British Journal of Pharmacology (1995) 116, 2391-2400

Comparison between novel steroid-like and conventional nonsteroidal antioestrogens in inhibiting oestradiol- and IGF-I-induced proliferation of human breast cancer-derived cells

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1 This study has two specific aims: (a) to compare the antioestrogenic activity of two steroidal analogues of 17β-oestradiol, the 7α-alkylamide, ICI 164,384 and the 7α-alkylsulphinylamide, ICI 182,780, with that of the triphenylethylene-derived compound 4OH-tamoxifen on a pool of human breast cancer cell lines (HBCCL) with a range of hormonal responsiveness and acquired anti-oestrogen resistance and (b) to investigate the ability of such antioestrogens to modulate the potent breast carcinoma growth-stimulatory activity of the 'IGF-I system'.

2 For the chemosensitivity investigations we used a long-term colorimetric and the short-term thymidine incorporation assay; we analysed IGF-I in conditioned media by a radioimmunoassay, IGF-I mRNA in the cells by RT-PCR and molecular species of IGF-I-binding proteins, secreted in conditioned media, by Western ligand blot. IGF-I receptors were assayed on cell monolayers by binding studies and by Scatchard analysis, we calculated K_D, B_{max} and sites/cell.

3 Our results indicate that ICI 182,780 and ICI 164,384 are 1.5-5.5 fold more potent than 4OH-tamoxifen in inhibiting the basal proliferation of oestrogen-receptor positive (ER+) breast cancer cell lines. Moreover we demonstrate the capacity of ICI 182,780 and ICI 164,384 to reduce, in a time-dependent fashion, oestrogen- and/or IGF-I-stimulated growth of ER+ cell lines, possibly by negatively interfering with an IGF-I-like material secretion and IGF-I-receptor number.

4 Our data provide the first evidence that, on ER+ human breast carcinoma cell lines, steroidal antioestrogens inhibit cell growth and modulate the IGF-I mitogenic system. The mechanism of this latter effect has yet to be identified.

Keywords: Human breast cancer; oestrogens; antioestrogens; growth factors

Introduction

Breast cancer is one of the main causes of cancer-related deaths among women in western countries, with an age-standardized incidence of 60-90 per 100,000 (Tomatis *et al.*, 1990). A clearer understanding of the biological processes involved in the progression of this neoplasm would be of benefit in the identification of new targets for breast cancer therapy. Breast cancer cells express receptors for and are sensitive to a variety of steroids, polypeptide hormones (McGuire *et al.*, 1975; Osborne & Arteaga, 1990) and growth factors (Cullen *et al.*, 1989). In addition, breast cancer cells synthesize and secrete growth factors acting via autocrine and/or paracrine mechanisms. Transforming growth factors (Knabbe *et al.*, 1987; Bates *et al.*, 1988) and cathepsin D (Vignon *et al.*, 1986) have been reported to act as autocrine mitogens in breast cancer cell lines. Platelet-derived growth factor, thought to be synthesized by tumour cells, appears to have paracrine effects on stromal cells stimulating them to synthesize other growth factors for the epithelial cancer cells (Freiss *et al.*, 1990). Insulin-like growth factor-I (IGF-I) has been reported to be produced by a variety of tumour cell types (Nakanishi *et al.*, 1988) and, via binding to its specific membrane receptor, it could be directly or indirectly involved in the control of cell proliferation (Favoni *et al.*, 1994). In human breast cancer, although IGF-I is not produced by epithelial cells, it may function as a paracrine stimulator (Yee *et al.*, 1989; Stewart *et al.*, 1992). The interaction of IGF-I with its receptor (Rosen *et al.*, 1991) as well as its biological activity are probably modulated by the specific IGF binding proteins (BPs) to which the growth factor is complexed in plasma and other biological fluids. The precise role of these BPs in IGF-I physiology is still unclear; however, at the cellular level, IGF-BPs have been described as having both inhibitory and stimulatory effects (Elgin *et al.*, 1987). In the initial stages of breast cancer development, cell proliferation is under hormonal control: in oestrogen-responsive cells, oestrogens induce a variety of biosynthetic processes that lead to increased cell growth. The mechanisms by which oestrogens stimulate cell proliferation are not yet completely understood. Recent studies by Stewart and colleagues (Stewart *et al.*, 1990; 1992) demonstrate that oestrogens can influence the synthesis of growth factors, such as IGF-I, and their receptors, suggesting an indirect mechanism of action of oestrogen-stimulated growth. The blockade of and/or the interference with at least one of these biochemical pathways could represent a new targeted therapeutic strategy for the management of human breast cancer. At present, the use of the cytostatic rather than cytotoxic agent, tamoxifen, is the most common hormonal therapy in the management of breast cancer. The triphenylethylene-derived tamoxifen compound has been shown to be an effective treatment for both pre- and post-menopausal women with all stages of the disease and gives partial responses in patients with advanced disease. Moreover, significant increases in both disease-free and overall survival occur with adjuvant tamoxifen therapy (Rutqvist *et al.*, 1987; Baum *et al.*, 1988). It has also been reported that treatment with tamoxifen causes a reduction in IGF-I plasma levels but it enhances the level of IGF-BP1 in breast cancer patients (Lien *et al.*, 1992; Lonning *et al.*, 1992; Friedl *et al.*, 1993). In comparison, using the active metabolite of tamoxifen, 4-hydroxy-tamoxifen (4-

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OH-tamoxifen), Reed *et al.*, (1992) found no significant effect on either IGF-I and IGF-BP1 serum levels. Recently, experimental and clinical investigations have also suggested a potential and promising role of tamoxifen treatment in the prevention of breast cancer (Powles *et al.*, 1990; Nayfield *et al.*, 1991) showing the ability of tamoxifen to prevent development of contralateral breast tumours in both pre- and post-menopausal patients in randomized clinical trials. These observations indicate that tamoxifen may be an effective preventive option for women at increased risk of neoplasm. Unfortunately, tamoxifen and 4-OH-tamoxifen retain partial agonist activity on the oestrogen receptor both *in vitro* (Thompson *et al.*, 1988) and *in vivo* (Litherland & Jackson, 1987). In addition, the use of these compounds is limited by the possible development of acquired tamoxifen-resistance in many breast cancer patients. Some of these tamoxifen-resistant tumours remain sensitive to alternative hormonal manipulations, while others became refractory to any endocrine therapy (Muss, 1992). Tamoxifen-resistance could be due to several mechanisms: alteration in systemic metabolism or in drug uptake and/or excretion; modification or loss of the oestrogen-receptor as well as selection of hormone-independent breast cancer sub-clones (Osborne *et al.*, 1992; Wolf & Jordan, 1993). These observations led chemists and investigators to synthesize and study new molecules such as ICI 164,384 (Bowler *et al.*, 1989) and ICI 182,780 (Wakeling *et al.*, 1991) which bind the oestrogen receptor (ER) with high affinity, but without activating the normal transcriptional hormone responses.

Here we have compared the non-steroidal 4-OH-tamoxifen molecule with the prototype steroidal antioestrogen, ICI 164,384 and the novel ICI 182,780 in inhibiting 17β-oestradiol-(17β-E_2) and/or IGF-I-induced cell proliferation, using a series of established human breast carcinoma-derived cell lines. These cells were selected on the basis of the presence of oestrogen receptors, oestrogen-responsiveness and acquired antioestrogen resistance. Using an *in vitro* model which represents some of the peculiar characteristics of breast cancer, we have demonstrated a negative modulation of the IGF-I-growth-regulatory system by the oestrogen antagonist steroid-like molecule, ICI 182,780.

Methods

Cell Lines

The MCF-7 oestrogen-receptor positive (ER+) and oestrogen-dependent cell line was a kind gift of Dr Guy Leclercq (Institute J. Bordet, Brussel, Belgium). The stepwise *in vitro* selected MCF-7-variant LCC2 (ER+, oestrogen-independent, 4-OH-tamoxifen-resistant), the selected and cloned MCF-7-variant LY2 (ER+, oestrogen-dependent, 4-OH-tamoxifen- and ICI 164,384-resistant) and the MDA-MB231 (ER−) cell lines as well as the HBL100, an apparently normal human epithelial-like, non-tumorigenic cell line (Caron de Fromentel *et al.*, 1985) were obtained from Dr Marc E. Lippman and Dr Robert Clarke (Georgetown University, V.T. Lombardi Cancer Center, Washington DC, U.S.A.). CHP-100, a neuroepithelioma cell line used as a positive control in IGF-I mRNA detection by RT-PCR, was obtained from Dr Douglas Yee (University of Texas at San Antonio, San Antonio, TX, U.S.A.) and the neuroblastoma Lan-5 cell line, used as a negative control in the same RT-PCR, was kindly donated by Dr GianPaolo Tonini (Advanced Biotechnology Center, Genova, Italy). All breast cell lines (except LCC2) were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated foetal calf serum (HI-FCS), 2 mM glutamine, antibiotics (0.02 iu ml^{-1} penicillin, 0.02 $\mu g\ ml^{-1}$ streptomycin) and 1% non-essential aminoacids. The LCC2 cell line was grown in phenol-red (Phr)-free DMEM with 5% of dextran charcoal-steroid depleted (DCS)-FCS, glucose (3.5 g l^{-1}) and supplemented with glutamine and antibiotics as above. For preparation of conditioned media (CM) cells were grown in serum-free medium (SFM) which consisted of Phr-free DMEM supplemented with 20 mM HEPES, 2 mg l^{-1} transferin, 292 mg l^{-1} glutamine and 1% trace elements.

Chemosensitivity to antioestrogen compounds

The cell response to antioestrogens was evaluated with the simple and rapid MTT colorimetric assay, which measures cellular proliferation and survival (Carmichael *et al.*, 1987). Exponentially growing cells from subconfluent cultures were harvested by brief trypsinisation, washed twice and resuspended in the appropriate culture medium. Single cell suspensions were obtained by gentle aspiration through a 22G hypodermic needle; cells were then counted by trypan blue exclusion and plated, in quadruplicate, in 96-well microplates at a density of 2000–6000 cells/well in 200 μl of Phr-free DMEM + 5% (DCS)-FCS. In preliminary experiments, the cell density and the growth kinetics of each cell line were examined. Depending on the cell growth rate (doubling time), the cell size and duration of the experiment, we selected the optimal cell number so that the control cultures would remain in exponential growth phase during the entire experiment. Following overnight incubation at 37°C in a 5% CO_2 atmosphere, spent medium was removed and replaced with fresh medium containing the appropriate concentrations of the test compounds which were chosen on the basis of preliminary dose-response assays. For the growth inhibition studies we used concentrations of 4-OH-tamoxifen, ICI 164,384 (Gong *et al.*, 1992) or ICI 182,780 (Wakeling *et al.*, 1991) ranging from 0.01 to 100 nM. Antagonistic activity of the antioestrogens on 17β-E_2 (1 nM) (Gong *et al.*, 1992) and IGF-I (10 nM) (Karey & Sirbasku, 1988), used alone or in combination, was evaluated using 4-OH-tamoxifen, ICI 164,384 and ICI 182,780 at a 100 nM final concentration. After 48, 72 and 96 h of treatment, 50 μl of 2 mg ml^{-1} of MTT stock solution was added to each well for an additional 4 h at 37°C. The plates were then centrifuged at 450 *g* for 10 min and the medium was removed. Dimethylsulphoxide (DMSO, 150 μl) was added to each well and the plates were incubated for an additional 10 min at 37°C. The absorbance was measured in an automated microplate reader spectrophotometer (SLT-Labinstruments, Austria), at 540 nm.

Effects of antioestrogens on DNA synthesis

The evaluation of the effect of antioestrogens in inhibiting 17β-E_2- and/or IGF-I-induced cell proliferation was measured by thymidine incorporation as previously described (Favoni *et al.*, 1994). Viable single cell suspensions, obtained as above, were plated in triplicate in 16 mm diameter multiwell plates at a density of $30-80 \times 10^3$ in 1 ml of 5% (DCS)-FCS Phr-free DMEM and allowed to attach at 37°C and 5% CO_2. After 24 h the medium was removed and replaced with the appropriate concentrations of drugs (see previous paragraph) for an additional 24 h.

Conditioned media

Cells were cultured in 5% DCS-FCS Phr-free DMEM in T175 plastic flasks for 3 days. At 70–80% confluence, monolayers were washed twice with PBS, SFM was added and cells were incubated at 37°C in humidified atmosphere. After 24 h, SFM was removed and replaced with fresh SFM containing 1 nM 17β-E_2 or 100 nM ICI 182,780. After 48 h, protease inhibitors (100 μM PMSF; 0.5 $\mu g\ ml^{-1}$ leupeptin and 100 nM pepstatin-A) were added, the conditioned medium (CM) was collected on ice, clarified by centrifugation and concentrated by ultrafiltration at 4°C as previously described (Favoni *et al.*, 1994).

Influence of antioestrogens on secretion of IGF-I material

IGF-I is bound to several high affinity binding proteins (BP) which could interfere in the immuno-radiometric assay for its

quantitation in conditioned media. The addition of heparin in the radioimmunoassay (RIA) buffer provokes the dissociation of IGF-I from its specific BPs and allows a more accurate evaluation of the polypeptidic growth factor (Furlanetto & Marino, 1987). The non-equilibrium RIA of heparin-treated and non-treated CM, using Pansorbin-A (Pan-A) precipitation to separate antibody-bound and free IGF-I material, was performed using an anti-IGF-I rabbit polyclonal antibody (UBK 487) at the final dilution of 1 : 18,000. hr-IGF-I was used for the standard curve (0.025–4.00 ng ml^{-1}). Appropriate quantities of standards and unknowns, diluted with 300 μl RIA buffer (0.03 M $NaHPO_4$, 0.25% w/v RIA grade BSA, 0.02% w/v Na azide, with and without 1.0 u ml^{-1} Na-heparin respectively, pH = 7.4), were added to 50 μl of antiserum in polypropylene tubes and incubated for 96 h at 4°C. After incubation, 100 μl of iodinated IGF-I ($\approx$20–25000 c.p.m.) was added and the incubation continued overnight at 4°C. Antibody-bound and free [^{125}I]-IGF-I were separated by precipitation with 100 μl of Pan-A (1 : 10 diluted in RIA buffer) after centrifugation at 13,000 r.p.m. for 10 min at 4°C; the radioactivity in the pellet was then determined, in duplicate, in a Beckman 5500 B γ-counter.

IGF-I mRNA detection by reverse-transcription polymerase chain reaction

The mRNA from all cell lines under study and controls was isolated utilising the QuickPrep Micro mRNA Purification kit (Pharmacia S.p.A.) and quantitated spectrophotometrically. Two hundred ng of mRNA from each sample was reverse-transcribed using random hexanucleotides as primers following the manufacturer's instructions (Gene Amp RNA kit, Perkin Elmer). Polymerase chain reaction (PCR) amplification followed directly using the mRNA-cDNA hybrid as a template and as upper primer 5′-GGAAAAATCAGCAGTCTTCCAACC-3′ and lower primer 5′-GCAAT-ACATCTCCAGCCTCCTTAG-3′ (made on a 391 PCR-MATE DNA Synthesizer, Applied Biosystems). These primers were based on the sequence of the human IGF-I gene and identify a fragment of 324 basepairs (bp) located from bp 170 to 493. The amplification was performed over 40 cycles of melting (94°C, 30 s), annealing (58°C, 30 s) and extension (72°C, 1 min) with an additional final extension step of 12 min in a System 2400 Gene Amp PCR machine (Perkin Elmer). The PCR amplification product was analysed by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and photographed.

Western ^{125}I-labelled IGF-I ligand blot for binding proteins analysis

Western ligand blotting was used to detect the presence of IGF-BPs, synthesized and secreted in the cell culture medium, on the CM prepared as described above. At the time of CM collection, the cell number (determined by counting in a haemocytometer) and the total protein in the CM (determined by the Bradford protein assay) were measured so that the quantity of sample loaded per lane could be normalized. Proteins were resolved on a 15% sodium dodecyl-sulphate-polyacrylamide gel-electrophoresis (SDS-PAGE), along with pre-stained molecular-weight standards; 300 ng of purified IGF-BP1, as an additional specific marker, and CM alone were also loaded on the gel. After transfer of proteins to nitrocellulose and hybridisation with radiolabelled IGF-I, as previously described (Favoni *et al.*, 1994), the blots were exposed to X-ray film for 1 and 6 days at −80°C. Relative radiolabel in each band was determined by scanning the autoradiograph with an LKB Ultroscan XL laser densitometer (Pharmacia/LKB - Uppsala, Sweden) and expressed in arbitrary units.

Influence of antioestrogens on IGF-I binding to its receptor

For the binding studies, $2-3 \times 10^5$ cells, which had been previously treated for 2 days with antioestrogens (100 nM) or 17β-E_2 (1 nM), were plated in duplicate in 24 well-multiwell plates in 2 ml of 5% (DCS)-FCS Phr-free DMEM in the presence of the agents. After 24 h incubation at 37°C and 5% CO_2 atmosphere, the monolayers were washed and incubated for an additional hour with binding buffer (0.1% BSA, 40 mM HEPES in DMEM). Cells were then incubated with gentle agitation for 2 h at 4°C with radiolabelled IGF-I ($\approx$20,000 c.p.m./well of [^{125}I]-IGF-I) and increasing amounts of unlabelled peptide (1.95–400 ng ml^{-1}). After binding, cells were washed three times with ice-cold washing buffer (0.1% BSA in HBSS) and the monolayers were lysed with solubilizing buffer (20 mM HEPES, 1% Triton X-100, 10% glycerol) for 30 min at 37°C. The radioactivity released was measured in a Beckman 5500 B γ-counter. Specific binding, after subtraction of non-specific binding determined in the presence of excess of unlabelled growth factor (400 ng ml^{-1}), was calculated. The dissociation constant (K_D), the maximum binding capacity (B_{max}) and receptor-sites/cell, in the presence or absence of antioestrogens or 17β-E_2, were determined by Scatchard analysis.

Drugs and chemicals

The antioestrogen: *trans*-4′-hydroxy metabolite of tamoxifen (ICI 46,474: *trans*-1-(4β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene); ICI 164,384 {N-n-butyl-*N*-methyl-11-[3,17β-dehydroxyoestra-1, 3, 5 (10) - triene - 7α-yl] undecanamide} and ICI 182,780 {7α-[9-(4,4,5,5,5-pentafluoropentasulphinyl) nonyl]oestra-1,3,5(10)triene-3,17β-diol} were donated by Dr Alan E. Wakeling, Zeneca Pharmaceuticals (formerly ICI-Pharma) (Macclesfield, U.K.); 17β-oestradiol (17β–E_2) was purchased from Sigma (St. Louis, MO, U.S.A.). Stock solutions of these compounds were prepared in absolute ethanol, stored at −20°C and diluted in appropriate medium as required. Lyophilized pure human recombinant insulin-like growth factor-I (hr-IGF-I), from Amgen Biologicals (CA, U.S.A.), was reconstituted in 0.1 N acetic acid, aliquoted at 10 μg 100 μl^{-1} and stored at −20°C. 3-[4,5-dimethylthiazol - 2 - yl] - 2, 5 - diphenyl tetrazolium bromide (MTT), phenyl-methyl-sulphonyl fluoride (PMSF), leupeptin and pepstatin-A were purchased from Sigma. [Methyl-^{3}H]-thymidine ([^{3}H]-dThd) (TRA120, specific activity, Sp.Act., = 5 Ci $mmol^{-1}$, 185 GBq $mmol^{-1}$) was reconstituted with phosphate buffered saline (PBS), aliquoted at 160 μCi ml^{-1} and stored at 4°C as well as radiolabelled [^{125}I]-IGF-I (IM172, Sp.Act. $\approx$ 2000 Ci $mmol^{-1}$, 74 TBq $mmol^{-1}$), reconstituted with 0.1 N acetic acid, aliquoted at 2 μCi 20 μl^{-1} and stored at −20°C, were purchased from Amersham Int. (U.K.). The anti-IGF-I rabbit polyclonal antibody (UBK 487) was acquired from the National Hormone and Pituitary Program distributed by the Hormone Distribution Program of the National Institute for Diabetes, Digestive and Kidney Diseases (Bethesda, MD U.S.A.). Pansorbin cells (*Staphylococcus aureus*) were from Calbiochem (La Jolla, CA, U.S.A.). Bovine albumin RIA-grade, was obtained from Sigma. Tissue-culture medium, 4-(2-hydroxy-ethyl)-1-piperazine ethane sulphonic acid buffer (HEPES) and Dulbecco's PBS were obtained from ICN (Milan, Italy), Dulbecco's medium without phenol-red and foetal calf serum (mycoplasma screened) as well as trace elements were acquired from GIBCO (Milan, Italy). Tissue-culture plasticware was purchased from Becton-Dickinson (Parsippany, NJ, U.S.A.) and from Greiner (Nutrigen, Germany).

Statistical analysis of data

Results are given as a mean of percentages ± s.e.mean of three experiments performed in quadruplicate (MTT), triplicate ([^{3}H]-dThd) and duplicate (RRA and RIA). Analysis of variance between treated and untreated groups was evaluated by the non-parametric Wilcoxon-test.

Results

Cell growth studies

Inhibition of cellular proliferation after treatment with these antioestrogens at concentrations ranging from 0.01 to 100 nM was evaluated by the long-term MTT assay (Figure 1). After 96 h of treatment, 4-OH-tamoxifen produced only a marginal inhibition of MCF-7, LCC2, LY2 and HBL100, whereas ICI 164,384 and ICI 182,780 caused a significant (P ranging from 0.003 to 0.001), dose-dependent decrease of MCF-7, LCC2 and LY2 cell proliferation. At the higher doses, the novel steroid-like antioestrogen, ICI 182,780 was more potent in inhibiting LCC2 cell growth (range 12–47%) than that of the LY2 cell line (range 6–29%). Cell growth inhibition evaluated after 48 and 72 h (not shown) of drug treatment showed similar behaviour to that found at 96 h. In the short-term [^{3}H]-dThd incorporation assay, 4-OH-tamoxifen showed a dose-dependent inhibition of cell proliferation after 24 h of MCF-7 (45% inhibition at 100 nM) and LY2 (36% inhibition at 100 nM) cell lines. Twenty-four hours of treatment with ICI 164,384 and ICI 182,780 caused a significant ($P=0.001$) dose-dependent growth inhibition in all ER+ cell lines (ranging from 40–70% at 100 nM), whereas no effect was observed in the MDA-MB231 and HBL100 lines (data not shown).

The ability of these antioestrogens to inhibit 17β-E_2-and/or IGF-I-induced cell proliferation was also assayed using [^{3}H]-dThd incorporation (Figure 2) and MTT assays (data not shown). Proliferation was significantly ($P=0.001$) stimulated only in the MCF-7 and LY2 cell lines by treatment with 1 nM 17β-E_2 (50 and 43%, respectively) and IGF-I (65 and 23%, respectively) alone or in combination (116 and 45%, respectively). MCF-7 was the most sensitive cell line to oestrogen and growth factor stimulation, showing additive effects. Treatment with 100 nM 4-OH-tamoxifen inhibited 17β-E_2-induced cell growth in these two cell lines (Figure 2). The two 'pure' steroidal antioestrogens, ICI 164,384 and ICI 182,780, used at 100 nM, provoked an even stronger growth inhibition on 17β-E_2-stimulated proliferation on MCF-7 (75 and 80%, respectively) and LY2 (78 and 83%, respectively) cell lines and also showed significant inhibition of LCC2 (25 and 35%, respectively) cell growth. Similar results were observed with IGF-I-induced cell proliferation on all ER+ cell lines (Figure 2). However, the antioestrogens were more potent in affecting 17β-E_2- or combined 17β-E_2- and IGF-I-stimulated cell proliferation than in inhibiting growth induced by IGF-I alone. No drug effects were observed in the MDA-MB231 and HBL100 cell lines (Figure 2). These experiments do not show substantial differences in potency between ICI 164,384 and ICI 182,780, whereas they confirm that 4-OH-tamoxifen is significantly ($P=0.001$) less effective than the two steroid-like molecules.

Evaluation of IGF-I immunoreactive material secreted in cell culture media

We analysed the secretion of IGF-I material in the 30× concentrated conditioned media of cells incubated for 48 h in presence of 17β-E_2 (1 nM) and ICI 182,780 (100 nM) (Table 1). 17β-Oestradiol enhanced IGF-I material secretion only in MCF-7 and LY2 cell lines (20 and 41%, respectively). Treatment with ICI 182,780 caused a marked decrease in peptide production by MCF-7 (54%) and LCC2 (46%) cell lines, whereas it only slightly reduced (14%) production by the 4-OH-tamoxifen- and ICI 164,384-resistant LY2 cell line. Exposure of MDA-MB231 cells to ICI 182,780 had no effect on IGF-I material secretion, whereas it faintly enhanced its production (12%) by HBL100 cells. Samples in the absence of heparin showed similar behaviour (data not shown).

IGF-I mRNA detection in cultured cell lines

Oligonucleotide primers specific for the IGF-I mRNA amplified the expected 324 bp product from CHP-100 cells, which have previously been shown to contain IGF-I mRNA (Yee *et al.*, 1989). However, no amplified product was observed in cDNA derived from mRNA isolated from all the breast carcinoma cell lines, indicating that essentially no IGF-I mRNA is

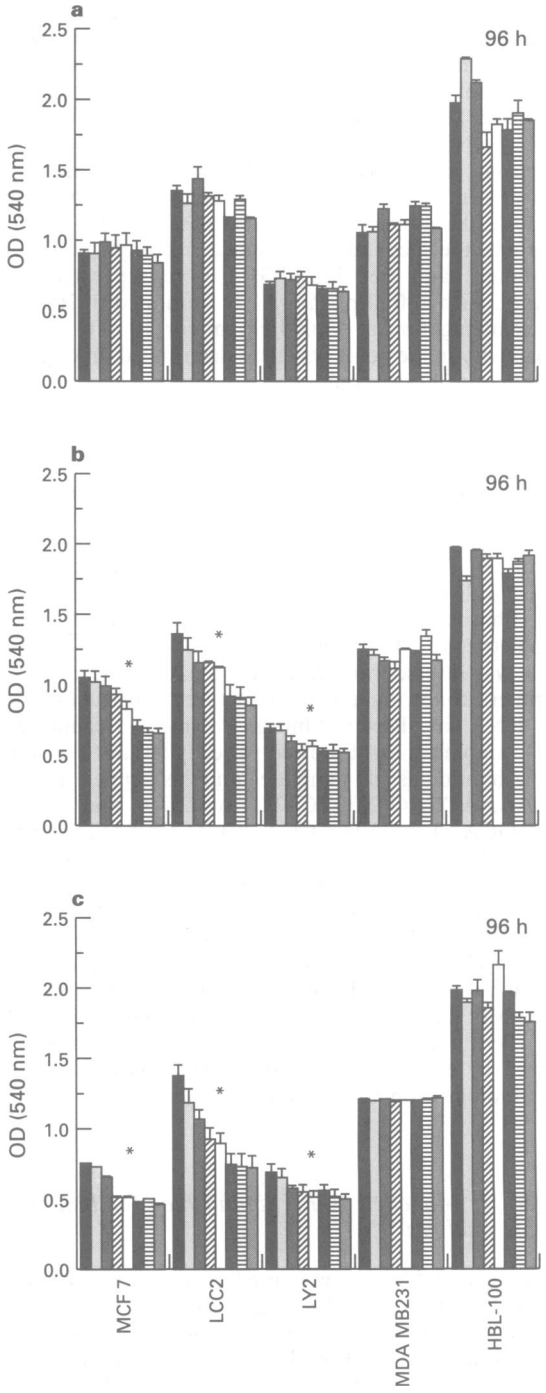

Figure 1 Effect of (a) 4-OH-tamoxifen (b) ICI 164,384 and (c) ICI 182,780 on the proliferation of oestrogen-dependent MCF-7, LY2; oestrogen-independent LCC2, MDA-MB231 and normal HBL-100 cells cultured in Phr-free DMEM + 5% (DCS)-FCS; $2-6\times10^3$ cells/well were plated in 96-well microplates and treated the next day (day 0) with 0.01 (■), 0.1 (■), 0.5 (▨), 1.0 (□), 5.0 (■), 10 (▤) and 100 (■) nM 4-OH-tamoxifen, ICI 164384 or 182,780. (■) = untreated cells. Cells from quadruplicate wells were added with 50 μl of 2 mg ml^{-1} MTT stock solution for 4 h at 37°C on days 2, 3 and 4. Data, expressed as % of control, represent the optical density (OD) (absorbance) which was measured at 540 nm in a spectrophotometer. Standard errors, indicated on the columns, were ranging from 0.1 to 3%; *Significant values (P values ranging from 0.001 to 0.003).

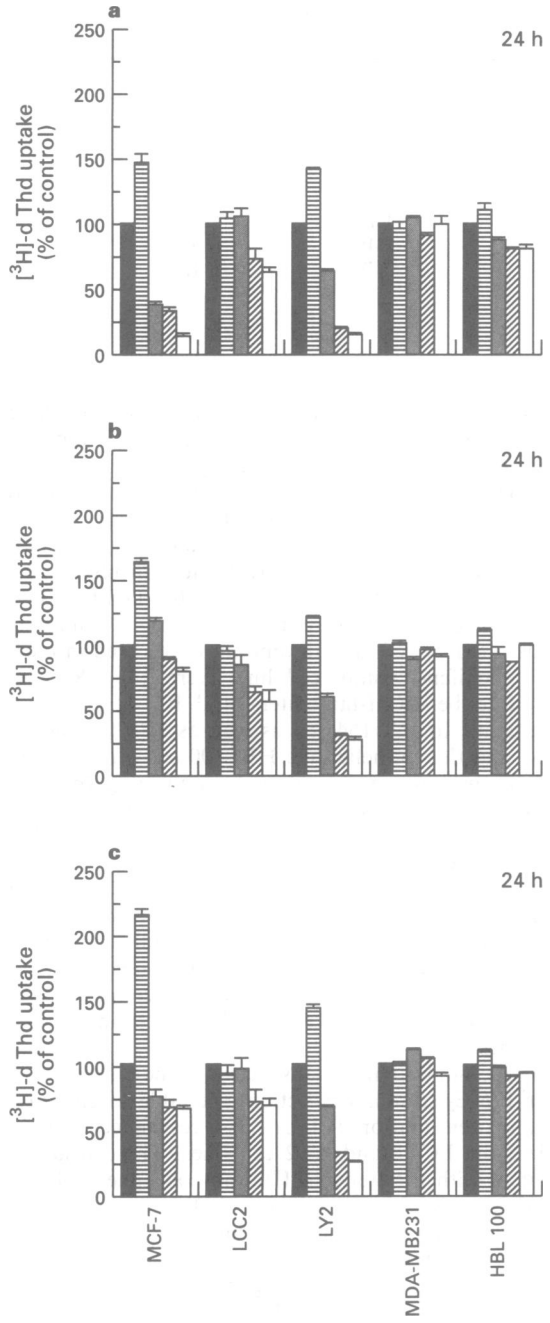

Figure 2 Effect of 4-OH-tamoxifen, ICI 164,384 and 182,780 alone or in combination with oestradiol (a), IGF-I (b) and oestradiol + IGF-I (c) on thymidine incorporation of human breast cancer and normal cell lines. (■ control; ▤ (a) 17β-E_2; (b) IGF-I; (c) 17β-E_2 + IGF-I; ▩ (a) 17β-E_2 + 4-OH-tamoxifen; (b) IGF-I + 4-OH-tamoxifen; (c) 17β-E_2 + IGF-I + 4-OH-tamoxifen; ▨ (a) 17β-E_2 + ICI 164,384; (b) IGF-I + ICI 164,384; (c) 17β-E_2 + IGF-I + ICI 164,384; □ (a) 17β-E_2 + ICI 182,780; (b) IGF-I + ICI 182,780; (c) 17β-E_2 + IGF-I + ICI 182,780). Thymidine incorporation into cellular DNA was measured by incubating various numbers of cells (range $3-8 \times 10^4$) with appropriate concentrations of drugs for 24 h and pulsing them for 2 h with 2 μCi ml^{-1} of [^{3}H]-dThd. Each column represents the mean ± s.e.mean of three replicates. Standard errors were all lower than 10%.

produced by these cells (Figure 3). This is in contrast to the result of IGF immunoreaction in RIA on CM from these cell lines, suggesting that these cells could be secreting immunoreactive IGF-I-like material or closely related peptides but not authentic IGF-I.

Ligand blot analysis

To determine whether ICI 182,780 modulates IGF-BP species secreted by ER+ and ER-HBCCL, ligand blotting was performed on aliquots of 30 × concentrated conditioned medium, normalised for total protein concentration, from 100 nM ICI 182,780-treated and -untreated cells. Ligand blot analysis showed that all tested breast cancer cell lines secreted a different pattern of multiple forms of IGF-BPs (Figure 4). Specifically, the ER positive MCF-7 tumour cell line secreted predominantly 24 and 34 kDa molecular weight species corresponding to the IGF-BP4 and -BP2 forms as well as a 29–30 kDa doublet which is most likely IGF-BP5 (Figure 4a, lane 1). The antioestrogen-resistant MCF-7-variants, LCC2 and LY2, secreted IGF-BP4 and -BP2 forms in smaller quantities and no IGF-BP5 (Figure 4a, lanes 4 and 7 respectively). The larger molecular weight (42–46 kDa) binding protein, known as IGF-BP3, is not displayed by either parental MCF-7 subclone and MCF-7-derived LCC2 and LY2 cells. The ER negative MDA-MB231 cells secreted three forms of IGF-BPs: IGF-BP4 (24 kDa), IGF-BP1 (30 kDa) and IGF-BP3 (42–46 kDa) (data not shown). Quantitative evaluation of IGF-BPs through scanning densitometry reveals that 48 h of 1 nM 17β-oestradiol exposure caused a reduction in two out of three BPs expressed by MCF-7 which was minimal for BP4 (12%) and more consistent for BP5 (31%) and in BP2 secreted by LY2 cells (22%) (Figure 4a, lanes 2 and 8 respectively, and 4b). In comparison, oestradiol provoked an increase of BP4 secretion in LY2 (31%) (Figure 4a, lane 8 and 4b) and an enhancement of expression of both BP4 and BP2 in LCC2 cell lines (8 and 3 fold, respectively) (Figure 4a, lane 5 and 4b). After 48 h of exposure to 100 nM ICI 182,780 a decrease below the basal level of all three BPs (IGF-BP4, -BP5 and -BP2) secreted by MCF-7 cells (Figure 4a, lane 3) (78, 99 and 52% respectively) (Figure 4b) and IGF-BP4 and -BP2 by LY2 cells (Figure 4a, lane 9) (60 and 37%, respectively) (Figure 4b) was observed. In contrast, ICI 182,780 strongly increased the IGF-BP4 and -BP2 secretion by the LCC2 cell line (Figure 4a, lane 6) (26 and 91%, respectively) (Figure 4b).

In the ER− MDA-MB231 as well as in the pseudonormal HBL-100 cell lines no consistent up and/or down regulation of IGF-BPs was observed after 17β-E_2 or ICI 182,780 treatment (data not shown).

Binding studies

Treatment for 72 h with 100 nM ICI 182,780 strongly inhibited [^{125}I]-IGF-I binding to cell surface receptors on the MCF-7 (47%), LYZ (41%) and LCC2 (35%) cell lines, whereas 1 nM 17β-E_2 only slightly increased it on the MCF-7 and LCC2 cell lines (Figure 5). No variations were observed on MDA-MB231 and HBL100 (data not shown). Table 2 presents the results of Scatchard analysis of the binding data performed with a Binding Analysis Computer Program (McPherson, 1985). After 2 h of co-incubation with labelled and unlabelled IGF-I, values of the dissociation constant (K_D) in untreated (K_D ranging from 0.29 to 0.85 nM), 17β-E_2-treated (K_D ranging from 0.29 to 1.10 nM) and drug-treated samples (K_D ranging from 0.31 to 0.90 nM) remained essentially identical. Differences were observed in maximum binding capacity (B_{max}) values and in the number of sites/cell. 17β-E_2 was able to enhance the B_{max} value only in LCC2 (54%) and MCF-7 (43%). In these cell lines an increase of IGF-I cellular binding sites (24% in LCC2 and 22% in MCF-7) was also observed. In contrast, antioestrogen treatment caused a strong decrease in the B_{max} value (ranging from 37 to 78%) and in the number of binding sites (ranging from 22 to 78%) in MCF-7, LCC2 and LY2 cell lines. Binding assays, performed using the older steroid-like compound ICI 164,384, showed that its potency in IGF-I binding inhibition, in all ER+ cell lines, is similar to that of the newer ICI 182,780 (data not shown). By comparison, on MCF-7 cell line, the widely used non-steroidal 4-OH-tamoxifen appeared to be a less effective IGF-I binding inhibitor

Table 1 Effect of 17β-oestradiol (1 nM) and ICI 182,780 (100 nM) on the secretion of IGF-I-like peptide in the conditioned medium of ER+, ER− human breast cancer and normal cell lines

	IGF-I like-material ng ml^{-1} (1 × CM)				
Cell lines	*Control*	*17β-E2* (1 nM)	*%* (of control)	*ICI 182,780* (100 nM)	*%* (of control)
MCF-7	1.95	2.35	+20	0.90	−54
LCC2	1.76	1.41	−20	0.95	−46
LY2	1.30	1.84	+41	1.12	−14
MDA-MB231	2.38	1.47	−38	2.45	+3
HBL100	3.36	2.90	−14	3.78	+12

Data represent the means of 2 experiments (s.e. range: 1–12%).

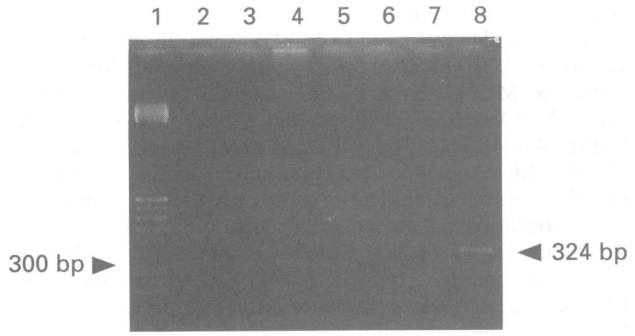

Figure 3 Reverse-transcriptase analysis of IGF-I mRNA content in breast cancer cell lines. Lane 1 = 100 base pair ladder marker (Pharmacia, S.p.A.). Lane 2 = MCF-7, lane 3 = LCC2, lane 4 = LY2, lane 5 = MDA-MB231, lane 6 = HBL-100, lane 7 = LAN-5 neuroblastoma cells (negative control), lane 8 = CHP-100 neuroepithelioma cells (positive control). The expected amplified product was observed only in the positive control cells. The other bands present are non-specific amplified material.

(≈1.5 fold). Finally, no modulation of IGF-I binding by 4-OH-tamoxifen was observed on the two antioestrogen-resistant LCC2 and LY2 cell lines (data not shown).

Discussion

Tamoxifen, the conventional non-steroid antioestrogen, represents the treatment of choice for the endocrine therapy of advanced breast cancer. This compound competes efficiently for oestrogen receptor binding, yet retains partial agonist activity. To circumvent this, new molecules with steroid-like structure, commonly identified as 'pure' antioestrogens, that conceptually should be devoid of any oestrogenic activity, have been synthesized. Representative of these are ICI 164,384 and ICI 182,780, which are characterized by an alkylamine side-chain at the 7α-position of the B ring in the steroid. ICI 182,780 differs from ICI 164,384 in having fluorine atoms in the side chain which enhance the solubility of the antioestrogen and also impart an increase in affinity for the oestrogen receptor (Wakeling *et al.*, 1991). Parker (1993) showed that 'pure' antioestrogens, in contrast to 4-OH-tamoxifen, increase oestrogen receptor turnover and suggested that this phenomenon is a consequence of impaired dimerisation of the receptor protein. Moreover, the author proposed that this inhibition of dimerisation could be responsible for the lack of nucleo–> cytoplasmic oestrogen-receptor shuttling observed with ICI 182,780 or ICI 164,384 treatment. Thus, in the absence of this nuclear uptake, the oestrogen receptor could be more rapidly degraded, probably by lysosomes.

In this paper we compare the activity of 4-OH-tamoxifen with that of ICI 164,384 and ICI 182,780 in inhibiting breast cancer cell proliferation. As a model system, we used several human breast cancer cell lines whose features (presence or lack of oestrogen receptors, oestrogen responsiveness, antioestrogen resistance) represent typical characteristics of breast cancer in patients. The steroidal-like compounds showed higher potency (ranging from 1.5 to 5.5 fold) than 4-OH-tamoxifen in inhibiting cell growth as evaluated by short- and long-term assays. This effect was more evident after 24 h of antioestrogen exposure than after longer treatment periods (48, 72 and 96 h). The non-steroidal antioestrogen showed only a marginal effect on cell growth inhibition, in agreement with results obtained by Mullick & Chambon (1990). 4-OH-tamoxifen resistance was overcome by both the 'pure' antioestrogen compounds, as demonstrated by cell growth inhibition observed in the LCC2 4-OH-tamoxifen-resistant cell line. Here, the LY2 cell line, reported to be 4-OH-tamoxifen- and ICI 164,384-resistant, was sensitive to ICI 164,384 as well as ICI 182,780. MDA-MB231 and the epithelial-like HBL100 cell lines were not affected by antioestrogen treatment, confirming that the presence of oestrogen receptor is necessary for antioestrogenic activity, and that the effect of the antisteroids on ER+ cell lines was not due to non-specific cytotoxicity. Our findings are in agreement with those of Wakeling *et al.* (1991) who reported a higher potency of ICI 164,384 and ICI 182,780 as compared to 4-OH-tamoxifen in cell proliferation studies on MCF-7 cell lines. However, we have not observed the substantially higher growth inhibitory potency of ICI 182,780 compared with ICI 164,384 reported, perhaps due to differences in the technical approaches used.

ICI 182,780, both in the absence or in the presence of IGF-I and/or 17β-E_2 stimulatory action, was able to circumvent 4-OH-tamoxifen and/or ICI 164,384 resistance which characterizes the LCC2 and LY2 cell lines. These results are in agreement with the study of Hu and colleagues (1993) where ICI 182,780 was able to overcome 4-OH-tamoxifen-resistance in a variant of the MCF-7 cell line and indicates that one potential mechanism for the increased effectiveness of ICI 182,780 may be related to its higher affinity for oestrogen receptor as compared with 4-OH-tamoxifen.

Many reports indicate that the 'insulin-like growth factor-I system' acts as a potent mitogen for breast cancer cells as well as for a wide variety of tumour cells (Westley & May, 1991). In breast cancer IGF-I, which is produced in the neighbouring stromal cells (Yee *et al.*, 1989), acts as a more potent growth stimulator than oestradiol. IGF-I activity is mediated by type I IGF transmembrane receptors and it is modulated by specific binding proteins (BP) which can either inhibit or potentiate IGF biological activity.

Recent studies indicate that the synthesis of IGF-I and its receptor is influenced by oestrogen (Stewart *et al.*, 1992). Freiss and colleagues (1990) have proposed that antioestrogens may inhibit cellular proliferation of ER+ cell lines not only by a direct interference with the natural ligand loop, but also as a consequence of the inhibition of growth factor production, growth factor receptor number, receptor affinity or receptor function. Vignon *et al.* previously (1987) showed that the antioestrogen tamoxifen is able to inhibit EGF and IGF-I mitogenic activities in the total absence of oestrogens. Newton *et al.* (1994) provided evidence that, in the absence of serum and oestrogens, a IGF-I-stimulated growth of a pituitary tumour cell line can be inhibited by these steroidal antioestrogens. This led us to verify the possible role of the new steroid-like com-

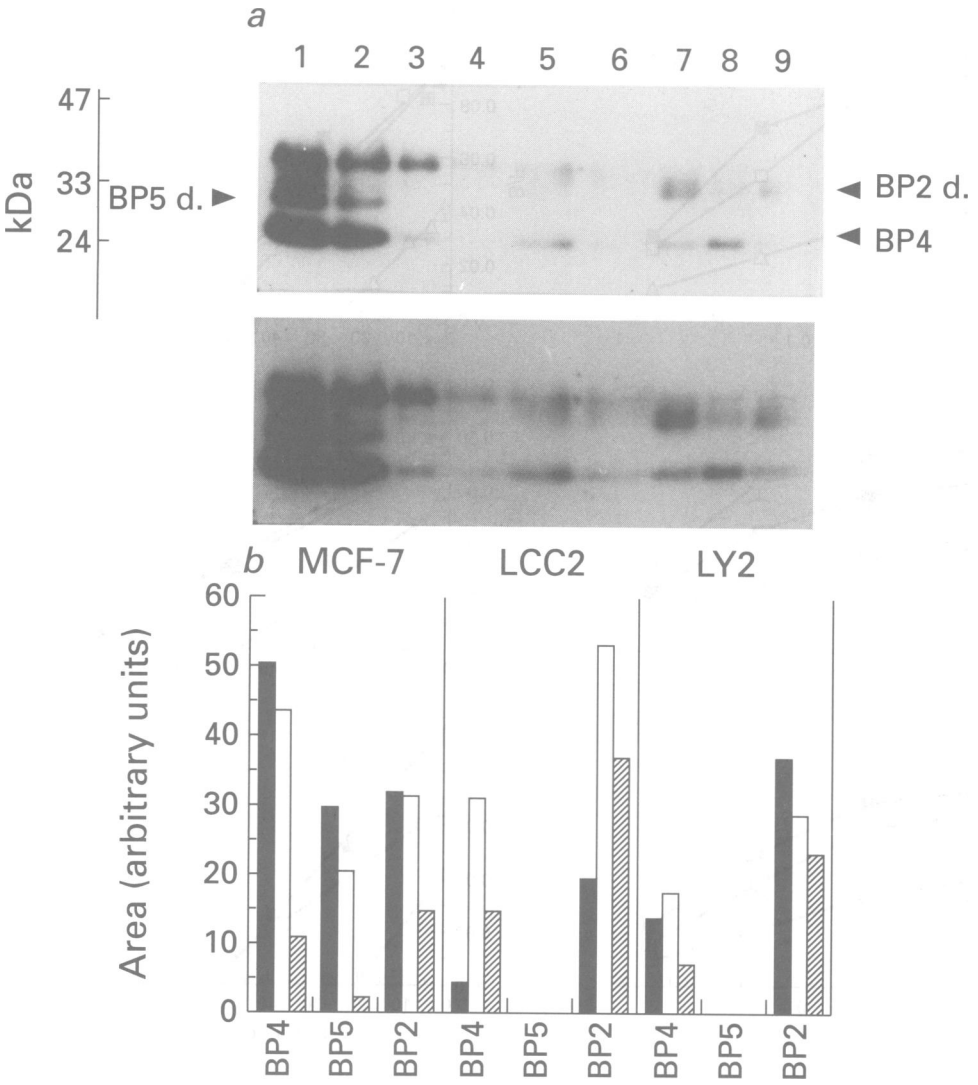

Figure 4 (a) Ligand blot of conditioned media (CM) from ER+ MCF-7, LCC2, LY2 human breast cancer cell lines. Equal total protein concentrations (10 μg), normalized in 7 to 61 μl of 30× concentrated CM from each untreated and 1 nM 17β-E_2- or 100 nM ICI 182,780-treated cell lines, were dried, redissolved in 30 μl of sample buffer, loaded onto 15% SDS-polyacrylamide gel and electrophoresed under non-reducing conditions. After transfer to 0.45 μm nitrocellulose filter, proteins were blotted overnight at 4°C with [^{125}I]-IGF-I ($\approx 4 \times 10^5$ c.p.m.). After washing and drying, the filter was exposed, at −80°C in the presence of intensifying screens, to X-ray film which was developed after 24 h (upper autoradiogram) and after 6 days (lower autoradiogram). Molecular weight (mol.wt.) standards are represented on the left vertical axis whereas IGF-BPs mol.wt. are identified by arrows on the both vertical axes. BP5d (in the MCF-7 samples) and BP2d (in the LCC2 and LY2 cell samples) indicate the binding proteins resolved as a doublet. Lanes 1, 4 and 7 = untreated cells; lanes 2, 5 and 8 = 17β-oestradiol-treated cells; lanes 3, 6 and 9 = ICI 182,780-treated cells. (b) Shows amount of IGF-BP4, BP5 and BP2 secreted by ER+ breast cancer cell lines at the basal level (■) and after 17β-oestradiol (□) or ICI 182,780 (▨) treatment. Values indicated by the columns, represent the areas (mm^2) of each BP expressed in arbitrary units, are obtained with a densitometric analysis of the autoradiography using a LKB Ultroscan XL Laser densitometer.

pounds, compared with the traditional non-steroidal 4-OH-tamoxifen, in modulating the IGF-I mitogenic system on human breast cancer cell lines and the action of these drugs on the IGF-BPs biochemical cascade.

Both ICI 164,384 and ICI 182,780 are significantly more potent than 4-OH-tamoxifen in inhibiting the single or combined IGF-I- and 17β-E_2-induced cell proliferation in hormone- and growth factor-sensitive cell lines. Our data confirm preliminary results obtained by Wakeling (1989) indicating that ICI 164,384 and 4-OH-tamoxifen attenuated IGF-I growth stimulation of MCF-7 cells. ICI 182,780 reduced IGF-I-like secretion by ER+ cells. Yee *et al.* (1989) did not find IGF-I mRNA in breast cancer cell lines by Northern blot and RNase protection assays, whereas IGF-I was identified in the stromal cells surrounding the tumours by *in situ* hybridisation of breast cancer tissues. Even using the much more sensitive RT-PCR assay, we are unable to detect IGF-I mRNA in these breast cancer cell lines, suggesting that the IGF-I immunoreactive proteins are unlikely to be authentic IGF-I. However, these results do not rule out the possibility that the molecules immunologically consistent with IGF-I might be due to IGF-I-related proteins that are not yet identified.

It is notable that the ICI 182,780-induced reduction of the immunoreactive IGF-I-like material correlates with anti-oestrogen-resistant characteristics. In the LY2 cell line the novel antioestrogen is less effective than in the LCC2 cell line in overcoming the acquired resistance. Huynh and colleagues (1993) showed tamoxifen-induced inhibition of IGF-I gene expression in common target organs for breast cancer metastasis *in vivo*. On the other hand, Huynh & Pollack (1993) reported that tamoxifen increases IGF-I gene expression in the rat uterus whereas ICI 182,780 inhibits it. IGF-I synthesis appears to be oestrogen-mediated in ER+ cells, whereas it becomes constitutive in ER negative breast tumour cells.

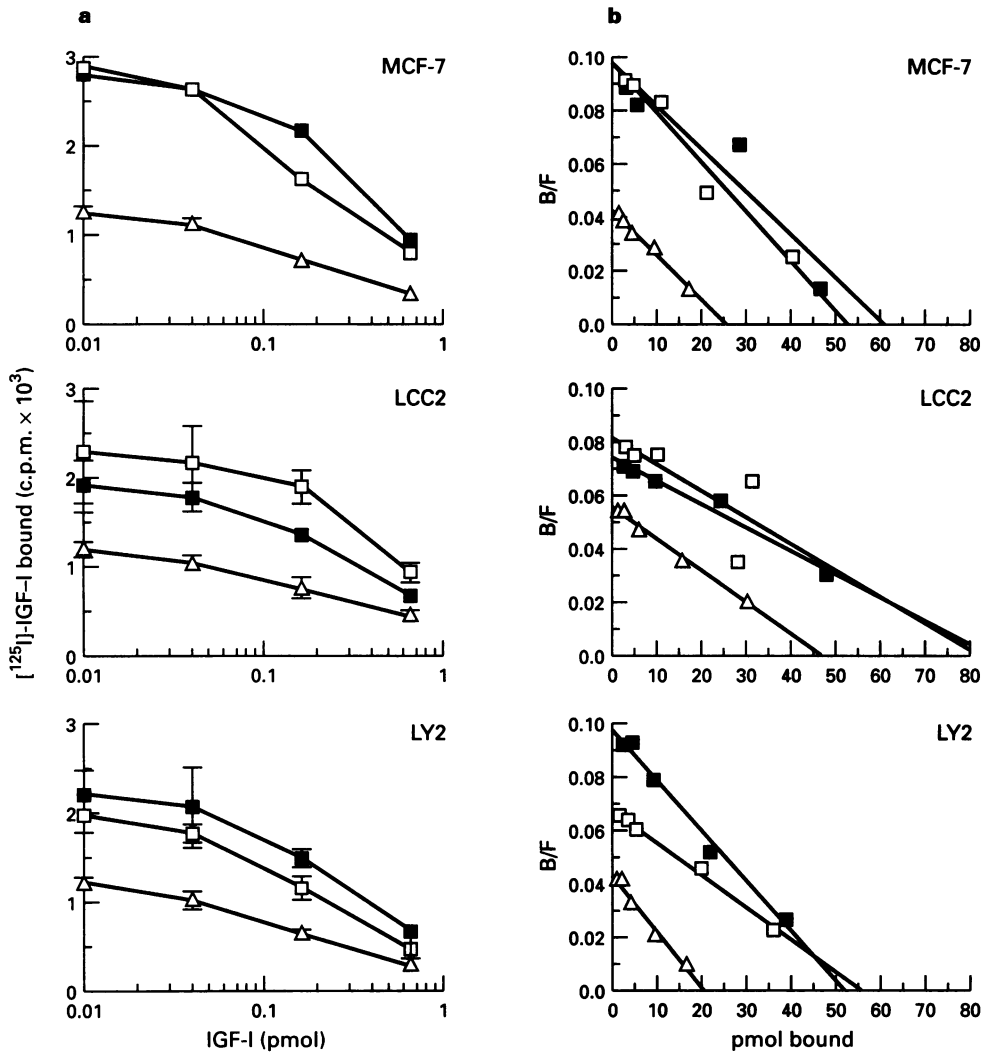

Figure 5 (a) Competitive binding analysis of IGF-I binding to untreated and 17β-E_2-, and ICI 182,780-treated MCF-7, LCC2 and LY2 cell lines: 2–3 x 10^5 cells, pretreated with 1 nM oestradiol or 100 nM ICI 182,780 for 2 days in flask, were seeded in duplicate in 24-well multiwells in presence of the drugs for an additional 24 h. After washing with ice-cold binding buffer, cells were simultaneously incubated in 400 ml of binding buffer, for 2 h at 4°C, with iodinated IGF-I (20,000 c.p.m./well) and varying amounts (1.95 to 400 ng ml^{-1}) of unlabelled peptide. After binding, monolayers were washed and lysed: the [^{125}I]-IGF-I bound to cells was then measured in a Beckman γ-counter. Each data point is the mean of duplicate determinations; s.e. between 0.011 and 0.450. (b) Scatchard analysis of competitive binding data of MCF-7, LCC2 and LY2 cell lines to determine dissociation constants (K_D), maximum binding capacity and number of sites/cell. Specific bound/free peptide is plotted against specific bound. The data points are the mean of two independent determinations. (■) Control; (□) 17β-oestradiol; (△) ICI 182,780.

Table 2 Effect of 17β-oestradiol (1 nM) and ICI 182,780 (100 nM) on IGF-I/receptor affinity, maximum binding capacity and number of sites/cell of ER+, ER– human breast cancer and normal cell lines

	Control			*17β-Oestradiol* (1 nM)			*ICI 182,780* (100 nM)		
Cell lines	K_D[a]	B_{max}[b]	*sites/cell*[c]	K_D[a]	B_{max}[b]	*sites/cell*[c]	K_D[a]	B_{max}[b]	*sites/cell*[c]
MCF-7	0.83	49	18	0.65	70	22	0.80	11	4
LCC2	0.85	65	14	1.10	100	18	0.90	41	10
LY2	0.65	54	19	0.77	54	20	0.45	20	13
MDA-MB231	0.57	34	8	0.48	32	8	0.53	37	10
HBL100	0.29	18	4.5	0.29	16	4.5	0.31	15	5

Data represent the means of 2 experiments (s.e. range: 1–21%)
[a]nM; [b]pM; [c]sites x 10^4/cell

Competitive binding studies indicate that ICI 182,780, as well as ICI 164,384, lowered binding of IGF-I to its cell surface receptor. Scatchard analysis reveals a decreased maximum binding capacity and a reduction in the number of sites/cell after cell exposure to antioestrogen. These data suggest that 'pure' antioestrogens-induced growth inhibition is partially due to reduced type I IGF-receptor numbers. It is possible that the growth stimulation induced by 17β-E_2 might also partially depend on a resultant increase in IGF receptors. A three day treatment with 1 nM oestradiol only slightly increased IGF-I binding on MCF-7 and LCC2 cell lines. However, Stewart and colleagues (1990) demonstrated, by cross-linking experiments, a striking increase in IGF-I binding upon 17β-E_2 treatment at higher concentration (10 nM) and after an extended oestrogen withdrawal period (five days). Interactions among oestrogens, antioestrogens and the 'IGF-I system' have been described

previously. Parker (1993) reported that ICI 182,780 and ICI 164,384 reduce oestrogen receptor cellular content by decreasing its half life ($t_{½}$), which in turn increases receptor turnover. Wiseman and colleagues (1993), have found that tamoxifen-enhanced oestrogen agonistic activity in one MCF-7 tamoxifen-resistant variant appeared to be due to positive induction of the type I IGF-receptor. This observation is in partial accordance with the results of our binding assays which show no 4-OH-tamoxifen-induced growth-inhibition of the antioestrogens-resistant cell lines under study.

The behaviour of the third component of the 'IGF-I system', IGF-binding proteins, which may positively or negatively cooperate with IGF-I in regulating breast cancer cell growth, is still a subject of controversy. Treatment with ICI 182,780 modified IGF-BP production, selectively down-regulating BP4, BP5 and BP2 secretion by the MCF-7 and LY2 lines. Cell growth inhibition provoked by ICI 182,780 could be partially related to this action as well. In contrast, ICI 182,780 increased BP4 and BP2 production by LCC2 cells. This phenomenon, which could be due to an attempt of the LCC2 cell line to bypass the inhibitory action of the antioestrogen, may not necessarily be related to the hormonal status of these cells. Based on our Western blot results, untreated MCF-7 and MCF-7-derived LCC2 and LY2 cell lines do not secrete significant amounts of IGF-BP3, even following treatment with ICI 182,780. DeLeon and colleagues (1990) were also unable to detect IGF-BP3 or its mRNA in MCF-7 cells by immunoprecipitation and Northern blot analysis. In contrast, low levels of the IGF-BP3 transcript were found by RNase protection assays (Yee *et al.*, 1991) and Pratt & Pollak (1993) have observed, by Western ligand blot analysis, IGF-BP3 in MCF-7 cells treated with tamoxifen or ICI 182,780. Such differences, which may also be due to heterogeneity in MCF-7 subclones, suggest that IGF-BP3 does not play a significant role in the inhibition of the IGF-I system by ICI 182,780. However, it is clear that ICI 182,780 inhibits the IGF-I-stimulated cell proliferation by acting on different targets within the 'IGF-I system'.

Recently, clinical trials have been conducted to assess ICI 182,780 tolerance, pharmacokinetics and short term biological effects in 56 women with primary breast cancer (DeFriend *et al.*, 1994). No serious drug-related side effects, sensitivity to antioestrogens or agonistic activity in breast tumours *in vivo* have been found. Moreover, Howell and colleagues (1995) demonstrated a 69% response rate in 19 patients with advanced tamoxifen-resistant breast cancer upon treatment with ICI 182,780. It would be interesting to analyse the IGF-I and IGF-BP serum levels in ICI 182,780-treated patients to compare the results obtained with those showed by Friedl *et al.* (1993) and Lonning & Reed (1992). Both clinical and our experimental studies, as well as other studies in different systems *in vitro* indicate that ICI 182,780 is an effective compound against breast cancer and it belongs to a new generation of agonist activity-free antioestrogens. In this report we also show the ability of the steroid-like antioestrogens to block the potent IGF-I mitogenic system which can potentially be a target for a more specific therapeutic approach in the control of malignant breast proliferation.

This research project was partially supported by the Associazone Nazionale per la Ricerca sul Cancro (A.I.R.C.). A. de C. was also supported by a grant from A.I.R.C. The authors would like to thank Dr A.E. Wakeling (Zeneca Pharmaceuticals, Cancer Res. Dept., Macclesfield, U.K.) for supplying antioestrogens and for helpful suggestions; Drs G. Isola, R. Russo and A. Lazzarini (Zeneca S.p.A., Div. Farmaceutici, Milano) for assistance and Dr R. Adatia for reading the manuscript.

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(Received February 22, 1995
Revised June 30, 1995
Accepted July 4, 1995)