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Carcinogenic antioxidants

Diethylstilboestrol, hexoestrol and 17 α-ethynyloestradiol

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The synthetic oestrogens diethylstilboestrol, hexoestrol and 17 α-ethynyloestradiol are known to be carcinogenic, yet they all exert antioxidant properties in vitro in that they are good inhibitors of iron ion-dependent lipid peroxidation. In rat liver microsomes incubated with Fe(III)-ascorbate or Fe(III)-ADP/NADPH and also in ox-brain phospholipid liposomes incubated with Fe(III)-ascorbate; the overall order of effectiveness of the compounds tested as inhibitors of lipid peroxidation was diethylstilboestrol > hexoestrol > 17 α-ethynyloestradiol > 4-hydroxytamoxifen > 17 β-oestradiol > tamoxifen. Compounds acting as antioxidants towards lipids may also exert pro-oxidant effects towards other molecules such as DNA and thus must never be assumed to be safe for human use.

Antioxidant; Lipid peroxidation; Carcinogenic action; Diethylstilboestrol; Anticancer action; Tamoxifen

1. INTRODUCTION

Tamoxifen is used extensively in the treatment of breast cancer [1–4] and is now being assessed in clinical trials as a prophylactic agent against this disease [5–9]. However, before tamoxifen became available diethylstilboestrol, a non-steroidal synthetic oestrogen, was the primary hormonal treatment in postmenopausal women [10]. Furthermore, diethylstilboestrol has been extensively used as an antiandrogenic agent for the treatment of patients with prostatic carcinoma [11,12]. Unfortunately, diethylstilboestrol is also a known transplacental carcinogen in humans [13]. The incidence of genital tract cancer increased significantly in the daughters of women treated with large doses of diethylstilboestrol for the stabilization of pregnancies [14]. Furthermore, there is clear evidence that administration of diethylstilboestrol in large doses during pregnancy increases the subsequent risk of breast cancer and that diethylstilboestrol increases the risk of testicular cancer in males exposed in utero [14]. In addition, diethylstilboestrol and synthetic steroidal oestrogens such as 17 α-ethynyloestradiol, the oestrogenic component of many contraceptive medications, can induce tumours in laboratory animals at high doses [15,16]. Although the exact mechanism of their carcinogenicity is unclear, damage to DNA bases by free radicals arising from the metabolism and redox cycling of these compounds may be involved [17–21]. There is increasing interest in the role of oxidant DNA damage in carcinogenesis [17–22].

We have already observed that tamoxifen, which is structurally related to diethylstilboestrol (and its derivative hexoestrol) and 17 β-oestradiol (related to 17 α-ethynyloestradiol) can exert antioxidant effects in vitro, in that they inhibit metal ion-dependent lipid peroxidation in a range of membrane systems [23–26]. We, therefore, decided to test diethylstilboestrol, its derivative hexoestrol and also the synthetic oestrogen 17 α-ethynyloestradiol (structures shown in Fig. 1) in similar systems. The results are compared with those obtained for tamoxifen, its 4-hydroxy metabolite and 17 β-oestradiol and are discussed in relation to both the carcinogenic and anticancer actions of diethylstilboestrol and related compounds.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Diethylstilboestrol, hexoestrol, 17 α-ethynyloestradiol, 17 β-oestradiol, tamoxifen and ox-brain phospholipids were from the Sigma Chemical Co. (Poole, UK). 4-Hydroxytamoxifen was kindly donated by ICI Pharmaceuticals (Macclesfield, UK). All other reagents were of the highest quality from the Sigma Chemical Co. (Poole, UK) or from BDH Ltd. (Dagenham, UK).

2.2. *Preparation and peroxidation of microsomes and liposomes*

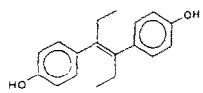
Rat liver microsomes were prepared from the livers of adult male rats by standard differential-centrifugation techniques as described in [27]. Ox-brain phospholipid liposomes were prepared as described previously [25].

Microsomal and liposomal lipid peroxidation in the presence of Fe(III) and ascorbate or ADP/NADPH, was measured by the formation of thiobarbituric acid-reactive substances (TBARS) as described previously [22]. The reaction mixtures (final volume of 1.0 ml) contained either microsomes (0.25 mg of microsomal protein) or liposomes (0.5 mg in 0.1 ml of phosphate buffered saline at pH 7.4); 10 mM KH_2PO_4-KOH buffer pH 7.4 was used for microsomal assays and

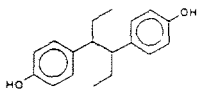
phosphate-buffered saline pH 7.4 was used for liposomal assays; 5 μl of ethanol or test compound dissolved in the same volume of ethanol was added. Peroxidation was started by adding aqueous solutions of $FeCl_3$ (0.1 ml) and ascorbate (0.1 ml) to give a final concentration of 100 μM of each. In some experiments, microsomal lipid peroxidation was started by the addition of $FeCl_3$ (100 μM), ADP (1.7 mM) and NADPH (0.4 mM) to give the final concentrations stated. Freshly prepared ADP and $FeCl_3$ solutions were premixed just before addition to the reaction mixture. Ascorbate or NADPH were added to start the reaction, and incubations were carried out at 37°C for 20 min (unless stated otherwise). The reactions were terminated by the addition of 100 μl BHT (butylated hydroxytoluene: 0.2% w/v) dissolved in ethanol to suppress further peroxidation during the heating stage of the TBA reaction. The amount of lipid peroxidation was determined by the TBA test [27]. HCl (0.5 ml, 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) and heating at 80°C for 30 min. The chromogen was extracted with 2 ml of butan-1-ol and the A_{532} of the upper (organic) layer was measured.

3. RESULTS

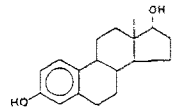
Diethylstilboestrol, hexoestrol, 17 α-ethynyloestradiol, 17 β-oestradiol, tamoxifen or 4-hydroxytamoxifen were each added to rat liver microsomes or ox-brain phospholipid liposomes at micromolar concentrations, in the range 0–30 μM, dissolved in ethanol. Ethanol itself when used at a final concentration of 0.5% (v/v) had no effect on microsomal or liposomal lipid peroxidation.



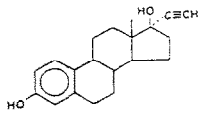
DIETHYLSTILBOESTROL



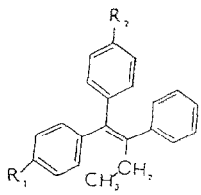
HEXOESTROL



17 β-OESTRADIOL



17 α-ETHYNYLOESTRADIOL



TAMOXIFEN R_1=H, R_2=$OCH_2CH_2N(CH_3)_2$

4-HYDROXYTAMOXIFEN R_1=OH, R_2=$OCH_2CH_2N(CH_3)_2$

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

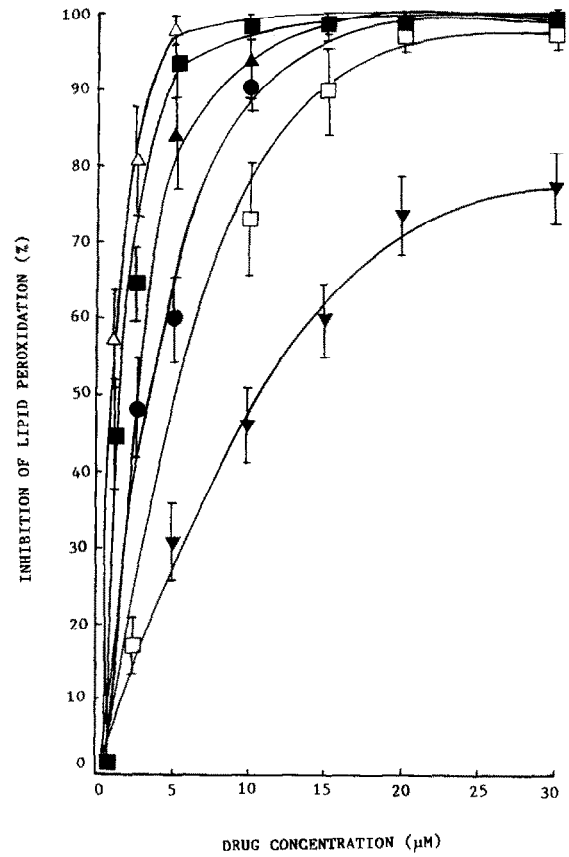


Fig. 2. Concentration-dependent inhibition of iron-ion dependent lipid peroxidation induced by Fe(III)-ascorbate in rat liver microsomes. (△) diethylstilboestrol, (■) hexoestrol, (▲) 17 α-ethynyloestradiol, (□) 17 β-oestradiol, (▼) tamoxifen, (●) 4-hydroxytamoxifen. Results are mean ± S.D., n = 3–6 tests.

Fig. 2 shows that when microsomes were incubated with Fe(III)-ascorbate, diethylstilboestrol and hexoestrol were powerful inhibitors of peroxidation, with very low IC_{50} values (Table I). 17 α-Ethynyloestradiol and 4-hydroxytamoxifen were of similar effectiveness and both were more effective than 17 β-oestradiol and much more effective than tamoxifen. These differences in potency are reflected in their IC_{50} values (see Table I). Table I shows that each of the compounds tested appeared to be of similar effectiveness whether microsomal peroxidation was started by adding Fe(III)-ascorbate or Fe(III)-ADP/NADPH, except that tamoxifen was more effective in the Fe(III)-ascorbate system, as observed previously [23]. The time courses of peroxidation in the presence of these compounds, at their IC_{50} concentrations (see Fig. 3) show that they inhibited microsomal lipid peroxidation throughout the incubation

Table I

IC_{50} values for the inhibition of microsomal and liposomal lipid peroxidation by diethylstilboestrol, hexoestrol, 17 α-ethynyloestradiol, 17 β-oestradiol, tamoxifen and 4-hydroxytamoxifen

Compound/Drug	Systems		
	Microsomal		Liposomal
	FeIII/asc (μM)	FeIII-ADP/ NADPH (μM)	FeIII/asc (μM)
Diethylstilboestrol	1	1	2.5
Hexoestrol	1.5	1.25	2.75
17 α-Ethynyloestradiol	2.5	2.75	4
17 β-Oestradiol	5	5	5
Tamoxifen	10.5	23.25	28.75
4-Hydroxytamoxifen	3	4.25	3.25

asc, ascorbate. Values are deduced from the graphs shown in Figs. 2 and 4 in which each point represents the mean ± S.D. of 3–6 and 4–8, respectively, separate assays.

period: there was no clear evidence of a lag period followed by an acceleration of peroxidation to the control rate.

Fig. 4 shows that when liposomes were incubated with Fe(III)-ascorbate, diethylstilboestrol and hexoestrol were approximately equally potent as inhibitors of lipid peroxidation and were more effective than 4-hydroxytamoxifen, 17 α-ethynyloestradiol, 17 β-oestradiol or tamoxifen. Tamoxifen was, as in the microsomal system, the least effective compound tested. This is also

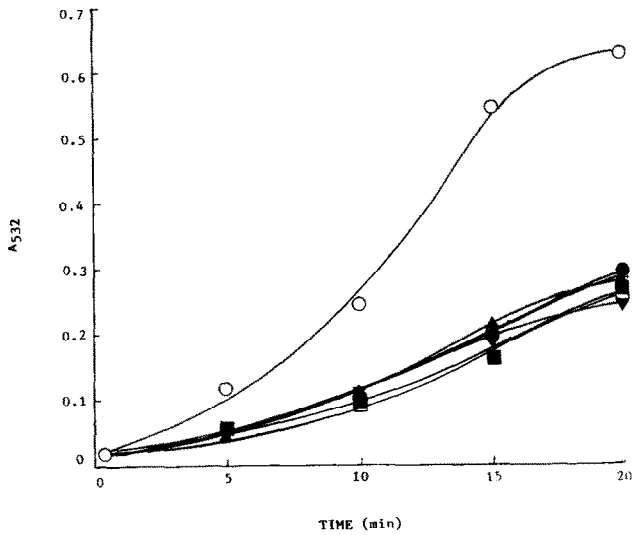


Fig. 3. Time course of microsomal lipid peroxidation induced by Fe(III) and ascorbate: the effect of test compounds added at their IC_{50} concentrations. (○) control (ethanol only added), (△) 1 μM diethylstilboestrol, (■) 1.5 μM hexoestrol, (▲) 2.5 μM 17 α-ethynyloestradiol, (□) 5 μM 17 β-oestradiol, (▼) 10.5 μM tamoxifen, (●) 3 μM 4-hydroxytamoxifen. Results shown are the means of duplicate determinations. Concentrations quoted are the final concentrations in the reaction mixtures.

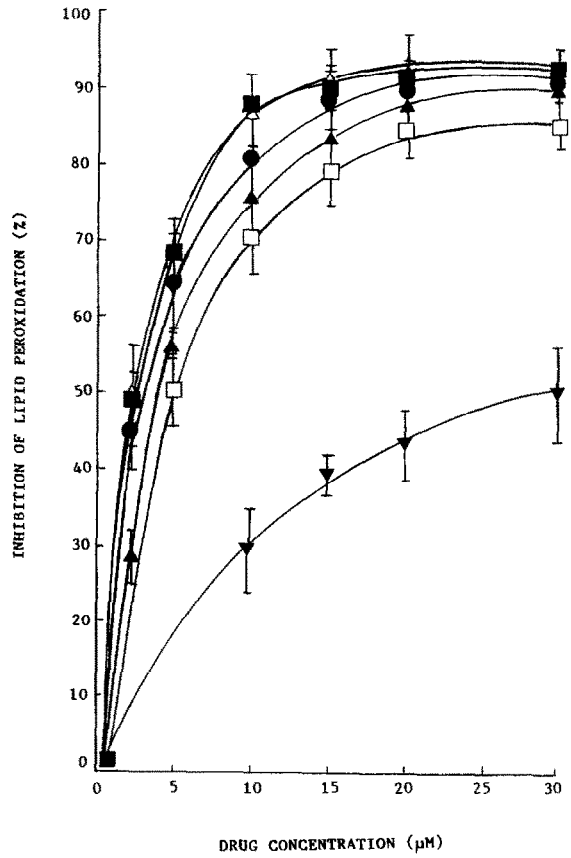


Fig. 4. Concentration-dependent inhibition of iron-dependent lipid peroxidation induced by Fe(III)-ascorbate in ox-brain phospholipid liposomes. (△) Diethylstilboestrol, (■) hexoestrol, (△) 17 α-ethynyloestradiol, (□) 17 β-oestradiol, (▼) tamoxifen, (●) 4-hydroxytamoxifen. Results are mean ± S.D., n = 4–8 tests.

reflected in the IC_{50} values of the compounds (Table I). Fig. 5 shows the time-courses of liposomal lipid peroxidation, again indicating that the compounds excerted inhibitory effects throughout the incubation.

Control experiments showed that none of the compounds tested interfered with the TBA test, in that no inhibition was observed when the compounds were added with the TBA reagents instead of at the beginning of the incubations.

4. DISCUSSION

These results show that diethylstilboestrol, hexoestrol and 17 α-ethynyloestradiol are all powerful antioxidants, in that they inhibit iron ion-dependent lipid peroxidation in microsomal and liposomal systems. In rat liver microsomes peroxidized with Fe(III)-ascorbate, diethylstilboestrol was the most effective inhibitor of lipid peroxidation of the compounds tested and the overall order of effectiveness was diethylstilboestrol > hexoestrol > 17 α-ethynyloestradiol > 4-hydroxytamoxifen > 17 β-oestradiol > tamoxifen. These compounds had a similar order of effectiveness when the microsomes were

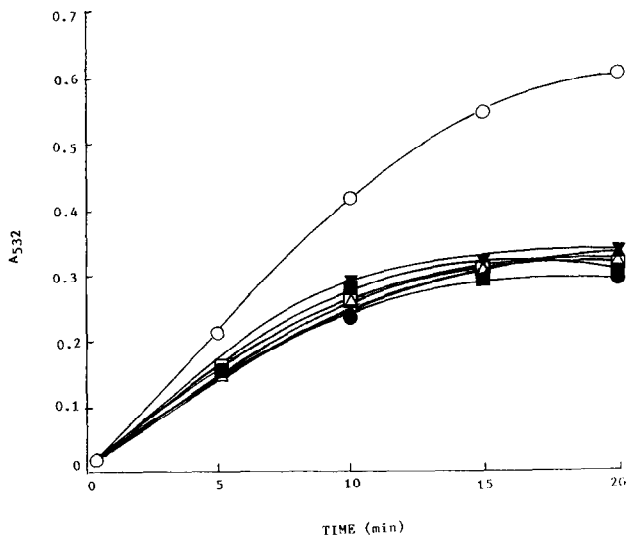


Fig. 5. Time course of liposomal lipid peroxidation induced by Fe(III) and ascorbate: the effect of test compounds added at their IC_{50} concentrations. (○) control (ethanol only added), (△) 2.5 μM diethylstilboestrol, (■) 2.75 μM hexoestrol, (▲) 4 μM 17 α-ethynyloestradiol, (□) 5 μM 17 β-oestradiol, (▼) 28.75 μM tamoxifen, (●) 3.25 μM 4-hydroxytamoxifen. Results shown are the means of duplicate determinations. Concentrations quoted are the final concentrations in the reaction mixtures.

peroxidized with Fe(III)-ADP/NADPH, and also in a much simpler lipid system, ox-brain phospholipid liposomes, peroxidized with Fe(III)-ascorbate. The ethynyl group possessed by 17 α-ethynyloestradiol, but not 17 β-oestradiol (see Fig. 1), appears to make it a somewhat more effective antioxidant than 17 β-oestradiol and this may reflect an increase its lipophilic nature compared to 17 β-oestradiol. Diethylstilboestrol and hexoestrol were similarly potent as antioxidants even though they differ structurally in that diethylstilboestrol has a double bond, which holds it in a particular configuration analogous to ring B in the steroid nucleus (see Fig. 1).

The time-course experiments suggest that diethylstilboestrol, hexoestrol and 17 α-ethynyloestradiol do not exert their antioxidant action via a chain-breaking mechanism, even though hydroxy groups with potentially donatable hydrogen atoms are present (see Fig. 1). There was no clear evidence of a lag period followed by an acceleration of peroxidation to the control rate that is usually observed with chain-breaking antioxidants. Perhaps these compounds act, in whole or in part, by stabilizing membranes against peroxidation [24,28]. We have suggested previously that this mechanism of antioxidant action of tamoxifen via decreased membrane fluidity may contribute to its known oestrogen receptor-independent anticancer action by antagonizing cell division in cancer cells [25,28] and a similar action is possible for diethylstilboestrol.

There is considerable current interest in the role of antioxidants as food additives and therapeutic agents (reviewed in [29–31]). However, such compounds are often styled as 'antioxidants' merely on the basis of ability to inhibit lipid peroxidation. The compounds studied in this paper, diethylstilboestrol, hexoestrol and 17 α-ethynyloestradiol, clearly illustrates how some compounds can be powerful inhibitors of lipid peroxidation whilst actually accelerating oxidative damage to DNA via their metabolism [17–21] and causing cancer in vivo. The characterization of antioxidants for therapeutic and food use, whether they are synthetic or of natural origin, should include much more than just studies upon lipids [31–34].

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