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THE ANTIOXIDANT ACTION OF SYNTHETIC OESTROGENS INVOLVES DECREASED MEMBRANE FLUIDITY: RELEVANCE TO THEIR POTENTIAL USE AS ANTICANCER AND CARDIOPROTECTIVE AGENTS COMPARED TO TAMOXIFEN?

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The synthetic oestrogens diethylstilboestrol, hexoestrol and 17 α-ethynyloestradiol are known to be good antioxidants, and we now report that they decrease membrane fluidity, in ox-brain phospholipid liposomes. The order of effectiveness was diethylstilboestrol > hexoestrol > 17 α-ethynyloestradiol and a good positive correlation was demonstrated between decreased membrane fluidity and antioxidant ability (measured as inhibition of liposomal lipid peroxidation: correlation coefficient, $r = 0.99$). This ability of diethylstilboestrol, hexoestrol and 17 α-ethynyloestradiol to decrease membrane fluidity is suggested, therefore, to be the mechanism of their antioxidant action. The membrane-modulating antioxidant action of these synthetic oestrogens is compared to that of tamoxifen and their potential use as anticancer and cardioprotective agents is discussed.

KEY WORDS: Synthetic oestrogens, antioxidant, tamoxifen, anticancer action, cardioprotection, membrane fluidity.

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INTRODUCTION

The synthetic oestrogens, diethylstilboestrol, hexoestrol and 17 α-ethynyloestradiol (oestrogenic component of many contraceptive medications) have been shown to exert antioxidant effects *in vitro*, in that they inhibit metal-ion dependent lipid peroxidation in liposomal and microsomal membrane systems.[1] Tamoxifen, which is structurally related to diethylstilboestrol (and its derivative hexoestrol) is widely used in the treatment of breast cancer[2–4] and is being assessed in clinical trials as a prophylactic agent against this disease.[5–8] Tamoxifen is also being used to treat cancers of the liver,[9] and brain[10,11] and may have a general application in the prevention and treatment of cancer.[12] Tamoxifen, 4-hydroxytamoxifen[13,14] and 17 β-oestradiol[13–15] have also been reported to have antioxidant abilities *in vitro* including the ability to protect human LDL (low density lipoproteins) against oxidative damage.[14,16,17]

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It is of interest that diethylstilboestrol was the primary hormonal treatment for breast cancer in postmenopausal women[18] before tamoxifen became available and has been extensively used as an antiandrogenic agent for the treatment of prostatic carcinoma.[19] It is unfortunate, therefore, that synthetic oestrogens have been reported to induce tumours in laboratory animals at high doses:[20] diethylstilboestrol has been shown to promote tumorigenesis in rat mammary glands.[21] Furthermore, diethylstilboestrol is also a known trans-placental carcinogen in humans:[22] the incidence of genital tract cancer increases significantly in the daughters of women treated with large doses of diethylstilboestrol for the stabilization of pregnancies.[23] The exact mechanism of the carcinogenicity of synthetic oestrogens remains unclear: however, damage to DNA bases by free radicals arising from the metabolism and redox cycling of these compounds may be involved.[24-26]

Tamoxifen (and 4-hydroxytamoxifen) appears to act as a membrane antioxidant by stabilising the membrane against lipid peroxidation through a mechanism involving a decrease in membrane fluidity.[27] Furthermore, diethylstilboestrol, hexoestrol and 17 α-ethynyloestradiol do not appear to exert their antioxidant action via a chain-breaking mechanism,[1] even though hydroxyl groups with potentially donatable hydrogen atoms are present (structures shown in Figure 1). We have investigated, therefore, the ability of diethylstilboestrol, hexoestrol and 17 α-ethynyloestradiol to decrease membrane fluidity, in a model membrane system consisting of liposomes formed from ox-brain phospholipid and the results were correlated with the known membrane antioxidant abilities of these compounds in liposomes.[1]

Fluidity was measured using the fluorescent probe diphenylhexatriene (DPH).[28] Although DPH is considered to have the disadvantage of not occupying a single, well defined position in the bilayer compared to its phosphatidylcholine derivative, which becomes intercalated between the phospholipids in the bilayers,[28,29] an earlier study gave comparable results when these two probes were used to determine the ability of tamoxifen to alter membrane fluidity in liposomes:[27] thus DPH was used in the study reported here.

MATERIALS AND METHODS

Chemicals

Diethylstilboestrol, hexoestrol, 17 α-ethynyloestradiol, ox-brain phospholipids and DPH were from the Sigma Chemical Co. (Poole, UK). All other reagents were of the highest quality from the Sigma Chemical Co. (Poole, UK) or from BDH Ltd. (Dagenham, UK).

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.

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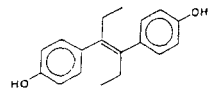
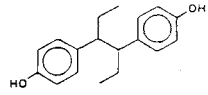
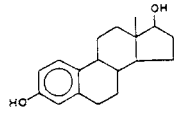


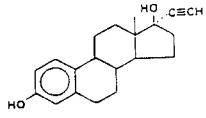
DIETHYLSTILBOESTROL



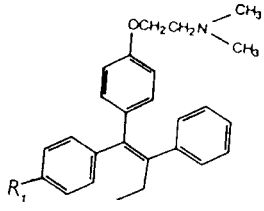
HEXOESTROL



17 β-OESTRADIOL



17 α-ETHYNYLOESTRADIOL



TAMOXIFEN R_1=H

4-HYDROXYTAMOXIFEN R_1=OH

FIGURE 1 Structures of diethylstilboestrol, hexoestrol, 17 α-ethynyloestradiol, tamoxifen, 4-hydroxytamoxifen and 17 β-oestradiol.

Fluorescence Measurements

Incubation mixtures contained (in a final volume of 3 ml) 1.5 ml of distilled water, 1.4 ml of PBS pH 7.4, and 0.1 ml of ox-brain phospholipid liposomes at a final concentration of phospholipid of 0.3 mM. Diethylstilboestrol, hexoestrol or 17 α-ethynyloestradiol 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.

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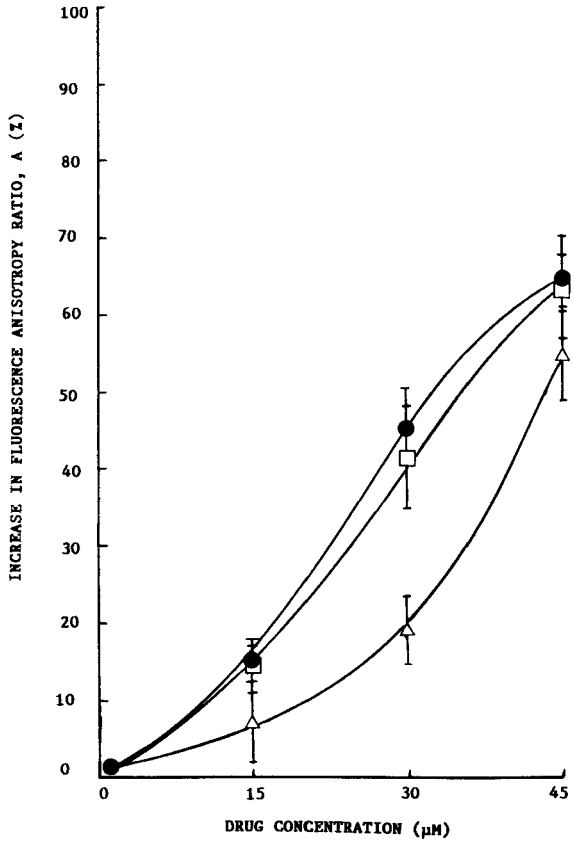

FIGURE 2 Concentration-dependent increase in fluorescence anisotropy ratios, A, by (●) diethylstilboestrol, (□) hexoestrol and (△) 17 α-ethynyloestradiol, all in the range 0–45 μM. Results are shown as mean ± S.D., $n = 3$.

The excitation (8 nm slit) and emission (8 nm slit) wavelengths were 348 nm and 428 nm respectively for DPH. 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[28,29] were calculated.

Inhibition of Liposomal Lipid Peroxidation

The addition of diethylstilboestrol, hexoestrol or 17 α-ethynyloestradiol 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 previously.[1] Graphs plotted from these results were used to obtain the IC_{50} and IC_{25} values for inhibition of liposomal lipid peroxidation used for purposes of comparison (in Tables 1 and 2) and correlation with those for decreased membrane fluidity.

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TABLE 1

IC_{50} values for the inhibition of liposomal lipid peroxidation and for the increase in the fluorescence anisotropy ratio, A, indicating decreased membrane fluidity by synthetic oestrogens

Compound	IC_{50}[a] Membrane fluidity[b] μM	IC_{50}[a] Lipid peroxidation in liposomes[c] μM
Diethylstilboestrol	33	2.5
Hexoestrol	36	2.8
17 α-Ethynyloestradiol	44	4.0

[a] IC_{50} was the concentration (in μM) of each compound required to either inhibit liposomal lipid peroxidation or to decrease membrane fluidity (increase the fluorescence anisotropy ratio, A) by 50%.

[b] Values are deduced from the graphs shown in Figure 2 in which each point represents the mean ± SD of three separate experiments.

[c] Values are deduced from the graphs shown in.[1]

TABLE 2

IC_{25} values for the increase in the fluorescence anisotropy ratio, A, indicating decreased membrane fluidity by synthetic oestrogens: compared to tamoxifen, 4-hydroxytamoxifen and 17 β-oestradiol

Compound	IC_{25}[a] Membrane fluidity[b] μM
Diethylstilboestrol	20
Hexoestrol	22
17 α-Ethynyloestradiol	33
4-Hydroxytamoxifen	36
17 β-Oestradiol	40
Tamoxifen	45

[a] IC_{25} was the concentration (in μM) of each compound required to decrease membrane fluidity (increase the fluorescence anisotropy ratio, A) by 25%.

[b] Values are deduced from the graphs shown in Figure 2 (or for tamoxifen, 4-hydroxytamoxifen and 17 β-oestradiol quoted from[27]) in which each point represents the mean ± SD of three separate experiments.

RESULTS

Diethylstilboestrol, hexoestrol and 17 α-ethynyloestradiol, 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. Figure 2 shows that these compounds all decreased membrane fluidity (increased the fluorescence anisotropy ratio, A) in a concentration dependent manner and that diethylstilboestrol, was somewhat more effective than hexoestrol and both were much more effective than 17 α-ethynyloestradiol. The differences in the ability of these compounds to decrease membrane fluidity (increase the fluorescence anisotropy ratio, A) are reflected in their IC_{50} values (see Table 1). In addition, IC_{25} values were calculated for diethylstilboestrol, hexoestrol and 17 α-ethynyloestradiol to make a comparison with the previously reported abilities of tamoxifen, 4-hydroxytamoxifen and 17 β-oestradiol to decrease membrane fluidity because none of the latter compounds, over the concentration range used, increased A by as much as 50% (see Table 2). Figure 2 and Tables 1 and 2 show that the order of effectiveness of these

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compounds in decreasing membrane fluidity is diethylstilboestrol > hexoestrol > 17 α-ethynyloestradiol. A good positive correlation was found between the IC_{50} values for decreased membrane fluidity and the IC_{50} values for the inhibition of liposomal lipid peroxidation by diethylstilboestrol, hexoestrol and 17 α-ethynyloestradiol (correlation coefficient, $r = 0.99$).

DISCUSSION

These results show that diethylstilboestrol, hexoestrol and 17 α-ethynyloestradiol all decreased membrane fluidity in ox-brain phospholipid liposomes, and that their order of effectiveness was diethylstilboestrol > hexoestrol > 17 α-ethynyloestradiol (see Figure 2 and Table 1). This order parallels the antioxidant ability of diethylstilboestrol, hexoestrol and 17 α-ethynyloestradiol as inhibitors of liposomal lipid peroxidation, and this is reflected in the good positive correlation coefficients ($r = 0.99$) between the two parameters.

It has been shown that diethylstilboestrol, hexoestrol and 17 α-ethynyloestradiol do not appear to exert their antioxidant action *via* a chain-breaking mechanism, because they do not show the kinetics characteristic of such an antioxidant mechanism[1] even though hydroxyl groups with potentially donatable hydrogen atoms are present (see Figure 1). The results presented here indicate that these compounds act as antioxidants by stabilizing membranes against peroxidation through the decreased membrane fluidity observed here. Cholesterol is thought to stabilize membranes through decreased membrane fluidity *via* interactions between the rigid hydrophobic ring structure of cholesterol and the saturated, monounsaturated, and to a much lesser extent the polyunsaturated, fatty acid side-chains of phospholipids:[30] and tamoxifen[27] and synthetic oestrogens, which show some structural similarity to cholesterol may act in a similar way. The antifluidity effect of diethylstilboestrol could contribute to its known action against cancer cells 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.[31] This could lead to decreased levels of cellular cAMP levels thereby causing inhibition of the growth of some cancer cells.[32]

Diethylstilboestrol, hexoestrol and 17 α-ethynyloestradiol thus have the potential to be useful anticancer agents, and indeed, diethylstilboestrol has been used clinically as an anticancer agent. These compounds are all highly lipophilic and they may accumulate in the plasma membrane of cancer cells *in vivo* to achieve the concentrations required. It is the formation of active metabolites of these compounds that results *in vivo* in their ability to cause oxidative damage to DNA and to thus display carcinogenicity. These metabolic reactions include the oxidation of oestrogens to catechol oestrogens and further to their respective quinones that can result in the damaging generation of free radicals by redox cycling between catechol oestrogens or diethylstilboestrol and their quinones.[24–26]

The molecular redesign of these synthetic oestrogens (see Figure 1), to prevent redox cycling by the hydroxyl and quinone group systems generated by these metabolic events, may enable clinical anticancer use for these potent membrane antifluidity agents, with a reduced risk of carcinogenic side-effects. The cardioprotective benefits of oestrogens[33] and tamoxifen[34,35] in postmenopausal women are well established and this is thought to be at least in part as a result of their antioxidant ability.[14–17] 17 β-Oestradiol[16,36] and tamoxifen[16] can protect LDL

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against oxidative damage *in vitro* and 17 β-oestradiol has now been reported to protect LDL against oxidation *in vivo* in postmenopausal women.[37] The potent antioxidant action of synthetic oestrogens[1] arising from the mechanism reported here suggests that they may be useful as cardioprotective agents provided that the problem of the carcinogenicity of these compounds can be overcome by appropriate redesign as discussed above. Studies on the ability of synthetic oestrogens to protect LDL against oxidation would thus be of considerable interest.

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Accepted by Professor J.M.C. Gutteridge

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