin, which damage the heart by free radical mediated mechanisms [37, 38]. Protection by tamoxifen could therefore be an important factor in the safe use of such cardiotoxic drugs. The recent remarkable finding that tamoxifen causes cells surrounding cancer cells to produce transforming growth factor β, which has an antiproliferative effect on the cancer cells [12] means that tamoxifen might in the future be used more widely in the treatment of all cancers.

We are currently investigating the ability of tamoxifen to protect low density lipoproteins against oxidation, which is an important step in the development of atherosclerosis [26–29]. The prophylactic use of tamoxifen to prevent breast cancer in women could additionally provide long term cardiovascular benefits including prevention of heart disease.

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