The anti-oestrogen ICI 182,780, but not tamoxifen, inhibits the growth of MCF-7 breast cancer cells refractory to long-term oestrogen deprivation through down-regulation of oestrogen receptor and IGF signalling

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Abstract

Long-term culture of MCF-7 wild-type (wt) cells in steroid-depleted medium (LTED) results in hypersensitivity to oestradiol (E2) coinciding with elevated levels of ER α and enhanced growth factor signalling. In this study, we aimed to compare the effects of the pure anti-oestrogen ICI 182,780 (ICI) with the competitive anti-oestrogen tamoxifen (TAM) on oestrogen and IGF signalling in these cells. Wt MCF-7 and LTED cells were treated with a log 7 concentration range of E2, TAM or ICI. Effects on cell growth, ER α transactivation, expression of ER α , ER β and components of the IGF pathway were measured with and without insulin. In the presence of insulin, growth of LTED cells was refractory to TAM but inhibited by ICI and E2. In the absence of insulin, LTED cells showed persistent hypersensitivity to E2, and remained inhibited by ICI but were largely unaffected by TAM. ICI but not TAM inhibited ER-mediated gene transcription and treatment with ICI resulted in a dose-dependent reduction in ER α levels whilst having no effect on ER β expression. IGF-I receptor and insulin receptor substrate 2 levels were increased in LTED versus the Wt MCF-7 cells, and ICI but not TAM reduced their expression in a dose-dependent fashion. Thus IGF signalling as well as ER α expression and function are enhanced during LTED. While the resultant cells are resistant to TAM, ICI down-regulates ER α , reducing IGF signalling and cell growth. These results support the use of ICI in women with ER-positive breast cancer who have relapsed on an aromatase inhibitor.

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Introduction

Most patients with oestrogen receptor (ER)-positive advanced breast cancer who have relapsed on tamoxifen (TAM) are prescribed aromatase inhibitors. Unlike TAM, which competes with oestrogen for the ER, aromatase inhibitors block the conversion of androgens to oestrogens removing the proliferative

stimuli of the tumour (Buzdar *et al.* 1998, Dombernowsky *et al.* 1998). Randomised trials in postmenopausal women with breast cancer have shown that oestrogen deprivation with aromatase inhibitors is superior to TAM as first-line therapy in metastatic disease (Nabholtz *et al.* 2000, Mouridsen *et al.* 2001). In the adjuvant setting, preliminary data in ER-positive postmenopausal primary breast cancer

have shown improved disease-free survival for the aromatase inhibitor anastrozole in comparison with TAM (ATAC Trialists' Group 2002). As a consequence, it is likely that an increasing number of ER-positive postmenopausal women may be treated by oestrogen deprivation as their initial endocrine therapy of choice (Buzdar & Howell 2001). Despite these advances the majority of women treated with aromatase inhibitors will eventually relapse (for review see Johnston & Dowsett 2003). As a consequence, understanding the mechanisms involved in resistance to oestrogen deprivation is of major clinical relevance for optimal treatment of breast cancer patients and the development of new therapeutic regimes.

Currently emerging data suggest that endocrine sensitivity may not only be maintained but enhanced when cells become refractory to long-term oestrogen deprivation (LTED). We and others have shown that hormone-sensitive MCF-7 breast cancer cells treated by LTED eventually adapt to residual oestradiol (E2) within their maintenance medium, becoming hypersensitive and maximally growth stimulated at doses as low as 10^{-13} M versus 10^{-9} M for parental cells (Masamura et al. 1995, Santen et al. 2001, Chan et al. 2002, Martin et al. 2003). Thus, in the clinical setting, tumour cells may become growth stimulated by the low E2 levels induced by LTED, which would imply that a functional ER signalling pathway remains in these cells. Jeng et al. (1998) previously demonstrated that ER expression and function was up-regulated in MCF-7 LTED cells, although not all oestrogen-responsive genes were activated.

More recent data suggest that an adaptive and complex 'cross-talk' network develops between various growth factor receptor pathways and ER signalling to account for refractory growth (Nicholson et al. 1999, Martin et al. 2003). Cells may utilise these alternative pathways to escape from the constraints of initial oestrogen deprivation therapy, with either the MAP kinase (p42/p44 MAPK) pathway (Coultts & Murphy 1998, Shim et al. 2000, Martin et al. 2003) or the insulin-like growth factor (IGF) pathway (Stephen et al. 2001) being used to activate ER signalling. We have previously shown that ERBB2 activation of p42/p44 MAPK results in enhanced transcriptional activity of ER in LTED cells (Martin et al. 2003). Evidence for the role of such cross-talk in causing enhanced cell growth and ER signalling was demonstrated by the ability of both an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (ZD1839) and a MEK inhibitor (U0126) to inhibit cell proliferation and oestrogen-regulated gene

transcription in LTED cells. Further analysis showed that ablation of MAPK reduced the sensitivity of these cells to added E2 but had no effect on phosphorylation of the ER, suggesting that these cells remained ligand dependent (Martin et al. 2003).

An important but unanswered question remains, namely that if ER is expressed and hypersensitive in LTED cells, would an anti-oestrogen still be effective in this setting? It is unclear whether such cells would be cross-resistant with TAM, possibly perceiving TAM as an agonist which might stimulate rather than inhibit growth. It is known that, in general, ER requires a ligand (E2 or TAM) to allow receptor dimerisation and interaction with DNA and, given the enhanced sensitivity of LTED cells to E2, concern might exist about the effect of TAM in this scenario, particularly in view of the fact that ERBB2 may also be up-regulated (Martin et al. 2003). ICI 182,780 (ICI) is a steroidal pure anti-oestrogen devoid of agonist activity (Wakeling & Bowler 1992). *In vitro*, ICI's binding affinity for the ER is approximately 100-fold greater than TAM, and in ER-positive MCF-7 breast cancer cells, ICI unlike TAM is a pure antagonist of oestrogen-regulated gene expression (Rajah et al. 1996). ER expression is suppressed and down-regulated by ICI, without any concomitant rise in EGFR or transforming growth factor- α (TGF- α) expression (McClelland et al. 1996).

Other important anti-proliferative effects of ICI include suppression of IGF receptor (IGF-IR) signalling, and previous reports have suggested that ICI, either directly or indirectly, may modulate expression of IGF-IR, IGF-binding proteins (IGFBPs) or the IGF-IR substrate insulin receptor substrate (IRS)-1 (Stewart et al. 1990a, Huynh et al. 1996a,b, Lee et al. 1999, Salerno et al. 1999, Molloy et al. 2000, Chan et al. 2001).

The main aim of this study was to determine the responsiveness of both wild-type (wt) and LTED MCF-7 breast cancer cells to two different anti-oestrogens, TAM and ICI. In particular, we wished to compare the effect of increasing doses of E2, TAM and ICI on the inhibition of cell growth in both the presence and the absence of exogenous insulin. We investigated the effects of both anti-oestrogens on ER expression (ER α and ER β), and measured changes in ER function in response to TAM and ICI using transient transfection with an oestrogen response-element (ERE) reporter gene. Finally, we examined the changes in IGF signalling in LTED compared with Wt MCF-7 cells, and the effects of TAM and ICI on components of the IGF signalling pathway.

Materials and Methods

Materials

ER α -6F11 antibody was obtained from Novocastra (Newcastle, Tyne and Wear, UK). The ER β , IRS-1 and IRS-2 antibodies were obtained from Upstate Biotechnology (Dundee, UK). The IGF-IR antibody was purchased from Autogene Bioclear and horse-radish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Amersham, Bucks, UK). 17 β -oestradiol (E2) and TAM were obtained from Sigma (Poole, Dorset, UK). Dr A Wakeling (AstraZeneca Pharmaceuticals, Alderley Edge, Cheshire, UK), kindly provided ICI 182,780. All chemicals, unless otherwise stated, were molecular grade and purchased from Sigma. All tissue culture grade plastics were purchased from Nunc (VWR, Poole, UK).

Tissue culture

Wt MCF-7 human breast cancer cells were maintained in phenol red-free RPMI 1640 supplemented with 10% foetal bovine serum (FBS), 10 μ g/ml insulin, 2 mM glutamine, 100 units penicillin–streptomycin and 10^{−9} M E2. LTED MCF-7 cells were maintained in medium depleted of steroids and referred to as DCC medium. This was comprised of RPMI 1640 medium containing 2 mM glutamine, 100 units penicillin–streptomycin, 10 μ g/ml insulin, 10% (v/v) dextran charcoal stripped (DCC) FBS. DCC FBS was prepared as previously described (Darbre *et al.* 1983). The level of E2 after charcoal stripping was quantified by radioimmunoassay (Dowsett *et al.* 1987) and was routinely less than 4 pmol/l (4 $\times$ 10^{−12} M) which, when added to RPMI 1640, equated to less than 4 $\times$ 10^{−13} M E2 in the final DCC medium. Cells were passaged weekly and medium was replenished every 2–3 days.

Cell growth assays

Wt MCF-7 cells were depleted of steroids for 3 days and then seeded into 12-well plates at a density of approximately 1 $\times$ 10⁴ cells per well in DCC medium. LTED cells were treated similarly. The cells were left for a further 2 days to acclimatise. Cell monolayers were subsequently treated with E2, TAM or ICI for 6 days with daily changes. The cell number was determined using a Z1 Coulter Counter (Beckman Coulter, High Wycombe, Bucks, UK). In studies determining the effect of insulin on cell growth, both wt and LTED cells were cultured in DCC medium minus insulin for 3 days prior to seeding on 12-well plates. The cells were allowed to acclimatise in DCC

medium minus insulin for a further 2 days prior to stimulation with increasing doses of E2, TAM, ICI, vehicle (0.1% (v/v) ethanol) or combinations of the drugs plus or minus 10 μ g/ml insulin.

Apoptosis assay

Apoptosis was measured using the Cell Death Detection ELISA^{PLUS} (Roche) according to the manufacturer's instructions. In essence, Wt MCF-7 cells (depleted of steroids for 3 days) and LTED cells were seeded into six-well plates at a density of 2 $\times$ 10⁵ cells per well in DCC medium. After 48 h, cells were transferred to serum-free medium for 24 h then treated for 72 h with DCC medium containing 10 μ g/ml insulin plus or minus E2 (10^{−10} M).

Real-time quantitative RT-PCR

Gene expression was assessed using a TaqMan ABI 7900HT (Applied Biosystems, Warrington, Cheshire, UK). mRNA from treated LTED and Wt MCF-7 cells was extracted using RNeasy (Qiagen, UK). The sequences of the primer/probe sets were as follows: ER α : forward (F), 5'-TTCTTCAAGAGAAGTATT-CAAGGACATAAC-3', reverse (R), 5'-TCGTATCC-CACCTTTCATCATTC-3', Probe (P), 5'-FAM-CCAGCCACCAACCAGTGCACCAT-TAMRA-p-3'; progesterone receptor (PR): F, 5'-ACCTGAGGCCG-GATTCAGAA-3', R, 5'-CCACAGGTAAGGACAC-CATAATGAC-3', P, 5'-FAM-CCAGAGCCCCACAA-TACAGCTTCGAGTCATT-TAMRA-p-3'; pS2, F, 5'-GCCCAGACAGAGACGTGTACAG-3', R, 5'-GTCGAAACAGCAGCCCTTATTT-3', P, 5'-FAM-CCCCCGTGAAAGACAGAATTGTGGTTT-TAMR-p-3'; c-myc, F, 5'-GCTGCTTAGACGCTGGATTTT-3', R, 5'-TTCCTGTTGGTGAAGCTAACGTT-3', P, 5'-FAM-TAGTGGAAAACCAGCAGCCTCCCGC-GACGA-TAMR-p-3'; cathepsin D, 5'-ACATCGC-TTGCTGGATCCA-3', R, 5'-GCTGCCCCGAGCCA-TAGTG-3', P, 5'-FAM-ACAAGTACAACAGCGA-CAAGTCCAGCACCTA-TAMR-p-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Applied Biosystems) was used as a housekeeping gene to normalise the data. Analysis was performed in standard 96-well plates. Reactions were carried out in triplicate using 50 ng mRNA. The relative quantity was determined according to the manufacturer's instructions (Applied Biosystems). In essence, the difference in cycle threshold (Δ ct) was determined by normalising against GAPDH. $\Delta\Delta$ ct was then established by normalising against the corrected DCC control.

Transcriptional assay

LTED and Wt MCF-7 cells (previously stripped of steroids for 3 days) were seeded in 24-well plates at a density of approximately 4×10^4 with about 8×10^4 cells per well respectively in DCC medium. The following day, the cells were transfected by lipofectin (Invitrogen, UK) with 0.25 µg ERE luciferase reporter construct (EREItkluc) or activator protein-1 (AP-1) reporter construct (collΔ73-luc), kindly supplied by Dr Paul Webb, University of California, San Francisco, CA, USA) and 0.25 µg β-galactosidase for normalising luciferase data (pCH110) for 4 h. The cells were subsequently fed with DCC medium and left to recover overnight, before treatment with the appropriate concentration of E2, TAM, ICI or vehicle. In the case of AP-1 studies, cells were stimulated with tetradecanoyl phorbol acetate (TPA) as an internal control to monitor AP-1 activity. After treatment for 24 h, the luciferase (Promega, UK) and β-galactosidase (GalactoStar; Applied Biosystems, UK) activity were measured using a luminometer.

Effect of E2, TAM and ICI on ER and IGF signalling

Wt MCF-7 cells (which had previously been stripped of steroids for 3 days by culturing in DCC medium) and LTED cells were seeded at a density of 5×10^5 cells per 25 cm² culture flat. The following day, monolayers were washed once with phosphate-buffered saline (PBS) then replaced with DCC medium and increasing doses of E2, TAM, ICI or vehicle. The medium was changed daily over 6 days prior to harvesting the monolayers for analysis as indicated in the figure legends.

Preparation of whole cell extracts for immunoblots

Cell monolayers were washed with ice-cold PBS then lysed in extraction buffer (1% (v/v) Triton X100, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM sodium fluoride, 2 mM Na₃VO₄ and 1 tablet of complete inhibitor mix (Roche; per 10 ml buffer) and homogenised by passage through a 26-gauge needle six times. The lysate was incubated on ice for 10 min then clarified by centrifugation (14 000 g for 10 min at 4°C). The protein concentration was then quantified using BioRad protein assay kit (BioRad, UK). Equal amounts of protein (50 µg) were resolved by SDS-PAGE and transferred to nitrocellulose filters (Schleicher and Schuell, London, UK). Filters were blocked (10 mM Tris-HCl, pH 8.3 and 150 mM NaCl

containing 4% milk powder), washed twice in PBS containing 0.025% Tween-20 and probed with specific antibodies. The antibodies were diluted in 1.5% bovine serum albumin, 10 mM Tris-HCl, pH 8.3, 150 mM NaCl, 0.025% Tween-20 and 0.01% sodium azide. Immuno complexes were detected using Ultra-Signal chemiluminescence kit from Pierce (Chester, Cheshire, UK). Chemiluminescence was quantified using Fluro-S and analysed using Quantity One software (BioRad).

Results

Growth of Wt MCF-7 and LTED cells in response to E2, TAM and ICI with or without added insulin

Evidence suggests that insulin and IGFs are potent breast cancer cell mitogens able to act synergistically with E2 (Stewart *et al.* 1990b). Studies have also shown that although IGF-IR has a 70-fold lower affinity for insulin compared with IGF-I, high doses of insulin can directly activate IGF-IR (Smith *et al.* 1988). Since the Wt MCF-7 and LTED cells were maintained in medium containing high concentrations of insulin and we had previously demonstrated the LTED cells had a twofold higher expression level of IGF-IR compared with Wt MCF-7 cells (Martin *et al.* 2003), we examined whether removal of insulin would affect the growth rate of the two cell lines in the presence of escalating doses of E2, TAM and ICI. E2 stimulated the proliferation of Wt MCF-7 cells in a dose-dependent manner both in the presence and the absence of insulin, with maximum growth occurring at 10^{-9} M in both settings (Fig. 1A and B). In contrast, there was no growth stimulation by E2 in LTED cells in the presence of insulin, but a significant growth inhibition by E2 at concentrations $>10^{-10}$ M (Fig. 1C). As previously reported, the baseline growth of the LTED cells in the absence of added E2 was tenfold higher than wt cells (data not show). In the absence of insulin, LTED cells revealed a persistent hypersensitivity to E2, being maximally stimulated at concentrations of E2 as low as 10^{-13} M (Fig. 1D).

In Wt MCF-7 cells, TAM alone at maximal concentration (10^{-6} M) caused a slight, but significant, enhancement of proliferation in the presence and absence of insulin (Fig. 1A and B). There was no significant growth inhibition by ICI (in the absence of exogenous E2) in Wt MCF-7 cells plus or minus insulin. In LTED cells plus or minus insulin, TAM had no inhibitory or stimulatory effects, except at the highest concentration (10^{-6} M) where some growth inhibition

Figure 1 Growth response of wt (Wt) MCF-7 and LTED cells to E2 and anti-oestrogens. Wt MCF-7 and LTED cells were seeded into 12-well plates and treated with a log 7 (10^{-13} M to 10^{-6} M) dose range of E2, TAM, ICI or vehicle (0.1% ethanol) for 6 days. (A and C) In the presence of 10 µg/ml insulin (ins) compared with (B and D) in the absence of insulin. The data represent the average of triplicate readings expressed as fold change relative to the vehicle treated control in each case. The means $\pm$ S.E. are shown. Data were confirmed in three independent experiments. Cont, control.

of approximately 50% was observed in the absence of insulin (Fig. 1C and D). In contrast, ICI significantly inhibited the cell growth of LTED cells in a dose-dependent manner to approximately 70% of the control. This effect of ICI in LTED cells was seen both with and without insulin (Fig. 1C and D).

LTED cells were sensitive to the anti-proliferative effects of the pure anti-oestrogen ICI and could be rescued by the addition of E2

To further characterise the sensitivity of the LTED cells to ICI, we compared their growth profile in the presence of increasing doses of ICI alone, versus Wt MCF-7 cells treated similarly but in the presence of a standard dose of E2 (10^{-9} M). Both cell lines revealed similar profiles with IC_{50} values of approximately 10^{-9} M and 5×10^{-9} M respectively (Fig. 2A). These data were consistent with the hypothesis that the LTED cells remain ligand dependent and hypersensitive to residual E2 in the DCC medium. Nonetheless, studies have suggested that ICI may influence proliferation by directly inhibiting growth factor-mediated signalling (Huynh *et al.* 1996a). If this were the case, E2 would be unable to reverse the inhibitory effect of ICI in the LTED cells. To test this hypothesis, LTED and Wt MCF-7 cells were cultured in the presence of a standard dose of 10^{-9} M, 10^{-8} M or 10^{-7} M ICI (demonstrated to inhibit cell growth) and increasing concentrations of E2 (Fig. 2B and C). Doses in excess of 10^{-11} M E2 reversed the inhibitory effect of 10^{-9} M ICI in the LTED cells (maximum stimulation 10^{-10} M) whilst in the Wt MCF-7 cells a dose of 10^{-10} M E2 was required (maximum stimulation 10^{-9} M). Most noteworthy, doses in excess of 10^{-10} M E2 were inhibitory in the LTED cells. In the presence of 10^{-8} M ICI, similar doses of E2 were required to rescue the inhibitory effect of ICI in both the Wt MCF-7 and LTED cells. However, a full log 2 increase in E2 was necessary to rescue the Wt MCF-7 cells treated with 10^{-7} M ICI compared with a single log increase for the LTED.

The pure anti-oestrogen ICI revealed the hypersensitive phenotype of the LTED cells

In an attempt to further reveal the hypersensitive nature of the LTED cells, a similar set of experiments in which LTED cells were treated with a standard dose of E2 (10^{-9} M) and escalating doses of ICI (Fig. 3A) was carried out. E2 (10^{-9} M) alone inhibited LTED cell growth 50% compared with the DCC control as

noted previously (Fig. 1C); however, this inhibition was reversed by increasing doses of ICI with cell proliferation restored almost to control levels by 10^{-8} M ICI. Doses in excess of 10^{-7} M ICI once again inhibited LTED cell proliferation in accordance with our previous findings. To test this further, a reverse experiment was carried out in which the LTED cells were treated with a standard dose of ICI (10^{-9} M) plus or minus insulin and increasing doses of either E2 or TAM (Fig. 3B–E) and their proliferation profile was compared with Wt MCF-7 cells treated with escalating doses of E2 or TAM plus or minus insulin but in the absence of ICI. In the presence of insulin and ICI (10^{-9} M), the inhibitory effect of E2 was reversed in the LTED cells (Fig. 3B) with maximum proliferation evident at 10^{-10} M E2 whilst doses in excess of 10^{-9} M E2 were now required to decrease proliferation by 50% (as opposed to 10^{-10} M in the absence of ICI; see Fig. 1C). In the absence of insulin, however, the LTED cells showed a similar proliferation profile to Wt MCF-7 cells with maximum growth occurring at 10^{-9} M E2 (Fig. 3C). Most notably, treatment of the LTED cells with increasing doses of TAM in the presence of 10^{-9} M ICI (plus or minus insulin) revealed the agonist effect of TAM, in keeping with the responses seen in the Wt MCF-7 cells (Fig. 3D and E). The ability of ICI to reverse the inhibitory effect of E2 is indicative of the hypersensitivity of the LTED cells and provides further evidence of their ligand-dependent nature. We postulate that in this setting ICI reduces the amount of ER to similar levels as those seen in wt cells and hence reveals the stimulatory effect of E2 and agonist activity of TAM. Taken together, these data suggested that the LTED cell proliferation was mediated wholly or in part via the ER/ERE pathway.

High doses of E2 resulted in enhanced apoptosis in LTED cells

As demonstrated in Fig. 1C, E2 doses in excess of 10^{-10} M caused a marked decrease in the proliferation of the LTED cells. Previous studies have suggested that high doses of E2 can result in increased apoptosis (Song *et al.* 2001, 2005, Liu *et al.* 2003, Osipo *et al.* 2003, Lewis *et al.* 2005). To investigate the possibility that this was responsible for the decrease in cell proliferation noted in this setting, LTED and Wt MCF-7 cells were treated with or without 10^{-10} M E2 for 72 h in the presence of insulin and the number of apoptotic cells determined. Although E2 appeared to have little effect on the Wt MCF-7 (Fig. 4), there was a threefold increase in apoptosis in the LTED cells.

Figure 2 The pure anti-oestrogen ICI inhibits LTED cell growth but can be rescued by E2. (A) Cell growth assays were performed as described in the Materials and Methods. LTED and Wt MCF-7 cells were cultured in the presence of increasing doses of ICI alone or, in the case of Wt MCF-7 cells, in combination with 10^{-9} M E2. Cells were incubated for 6 days. The data represent the average of triplicate readings. The means $\pm$ s.e. are shown. (B and C) Wt MCF-7 and LTED cells were cultured as previously described in the presence of 10^{-9} M (-9 M), 10^{-8} M (-8 M) or 10^{-7} M (-7 M) ICI and increasing doses of E2. The data represent the average of triplicate readings. The means $\pm$ s.e. are shown. Data were confirmed in two independent experiments.

LTED appeared to use the classical ER/ERE pathway for proliferation and transcription

The proliferation data depicted in Figs 1 and 2 suggested that the LTED cells used the classical ER/ERE

pathway for proliferation and transcription. To test this further, the level of five endogenous genes known to be responsive to E2 were screened by quantitative real-time RT-PCR. Both LTED and Wt MCF-7 cells were treated with escalating doses of E2 for 24 h in the

Figure 3 The pure anti-oestrogen ICI reveals the hypersensitive phenotype of the LTED cells. (A) Addition of ICI reduced the anti-proliferative effects of E2. LTED cells were cultured as previously described in the presence of 10⁻⁹ M E2 and increasing doses of ICI. The data represent the average of triplicate readings. The means $\pm$ S.E. are shown. (B and C) Addition of ICI reveals the hypersensitivity of the LTED cells to E2. LTED cells were cultured with or without insulin (ins) in the presence or absence of 10⁻⁹ M ICI and increasing doses of E2. Wt MCF-7 cells were treated similarly but in the absence of ICI. The data represent the average of triplicate readings. The means $\pm$ S.E. are shown. (D and E) Addition of ICI reveals the agonist activity

Figure 4 High doses of E2 result in enhanced apoptosis in LTED cells. LTED (solid bars) and Wt MCF-7 (dotted bars) cells were cultured in DCC medium plus or minus 10^{-10} M E2 (-10 E2) for 72 h. The number of apoptotic cells was determined using a cell death detection ELISA. The data represent the average of duplicate readings. The means $\pm$ S.E. are shown. Data were confirmed in two independent experiments.

presence of insulin. RNA was extracted from the cell lines and the levels of expression of ER, PR, cathepsin D, c-myc and pS2 were measured. Treatment with E2 resulted in a largely dose-dependent decrease in ER (Fig. 5) in both Wt MCF-7 and LTED cells. However, in the LTED cells, doses as low as 10^{-13} M caused a 50% decrease in ER mRNA whilst having no effect on the Wt MCF-7 cell line. Treatment with 10^{-8} M E2 caused an increase in ER mRNA expression in three independent experiments in both cell lines and as yet we have no explanation for this finding. Expression of pS2, cathepsin D, c-myc and PR (Fig. 5) all increased in a dose-dependent manner in both cell lines in response to added E2.

To further assess the effects of ER/ERE activity in the LTED versus the Wt MCF-7, transient transfections with an ERE-linked luciferase reporter construct were carried out. The basal transcription activity within the Wt MCF-7 and LTED cells in the presence and absence of insulin was monitored. As we have documented previously (Martin *et al.* 2003) there was an approximate tenfold increase in ER transcriptional activity in LTED compared with Wt MCF-7 cells. This was observed to a similar extent with or without added insulin (Fig. 6A).

A dose-dependent increase in ER-mediated transcription was observed with E2 both in Wt MCF-7 and LTED cells (Fig. 6B). The increase in response to E2 was approximately 20-fold over control for both the Wt MCF-7 and LTED cells. However, as shown in Fig. 6A, LTED cells started with a significant increase in basal transcriptional activity compared with Wt cells. Thus for 10^{-13} M E2 the LTED cells transcription is approximately tenfold higher than the Wt MCF-7 cells in absolute terms. It should be noted that while it is reasonable to describe this response to low doses of E2 as hypersensitive it does not comply with the strict definition of a significantly reduced dose required to achieve 50% maximal stimulation. Analysis of the effect of insulin on transcriptional activity in response to E2 revealed no difference in Wt or LTED cells plus or minus insulin (data not shown).

In Wt MCF-7 cells (in the absence of added E2), TAM alone caused no significant stimulation or inhibition of ER transcriptional activity (Fig. 6C) and was independent of insulin (data not shown). Although TAM had a partial agonist effect on the growth of Wt cells at high concentrations (10^{-6} M), no such effect on the ER α transactivation was seen (Fig. 6C). Likewise, in Wt MCF-7 cells, ICI (in the absence of added E2) had no inhibitory effect on ER α transactivation (Fig. 6D). As such, these transcriptional results with both anti-oestrogens broadly mirrored the effects seen in cell growth assays (Fig. 1).

In LTED cells, TAM had no effect on ER α transactivation (Fig. 6C). In contrast, ICI significantly inhibited (by >90%) the transcriptional activity of ER in a dose-dependent manner (Fig. 6D). Again this inhibitory effect of ICI in LTED cells was seen both in the presence and the absence of insulin (data not shown) and mirrored the growth-inhibitory effects seen with ICI.

We next investigated the ability of ICI to antagonise E2-stimulated transcription in both Wt MCF-7 and LTED cells. In the presence of a standard dose of 10^{-9} M E2 and increasing doses of ICI, the Wt MCF-7 cells were inhibited by ICI in a dose-dependent manner and showed a similar ER α transactivation profile to the LTED cells treated with ICI in the absence of E2 (compare Fig. 6D with 6E). In the presence of a standard dose of E2, the LTED cells showed a reduced sensitivity to ICI compared with the Wt cells (Fig. 6E). This, combined with our previous cell growth data, suggests that elevated levels of ER and the resultant

of TAM in LTED cells. LTED cells were cultured with or without insulin in the presence or absence of 10^{-9} M ICI and increasing doses of TAM. Wt MCF-7 cells were treated similarly but in the absence of ICI. The data represent the average of triplicate readings. The means $\pm$ S.E. are shown. All data were confirmed in three independent experiments.

Figure 5 LTED appear to use the classical ER/ERE pathway for proliferation and transcription. LTED and Wt MCF-7 were cultured in the presence of DCC medium containing insulin and plus or minus increasing doses of E2. The level of expression of five endogenous E2 responsive genes was measured by quantitative real-time RT-PCR. The data represent the average of triplicate samples. The means $\pm$ s.e. are shown. The data were confirmed in three independent experiments.

ER-driven transcription are important components of the LTED cell phenotype.

LTED did not appear to use non-classical AP-1 pathways as part of their adaptive mechanism

Recent studies have suggested that resistance to endocrine therapies may involve the non-classical interaction of ER with the Fos/Jun (AP-1) complex, allowing the ER to exert its action by binding indirectly to AP-1 sites in target genes influencing proliferation and cell survival (Webb *et al.* 1999, Shaulian & Karin 2001, DeNardo *et al.* 2005). To determine if AP-1 played a role in the LTED phenotype, Wt MCF-7 and LTED cells were transfected with an AP-1 luciferase reporter construct followed by exposure to TPA and increasing doses of E2, TAM or ICI (Fig. 7). Assessment of the basal AP-1 activity in the LTED versus the Wt MCF-7 cells showed markedly less activity in the LTED cell line (approximately 50% in the presence of insulin and approximately 70% less in the absence of insulin) compared with the parental cell line (Fig. 7A). TPA caused a twofold increase in AP-1 transactivation as expected. However, none of the other endocrine agents revealed any effect (Fig. 7B–D) plus or minus insulin (data minus insulin not shown). This suggested that AP-1 did not play a major role in this setting.

Expression of ER α , ER β , IGF-IR, IRS-1 and IRS-2 in Wt MCF-7 and LTED cells

The basal level of expression of ER α , ER β and components of the IGF signalling pathway in Wt MCF-7 and LTED cells was assessed in untreated cells growing in their maintenance medium. In LTED cells, expression of ER β and IRS-1 were similar to the Wt cells, whilst levels of ER α , IRS-2 and IGF-IR protein were all significantly increased (Fig. 8).

Effect of E2, TAM and ICI on expression of ER α and ER β in Wt MCF-7 and LTED cells

To investigate the basis by which ICI, but not TAM, can inhibit both the cell growth and the transcriptional activity of the LTED cells (with insulin), both Wt MCF-7 and LTED cells were treated for 6 days in the presence of increasing doses of E2, TAM or ICI plus insulin. Western blots of whole cell extracts were probed for ER α and ER β expression. ICI (in the absence of E2) down-regulated ER α protein expression in a dose-dependent manner, both in Wt and

LTED cell lines. It is noteworthy that higher doses of ICI were necessary to reduce ER α levels in the LTED compared with Wt MCF-7 (10^{-8} M versus 10^{-10} M) (Fig. 9A and B). ICI had no effect on ER β expression in either Wt or LTED cells and to our knowledge this is the first time this observation has been reported. There was no effect of TAM on expression of ER α or ER β in either cell type (compare Fig. 9A with 9B). E2 had a dose-dependent inhibitory effect on ER α expression in the Wt cells and most markedly in the LTED cells where doses in excess of 10^{-9} M resulted in complete loss of ER α (Fig. 9A).

Effect of E2, TAM and ICI on expression of IGF signalling components in Wt MCF-7 and LTED cells

Addition of E2 resulted in an increase in IGF-IR expression in both Wt MCF-7 and LTED cells particularly at doses in excess of 10^{-9} M (Fig. 9A and B). IRS-1 decreased in the LTED cells whilst IRS-2 remained unchanged. Most notably, this decrease in IGF-IR signalling in the LTED cells coincides with high doses of E2, which we previously showed (Fig. 1) decreased proliferation and increased apoptosis (Fig. 4). Conversely, in the Wt MCF-7 cells, IRS-1 remained unchanged whilst IRS-2 expression was increased, in keeping with the stimulatory response to high doses of E2 and enhanced proliferation. TAM (in the absence of exogenous E2) had no appreciable effect on expression of IRS-1, IRS-2 or IGF-IR expression in Wt MCF-7 cells (Fig. 9B). Similarly, the LTED cells treated with TAM showed no significant changes in IRS-1 or IRS-2 expression. However, TAM doses in excess of approximately 10^{-7} M had an inhibitory effect on IGF-IR expression in the LTED cells (Fig. 9A).

ICI (in the absence of added E2) had no effect on the expression of IRS-1, IRS-2 or IGF-IR in the Wt MCF-7 cells. However, similar treatment of the LTED cells resulted in a dose-dependent decrease in the expression of IRS-1, IRS-2 and IGF-IR (Fig. 9A).

Effect of TAM and ICI on expression of IGF signalling components in Wt MCF-7 in the presence of exogenous E2

We hypothesised that in the absence of exogenous E2 the un-liganded ER within the Wt MCF-7 cells would be largely inactive and unable to drive ER/ERE-directed transcription. As a consequence, although ICI would decrease ER α protein expression, its inhibitory effects on proliferation would be negligible. This view is supported by the lack of effect of ICI on IGF-IR

Figure 6 Effects of E2, TAM and ICI on transcriptional activity of an ERE-reporter gene in wt and LTED cells. (A) Basal ER-mediated transcription in the presence and absence of insulin. Cells were co-transfected with EREII_{tk} and pCH110. After transfection, cells were treated with DCC medium plus or minus insulin for 24 h. Cells were harvested and luciferase and β -galactosidase activities measured. To correct for differences in transfection efficiency, the luciferase activities were normalised to β -galactosidase activities. Normalised luciferase activity from triplicate wells was expressed relative to the Wt MCF-7 control. Bars represent $\pm$ S.E. of the means. (B) The effect of E2 on ER-mediated transcription. Wt and LTED cells were transiently co-transfected with EREII_{tk} and pCH110 in serum-free medium followed by 24-h incubation with DCC medium

Figure 7 LTED cells do not appear to use non-classical AP-1 pathways as part of their adaptive mechanism. (A) Basal AP-1-mediated transcription in the presence and absence of insulin. Cells were co-transfected with coll Δ 73-luc and pCH110. After transfection, cells were treated with DCC FBS medium plus or minus insulin for 24 h. Cells were harvested and luciferase and β -galactosidase activities measured. To correct for differences in transfection efficiency, the luciferase activities were normalised to β -galactosidase activities. Normalised luciferase activity from triplicate wells was expressed relative to the wt MCF-7 control. Bars represent $\pm$ s.e. of the means. (B) LTED and Wt MCF-7 cells were co-transfected with coll Δ 73-luc and pCH110. After transfection, cells were treated with 10^{-7} M TPA or increasing doses of E2 for 24 h. Cells were harvested and luciferase and β -galactosidase activities measured. The data are reported as the fold increase compared with vehicle-treated control. (C) The effect of TAM on AP-1-mediated transcription. Wt and LTED cells were treated for 24 h with DCC medium containing increasing doses of TAM in the absence of exogenous E2. (D) The effect of ICI on AP-1-mediated transcription. Wt and LTED cells were transfected as described previously followed by treatment for 24 h with increasing doses of ICI in the absence of exogenous E2. All data shown were normalised against the β -galactosidase control. Luciferase activity from triplicate wells was expressed relative to the vehicle-treated control. Bars represent $\pm$ s.e. of the means. Results were confirmed in three independent experiments.

containing increasing doses of E2 plus or minus insulin (minus insulin data not shown). Normalised luciferase activity from triplicate wells was expressed relative to the vehicle-treated control. Bars represent $\pm$ s.e. of the means. (C) TAM has no significant effect on Wt MCF-7 and LTED cells. Cell lines were transfected as described and treated for 24 h with increasing doses of TAM in the absence of exogenous E2. (D and E) ICI inhibits basal ER-mediated transcription in LTED cells compared with Wt MCF-7. Wt and LTED cells were transfected as described then treated for 24 h with increasing doses of ICI plus or minus E2. Normalised luciferase activity from triplicate wells was expressed relative to the vehicle-treated control. Bars represent $\pm$ s.e. of the means. Results shown were confirmed in four independent experiments.

Figure 8 Basal expression of ERs (ER α and ER β), IGF-IR and IRS-1 and IRS-2 proteins in Wt MCF-7 and LTED cells. Cell lysates from Wt and LTED cells growing in their maintenance medium were resolved through 10% SDS-PAGE gels. Following western blotting, filters were subsequently probed with antibodies raised against ER α , ER β , IGF-IR, IRS-1 and IRS-2.

expression. Conversely, in the LTED cells, our current data suggest that ER/ERE activity is enhanced by hypersensitivity to the residual E2 within the DCC medium. As a consequence, the inhibitory effects of ICI were apparent in both transcription and proliferation assays and supported by the concomitant decrease in ER α and IGF-IR expression. To support our view further, that the LTED cells were hypersensitive to E2 and that inhibition by ICI was a direct result of this, we treated Wt MCF-7 cells with a standard dose of E2 (10^{-9} M) and increasing doses of ICI and TAM in the presence of insulin (Fig. 9C). TAM had no effect on ER, IRS-1, IRS-2 or IGF-IR protein expression. However, increasing doses of ICI resulted in a decrease in ER, IRS-1, IRS-2 and IGF-IR expression, which was particularly marked at doses in excess of 10^{-8} M. When compared with the LTED cells (Fig. 9A) the profile was similar but in this case doses in excess of 10^{-9} M resulted in almost complete loss of IGF-IR, confirming the hypersensitivity of the LTED cells.

Discussion

There is increasing evidence for adaptive changes in the hormone responsiveness of breast cancer cells during therapy with LTED with aromatase inhibitors. With the greater use of first-line LTED for most breast cancer patients who have ER-positive tumours (Nabholtz *et al.* 2000, Mouridsen *et al.* 2001, ATAC Trialists' Group 2002), an understanding of the mechanisms involved in resistance to this approach is of paramount importance for optimal patient care. It is clear that these cells often remain steroid receptor

positive once they become refractory to LTED and that expression and activation of ER is significantly enhanced in these cells (Jeng *et al.* 1998, Chan *et al.* 2002, Martin *et al.* 2003). Some criticism of these models has been made based on recent findings that suggest that culture dishes used to maintain these cell lines contain oestrogenic substances which may contribute to the growth of these LTED cell lines (Ishikawa *et al.* 2001). However, several groups have documented hypersensitivity to low levels of E2 in LTED cells, with growth stimulation at concentrations as low as 10^{-13} M E2 (Masamura *et al.* 1995, Santen *et al.* 2001, Chan *et al.* 2002, Martin *et al.* 2003), less than the levels of residual E2 (10^{-12} M) detected in postmenopausal women receiving aromatase inhibitors (Geisler *et al.* 2002).

Here we have shown that removal of insulin appears to reveal a persistent hypersensitivity in the LTED cells, which is imperceptible in the presence of insulin, as the cells have reached their maximum growth rate. In fact, in the presence of insulin, doses of E2 in excess of 10^{-10} M (which provide a stimulatory effect in the Wt cells) are inhibitory to a similar level as ICI and resulted in enhanced apoptosis in the LTED versus the Wt MCF-7 cells. Previous studies have also reported the inhibitory effect of high doses of E2 and observed a concomitant increase in the expression of Fas ligand. As a consequence, it has been postulated that tumour regression in response to high doses of E2 may result via Fas-mediated apoptosis and that E2 significantly inhibits cell growth primarily through a proapoptotic action involving caspase 7 and 9 (Song *et al.* 2001, 2005, Liu *et al.* 2003, Osipo *et al.* 2003, Lewis *et al.* 2005).

Overall, these data suggest that cross-talk between the IGF and steroid signalling pathways may be involved in the adaptation of the cells to E2 deprivation.

Although changes in the molecular cross-talk allowing adaptation to LTED appear complex (Coutts & Murphy 1998, Jeng *et al.* 1998, Nicholson *et al.* 1999, Shim *et al.* 2000, Stephen *et al.* 2001, Chan *et al.* 2002, Martin *et al.* 2003) it is clear that ER signalling remains an integral part of this mechanism driving proliferation of these cells. Hence evaluating whether anti-oestrogens remain effective in this setting is of major importance.

We have previously demonstrated that LTED cells have a fourfold higher level of ER, which is phosphorylated on ser¹¹⁸ compared with the Wt cells. Phosphorylation of ER in the LTED setting remained ligand dependent, as evidenced by the fact that complete abrogation of p42/p44 MAPK (shown to phosphorylate ER ser¹¹⁸ in a ligand-independent

A LTED **B** Wt MCF-7 **C** Wt MCF-7 + E2 (10^{-9} M)

Figure 9 Effect of E2, TAM and ICI on expression of ERs (ER α and ER β), IGF-IR, IRS-1 and IRS-2 protein expression in Wt MCF-7 and LTED cells. Cells lysates prepared from wt and LTED cells treated for 6 days with increasing concentration of E2 (control (0.1% (v/v) vehicle), 10^{-13} , 10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} M), TAM (control (0.1% (v/v) vehicle), 10^{-12} , 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} M) or ICI (control (0.1% (v/v) vehicle), 10^{-12} , 10^{-11} , 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} M) plus insulin were resolved through 10% SDS-PAGE gels. Western blot analysis with antibodies raised against ER α , ER β , IGF-IR, IRS-1 and IRS-2 was used to profile protein expression. Panels (A and B) show the effect of the treatments on LTED and Wt cells respectively. ICI and TAM treatments are in the absence of exogenous E2. (C) Wt MCF-7 cells in the presence of a standard dose of exogenous E2 (10^{-9} M) and increasing doses of TAM or ICI show similar signalling profile to LTED cells. Cells lysates were prepared from wt cells treated for 6 days as previously described but in the presence of exogenous E2 and insulin. Western blots show the effect on ER α , ER β , IGF-IR, IRS-1 and IRS-2 expression in wt cells. The data shown in each panel are representative of two independent experiments.

manner (Bunone *et al.* 1996)) had no effect on phosphorylation. We have postulated that the observed increase in basal transcription and E2 sensitivity may relate to the elevated level of activated ER (Martin *et al.* 2003).

The data in this study from transient transfection assays with an ERE-reporter gene similarly illustrate a significant increase in ER α transactivation in LTED cells compared with Wt cells (Fig. 3). It has been suggested that enhanced p42/p44 MAPK signalling may be responsible for this sensitisation and activation of ER signalling in the LTED setting (Shim *et al.* 2000, Martin *et al.* 2003). More recently, we reported that enhanced ERBB2 expression and subsequent p42/p44 MAPK activation may be one of the key underlying adaptive changes in these cells (Martin *et al.* 2003). Other polypeptide growth factors may also be important in the growth regulation of breast cancer cells, including insulin and IGF (Stewart *et al.* 1990a, Ando *et al.* 1998). E2 can affect IGF signalling at several levels, increasing the expression of IGF-IR (Stewart *et al.* 1990a), in addition to IGF activators such as IRS-1 (Jackson *et al.* 1998, Lee *et al.* 1999, Molloy *et al.* 2000), and decreasing expression of IGFBPs such as IGFBP-3 (Huynh *et al.* 1996b). In addition, E2 activation of ER may cause a direct interaction between ER and IGF-IR, resulting in activation and phosphorylation of IGF-IR and downstream signals such as p42/p44 MAPK via a non-genomic mechanism (Kahlert *et al.* 2000, Marquez & Pietras 2001, Powell *et al.* 2001). More recently, studies have suggested a role for shc and IGF-IR in mediating the translocation of ER to the plasma membrane leading to activation of p42/44 MAPK (Song *et al.* 2002, 2004). Although our previous studies have suggested that elevated ERBB2 is involved in the increased p42/44 MAPK activity and hence E2 hypersensitivity (Martin *et al.* 2003) it is possible that the non-genomic interaction of ER with IGF-IR may provide an alternate explanation for the enhanced p42/44 MAPK activity associated with this hypersensitive phenotype.

In this study we have shown that in LTED cells both the IGF-IR and, to a lesser extent, IRS-2 levels are increased two- to threefold in comparison with Wt cells (Fig. 4). The increase in IGF-IR levels might be a direct consequence of the significant increase in ER α content and ER-mediated gene transcription which has been observed in LTED cells (Jeng *et al.* 1998, Martin *et al.* 2003) and confirmed in this study. It is known that ER α rapidly activates the IGF pathway (Kahlert *et al.* 2000). In similar studies increases in IGF-IR levels in LTED cells have been noted and shown to correlate with a reversal of response to exogenously

added ligand (Stephen *et al.* 2001). Insulin, IGF-I and IGF-II, which have a stimulatory effect on the proliferation of oestrogen-maintained cells, became inhibitory in LTED cells. Our observations on proliferation of LTED cells in response to various ligands are in agreement with this previous report, with E2 having an inhibitory effect on LTED cell growth in the presence of insulin. Daws *et al.* (1996) also reported that over-expression of IGF-IR may alter the proliferative response to E2, but at the same time demonstrated little effect on oestrogen-regulated gene expression. This would be consistent with our finding that, despite the change in proliferative response to oestrogen, we failed to demonstrate a similar shift in ERE gene transcription in LTED cells in the absence of insulin (Fig. 6A). These data imply that in LTED cells where IGF signalling may become enhanced, E2 may have paradoxical inhibitory effects on cell growth in the presence/absence of insulin, but that not all oestrogen-regulated genes may respond in the same way.

The effect of the two anti-oestrogens TAM and ICI on growth and ER-mediated gene transcription in LTED cells was also investigated. In LTED cells, TAM was largely ineffective and had only a minor inhibitory effect on growth and gene transcription at doses in excess of 10^{-7} M. ICI, however, inhibited cell growth in a dose-dependent manner (Fig. 1) with a matching reduction in ER-mediated gene transcription (Fig. 6) compared with the Wt cells, consistent with the drug's known effect in reducing levels of functioning ER as demonstrated in Fig. 5. Of interest, there was no significant effect of ICI on expression of the second isoform ER β (Fig. 5) and to our knowledge this is the first time this observation has been made. Competition of ICI with E2 at the ER β ligand-binding domain is nonetheless expected to block signalling through ER β . Previous studies have associated enhanced expression of ER β with resistance to TAM (Speirs *et al.* 1999); however, we found no evidence of any change in ER β expression in the LTED cells versus the Wt cells.

In LTED cells, expression of IGF-IR and IRS-1/IRS-2 was modulated by ICI, but not by TAM. In Wt MCF-7 cells, TAM is known to modulate IGF signalling by down-regulation of tyrosine phosphorylation of IGF-IR without affecting basal levels of receptor protein (Guvakova & Surmacz 1997). The same group showed that cells engineered to over-express IGF-IR became oestrogen independent, but that TAM could still modulate IGF signalling without affecting levels of IGF-IR protein (Surmacz & Burgaud 1995, Guvakova & Surmacz 1997). Likewise, others have shown that TAM on its own may not alter

IRS-1 levels, although it may inhibit the induction of IRS-1 by E2 (Molloy *et al.* 2000). In contrast, several groups have previously demonstrated that ICI inhibits IGF signalling pathways in breast cancer cells, reducing expression of IGF-I (Huynh *et al.* 1996a), IRS-1 (Salerno *et al.* 1999), and downstream signalling including phosphorylation of phosphoinositide 3-kinase (PI-3-K) and Akt-1 (Chan *et al.* 2001). Our data in LTED cells are supportive of the concept that ER α remains a critical component for IGF signalling. For instance, in the absence of exogenous E2, ICI causes a decrease in ER α and IGF-IR expression in the LTED cells whilst in the Wt MCF-7 cells ICI causes a decrease in ER α but has no effect on IGF-IR (Fig. 5A and B). We propose that this results from the hypersensitivity of the LTED cells to residual E2 in the DCC medium, hence ER signalling continues. However, in the Wt cells, ER is unliganded and thus cross-talk between ER and IGF-IR is absent. Conversely, in Wt cells treated with exogenous E2, ICI reduces expression of both ER α and IGF-IR (Fig. 9C). This view was further corroborated by experiments in an ER-negative breast cancer cell line where re-expression of ER α restored both oestrogen and IGF-mediated signalling and growth (Oesterreich *et al.* 2001).

Ultimately the basis for the differential effect of TAM versus ICI in the LTED cells relates to the different mechanisms of action of these two anti-oestrogens. In LTED cells, enhanced ER α expression and signalling appears to be central to the resistance mechanism. TAM alone is unable to have a significant effect, probably in part due to cross-talk and activation of ER in this system by peptide growth factors including the IGF pathway. TAM still allows ER dimerisation and DNA binding which may facilitate rather than antagonise ER-dependent growth in LTED cells; this is to some extent supported by our data (Fig. 3D and E) which indicate that in the presence of low doses of ICI (decreasing the amount of available ER) the agonist activity of TAM is revealed in the LTED cells. In contrast, the mode of action of ICI results in the targeted degradation of the ER α protein and hence is able to modulate endocrine-dependent signalling, thus inhibiting growth in these LTED cells.

In summary, we have shown that while LTED cells are clearly resistant to TAM, they remain sensitive to the pure anti-oestrogen ICI. Our data suggest that inhibition by ICI occurs by negating ER α signalling and not as a result of inhibition of growth factor receptor pathways as confirmed by our E2 rescue experiments (Fig. 2). We have shown that the LTED

cells have enhanced ER α and IGF-IR/IRS-2 expression and signalling, and that the ability of ICI but not TAM to modulate this in LTED cells may account for the differential sensitivity to these two anti-oestrogens. This may have important implications for the therapeutic options utilised for women with ER-positive breast cancer that becomes resistant to long-term therapy with aromatase inhibitors. Although ICI appears particularly effective in this setting and clinical trials have shown it to be as efficient as anastrozole as a second-line therapy (for review see Buzdar 2003), concerns have been raised with regard to the escalating dose regime necessary to achieve maximal inhibition. As a result of these studies we believe that ICI in combination with an aromatase inhibitor will provide a more effective therapy for women who have relapsed on aromatase inhibitors, rather than ICI alone. In this setting where E2 levels remain suppressed by the aromatase inhibitor, lower doses of ICI would be required to give maximum benefit. Based upon these data we have initiated a clinical trial for treatment of women with advanced ER-positive disease who have relapsed on aromatase inhibitors. Patients will be randomised and treated with ICI, ICI plus anastrozole or exemestane. In conclusion, we believe that this study may provide a realistic alternative to current clinical practice.

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