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# THE ANTIOXIDANT ACTION OF A PURE ANTIOESTROGEN: ABILITY TO INHIBIT LIPID PEROXIDATION COMPARED TO TAMOXIFEN AND 17β-OESTRADIOL AND RELEVANCE TO ITS ANTICANCER POTENTIAL

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**Abstract**—The pure antioestrogen ICI 164,384, and nafoxidine (structurally related to tamoxifen) were good inhibitors of iron ion-dependent lipid peroxidation. In rat liver microsomes incubated with Fe(III)–ascorbate the overall order of effectiveness of the compounds tested as inhibitors of lipid peroxidation was 4-hydroxytamoxifen > 17β-oestradiol > nafoxidine ≥ tamoxifen > ICI 164,384. When the microsomes were incubated with Fe(III)–ADP/NADPH, a similar order of effectiveness was observed. In ox-brain phospholipid liposomes incubated with Fe(III)–ascorbate the order was 4-hydroxytamoxifen > 17β-oestradiol > ICI 164,384 > tamoxifen ≥ nafoxidine. The antioxidant ability of ICI 164,384, a steroidal oestrogen antagonist, is compared to that of tamoxifen (a non-steroidal antioestrogen and partial oestrogen agonist) and 17 β-oestradiol and is discussed in relation to its anticancer action.

*Key words:* Antioxidant; anticancer; lipid peroxidation; pure antioestrogen ICI 164,384; tamoxifen; 17β-oestradiol

Tamoxifen, an antioestrogen with other mechanisms of action, 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, tamoxifen is not a pure oestrogen antagonist and possesses partial oestrogen agonist activity both *in vivo* and *in vitro*. Tamoxifen and its active metabolite, 4-hydroxytamoxifen stimulate uterine growth in ovariectomized rats and mice [10] and although tamoxifen is generally tumouristatic to MCF-7 tumours grown in the nude mouse [11] prolonged tamoxifen exposure can lead to tamoxifen-resistant [12] and tamoxifen stimulatable tumours [13]. Furthermore, occurrence of tamoxifen-stimulated cancer cell growth in patients as a type of drug resistance resulting in relapse could be blocked with pure antioestrogens [14]. ICI 164,384, a pure antagonist of oestrogen-stimulated MCF-7 cell proliferation and invasiveness [15] has been shown to inhibit the growth of an MCF-7 variant, dependent on tamoxifen for growth stimulation [16]. In addition, in intact rats ICI 164,384 is almost as effective as ovariectomy at reducing uterine weight, whereas tamoxifen is much less effective than ovariectomy [17, 18].

It is of interest therefore, that contrary to some suggestions that ICI 164,384 inhibits oestrogen receptor dimerization and thus DNA binding [19], further studies have shown that this compound does not inhibit DNA binding of the oestrogen receptor *in vivo* [20] although it may reduce the affinity of the oestrogen receptor for its cognate response element; therefore ICI 164,384 belongs to the same class of antihormones as tamoxifen (and 4-hydroxytamoxifen) [21]. Furthermore, ICI 164,384 and 4-hydroxytamoxifen are both potent inhibitors of insulin-induced cell cycle progression in MCF-7 cell in a steroid-free environment [22]. Similarities between the non-oestrogen dependent mechanisms of anticancer action of ICI 164,384 and tamoxifen are thus worth investigating.

We have already observed that tamoxifen, 4-hydroxytamoxifen and 17β-oestradiol can exert antioxidant effects *in vitro*, in that they inhibit metal ion-dependent lipid peroxidation in a range of membrane systems [23–25] and protect human low density lipoproteins against oxidative damage [26]. It was decided, therefore, to test ICI 164,384 (structurally related to 17β-oestradiol) and nafoxidine (structurally related to tamoxifen: structures shown in Fig. 1) in similar systems. The results are compared with those obtained for tamoxifen, its 4-hydroxy metabolite and 17β-oestradiol, and are discussed in relation to the anticancer action of ICI 164,384.

## MATERIALS AND METHODS

17β-Oestradiol, tamoxifen, nafoxidine and ox-brain phospholipids were from the Sigma Chemical Co. (Poole, U.K.). 4-Hydroxytamoxifen was kindly donated by ICI Pharmaceuticals (Macclesfield, U.K.) and ICI 164,384 was a kind gift from Dr Alan Wakeling, ICI Pharmaceuticals (Macclesfield, U.K.). All other reagents were of the highest quality from

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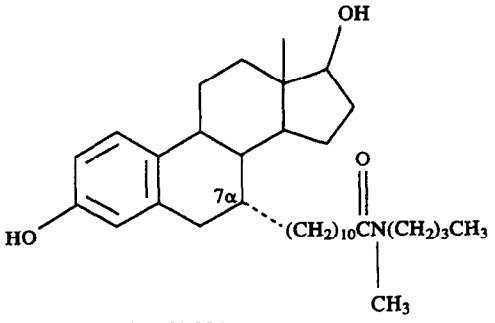
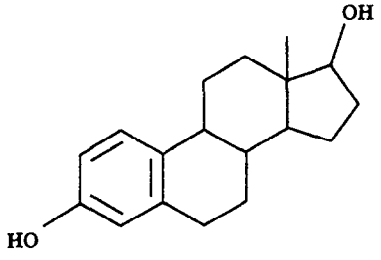



ICI 164,384

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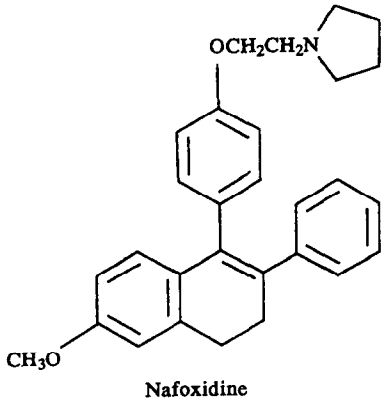
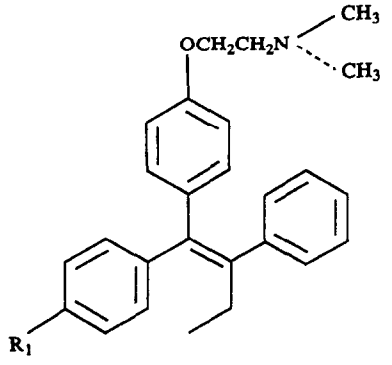



Nafoxidine




Tamoxifen $R_1$=H
4-Hydroxytamoxifen $R_1$=OH

Fig. 1. Structures of ICI 164,384, nafoxidine, 17β-oestradiol, tamoxifen and 4-hydroxytamoxifen.

the Sigma Chemical Co. or from BDH Ltd (Dagenham, U.K.).

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 TBARS* as described previously [27]. The reaction mixtures (final volume 1.0 mL) contained either microsomes (0.25 mg of microsomal protein) or liposomes (0.5 mg in 0.1 mL of PBS at pH 7.4); 10 mM $KH_2PO_4$-KOH buffer pH 7.4 was used for microsomal assays and PBS 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 addition of $FeCl_3$ (100 μM), ADP (1.7 mM) and NADPH (0.4 mM) to give the final concentrations stated. In these experiments freshly prepared ADP and $FeCl_3$ solutions were mixed just before addition to the reaction mixture. The reactions were started by the addition of ascorbate or NADPH, and incubations were carried out at 37° for 20 min (unless stated otherwise). 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. 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 TBA solution (1% w/v in 50 mM sodium hydroxide) and heating at 80° 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.

## RESULTS

### *The effect of ICI* 164,384, *nafoxidine*, 17-β-*oestradiol, tamoxifen and* 4-*hydroxytamoxifen on iron-dependent microsomal lipid peroxidation*

Lipid peroxidation, which can be measured by the TBA test, occurs when rat liver microsomes are incubated with Fe(III)–ascorbate [28] or Fe(III)–ADP/NADPH [29] at pH 7.4. ICI 164,384, nafoxidine, 17β-oestradiol, tamoxifen or 4-hydroxytamoxifen were added to these systems 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 lipid peroxidation. Figure 2 shows that in the Fe(III)–ascorbate system, ICI 164,384 was almost as effective as tamoxifen and nafoxidine but was less effective than 17β-oestradiol and much less effective than 4-hydroxytamoxifen. These differences in potency are reflected in their $IC_{50}$ values (see Table 1). Table 1 shows that although 17β-oestradiol

* Abbreviations: TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances.

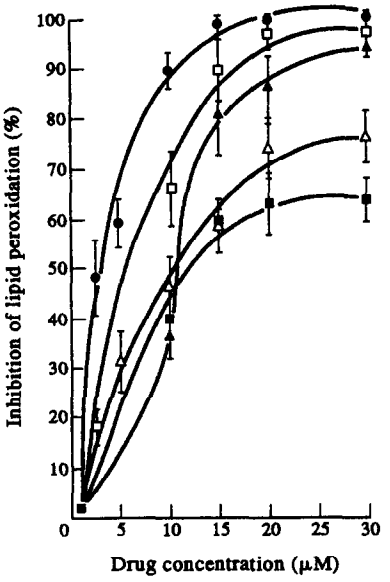



Fig. 2. Concentration-dependent inhibition of iron ion-dependent lipid peroxidation induced by Fe(III)–ascorbate in rat liver microsomes. (■) ICI 164,384; (▲) nafoxidine; (□) 17β-oestradiol; (△) tamoxifen; (●) 4-hydroxytamoxifen. Results are means ± SD, N = 3–6 tests.

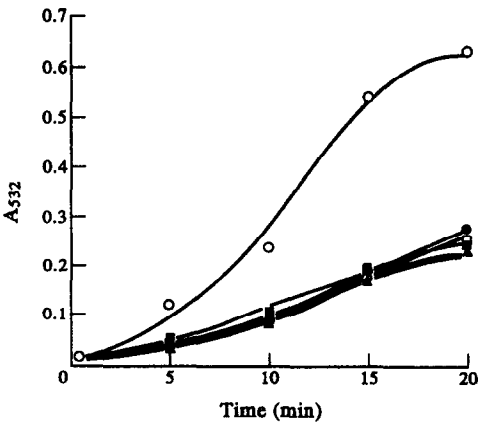



Fig. 3. Time–course of microsomal lipid peroxidation induced by Fe(III) and ascorbate: the effect of test compounds added at their $IC_{50}$ concentrations. (○) Control (ethanol only added); (■) 11.9 μM ICI 164,384; (▲) 10.5 μM nafoxidine; (□) 5 μM 17β-oestradiol; (△) 10.5 μM tamoxifen; (●) 3 μM 4-hydroxytamoxifen. Results shown are the means of duplicate determinations. Concentrations quoted are the final concentrations in the reaction mixtures.

and 4-hydroxytamoxifen appeared to be of similar effectiveness whether peroxidation was started by adding Fe(III)–ascorbate or the Fe(III)–ADP/NADPH system tamoxifen, nafoxidine and ICI 164,384 were more effective in the Fe(III)–ascorbate system.

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

*The effect of ICI 164,384, nafoxidine, 17β-oestradiol, tamoxifen and 4-hydroxytamoxifen on iron-dependent liposomal lipid peroxidation*

Liposomes formed from ox-brain phospholipids have been shown to provide a model membrane (lipid bilayer) system, which in the presence of Fe(III)–ascorbate at pH 7.4 is rapidly peroxidized [30] as measured by the TBA test. ICI 164,384, nafoxidine, 17β-oestradiol, tamoxifen or 4-hydroxytamoxifen, dissolved in ethanol, were added to ox-brain phospholipid liposomes to give final concentrations in the range 0–30 μM. Figure 4 shows that at low concentrations, ICI 164,384 was as effective as 17β-oestradiol as an inhibitor of lipid peroxidation and both were more effective than

Table 1. $IC_{50}$ values for the inhibition of microsomal and liposomal lipid peroxidation by ICI 164,384, nafoxidine, 17β-oestradiol, tamoxifen and 4-hydroxytamoxifen

| | Systems | | |
|---|---|---|---|
| | Microsomal | | Liposomal |
| Compound/drug | Fe(III)–ascorbate (μM) | Fe(III)–ADP/NADPH (μM) | Fe(III)–ascorbate (μM) |
| ICI 164,384 | 11.9 | 28.8 | 5.6 |
| Nafoxidine | 10.5 | 20.0 | 28.8 |
| 17β-Oestradiol | 5.0 | 5.0 | 5.0 |
| Tamoxifen | 10.5 | 23.3 | 28.8 |
| 4-Hydroxytamoxifen | 3.0 | 4.3 | 3.3 |

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

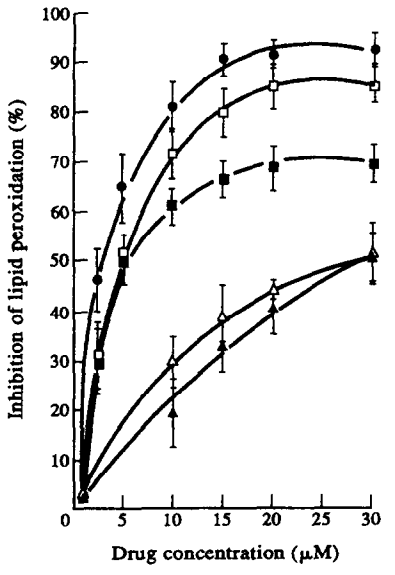



Fig. 4. Concentration-dependent inhibition of iron-dependent lipid peroxidation induced by Fe(III)–ascorbate in ox-brain phospholipid liposomes. (■) ICI 164,384; (▲) nafoxidine; (□) 17β-oestradiol; (△) tamoxifen; (●) 4-hydroxytamoxifen. Results are means ± SD, N = 4–8 tests.

tamoxifen and nafoxidine, though still less effective than 4-hydroxytamoxifen. This is also reflected in the $IC_{50}$ values of the compounds (Table 1). Figure 5 shows the time-courses of lipid peroxidation again indicating that the compounds exerted inhibitory effects throughout the incubation.

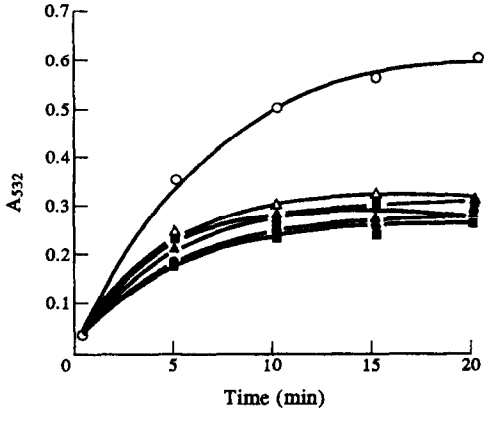



Fig. 5. Time-course of liposomal lipid peroxidation induced by Fe(III) and ascorbate: the effect of test compounds added at their $IC_{50}$ concentrations. (○) Control (ethanol only added); (■) 5.6 μM ICI 164,384; (▲) 28.8 μM nafoxidine; (□) 5 μM 17β-oestradiol; (△) 28.8 μM tamoxifen; (●) 3.3 μM 4-hydroxytamoxifen. Results shown are the means of duplicate determinations. Concentrations quoted are the final concentrations in the reaction mixtures.

## DISCUSSION

These results show that ICI 164,384 and nafoxidine are antioxidants, in that they inhibit iron ion-dependent lipid peroxidation in microsomal and liposomal systems. In rat liver microsomes peroxidized with Fe(III)–ascorbate, ICI 164,384 was almost as effective as tamoxifen and nafoxidine as an antioxidant, though less effective than 17β-oestradiol and much less effective than 4-hydroxytamoxifen. When the microsomes were peroxidized with Fe(III)–ADP/NADPH instead of Fe(III)–ascorbate, although 17β-oestradiol and 4-hydroxytamoxifen were similarly effective in both systems, ICI 164,384, nafoxidine and tamoxifen were much less effective in the Fe(III)–ADP/NADPH system, a result observed previously for tamoxifen [23]. Interestingly, in a much simpler lipid system, ox-brain phospholipid liposomes peroxidized with Fe(III)–ascorbate, ICI 164,384 was much more effective than in the microsomal systems and indeed its antioxidant ability, at low concentrations, approached that of 17β-oestradiol.

The chemical structure of nafoxidine (see Fig. 1) indicates that it is unlikely to act as a chain-breaking antioxidant, because like tamoxifen it has no potentially donatable hydrogen atoms (such as phenolic hydrogens), and this is in agreement with the time-course data. ICI 164,384 like 17β-oestradiol and 4-hydroxytamoxifen does have potentially donatable hydrogen atoms (see Fig. 1) but again the time-course data indicate that ICI 164,384 does not appear to be acting as a chain-breaking antioxidant. There was no clear evidence of a lag phase followed by an acceleration of peroxidation to the control rate. It has been reported that the mechanism of the membrane antioxidant action of tamoxifen and related compounds, like that of cholesterol, is through their ability to decrease membrane fluidity [31] and thus stabilize the membrane against lipid peroxidation and a similar action for ICI 164,384 and nafoxidine is likely.

ICI 164,384 was less effective than 17β-oestradiol as an inhibitor of lipid peroxidation in the Fe(III)–ascorbate microsomal system and much less effective in the Fe(III)–ADP/NADPH system. In the liposomal system although ICI 164,384 was as effective as 17β-oestradiol at low concentrations (≤5 μM) it was again less effective at higher concentrations. This reduced antioxidant ability compared to 17β-oestradiol results from the side-chain at the 7α position of the sterol ring B in ICI 164,384. This side-chain is highly hydrophobic and thus should increase uptake of the compound into the membrane, however, it could well interfere with the orientation required for membrane stabilization against lipid peroxidation.

Nafoxidine differs structurally from tamoxifen in that it has a $CH_3O$ group instead of a hydrogen at position $R_1$, a side-chain with an N-containing heterocyclic ring and a complete ring B similar to that found in sterols. This ring might be expected to increase the ability of nafoxidine to inhibit lipid peroxidation compared to tamoxifen because it makes nafoxidine structurally similar to the membrane stabilizing sterol cholesterol than tamoxifen.

Indeed, in the Fe(III)–ADP/NADPH microsomal system and in the Fe(III)–ascorbate microsomal system (but only at concentrations of $>10.5\ \mu M$) nafoxidine was more effective than tamoxifen as an inhibitor of lipid peroxidation, however, in the liposomal system they were similarly effective as inhibitors of lipid peroxidation (see Table 1).

The antioxidant ability of tamoxifen and related compounds, via their ability to decrease membrane fluidity [31] may also contribute to their antimitotic action. The antifluidity action of these compounds, which are all highly lipophilic and thus may accumulate in the plasma membrane of cancer cells *in vivo* to achieve the concentrations required, could inhibit the action of membrane enzymes, receptors and channels and thus inhibit the growth of cancer cells [25, 31]. A similar antimitotic action is possible for ICI 164,384 and the observed antioxidant ability of these compounds via decreased membrane fluidity is likely to be of relevance to their clinical anticancer action.

Unfortunately, the oral bioavailability of ICI 164,384 is poor and indeed poor oral availability is a common clinical problem with steroidal compounds. However, studies are now underway using injection of oil-based formulations of a chemically modified version of ICI 164,384 (ICI 182,780: improved oral bioavailability compared to ICI 164,384), which may be of use for patients with advanced breast cancer who have relapsed during tamoxifen therapy [14, 32]. In addition, a new series of pure antioestrogens, the 11$\beta$-amidoalkyl oestradiols, have been reported to have a potent ability to inhibit the growth of human breast cancer cell lines [33]. However, these compounds are likely to have the same clinical oral bioavailability problems as found for ICI 164,384 (and to a lesser extent ICI 182,780).

The partial oestrogen agonist actions of tamoxifen confer some very beneficial clinical side-effects particularly its cardioprotective action [34]; tamoxifen acts like oestrogen in liver to inhibit cholesterol synthesis [35]. Furthermore, tamoxifen and 4-hydroxytamoxifen protect cardiac membranes against lipid peroxidation [25] and human low density lipoproteins against oxidative damage [26], and such oxidative damage to low density lipoproteins is known to contribute to the development of atherosclerosis [36]. Therefore, the antioxidant ability of any future pure antioestrogens used clinically in long-term therapy is likely to be crucial to achieving the cardioprotection reported with tamoxifen.

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