Influence of Anti-Oestrogens on the Specific Binding *in vitro* of [³H]Oestradiol by Cytosol of Rat Mammary Tumours and Human Breast Carcinomata

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The anti-oestrogenic potential of two nitrogen-mustard-containing compounds, I.C.I. 79792 and I.C.I. 85966, was studied. I.C.I. 85966 usually did not decrease specific binding of [³H]oestradiol by breast-tumour cytoplasmic proteins. I.C.I. 79792 decreased specific [³H]oestradiol binding, but not to the same extent as similar concentrations of I.C.I. 46474, diethylstilboestrol or dibutylidihydrostilboestrol.

Certain compounds are able to decrease the specific uptake of oestradiol *in vivo* and *in vitro* by various target tissues of the rat (Terenius & Ljungkvist, 1972; Jensen *et al.*, 1972; Geynet *et al.*, 1972; Skidmore *et al.*, 1972) and man (Jensen *et al.*, 1972; Hahnel *et al.*, 1973; Lunan & Green, 1974). These substances, some steroids and synthetic anti-oestrogens, are able to inhibit the selective transfer of oestrogens by specific receptor macromolecules to the chromatin of target tissues (Jensen *et al.*, 1971; Jensen & DeSombre, 1972, 1973), and have been found useful in distinguishing between the interactions of oestradiol with specific and non-specific binding proteins (Jensen *et al.*, 1972). Such anti-oestrogens have an important therapeutic role in the treatment of women with advanced carcinoma of the breast (Terenius, 1971). This present communication evaluates the anti-oestrogenic potential of several substances, in particular two compounds containing nitrogen-mustard groupings, by their comparative ability to inhibit specific binding of [³H]oestradiol by cytoplasmic proteins of human breast tumours and rat mammary carcinomas induced by 7,12-dimethylbenz[*a*]anthracene.

Materials and Methods

Animals and tissue. Mammary tumours were induced in virgin female Sprague-Dawley rats by intubation of 7,12-dimethylbenz[*a*]anthracene (20 mg in 1 ml of sesame oil). Tumours reached a suitable size for experimentation (approx. 2 cm × 2 cm) 7-12 weeks after intubation. Rats bearing such tumours were killed by cervical dislocation and tumours were removed, placed in vessels surrounded with crushed ice, and used immediately.

Samples of human primary breast-tumour tissue were provided by the Department of Surgery, University of Nottingham, where they were stored

at -20°C until collection. Frozen tissues were transported to the Tenovus Institute where they were retained at -20°C until used for analysis.

Chemicals. [2,4,6,7-³H]Oestradiol (specific radioactivity 85 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Non-radioactive oestradiol, diethylstilboestrol [3,4-bis-(*p*-hydroxyphenyl)hex-3-ene] and dithiothreitol were provided by Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. *meso*-Dibutylidihydrostilboestrol [5,6-bis-(*p*-hydroxyphenyl)decane], I.C.I. 46474 {the *trans* isomer of 1-[*p*-(β-dimethylaminoethoxy)phenyl]-1,2-diphenylbut-1-ene}, I.C.I. 79792 (1-{4-[β-bis-(2-chloroethyl)amino]ethoxyphenyl}-*trans*-diphenylbut-1-ene hydrochloride) and I.C.I. 85966 (3,4-bis-{*p*-(*N*-bis-2-chloroethyl)carbamoyl}phenyl)hex-3-ene) were gifts from Dr. A. L. Walpole and Dr. D. N. Richardson of I.C.I. Pharmaceuticals, Alderley Park, Cheshire, U.K. 7,12-Dimethylbenz[*a*]anthracene was supplied by Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. All other compounds were of AnalaR grade and were supplied by British Drug Houses Ltd., Poole, Dorset, U.K.

Preparation of soluble supernatants. N₂-frozen human breast-tumour tissue was pulverized in a spring-loaded plunger gun and mortar previously cooled in liquid N₂. The pulverized tissue was quickly transferred to a Potter-Elvehjem homogenizer and homogenized in 10 mM-Tris-HCl buffer, pH 7.4, containing 1 mM-EDTA and 0.25 mM-dithiothreitol (medium A: 5 ml/2 g of original tissue) at 4°C.

Rat mammary tumours were dissected free from adhering connective tissue and obvious necrotic material, and washed in medium A. Tissue was then minced with scissors and homogenized in medium A (5 ml/2 g of tissue) in a Potter-Elvehjem ground-glass homogenizer with a Teflon pestle driven by a motor (TriR Instruments, Jamaica, N.Y., U.S.A.) operating at 3000-3500 rev./min at 4°C.

Fig. 1. Effects of various substances on the specific binding of [^3H]oestradiol in cytosol of rat 7,12-dimethylbenz[a]anthracene-induced mammary tumours

Samples of cytosol prepared from rat mammary tumours were labelled at 0°C with [^3H]oestradiol (0.5 nM) in the absence or presence of various other compounds, and analysed by sucrose-density-gradient centrifugation (from left to right). The sedimentation marker (arrows) was bovine serum albumin ($S_{20,w}$ 4.6S) in each case. (a) Cytosol labelled with [^3H]oestradiol alone (○) or in the presence of 50 nM oestradiol (●) or I.C.I. 85966 (■); (b) cytosol labelled with [^3H]oestradiol alone (○) [curve from (a) for comparison] or in the presence of 50 nM diethylstilboestrol (●) or dibutylidihydrostilboestrol (■); (c) cytosol labelled with [^3H]oestradiol alone (○) [curve from (a) as comparison] or in the presence of 50 nM I.C.I. 46474 (●) or I.C.I. 79792 (■); (d) cytosol labelled with [^3H]oestradiol alone (○) or in the presence of 50 nM I.C.I. 79792 (●) or I.C.I. 85966 (■).

Fig. 2. Effects of various substances on the specific binding of [3 H]oestradiol in cytosol of rat 7,12-dimethylbenz[a]anthracene-induced mammary tumours and human primary breast carcinoma

Soluble supernatants (cytosol) were prepared by centrifugation of crude homogenates at $100\,000g_{av}$ for 1 h at $3^{\circ}C$ in the SW50.1 (6×5 ml) swinging-bucket rotor (r_{av} 8.35 cm) in a Beckman L2-65B preparative ultracentrifuge.

Labelling of cytosol receptor proteins with [3H]oestradiol. Portions of cytosol (0.5–1.0 ml) were incubated for 1 h at $0^{\circ}C$ with [3H]oestradiol at a final concentration of 500 pM in the absence and presence of an excess of various anti-oestrogens (as detailed in the text). In some experiments, free and non-specifically bound steroid were removed from the cytosol by treatment with dextran-coated charcoal (0.5% Norit-A, 0.05% dextran T-70, sedimented from 1 ml of medium A).

Linear 5 ml (5–20%, w/v) sucrose density gradients in a uniform concentration of medium A were prepared as described by Martin & Ames (1961). Samples of 3H -labelled cytosol (400 μ l) were layered over the gradients and centrifuged at $100\,000g_{av}$ for 18 h at 3 – $4^{\circ}C$. One gradient in each set was layered with bovine serum albumin ($s_{20,w}$ 4.6S; 4 mg in 400 μ l) as a sedimentation marker. Gradients were fractionated by upward displacement by sucrose ($\geq 40\%$, w/v) and three-drop fractions collected and analysed as described by Davies & Griffiths (1973).

Results and Discussion

Analysis of 3H -labelled cytosol preparations from rat mammary tumours showed two peaks of protein-bound radioactivity corresponding to [3H]oestradiol-receptor complexes of approximate sedimentation coefficients 4S and 8S (Fig. 1a). The low capacity of the 8S peak was demonstrated by the displacement of radioactivity to the high-capacity 4S peak by the presence of a 100-fold excess of unlabelled oestradiol (Fig. 1a). Whereas a similar concentration (50 nM) of either diethylstilboestrol or dibutylidihydrostilboestrol produced an identical displacement (Fig. 1b), diethylstilboestrol, but not dibutylidihydrostilboestrol, caused some depression of the 8S peak at a concentration of 5 nM (Fig. 2). I.C.I. 46474 abolished the 8S peak at a concentration of 50 nM (Fig. 1c) and caused a significant depression at 5 nM (Fig. 2b). I.C.I. 79792 (50 nM) was not as effective as I.C.I. 46474 at the same concentration (Fig. 1c) and had no effect at 5 nM (Fig. 2b). The other nitrogen-mustard-containing compound, I.C.I. 85966, generally failed to diminish [3H]oestradiol binding to the 8S receptor (Fig. 1a), although it should be noted that in two of the rat mammary-tumour cytosols studied (Fig. 1d) this compound was more effective than I.C.I. 79792 and decreased specific binding of [3H]oestradiol almost as efficiently as diethylstilboestrol, dibutylidihydrostilboestrol and I.C.I. 46474. This effect may be due to differing affinities of receptors for oestrogen and anti-oestrogens in different tumour cytosols, or to some entirely independent factor.

Cytosol prepared from samples of human breast carcinoma and labelled with [3H]oestradiol (0.5 nM) also showed two peaks of radioactivity when analysed by sucrose-density-gradient centrifugation. Removal with charcoal of free and loosely bound steroid indicated, however, high-affinity binding in both the 4S and 8S peaks (Fig. 2c), as previously reported (McGuire & DeLaGarza, 1973). Preliminary studies showed that this specific binding could be decreased by a 50 nM concentration of either diethylstilboestrol, dibutylidihydrostilboestrol and I.C.I. 46474 (Fig. 2c), but only diethylstilboestrol was successful at 5 nM (Fig. 2d).

Diethylstilboestrol and I.C.I. 46474, therefore, were the two most active anti-oestrogens studied, whereas dibutylidihydrostilboestrol and I.C.I. 79792 were quite effective at higher concentrations. Dibutylidihydrostilboestrol may, however, prove useful in the clinical management of breast cancer, since it displays only 1% of the oestrogenicity of diethylstilboestrol (Grundy, 1957). This latter compound was more efficient than I.C.I. 46474 in displacing [3H]oestradiol from human breast-carcinoma receptor proteins, whereas the converse was true for rat mammary-carcinoma receptor proteins; this may be due to a species variation in the protein. However, I.C.I. 46474 has been used clinically in the management of breast cancer (Cole *et al.*, 1971; Ward, 1973).

The binding of oestradiol to receptor proteins has been postulated to occur by the initial attachment of the C-3 phenolic hydroxyl group to a highly specific site facilitating the attraction of the C-17 β -hydroxyl function to a less specific binding site (Hahnel & Twaddle, 1974). It is obvious that diethylstilboestrol and dibutylidihydrostilboestrol could also bind through the two phenolic hydroxyl groupings. The nitrogen-mustard derivative of diethylstilboestrol, I.C.I. 85966, may lose its ability to compete with [3H]oestradiol because of the dichloroethylcarbonyl grouping. It is possible that its occasional effect (Fig. 1d) may be due to some metabolic process resulting in the loss of these groups. It has been suggested, however, that the inhibition of specific [3H]oestradiol binding of I.C.I. 46474 is due to allosteric competition (Hahnel *et al.*, 1973). This would be brought about by the association of the aromatic *N*-ethyl ether grouping to a site on the receptor protein other than that specific for oestradiol. Whether the inhibition is of a simple competitive or allosteric type, replacement of the *NN*-dimethyl group by the *NN*-(2-chloroethyl) group to produce I.C.I. 79792 may decrease the affinity at this site and may account for the lower anti-oestrogenic properties of this compound.

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