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The Steroidal Antiestrogen ICI 182,780 is an Inhibitor of Cellular Aromatase Activity

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Two types of endocrine therapy that have been successfully applied to patients with hormone-dependent breast cancer are the non-steroidal antiestrogen tamoxifen, and inhibitors of aromatase, the enzyme that synthesizes estrogens. The major drawback with tamoxifen is that it acts as a partial estrogen-agonist and this is believed to mediate, at least in part, acquired tumor resistance to the drug as well as endometrial hyperplasia and carcinoma in some patients. The newer and more potent antiestrogen ICI 182,780 is a steroidal molecule that is devoid of estrogenic activity. We now report that ICI 182,780 is also an inhibitor of aromatase activity in fibroblasts isolated from the normal human breast as well as other carcinoma cell lines that express aromatase (MCF-7Ca breast cancer and JEG-3 choriocarcinoma). ICI 182,780 (1 μM) did not affect aromatase activity levels in human placental microsomes and only inhibited aromatase activity in each of the cell lines following a prolonged incubation period. In the fibroblasts, inhibition of aromatase activity by ICI 182,780 was shown to be time and dose-dependent. In contrast, tamoxifen and 17β-estradiol were shown to have no effect on aromatase activity levels. ICI 182,780 inhibited aromatase activity levels with IC_{50} values of 16.80 nM in MCF-7Ca cells, 125.50 nM in JEG-3 cells and 386.1 nM in breast fibroblasts. These values were compared to those for known aromatase inhibitors, and in each of the cell lines the order of potency was letrozole>4-OHA>anastrozole>ICI 182,780. The inhibition of aromatase activity by ICI 182,780 was sustained even after the antiestrogen was removed from the cells indicating that ICI 182,780 may be remaining bound to the enzyme. Although ICI 182,780 had no effect on the proliferation of the fibroblasts, or JEG-3 cells, it significantly inhibited the growth of MCF-7Ca cells. This growth inhibition appeared to be due to the antiestrogenic activity of ICI 182,780 and not to its aromatase inhibiting effects. ICI 182,780 did not inhibit aromatase activity by down-regulating levels of the aromatase transcript. These results show that in addition to being a potent antiestrogen, ICI 182,780 is also an inhibitor of cellular aromatase activity, and suggest that by interfering with the actions of estrogen by two distinct mechanisms, ICI 182,780 may be a suitable drug for treating patients with hormone-dependent breast cancer.
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INTRODUCTION

It is now well-established that estrogens play a fundamental role in promoting the growth of breast cancer cells. This understanding has led to the development of various endocrine therapies designed to block estrogen-mediated tumor growth. Tamoxifen is a non-steroidal, triphenylethylene antiestrogen (Fig. 1) that has been shown to be beneficial for patients with advanced breast cancer, as well as an adjuvant therapy for primary breast cancer patients [1, 2]. Tamoxifen acts by competitively binding to the estrogen receptor (ER) and blocking the growth-mediating effects of estrogen. However, in the breast, tamoxifen is only a partial antagonist and when complexed to the ER, retains some transcriptional activity [3]. Depending upon species, target tissue, and concentration, tamoxifen displays a range of biological activities varying from fully antagonistic to purely

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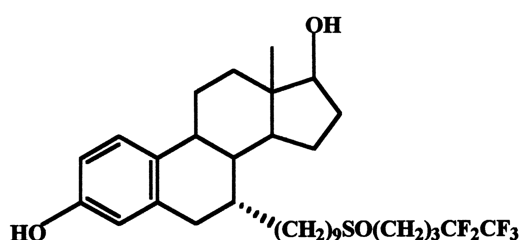
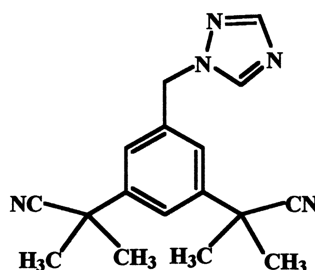


ICI 182,780 (Faslodex)



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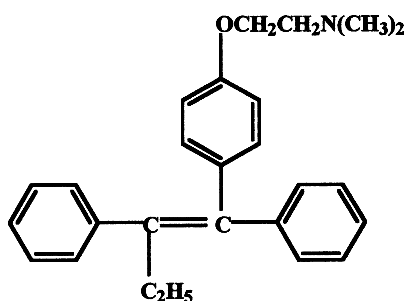
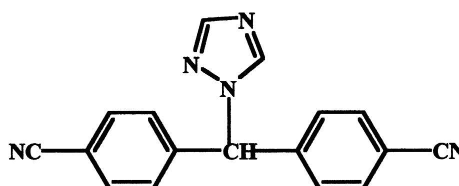


Tamoxifen



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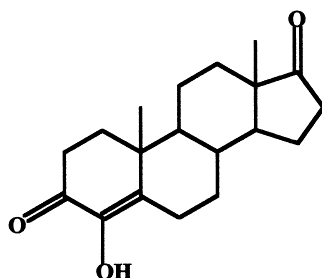
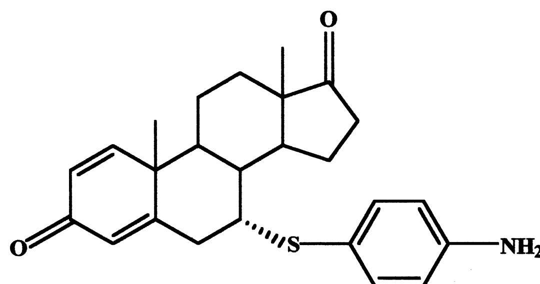


4-Hydroxyandrostenedione (Formestane)



7α-APTADD

Fig. 1. Structures of antiestrogens and aromatase inhibitors.

agonistic [4]. The partial estrogenic activity of tamoxifen is believed to mediate, at least in part, resistance to the drug, tumor progression, and patient relapse which occurs eventually in the majority of patients who were initially responsive to tamoxifen [5]. Tamoxifen's estrogenic effects are also responsible for the development of endometrial carcinoma in a small population of patients receiving the drug [6, 7]. Pure antiestrogens which display no estrogenic activity were developed with the hope of alleviating the estrogenic effects associated with tamoxifen therapy. ICI 182,780 is a steroidal antiestrogen (Fig. 1) that exhibits no estrogenic activity in breast cancer cell lines *in vitro* and breast tumor xenografts *in vivo* [8, 9]. In addition, it has been shown that breast cancer cells that acquire resistance to tamoxifen remain sensitive to the antiproliferative effects of ICI 182,780 [10, 11]. ICI 182,780 also competes with estrogen for binding to the ER, but once bound to the receptor, the long 7α-alkylamide side chain prevents receptor dimerization, which is prerequisite for the receptor to bind to an estrogen response element and initiate gene transcription [12]. Therefore, the mechanism of action in ICI 182,780 differs from that of tamoxifen

in that all gene transcription ceases. ICI 182,780 is currently in clinical trials as a potential alternative to tamoxifen for the treatment of breast cancer and the results from initial studies have yielded promising results [13–15].

Upon developing resistance to tamoxifen, breast cancer patients generally continue to receive endocrine therapies in the form of progestins or more recently, aromatase inhibitors [16, 17]. Aromatase is a cytochrome P_{450} enzyme that converts the androgen precursor, androstenedione, to the estrogens estrone (E1) and 17β-estradiol (E2). The first specific aromatase inhibitor, 4-hydroxyandrostensdione (4-OHA, Fig. 1), was shown to be effective in dramatically reducing serum estrogen levels and proved successful for treating breast cancer patients who had failed tamoxifen therapy [17, 18]. Recently, two non-steroidal aromatase inhibitors (letrozole and anastrozole, Fig. 1) have been approved for the treatment of breast cancer [19].

One of the major advantages of using aromatase inhibitors to treat breast cancer patients who have failed tamoxifen therapy is that the two types of drugs act through different mechanisms, and patients tend not to exhibit cross-resistance. Also, in contrast to tamoxifen therapy there are no serious side-effects to the endometrium associated with aromatase inhibitors. In the present study, we have found that the pure antiestrogen ICI 182,780 also inhibits aromatase in several types of cells; MCF-7Ca cells (MCF-7 cells constitutively expressing the aromatase gene) [20], JEG-3 choriocarcinoma cells which express high levels of aromatase [21] and fibroblasts isolated from the normal human breast.

MATERIALS AND METHODS

Materials

Aromatase substrate [1β-^{3}H]androstenedione (24.9 Ci/mmol, ^{3}HΔ^4A), [1,2,6,7-^{3}H]androstenedione (85.4 Ci/mmol), [4-^{14}C]estradiol (52.3 mCi/mmol) and [4-^{14}C]estrone (51.0 mCi/mmol) were purchased from New England Nuclear (Boston, MA). 17β-estradiol, 4-hydroxytamoxifen, dibutyryl cyclic adenosine monophosphate (cAMP), phorbol 12,13-diacetate (PDA), dexamethasone (Dex), collagenase type 1-A, hyaluronidase type 1-S, polymixin B sulfate, amphotericin B, sodium pyruvate, trichloric acetic acid (TCA), NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were from Sigma (St Louis, MO). Tissue culture media, antibiotics and all the reagents for RT-PCR were obtained from Gibco BRL (Grand Island, NY), while fetal bovine serum (FBS) was supplied by Summit Biotechnology (Fort Collins, CO). All tissue culture flasks and plates were from Corning (Corning, NY). Si-250F-PA thin layer chromatography (TLC) plates were from J.T. Baker. The oligonucleotide primers were synthesized in the Biopolymer Department, University of Maryland, using an Applied Biosystems DNA synthesizer (Foster City, CA) and the DNA molecular weight ladder was obtained from Promega (Madison, WI).

ICI 182,780 and anastrozole were kindly provided by Dr Alan Wakeling and Dr Michael Dukes (Zeneca Pharmaceuticals, Macclesfield, England). Letrozole was generously provided by Dr Ajay Bhatnager (Norvatis Pharmaceuticals, Basel, Switzerland). 4-OHA was synthesized in our laboratory as previously described [22].

Isolation of normal breast fibroblasts

Breast tissue from patients undergoing reduction mammaplasty was supplied by the Department of Pathology, University of Maryland Medical System. Gross fat was removed and the remaining tissue was cut into small pieces (0.5 cm^3) and incubated at 37°C, stirring overnight with collagenase type 1-A (1 mg/ml) and hyaluronidase type 1-S (1 mg/ml) in RPMI-1640 medium with 5% FBS, 2% penicillin/streptomycin, 2.5 μg/ml amphotericin B, 50 U/ml polymixin B sulfate and 50 μg/ml gentamicin sulfate. The following day the fat layer was decanted and the remaining organoids (ductal and lobulo-alveolar fragments) and cells were washed. Three 30 min sedimentation steps at 1*g* were performed to separate the majority of free blood cells and fibroblasts from the organoids. Fibroblasts were isolated as outgrowths from the organoid preparation following plating into 10 cm^2 plates in media which consisted of DMEM/F-12 medium containing 15 mM Hepes and 2 mM L-glutamine and supplemented with 10% FBS, 1 mM sodium pyruvate, 1% non-essential amino acids, 2% penicillin/streptomycin, 2.5 μg/ml amphotericin B, 50 U/ml polymixin B sulfate and 50 μg/ml gentamicin sulfate. Fibroblasts which grew from these preparations were removed by a brief treatment with trypsin/EDTA and cultured in the above medium. Cells remained viable for approximately 20 passages (1:7 split weekly); however, all experiments were performed between passages 1 and 10.

Cell lines and growth conditions

MCF-7 human breast cancer cells stably transfected with the aromatase gene (MCF-7Ca) were kindly provided by Dr S. Chen (City of Hope, Duarte, CA [20]) and were maintained in EMEM with 5% FBS, 1% penicillin/streptomycin, 1% non-essential amino acids and 600 μg/ml geneticin (G418). JEG-3 cells were purchased from the American Type Culture Collection and were cultured in the same medium as MCF-7Ca cells with 10% FBS and without the G418.

Radiometric aromatase (^3H_2O) release assay with placental microsomes

Preparation of placental microsomes and aromatase activity assays were performed as previously described [22]. Briefly, 200 μg of placental microsomes were mixed with 1.25 IU/ml of the NADPH generating system (NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase) in 1 ml 0.1 M phosphate buffer, pH 7.4. Samples were mixed with substrate (0.5 nM androstenedione containing 0.3 μCi of $^3H\Delta^4A$) and the indicated concentrations of inhibitors. Following a 30 min incubation at 37°C the assay was terminated by adding 2 ml of chloroform, which removed unconverted steroids. The aqueous phase (0.7 ml) was then removed and mixed with 0.7 ml of 2.5% activated charcoal suspension. Tritiated water (^3H_2O) formed during the aromatization of $^3H\Delta^4A$ to estrogen was measured by counting the radioactivity in 0.7 ml of the aqueous supernatant.

Radiometric aromatase (^3H_2O) release assay with fibroblasts

Semi-confluent flasks of cells (patient was a 22 year old female) were washed with DPBS and grown for 48 h in routine medium. Cells (100,000–300,000) were then plated into six-well plates and allowed to attach. Cells were washed and treated with Dex (1 μM) in the same medium or with cAMP (500 μM), PDA (100 nM) or a combination of cAMP and PDA in medium devoid of serum for 30 h. The following day the cells were washed again and incubated with 1 ml of either serum-containing or serum-free medium containing approximately 0.5 μCi of $^3H\Delta^4A$ with the appropriate treatments, for 18 h at 37°C. After incubation, medium was transferred to a glass test-tube and 300 μl of TCA was added to precipitate the proteins. After centrifugation, 1 ml of medium was mixed with 2 ml of chloroform to extract unconverted substrate and other steroids. An aliquot of 0.7 ml of the aqueous phase was treated with 2.5% activated charcoal suspension (0.7 ml) to remove any residual steroids. Tritiated water (^3H_2O) formed during the aromatization of $^3H\Delta^4A$ to estrogen was measured by counting the radioactivity in the aqueous supernatant. To determine the effect of ICI 182,780 on induced aromatase activity levels and the IC_{50} values for the known aromatase inhibitors, cells were treated with Dex (1 μM) as described above and co-incubated with vehicle or drugs at various concentrations for the duration of the experiment. For all experiments, one of the wells was not exposed to radioactivity and the cell number counted with a Coulter Counter (Coulter, Miami, FL) to standardize aromatase activity levels to cell number. Blanks were performed as described above, using six-well plates that did not contain cells. Aromatase activity levels were determined as fmol/100,000 cells/6 h. All experiments were repeated in triplicate and the results are expressed as mean $\pm$ SEM.

Radiometric aromatase (^3H_2O) release assay with MCF-7Ca and JEG-3 cells

Cells were plated into six-well plates (50,000 cells/well) and left overnight to attach. The following day cells were washed and treated with vehicle or ICI 182,780 in routine culture medium for 2 days, with fresh medium and drug added after 24 h. Following treatment, cells were washed again and incubated with 0.5 μCi of $^3H\Delta^4A$ in 1 ml of medium for 2 h. For determination of IC_{50} values cells were treated with letrozole, anastrozole and 4-OHA for 2 h only in the presence of 0.5 μCi of $^3H\Delta^4A$. Aromatase activity levels were then determined as described above. IC_{50} values for inhibitors were calculated from the linear regression line in a plot of % of activity vs log inhibitor concentration.

Thin layer chromatographic (TLC) analysis of aromatase products

JEG-3 cells were plated into six-well plates (50,000 cells/well) and left to attach overnight. The following day cells were treated with vehicle or the indicated concentrations of ICI 182,780 for 48 h, with medium replaced at 24 h. Cells were then incubated at 37°C for 1 h with [1,2,6,7-^{3}H]androstenedione and unlabeled substrate (0.1 μM). After incubation, the medium was transferred to a glass test-tube and [4-^{14}C]estradiol, [4-^{14}C]estrone and [4-^{14}C]androstenedione were added to each tube for the calculation of recovery. The corresponding unlabeled steroids were also added to locate the metabolic products after TLC. All steroids in the incubation medium were extracted by mixing the medium with ether, mixing the aqueous phase in dry ice–acetone, and transferring the organic phase to a fresh tube. The extraction was repeated three times and the ether phases pooled. The ether was evaporated and the remaining steroids were dissolved in ethanol and transferred to a TLC plate. The steroids on the TLC plate were separated by a solvent mixture of ether and hexane (3:1) and the areas containing estrone and estradiol or androstenedione were visualized by ultra violet illumination and exposure to iodine vapor. The stained areas were removed from the plate and the steroids were extracted from the silica products with ether. Following drying of the ether and reconstitution in ethanol the samples were added to a scintillation vial for radioactivity measurement. The protein content in each well was then determined to standardize aromatase activity levels.

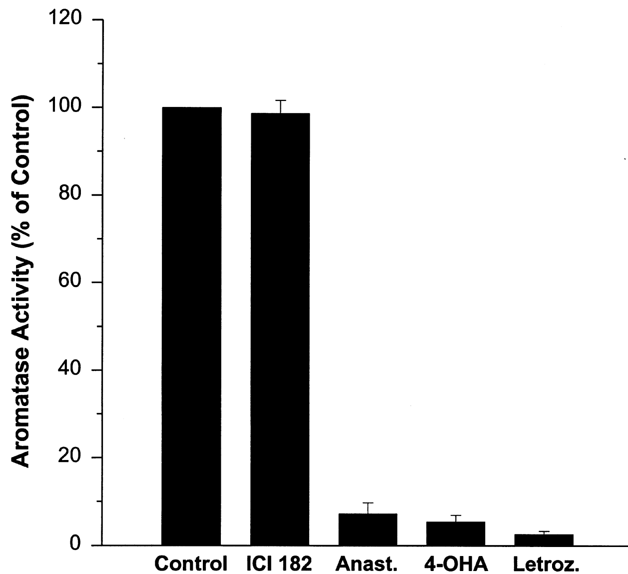


Fig. 2. Inhibition of aromatase activity levels in placental microsomes by ICI 182,780 (1 μM), anastrozole, 4-OHA and letrozole (100 nM). 200 μg of human placental microsomes were incubated with drugs in the presence of 1.25 IU of an NADPH generating system in 0.1 M phosphate buffer (pH 7.4), for 30 min at 37°C. Aromatase activity levels are presented as % of control and show the mean $\pm$ SEM of triplicate experiments.

Effect of ICI 182,780 on cell proliferation

Cells were plated into 24-multiwell dishes (20,000 cells/well) in routine culture medium and allowed to attach overnight. The following day the cells were washed and medium was replaced with routine culture medium (1 ml) containing the indicated concentrations of ICI 182,780. Cells were counted three and six days later using a Coulter Counter. The media was replaced in the duplicate plates on day 3.

Measurement of aromatase mRNA by RT-PCR

Flasks of cells (80% confluent) were washed and medium was replaced with routine culture medium containing ethanol vehicle, ICI 182,780 (0.1 or 1 μM), Dex (1 μM) or a combination of ICI 182,780 and Dex. Cells were treated for 2 days after which time total RNA was extracted using Trizol Reagent (Gibco BRL). RT-PCR was performed according to the previously described protocol [23]. Briefly, 2 μg of total RNA was reverse transcribed with 400 U of moloney leukemia virus reverse transcriptase for 1 h at 42°C in a total volume of 20 μl. PCR amplification of 5 μl of reversed transcribed sample was carried out in 25 μl of PCR buffer (10 mM Tris–HCl, pH 8.3) with 1.5 mM $MgCl_2$, 400 μM dNTPs, 1.5 U of Taq DNA polymerase and 1 μM of each primer. One PCR cycle (40 cycles total) consisted of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and polymerization at 72°C for 1.5 min. PCR products were electrophoresed on 1–1.2% agarose gels and visualized by ethidium bromide staining. Primers for aromatase were 5′-GAATATTGGAAGGATGCACAGACT-3′ and 5′-GGGTAAAGATCATTTCCAGCATGT-3′ which produced a 293 bp product [24]. Primers for the positive control, glyceraldehyde-phosphate dehydrogenase (GAPDH) were 5′-TGAAGGTCGGAGTCAACGGATTTG-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′ and produced a 983 bp product [25].

RESULTS

Effect of ICI 182,780 on placental microsome aromatase activity

ICI 182,780 (1 μM) had no effect on placental aromatase activity over the 30 min duration of the assay (Fig. 2). This was in contrast to the results obtained with the known aromatase inhibitors anastrozole, 4-OHA and letrozole which significantly inhibited placental aromatase activity by 93%, 95% and 98% respectively. ICI 182,780 also had no effect on aromatase activity levels when the assay time was extended to 60 and 90 min (data not shown).

Inhibition of aromatase activity in fibroblasts, MCF-7Ca cells and JEG-3 cells by 4-OHA and ICI 182,780

Fibroblasts, MCF-7Ca human breast cancer cells and JEG-3 choriocarcinoma cells were treated with 4-OHA and ICI 182,780 (both at 1 μM) for 2 h (6 h for fibroblasts as they had lower aromatase activity) and 48 h, at which time aromatase activity levels were determined (Table 1). Aromatase activity was highest

Table 1. The effect of incubation time on the inhibition of aromatase activity in normal breast fibroblasts, MCF-7Ca breast cancer cells and JEG-3 choriocarcinoma cells by 4-OHA (1 μM) and ICI 182,780 (1 μM)

	Aromatase activity (fmol/100,000 cells/unit time)					
	fibroblasts		MCF-7Ca		JEG-3	
	6 h	48 h	2 h	48 h	2 h	48 h
Control	2.83 $\pm$ 0.51	8.92 $\pm$ 1.98	77.66 $\pm$ 3.66	83.53 $\pm$ 4.27	358.82 $\pm$ 15.77	347.66 $\pm$ 14.48
ICI 182,780	1.75 $\pm$ 0.62	1.22 $\pm$ 0.49	81.83 $\pm$ 5.57	12.55 $\pm$ 2.91	361.99 $\pm$ 18.23	49.36 $\pm$ 6.91
4-OHA	0.87 $\pm$ 0.24	1.17 $\pm$ 0.38	10.28 $\pm$ 1.88	3.18 $\pm$ 1.07	26.91 $\pm$ 4.75	5.74 $\pm$ 2.80

Cells in six-well plates were treated with vehicle, 4-OHA (1 μM) or ICI 182,780 (1 μM) for the indicated time periods and aromatase activity levels determined. For the 48 h incubation, fresh medium and drug were added after 24 h. MCF-7Ca cells and JEG-3 cells were assayed with 0.5 μCi of labeled androstenedione for 2 h. Fibroblasts were assayed for 6 h. Results are expressed as the mean $\pm$ SEM of triplicate experiments.

in the JEG-3 and MCF-7Ca cells which express aromatase at high levels [20, 21] and lowest in the breast fibroblasts. Following a 2 h incubation period (6 h for fibroblasts) with 4-OHA, aromatase activity was lower in each of the cell lines and was reduced further after a 48 h incubation. In contrast, ICI 182,780 had no effect on aromatase activity levels following a 2 h incubation, but by 48 h aromatase was effectively inhibited. In each cell type 4-OHA was shown to be a more potent inhibitor of aromatase than ICI 182,780.

Inhibition of aromatase activity in normal breast fibroblasts

Fibroblasts grown in six-well plates in the presence and absence of 10% FBS were treated with drugs that have previously been reported to induce aromatase in adipose cells isolated from normal breast tissue [26, 27]. In untreated cells, aromatase activity levels were between 5–10 fmol/100,000 cells/6 h, with higher passaged cells having lower activity. When treated with the inducers for 48 h, activity levels were stimulated by cAMP (~six-fold) only in the absence of serum and this induction was potentiated by the phorbol ester, PDA. Dex (1 μM) had no effect on aromatase activity in the absence of serum but strongly induced activity levels (~five-fold) in the presence of 10% FBS (Fig. 3(A)).

The ability of ICI 182,780 to inhibit these induced levels was examined (Fig. 3(B)). Fibroblasts in six-well plates were co-treated for 30 h with the inducing agent(s) and ICI 182,780 (1 μM). Cells were then incubated with the labeled substrate in the presence of the inducers and drug. Untreated cells had low aromatase activity, which was further reduced by treatment with ICI 182,780. The antiestrogen also significantly lowered the induced activity levels. Regardless of whether aromatase was up regulated by Dex or cAMP, ICI 182,780 was able to significantly reduce activity to levels similar to the untreated cells.

In order to determine whether ICI 182,780 was capable of completely inhibiting induced aromatase activity levels, a time course was performed over a 72 h period. Fibroblasts in six-well plates were treated with ICI 182,780, Dex or a combination of the two and assayed for aromatase activity. New medium with fresh drugs was added to the cells daily. Induction of aromatase by Dex rose to a maximum following a 24 h treatment, after which time it slowly declined (Fig. 4(A)). Throughout the time period, aromatase activity levels were lowered by ICI 182,780, with maximal inhibition at the 24–48 h period. Following a 48 h treatment with the antiestrogen, Dex-induced aromatase activity levels were similar to those of untreated cells. Based on these results, a 48 h treatment period was utilized to determine the response of the fibroblasts to a range of concentrations of ICI 182,780. Cells were co-treated with Dex plus ICI 182,780 (1 pM–1 μM) for 30 h and then assayed with labeled substrate plus drugs for 18 h. ICI 182,780 concentrations of 1 nM to 1 μM inhibited aromatase activity levels in a dose-dependent manner but the antiestrogen was ineffective at inhibiting aromatase activity at concentrations below 1 nM (Fig. 4(B)).

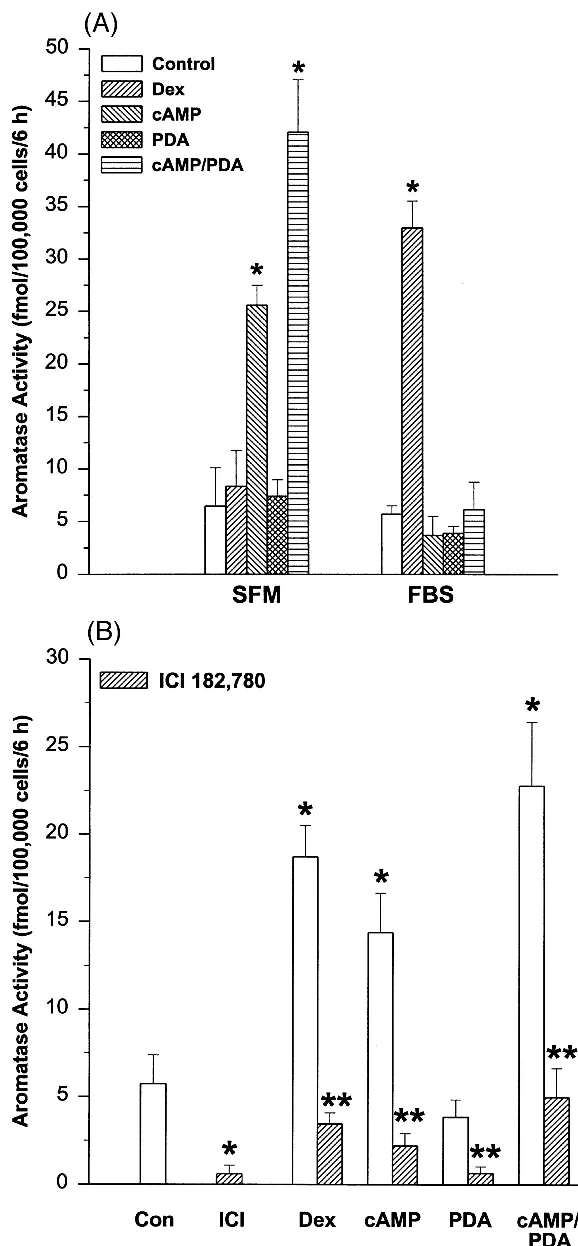


Fig. 3. (A) Induction of aromatase activity in normal human breast fibroblasts by Dex (1 μM), cAMP (500 μM), PDA (100 nM) and a combination of cAMP and PDA in the presence (FBS) and absence (SFM) of 10% serum. (B) Inhibition of aromatase activity induced by Dex, cAMP, and a combination of cAMP and PDA by 1 μM ICI 182,780. Fibroblasts in six-well plates were treated with either inducer alone or in combination with antiestrogen for 30 h, before being incubated with 0.5 μCi ^{3}HΔ^4A for 18 h in the presence of drugs. Aromatase activities were expressed as fmol/100,000 cells/6 h. Results show the mean ± SEM of triplicate experiments. *P < 0.01 vs control. **P < 0.01 vs treatment (Students t-test).

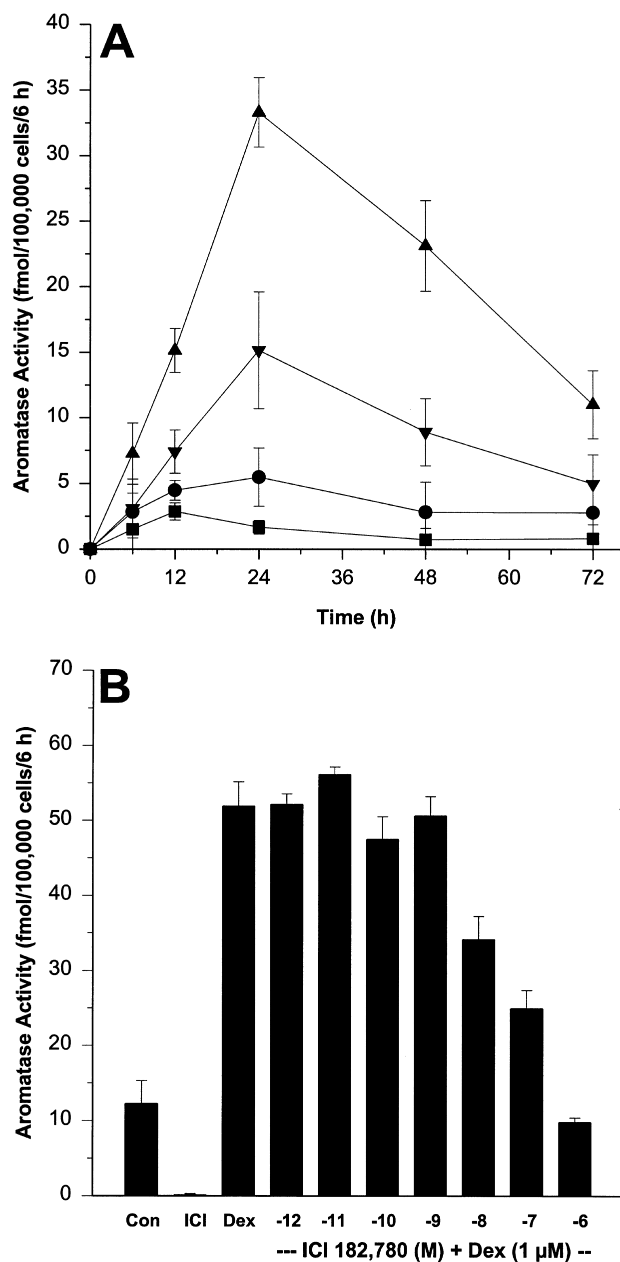


Fig. 4. Inhibition of aromatase activity levels in normal breast fibroblasts by ICI 182,780. (A) The effect of time on aromatase inhibition. Fibroblasts in six-well plates were treated with vehicle (●), 1 μM ICI 182,780 (■), 1 μM Dex (▲) or Dex plus ICI 182,780 (▼) for 6–72 h and aromatase activity levels were determined. For 6 and 12 h treatments drugs were added at the same time as $^3H\Delta^4A$, and for all other times the cells were incubated with 0.5 μCi $^3H\Delta^4A$ for 18 h after treatment with drugs. (B) Dose-dependent inhibition of aromatase activity in fibroblasts by ICI 182,780. Fibroblasts in six-well plates were treated with ICI 182,780 for 30 h, before being incubated with 0.5 μCi $^3H\Delta^4A$ for 18 h in the presence of antiestrogen (1 pM–1 μM). Aromatase activities were expressed as fmol/100,000 cells/6 h. Results show the mean ± SEM of duplicate experiments.

The specificity of ICI 182,780 as an inhibitor of aromatase activity in normal breast fibroblasts was established by determining the effects of E2 and 4-hydroxytamoxifen (4-OHT) on activity levels in these cells (Fig. 5). Using the assay conditions described above for ICI 182,780, fibroblasts were treated with E2 and 4-OHT (1 μM) either alone or in combination with Dex (1 μM). Neither E2 nor 4-OHT had an effect on aromatase activity levels in the fibroblasts, indicating that the ability of ICI 182,780 to inhibit activity was not due to the antiestrogenic nature of the drug nor due the estrogenic structure from which it was derived.

Determination of IC_{50} values for ICI 182,780, anastrozole, 4-OHA and letrozole

IC_{50} values were determined for ICI 182,780 and compared with those for the known aromatase inhibitors 4-OHA, letrozole and anastrozole in each of the cell lines (Table 2). Fibroblasts were co-treated with Dex (1 μM) for the duration of the experiment in order to maintain relatively high aromatase activity levels. The IC_{50} value for ICI 182,780 was highest in the fibroblasts at 386 nM. This value compared to 16.80 nM for MCF-7Ca cells, which were the most sensitive to the drug, and 125.5 nM for JEG-3 cells. In all three cell lines letrozole was the most potent aromatase inhibitor, with IC_{50} values ranging between 0.14–0.45 nM, while ICI 182,780 was the least effective aromatase inhibitor. Regardless of cell type, 4-OHA was a more effective inhibitor of aromatase than anastrozole.

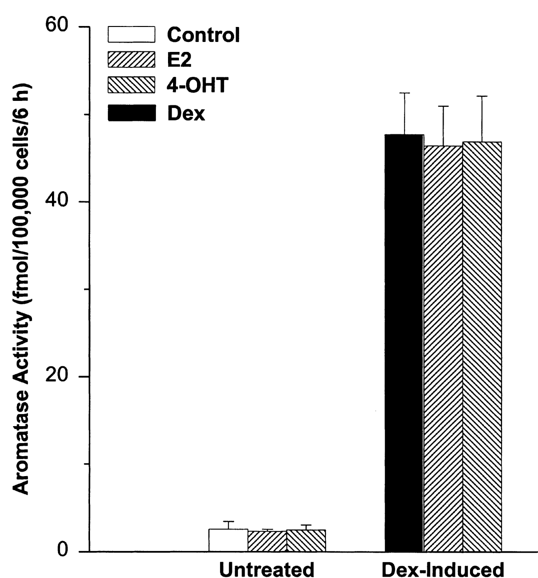


Fig. 5. The effect of 17β-estradiol (E2) and 4-hydroxytamoxifen (4-OHT) on aromatase activity levels in normal breast fibroblasts. Fibroblasts in six-well plates were treated with compounds and assayed for aromatase activity as described for Figs 2 and 3. Aromatase activities were expressed as fmol/100,000 cells/6 h. Results show the mean ± SEM of triplicate experiments.

Table 2. IC_{50} values (nM) of aromatase inhibitors ICI 182,780, 4-OHA, anastrozole, and letrozole

	Cell line		
	fibroblasts	MCF-7Ca	JEG-3
Letrozole	0.14 ± 0.03	0.35 ± 0.07	0.45 ± 0.07
Anastrozole	17.17 ± 2.13	3.62 ± 0.90	5.66 ± 2.13
4-OHA	0.72 ± 0.25	0.59 ± 0.10	1.60 ± 0.36
ICI 182,780	386.10 ± 45.70	16.80 ± 3.13	125.50 ± 28.45

IC_{50} values for the fibroblasts were obtained by co-treating the cells with Dex (1 μM) plus inhibitors for 30 h followed by an 18 h assay with 0.5 μCi $^3H\Delta^4A$ in the presence of drugs. The IC_{50} values for ICI 182,780 in MCF-7Ca and JEG-3 cells were obtained by incubating the cells with the antiestrogen for 46 h, followed by a 2 h incubation with labeled substrate in the presence of drug. IC_{50} values for the three specific aromatase inhibitors were obtained by incubating MCF-7Ca and JEG-3 cells with 0.5 μCi $^3H\Delta^4A$ for 2 h in the presence of drugs. Results are expressed in nM and show the mean $\pm$ SEM of triplicate experiments.

Effect of ICI 182,780 on the formation of aromatase products by JEG-3 cells

JEG-3 cells, which express the highest levels of aromatase activity, were treated with ICI 182,780 for 48 h and the ability of the antiestrogen to inhibit the production of estrogen was analyzed using the direct product isolation method. [1,2,6,7-^{3}H]androstenedione was used as the substrate because it is converted to both [6,7-^{3}H]estrone and [6,7-^{3}H]estradiol by aromatization. Following separation by TLC, the products were then quantitated by scintillation counting. As shown in Fig. 6, a 48 h incubation with ICI 182,780 significantly inhibited the conversion of androstenedione to both estrone and estradiol. This result is consistent with the result of the ^3H_2O release assay.

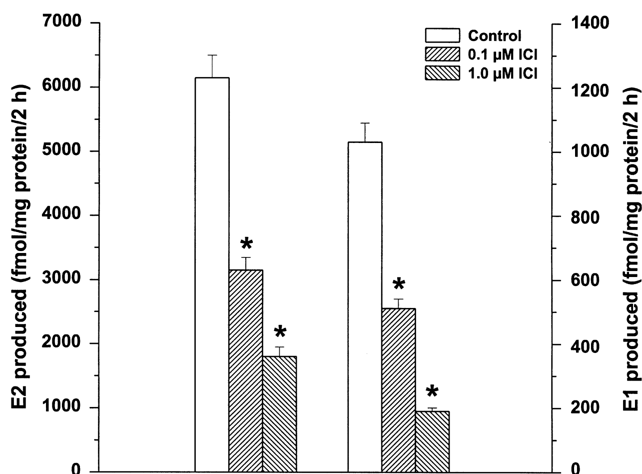


Fig. 6. JEG-3 cells in six-well plates were treated with ICI 182,780 (0.1 μM) and (1.0 μM) for 48 h, with medium and drug refreshed at 24 h. Cells were washed to remove residual ICI 182,780 and aromatase activity was measured by incubating the cells with [1,2,6,7-^{3}H]androstenedione and unlabeled substrate (0.1 μM) at 37°C for 2 h. Medium was then transferred to fresh tubes and following extraction of the steroids with ether, the products were separated by TLC. Aromatase activities were expressed as fmol of estrogen produced per mg of protein per 2 h. *$P < 0.01$ vs control (Students t-test).

Sustained inhibition of aromatase activity in normal breast fibroblasts by ICI 182,780

To determine whether the inhibitory effects of ICI 182,780 were sustained when the drug was removed from the culture medium, fibroblasts in six-well plates were treated with Dex (1 μM) or a combination of Dex and ICI 182,780 (1 μM) for 24 h. The following day the cells were washed three times with phosphate buffered saline and treated with Dex alone. Aromatase activity levels were determined following the 24 h treatment, and at 12, 24, 48 and 72 h following removal of the ICI 182,780 (Fig. 7). As observed in Fig. 3(A), aromatase activity in the fibroblasts was maximal following a 24 h treatment with Dex, and gradually declined over the next 72 h. Treatment of the fibroblasts with ICI 182,780 for 24 h significantly reduced aromatase activity levels and upon removal of the drug, activity levels remained significantly lower than in the cells treated with Dex alone for an additional 48 h. Over the 72 h time period that ICI 182,780 had been removed from the cells, aromatase activity levels rose in a time-dependent manner. However, it was not until 72 h

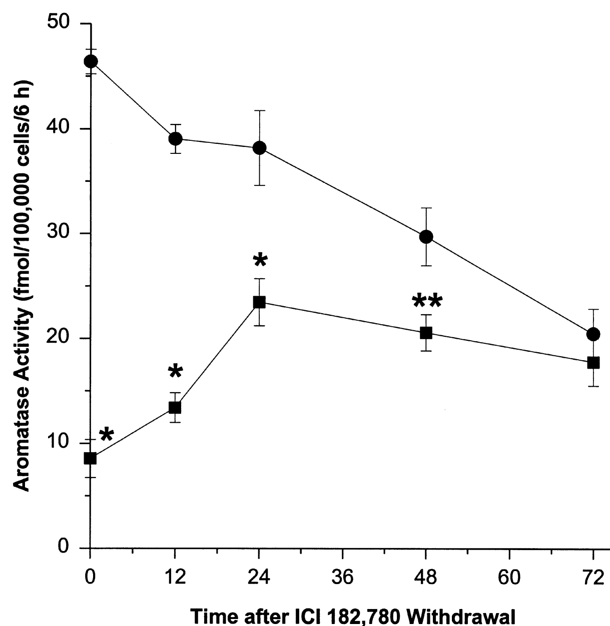


Fig. 7. Sustained inhibition of Dex-induced aromatase activity levels in normal breast fibroblasts following a 24 h treatment with ICI 182,780 (1 μM). Fibroblasts in six-well plates were treated with Dex (●), or Dex plus ICI 182,780 (■) for 24 h, after which time medium was removed, cells were washed and treated only with Dex. At the indicated times after removal of the antiestrogen cells were assayed for aromatase activity levels as described in Section 2. Aromatase activities were expressed as fmol/100,000 cells/6 h. Results show the mean $\pm$ SEM of duplicate experiments. *$P < 0.01$, **$P < 0.05$ (Students t-test).

after the antiestrogen had been withdrawn that the activity returned to levels similar to those of untreated cells.

Effect of ICI 182,780 on cellular proliferation

To determine whether the inhibition of aromatase activity in the cells treated with ICI 182,780 was being caused by reduced cell proliferation, normal breast fibroblasts, MCF-7Ca cells and JEG-3 cells in 24-well plates were treated with ICI 182,780 (0.1 and 1.0 μM) and counted following 3 and 6 days of treatment. Treatment with ICI 182,780 had no effect on the proliferation of normal breast fibroblasts or JEG-3 choriocarcinoma cells (Fig. 8) indicating that a cytotoxic effect on the cells was not responsible for the inhibition of cellular aromatase activity. Although ICI 182,780 significantly inhibited the proliferation of MCF-7Ca cells (which depend on estrogen for growth), it also inhibited the growth of MCF-7 cells without the aromatase gene to the same extent as the MCF-7Ca cell line (data not shown), indicating that growth inhibition was most likely due to the antiestrogenic nature of the drug and not to its aromatase inhibiting effect.

Effect of ICI 182,780 on aromatase mRNA levels

To determine whether ICI 182,780 was causing a reduction in aromatase activity levels by down-regulating aromatase mRNA expression, fibroblasts were treated with ICI 182,780 alone, Dex alone and a

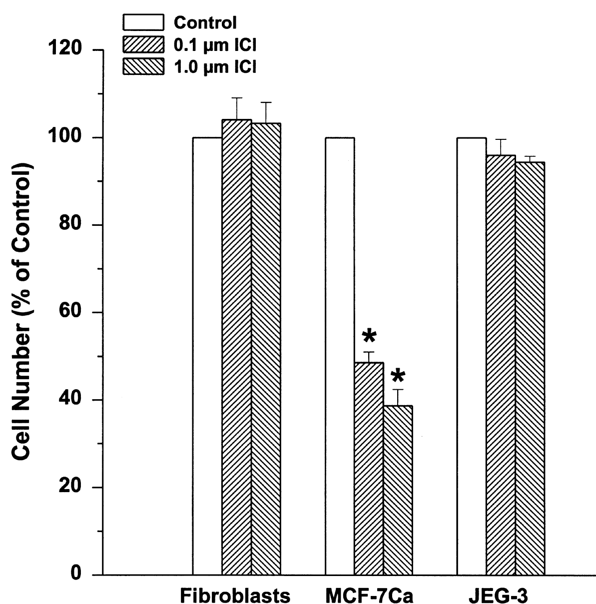


Fig. 8. The effect of ICI 182,780 on the proliferation of normal human breast fibroblasts, MCF-7Ca cells and JEG-3 cells. Cells were treated with ICI 182,780 for 6 days with fresh drug added after 3 days. Cells were counted 3 and 6 days after treatment. The results show cell number after 6 days of treatment as a % of vehicle treated cells and are expressed as the mean $\pm$ SEM of duplicate experiments. *$P < 0.01$ (Students *t*-test).

combination of Dex plus ICI 182,780 for 48 h, while MCF-Ca and JEG-3 cells were treated with 0.1 and 1.0 μM of ICI 182,780 for 48 h. RT-PCR was used to analyze mRNA expression with tRNA used as a negative control. The 293 bp fragment representing aromatase mRNA was present in the fibroblasts, and at a lower level than in MCF-7Ca cells (Fig. 9(A)). Levels of the 293 bp aromatase transcript were higher after treatment of the fibroblasts with Dex (1 μM; 48 h). However, ICI 182,780 did not appear to affect either basal or Dex induced aromatase mRNA expression in the fibroblasts, indicating that ICI 182,780 inhibits aromatase activity by interacting directly with the enzyme. This was confirmed by treating MCF-7Ca and JEG-3 cells with ICI 182,780 for 48 h, which again had no effect on intensity of the 293 bp band (Fig. 9(B)).

DISCUSSION

The results presented in this study show that the pure antiestrogen ICI 182,780 inhibits aromatase activity in fibroblasts isolated from normal human breast, MCF-7Ca human breast cancer cells, and JEG-3 human choriocarcinoma cells. We have demonstrated that each of these cell types express aromatase activity at varying levels and that the ability of ICI 182,780 to inhibit aromatase activity is not dependent on the origin of the cell or its malignant status (Table 1). However, the ability of ICI 182,780 to inhibit aromatase activity, is dependent upon the cells being exposed to the antiestrogen for an extended period of time (at least 12 h). ICI 182,780 did not reduce cellular aromatase activity levels following a 2 h incubation; a result that was in contrast to the specific aromatase inhibitor 4-OHA. This finding indicates that ICI 182,780 itself is not an aromatase inhibitor, but rather, it acts through an as yet undetermined mechanism to reduce aromatase activity levels, and does so only after an extended incubation period. This is further demonstrated in Fig. 2, which shows that ICI 182,780 has no effect on aromatase activity levels in placental microsomes, whereas 4-OHA, anastrozole, and letrozole all inhibited aromatase activity levels by greater than 90% following a 30 min exposure to drug.

ICI 182,780 was developed to be a pure antiestrogen devoid of the estrogenic activities associated with tamoxifen. Prolonged treatment with tamoxifen increases the risk of developing endometrial hyperplasia and carcinoma, where it acts as a full estrogen agonist [6, 7]. In animal studies, tamoxifen has also been shown to be carcinogenic and long-term exposure to the drug is linked to the formation of DNA adducts in the rat liver [28, 29]. Compared to tamoxifen, ICI 182,780 is a more potent antiestrogen that binds to the ER with a higher affinity [8], and also down-regulates ER levels by reducing intracellular

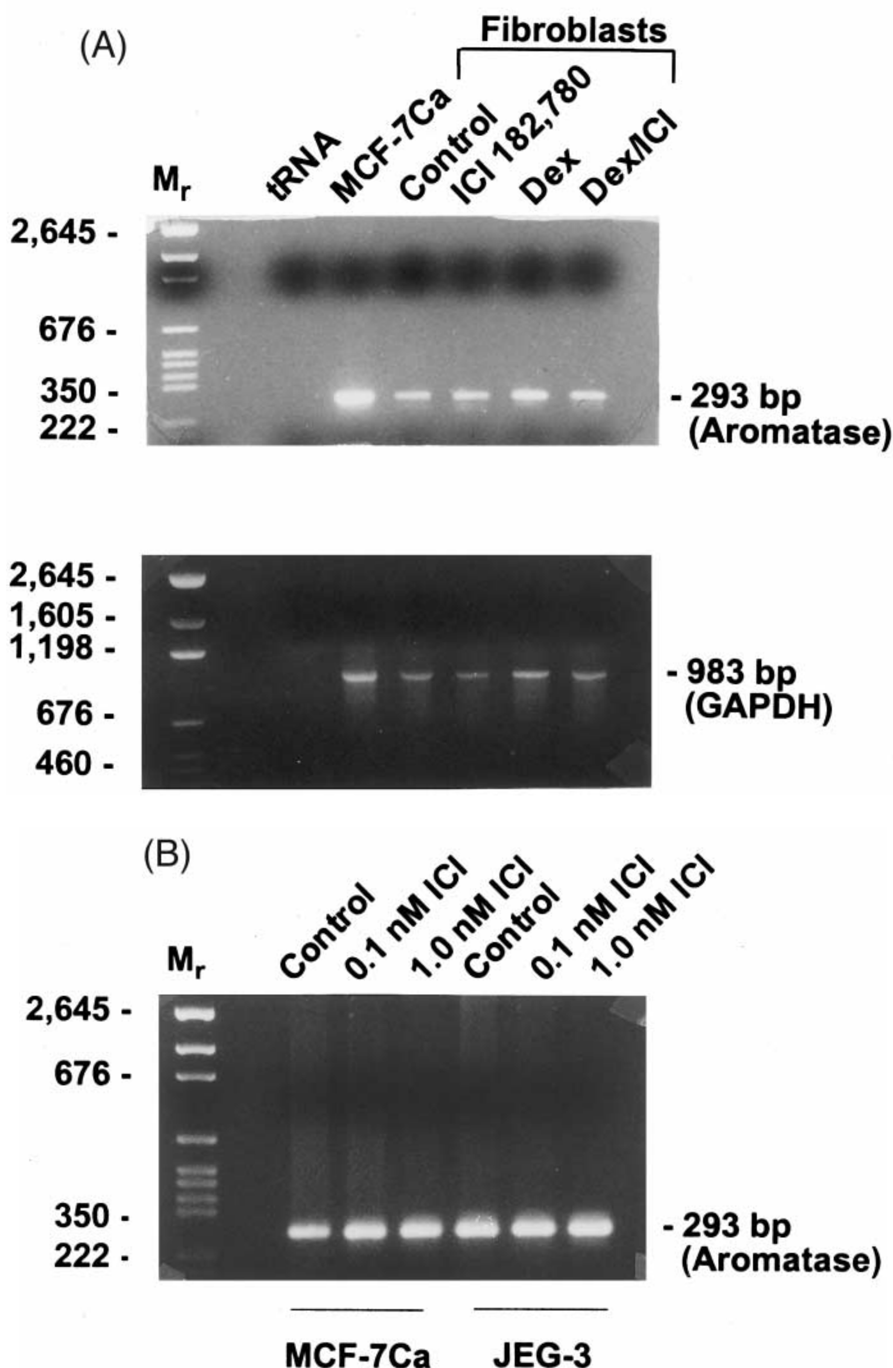


Fig. 9. RT-PCR amplification of aromatase mRNA from: (A) Normal breast fibroblasts that had been treated for 48 h with ICI 182,780 (1.0 μM), Dex (1.0 μM) and a combination of ICI 182,780 and Dex. The size and position of the expected 293 bp transcript is shown on the right and the molecular weight markers are shown on the left. tRNA was used as a negative control. RT-PCR analysis of glyceraldehyde-phosphate dehydrogenase (GAPDH; 983 bp fragment) confirmed integrity of the RNA samples and equivalency of loading. (B) MCF-7Ca and JEG-3 cells that had been treated with 0.1 and 1.0 μM ICI 182,780 for 48 h.

half-life [30]. Preclinical studies with ICI 182,780 and its predecessor ICI 164,384 have shown that they are both more effective than tamoxifen in inhibiting the growth of ER-positive human breast cancer cell lines *in vitro* and tumor xenografts *in vivo* [8, 9], and that they are also effective second line therapies for cells and tumors that have acquired resistance to tamoxifen [10, 11]. Clinically, ICI 182,780 has been shown to reduce endometrial thickness in premenopausal women [31], and is effective in patients with primary breast cancer [13], and with advanced breast cancer that has failed tamoxifen therapy [14, 15].

The development of compounds that specifically inhibit the enzyme aromatase (estrogen synthetase) has also provided an alternative approach for treating patients with hormone-dependent breast cancer. The

first specific aromatase inhibitor, 4-OHA, proved to be very effective in tamoxifen resistant breast cancer patients [17, 18] and by blocking estrogen synthesis, 4-OHA does not exhibit any of the agonist side-effects associated with tamoxifen. 4-OHA is an analog of androstenedione and acts by competing with the substrate for the enzyme. In this respect, 4-OHA is comparable to ICI 182,780 which is an analogue of 17β-estradiol, with a long 7α-alkylsulfinyl side chain and acts by competing with E2 for binding to the ER [8]. In addition to 4-OHA, there are a number of other androstenedione analogues which inhibit the aromatase enzyme and, interestingly, bulky substitutions at the C-7 position have yielded some very potent inhibitors [32, 33]. One of the most potent of these compounds, 7α-(4′-amino)phenylthio-1,4-androstadienedione (7α-APTADD, Fig. 1), was capable of suppressing DMBA-induced mammary carcinoma in rats by reducing serum estrogen levels. Studies with these 7α-inhibitors also provided evidence that a hydrophobic binding pocket exists near to this position in the active site of the aromatase enzyme [34]. Given that E2 has no effect on aromatase activity levels in normal breast fibroblasts (Fig. 5), the 7α-substitution on ICI 182,780 appears to be critical for the antiestrogen to function as an aromatase inhibitor. The 7α-acyl chain of ICI 182,780 may also produce an area of potential hydrophobicity which could interact with the hydrophobic site of the aromatase enzyme in a manner similar in principle to 7α-APTADD. The mechanisms of action of 4-OHA and 7α-APTADD are similar in that both bind irreversibly to aromatase and cause enzyme inactivation [34, 35]. In this study, we have shown that ICI 182,780 (or a potential metabolite) also appears to be remain bound to the aromatase enzyme, as a single dose (24 h) of the antiestrogen inhibited aromatase activity levels for an additional 48 h, following its removal from the cells (Fig. 7).

The 7α-side chain on ICI 182,780 appears to be critical for the inhibition of aromatase activity. However, as ICI 182,780 had no effect on aromatase activity levels in placental microsomes, it is likely that another mechanism of action is also involved in the ability of ICI 182,780 to inhibit aromatase. In addition, the IC_{50} values for ICI 182,780 in each of the cell lines are considerably higher than those of the three specific aromatase inhibitors tested (Table 2). In each of the cell lines the order of potency was letrozole>4-OHA>anastrozole>ICI 182,780. It is interesting to note that MCF-7Ca breast cancer cells were the most sensitive to ICI 182,780, and that these were the only cells which were growth inhibited by the antiestrogen. This reduction in cell number appears to be caused by the antiestrogenic nature of ICI 182,780. However, the growth studies performed cannot discriminate between the reduction in cell number caused by the antiestrogenic nature of ICI 182,780 and the reduction in cell number caused by its aromatase inhibiting properties. It is unclear why MCF-7Ca cells were more sensitive to the aromatase inhibiting effects of ICI 182,780, but the results suggest that of the various populations of cells within a breast tumor, the malignant epithelial cells may be the most sensitive to ICI 182,780 regardless of whether it is functioning as an antiestrogen or as an aromatase inhibitor. Our laboratory has shown that functionally active aromatase is localized in the breast cancer epithelial cells and contributes to the increased estrogen levels present in breast tumors [23]. It is possible that the clinical effectiveness of ICI 182,780 as a breast cancer drug may also be related to its ability to inhibit intratumoral aromatase, which we have reported to be localized in the same malignant epithelial cells that express ER [23]. This is also supported by the recent findings of Yue *et al.* who used the MCF-7Ca breast cancer xenograft model in nude mice to show that intratumoral aromatase is responsible for elevated tumor estrogen levels and that this local estrogen production caused increased cellular proliferation [36].

We have shown that the aromatase inhibiting effect of ICI 182,780 is not being mediated by inhibiting the growth of the cells (Fig. 8) nor by reducing levels of the aromatase transcript (Fig. 9). In addition, ICI 182,780 does not affect the uptake of aromatase substrate by JEG-3 cells, nor does it appear to down-regulate aromatase protein levels (data not shown). Also, we were unable to detect the presence of an active metabolite of ICI 182,780 in cell culture media which may have been responsible for its aromatase inhibiting properties (data not shown). Therefore, it appears that ICI 182,780 is interacting with the aromatase enzyme to reduce activity levels. However, the exact nature of this interaction remains unclear. Aromatase catalyzes the formation of 17β-estradiol from the androgen androstenedione, and therefore, estrogen will be present in the enzyme for a short period of time before being released as product. The results from this study indicate that ICI 182,780 (or a potential metabolite) is not being released from the enzyme, but remains bound and inhibits aromatase activity levels. Irrespective of the mechanism by which ICI 182,780 functions to inhibit aromatase, it is clear that when each of the cell lines is incubated with the antiestrogen for an extended period of time, aromatase activity levels are substantially reduced (Table 1), and that activity remains inhibited even after the drug is withdrawn (Fig. 7). In its dual capacity, primarily as an antiestrogen and secondarily as an aromatase inhibitor, ICI 182,780 is likely to be beneficial for the treatment of breast cancer patients. In particular, ICI 182,780 should be effective for patients who have developed resistance to tamoxifen, as these tumors are not expected to exhibit cross resistance to steroidal antiestrogens [10, 11].

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