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TAMOXIFEN INHIBITS GROWTH OF OESTROGEN RECEPTOR-NEGATIVE A549 CELLS

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Abstract—The non-steroidal anti-oestrogen tamoxifen inhibits proliferation of the A549 human lung adenocarcinoma cell line ($EC_{50} \simeq 10$ nM) yet there was no evidence of oestrogen receptor expression as determined by ligand binding assay and northern blotting. 17-β-Oestradiol had no effect on A549 cell proliferation (1 pM–1 μM) and moreover a 100-fold excess failed to reverse the effect of 10 nM tamoxifen as did a 100-fold excess of the steroidal anti-oestrogens ICI 164384 and ICI 182780. However, 4-hydroxytamoxifen which had no significant effect on A549 cell growth (1 pM–1μM) completely antagonized the effect of 10 nM tamoxifen when used at a 100-fold excess. In the presence of oleic acid and stearic acid (10 μM) the growth inhibitory effect of tamoxifen in A549 cells was greatly enhanced, unlike effects mediated by the anti-oestrogen binding protein described in other cells where these fatty acids had no effect. These results indicate the presence of a unique and highly sensitive mechanism in A549 cells whereby concentrations of tamoxifen relevant to classical receptor binding can inhibit cell growth in the absence of the oestrogen receptor.

Key words: anti-oestrogens, fatty acids, lung adenocarcinoma, binding protein, ICI 164384/ICI 182780

The classical explanation of the anti-proliferative action of the anti-oestrogen tamoxifen is that it competes with oestradiol for binding to its receptor and thereby blocks the mitogenic effects of this steroid [1, 2 for review]. This notion is supported by the fact that increasing levels of oestradiol can reverse these effects of tamoxifen [3, 4]. However, it is now apparent that anti-proliferative effects of tamoxifen can be mediated by other oestrogen receptor-(ER§) independent mechanisms (particularly when used at high doses) and that these effects are in general not reversed by oestradiol. Much interest has focused upon the discovery of an anti-oestrogen binding protein [5]. This is an intracellular protein associated with the endoplasmic reticulum and binds non-steroidal anti-oestrogens with high affinity and specificity and appears to have a ubiquitous distribution [6]. The physiological role of this protein remains unknown; however, its binding affinity for tamoxifen is reduced in the presence of certain fatty acids [7] and a correlation has been made between this control of binding by fatty acids and the regulation of lymphoid cell growth [8].

Other direct inhibitory effects of tamoxifen have also been reported including inhibition of calmodulin activity [9], histamine action [10], cyclooxygenase [11], 5-lipoxygenase [12], lipid peroxidation [13] and protein kinase C (PKC) [14]. However, high (>μM) concentrations of tamoxifen are required to produce these effects and the interpretation of this data is open to debate.

We have previously described a cell proliferation model using the A549 human lung adenocarcinoma cell line in which low concentrations (1 pM–1 μM) of dexamethasone inhibit cell growth through a glucocorticoid receptor-mediated mechanism [15]. This treatment results in an induction of the protein lipocortin-1 which suppresses the generation of the eicosanoid metabolites, such as prostaglandin E_2, necessary to maintain the proliferation of these cells [15]. The proliferation of A549 cells is inhibited by glucocorticoids but not by progesterone [15] and we have therefore decided to investigate in more detail the range of steroid responsiveness of this cell line using oestradiol and anti-oestrogens. A549 cells are also regulated by growth factors such as epidermal growth factor (EGF) [16] and the differential autocrine regulation of transforming growth factors, TGF-β_1 and TGF-β_2, production has been shown to modulate the growth effects of retinoic acid, glucocorticoids and EGF [17], providing further evidence of the value of this cell line in evaluating mechanisms of steroid action.

We now report that the proliferation of A549 cells is inhibited by nanomolar concentrations of tamoxifen and that this effect is insensitive to oestradiol. Furthermore, the characteristics of this response apparently distinguish it from other non-ER responses described in other systems. Moreover, we show that A549 cells do not express ER and therefore propose that this cell line constitutes a useful model for investigating the effects of tamoxifen that are not ER-dependent.

MATERIALS AND METHODS

Cell culture. A549 cells (Flow, Irvine, U.K.) were

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§ Abbreviations: ER, oestrogen receptor; EGF, epidermal growth factor; DMEM, Dulbecco's Modified Eagle's Medium; TGF, transforming growth factor; FCS, foetal calf serum; PKC, protein kinase C.

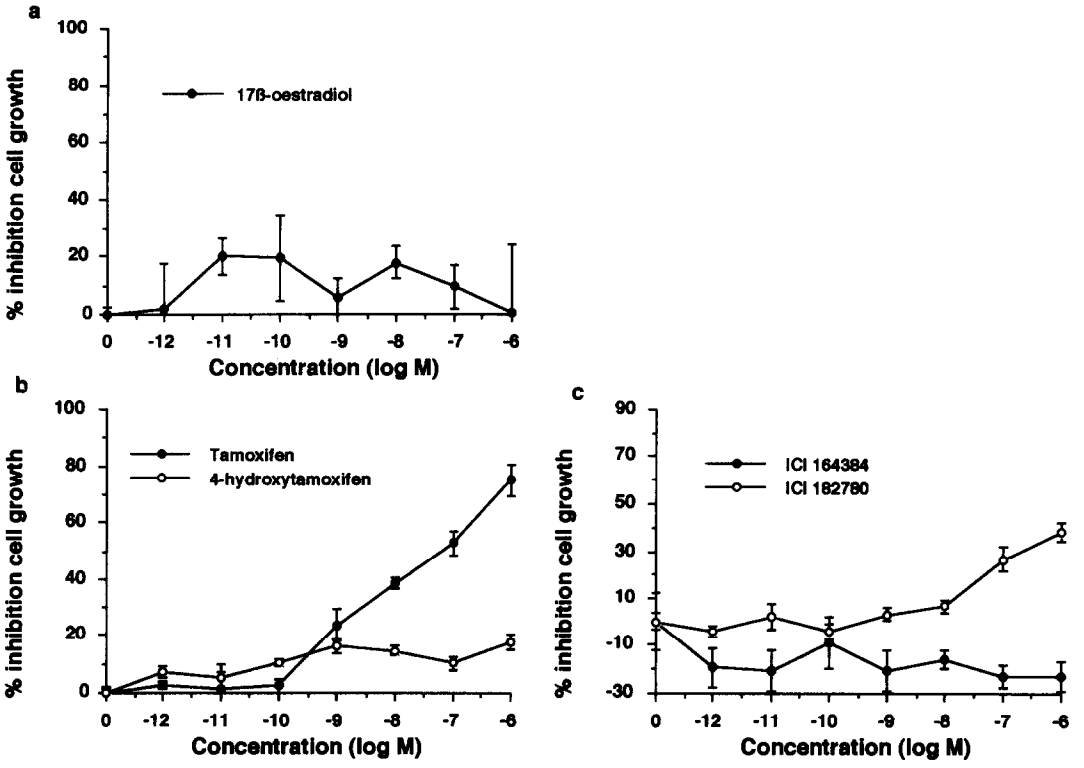

Fig. 1. (a) Oestradiol does not affect A549 cell growth in the concentration range 1 pM to 1 μM. (b) Tamoxifen significantly inhibits growth in the range 1 nM to 1 μM ($P < 0.001$ all points) but 4-hydroxytamoxifen does not effect growth significantly at any concentration between 1 pM and 1 μM. (c) ICI 164384 does not effect cell growth significantly whereas ICI 182780 inhibits cell growth at 0.1 and 1 μM ($P < 0.001$ both points). All cultures were treated for 3 days in DMEM without serum. Each point is the mean of three wells ± SD expressed as a percentage. Statistical significance was calculated from raw data. Each graph is a typical example of three experiments.

maintained in continuous log phase growth in Dulbecco's Modified Eagles Medium/F-12 (DMEM/F-12, Sigma, St Louis, MO, U.S.A.) containing Phenol red (Sigma) and 10% foetal calf serum (FCS, Sigma) in T-150 flasks (Greiner). The cells were not allowed to reach confluence at any time as this diminishes their response to steroids. The cells were routinely checked for the absence of mycoplasma contamination.

Cell proliferation experiments. Subconfluent A549 cells were washed with trypsin (0.05% w/v, Sigma)/EDTA (0.02% w/v, Sigma) solution in T-150 flasks for approximately 5 min. The detached cells were seeded into 12-place multi-well plates (Flow) at 5×10^4 cells/mL/well in DMEM/F-12, 10% FCS and incubated overnight. The medium was replaced with DMEM/F-12 (without Phenol red) containing dilutions of the appropriate steroid, fatty acid or vehicle control. Steroids were used in an ethanol vehicle which never exceeded 0.1% (v/v) in the final culture media. All experiments described here were performed under serum-free conditions. On the days indicated, cells were removed with trypsin and counted in triplicate using a Neubauer haemocytometer. Viability was assessed routinely using the Trypan exclusion assay.

Northern blotting. Total cellular RNA was extracted from sub-confluent cultures of A549 and ZR-75 cells using RNAzol B (Biogenesis, Bournemouth, U.K.) as specified by the manufacturer. RNA (20 μg) was resolved by agarose gel electrophoresis and ER transcripts were detected, following northern transfer, using a cDNA probe to the human mRNA (a gift from P. Chambon, Strasbourg, France).

Tamoxifen, 4-hydroxytamoxifen, ICI 164384 and ICI 182780 were all generously provided by Dr Alan Wakeling, ZENECA Pharmaceuticals, Cheshire, U.K.

RESULTS

Effect of oestrogen and anti-oestrogens on A459 cell growth

17-β Oestradiol had no effect on A549 cell proliferation at any concentration used in the range 1 pM–1 μM (Fig. 1a). Tamoxifen inhibited A549 cell growth in a dose-dependent fashion after 3 days of treatment (Fig. 1b) with a significant effect at concentrations of 1 nM and above ($P < 0.001$, all points). At 1 μM, the maximum dose used in these experiments, growth inhibition was high at 75%

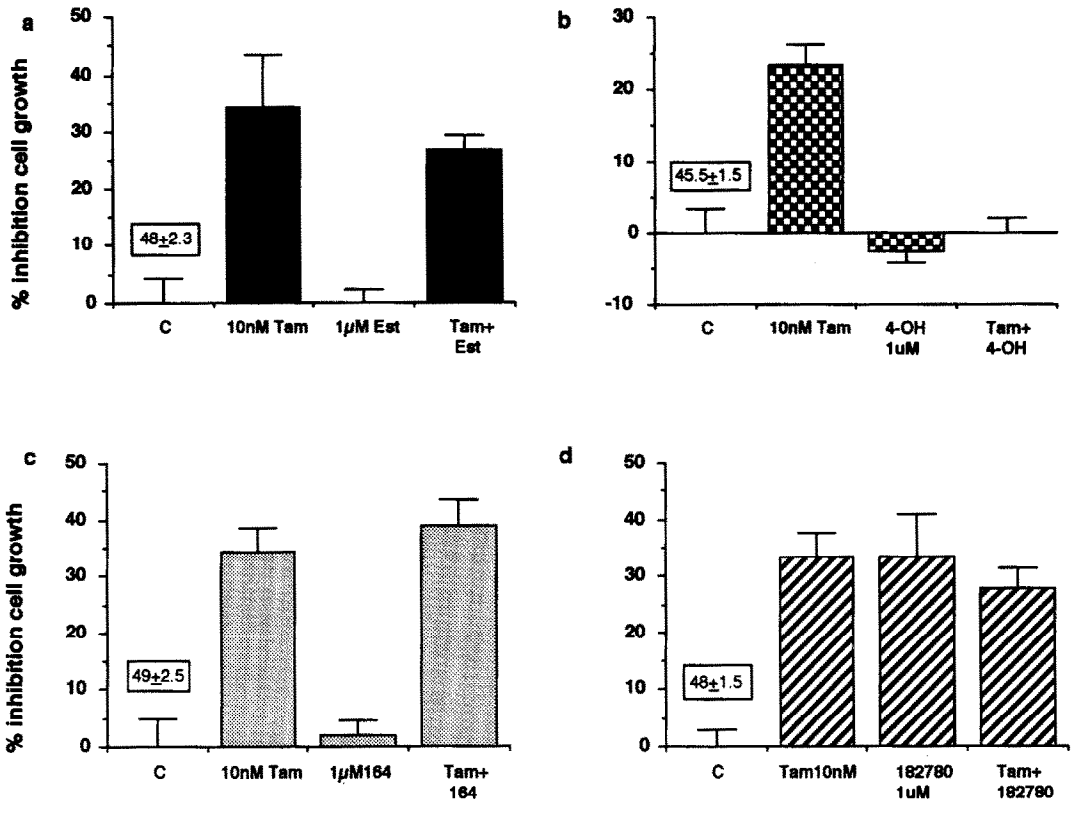

Fig. 2. (a) Oestradiol at 1 μM does not reverse the growth inhibition of 10 nM tamoxifen significantly. (b) 4-Hydroxytamoxifen at 1 μM completely reverses the growth inhibition of 10 nM tamoxifen ($P < 0.001$). (c) ICI 164384 at 1 μM does not effect growth inhibition of 10 nM tamoxifen significantly. (d) ICI 182780 at 1 μM inhibits cell growth significantly ($P < 0.001$) but does not reverse growth inhibition of 10 nM tamoxifen. All cultures were treated for 3 days in DMEM without serum. Each bar is the mean of three wells ± SD expressed as a percentage. Statistical significance was calculated from raw data. Actual control (C) cell number values are given in the panels ($\times 10^{-4}$). Each graph is a typical example of three experiments.

compared to control cell cultures. Cell viability assessment was >95% at all concentrations of tamoxifen used and therefore these results are unlikely to be explained by cytotoxic effects of the drug. The results presented are typical of at least three to six such experiments. All data are presented as percentage inhibition to enable comparisons between different experiments as plating densities and cell growth rates are always slightly variable. However, the statistical significance of response to treatments was calculated from raw data. The growth inhibitory effects of tamoxifen in A549 cells appear to be specific as 4-hydroxytamoxifen was only weakly active with a maximum inhibitory effect at 1 μM of 17.7% but this is not statistically sigificant (Fig. 1b). Similarly, the steroidal anti-oestrogen ICI 164384 had no significant effect on cell growth in the concentration range 1 pM–1 μM (Fig. 1c). However, the anti-oestrogen ICI 182780 did inhibit A549 cell growth in the range 10 nm–1 μM (EC_{50} 100 nM) being approximately 10-fold less active than tamoxifen itself (Fig. 1c). Tamoxifen also inhibited A549 cell growth in the presence of 10% FCS but with an EC_{50} reduced to 100 nM (24.4 ± 1.9% inhibition at 100 nM).

Reversal of tamoxifen growth inhibition by other steroids

A549 cells were cultured with a half-maximal concentration of tamoxifen (10 nM) for a period of 3 days in the presence of oestradiol and other anti-oestrogen drugs. Oestradiol did not reverse the growth inhibition of 10 nM tamoxifen when used at 100-fold excess (Fig. 2a). However, 4-hydroxy-tamoxifen which had no significant effect on cell growth itself, completely reversed the growth inhibitory effect of 10 nM tamoxifen when used at 100-fold excess (Fig. 2b). The steroidal anti-oestrogen ICI 164384 had no effect on tamoxifen growth inhibition when used at 100-fold excess (Fig. 2c) and ICI 182780, which was itself growth inhibitory at the concentration used, also did not reverse tamoxifen growth inhibition. However, there was no further increase in growth inhibition when tamoxifen and ICI 182780 were used together (Fig. 2d) which would be consistent with the two

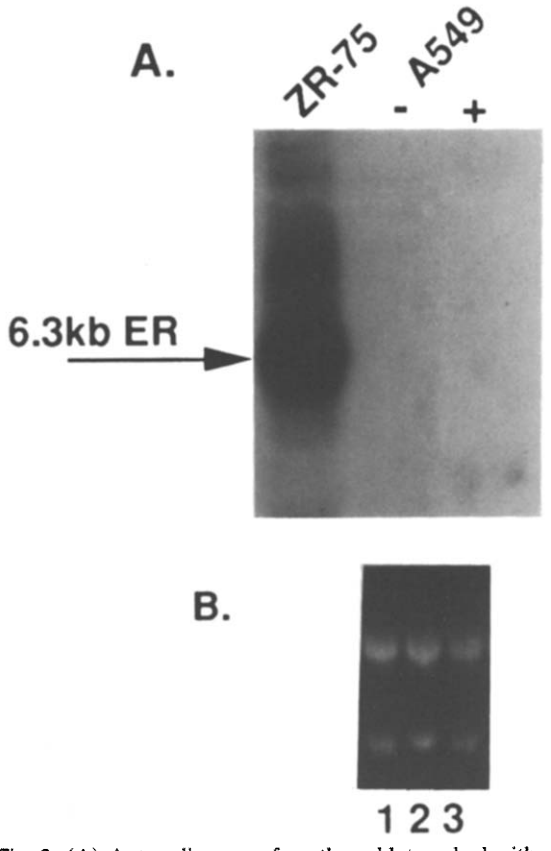

Fig. 3. (A) Autoradiogram of northern blot probed with radiolabelled hER cDNA. ZR-75 human breast cancer cells were used as a positive control. A549 +/− represents RNA extracted from cells grown in the presence and absence of serum, respectively. (B) Ethidium bromide staining of agarose gel prior to transfer to indicate equivalent loading of RNA.

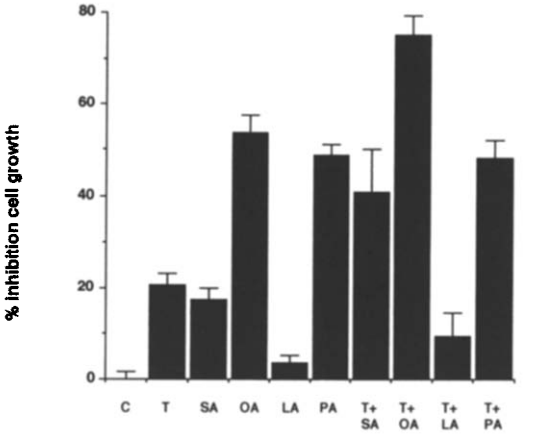

Fig. 4. Growth inhibition by the combined use of 10 nM tamoxifen (T) and 10 μM stearic acid (SA) or 10 μM oleic acid (OA) is significantly increased compared to each agent used alone ($P < 0.001$ compared to T, SA or OA alone). Linoleic acid (LA, 10 μM) partially, but not significantly reverses the growth inhibition of 10 nM tamoxifen. The combined use of 10 μM palmitic acid (PA) results in no further inhibition of growth than when PA is used alone. Each bar is the mean of three wells ± SD expressed as a percentage. Statistical significance was calculated from raw data. Each graph is a typical example of three experiments.

compounds acting through the same mechanism. Similar results were obtained using a 10-fold excess of each drug against 10 nM tamoxifen (data not shown).

Expression of ER in A549 cells

Ligand binding assays were performed as described previously [18] and failed to detect the presence of the oestrogen receptor (data not shown). This was confirmed by northern blotting where expression was detected in preparations of ZR-75 using a human cDNA probe; however, in A549 cell preparations no ER was visible (Fig. 3).

Effect of fatty acids and tamoxifen on A549 cell growth

Non-ER effects of tamoxifen mediated by the anti-oestrogen binding protein described in other cells have been shown to be influenced by fatty acids [8]. The same fatty acids have been used in this study and were all growth inhibitory when used at concentrations of 10 μM with an order of efficacy of linoleic < stearic < palmitic < oleic acid (Fig. 4). Growth inhibition by 10 nM tamoxifen was significantly increased when used in combination with oleic acid and stearic acid (Fig. 4). The combined use of tamoxifen and palmitic acid resulted in no further increase of growth inhibition than when palmitic acid was used alone (Fig. 4). Only linoleic acid decreased growth inhibition by tamoxifen; however, this effect was not statistically significant (Fig. 4).

DISCUSSION

In this paper we report that the non-steroidal anti-oestrogen tamoxifen substantially inhibited proliferation of the A549 cell line with a magnitude comparable to that previously reported for the action of glucocorticoids in these cells [15]. This is of considerable interest because we have shown that A549 cells appear not to express ER and yet still remain highly sensitive to the growth inhibitory effects of low concentrations of tamoxifen. Moreover, as this effect was not reversed by the presence of a large excess of either oestradiol or other specific steroidal anti-oestrogens, the action appears to be mediated by an ER-independent mechanism.

There have been several reports of inhibitory effects of tamoxifen that are not mediated by ER. These effects are, however, only achieved using micromolar concentrations of the drug. For example, tamoxifen inhibits the calmodulin activation of cAMP phosphodiesterase [9] but with an EC_{50} of 2 μM and 4-hydroxytamoxifen is active in this model [19]. Calmodulin does have another high affinity binding site for tamoxifen (K_d 6 nM) but there is no correlation with inhibitory activity at this

concentration [20]. Similarly, tamoxifen has been shown to antagonize the effects of histamine on tracheal muscle but at an optimum concentration of 30 μM and 4-hydroxytamoxifen was more potent in this model [10]. Inhibition of protein kinase C activity *in vitro* has also been described [14] but $>10\,\mu$M tamoxifen was required and again 4-hydroxytamoxifen had comparable activity. There are, of course, different sub-species of PKC which may respond differently to tamoxifen. However, a recent report identifying three sub-species of PKC in ER-positive and ER-negative human breast cancer cell lines demonstrated that micromolar concentrations of tamoxifen were required for inhibition of each species [21]. Tamoxifen also inhibits the ability of PKC to phosphorylate histones and lipocortin-1 [22] which we have shown is an important modulator of cell growth [15]. However, $>1\,\mu$M concentrations of tamoxifen were required for this effect also but we cannot exclude the possibility that alternative, more sensitive forms of PKC may exist.

Inhibition of cell growth by tamoxifen in an ER-negative cell line has been described before in the breast cancer cell line MDA-MB-330 [23]. However, high concentrations were required ($EC_{50} \simeq 10\,\mu$M) and following cell cycle analysis the authors concluded that this was a cytotoxic effect of the drug. These observations were made with 10% FCS present in the culture media which can reduce the efficacy of steroid action. Our observations on A549 cell growth have been carried out under serum-free conditions. However, even in the presence of 10% FCS, tamoxifen was still able to inhibit cell proliferation significantly, albeit with an approximate 10-fold reduction in efficacy.

Another potential mechanism of action for tamoxifen was highlighted with the discovery of specific high affinity binding sites distinct from the ER [5]. This so-called anti-oestrogen binding protein appears to have a ubiquitous distribution which does not follow the pattern of ER distribution [6]. However, the pharmacological actions of the anti-oestrogen binding protein in ER-positive or ER-negative tissues led to speculation that this protein is unlikely to mediate the growth inhibitory effects of tamoxifen [2]. Unfortunately the function of the anti-oestrogen binding protein remains unknown as does the identity of its natural ligand. However, it has been reported that certain polyunsaturated fatty acids are able to bind the anti-oestrogen binding protein and thus displace tamoxifen [7]. Oleic, arachidonic and linoleic acid have the highest affinity whilst no binding was detected with palmitic and stearic acid. A further report seemed to indicate that displacement of micromolar concentrations of tamoxifen by linoleic acid from the binding protein was responsible for blocking growth inhibition in cultured lymphoid cells [8]. Oleic and stearic acid had no effect in this model.

These results are quite contrary to those described in this report where identical concentrations of stearic and oleic acid significantly increase the effects of nanomolar concentrations of tamoxifen on cell growth and only linoleic acid partially reversed growth inhibition when used at a 10,000-fold molar excess. Clearly the properties of the anti-oestrogen binding protein have to be evaluated in a wider variety of cell systems but it seems unlikely that the observations of tamoxifen-induced growth inhibition in A549 cells are adequately explained by existing reports of the properties of this protein. Furthermore, as 4-hydroxytamoxifen is only weakly active in A549 cells yet still binds to the anti-oestrogen binding protein with high affinity [24, 25] this remains an unlikely mechanism of action.

The 4-hydroxy metabolite of tamoxifen has a 100-fold higher affinity for ER than tamoxifen itself which is reflected in an increased activity of cell growth inhibition in ER-positive cells [26]. Similarly ICI 164384 has a 10-fold higher affinity for ER and ICI 182780 has a 40–50-fold higher affinity for ER than tamoxifen and again these differences are manifest by increases in bioactivity of these compounds in ER-positive cells [27 for review]. This is contrary to the results we report here in ER-negative A549 cells where tamoxifen is the most active compound and at best ICI 182780 is weakly active but only at high concentrations.

A major advance in understanding the mechanism of action of steroids on cell growth came with the discovery that these were not necessarily direct effects, but could be mediated by the induction of growth factors that influenced cell growth in an autocrine or paracrine fashion [28 for review]. Initial reports demonstrated that tamoxifen treatment of ER-positive breast cancer cells resulted in the production of TGF-β, a growth factor that inhibits epithelial cell proliferation [29] although this is highly dependent on culture conditions [30]. However, the situation is now recognised to be more complex in that there are several forms of TGF-β and that it is the subtle regulation of expression of the relative levels of these different forms that is important for controlling cell growth [31 for review]. This regulation has been demonstrated in A549 cells where treatment with glucocorticoids, retinoic acid and EGF resulted in discrete changes in the expression of TGF-β_1 and TGF-β_2 [17]. The regulation of TGF-β production by tamoxifen was always assumed to be ER mediated; however, this view has now been challenged with the demonstration of induction in ER-negative fibroblasts [32] and human breast cancer *in vivo* [33]. How tamoxifen induces changes in the expression of TGF-β in such cells therefore remains to be determined but these observations imply the existence of other signalling systems.

In this report we have described a highly sensitive mechanism of growth control in A549 cells by tamoxifen that is ER-independent. Furthermore, these results are not adequately explained by previous reports of direct effects of tamoxifen requiring micromolar concentrations of the drug. The profile of tamoxifen effects that we have described is different to those mediated by the anti-oestrogen binding protein in other cells. However, we cannot exclude the possibility that the anti-oestrogen binding protein may exist in other forms or function in other ways in different cell types. Certainly, however, the A549 cell proliferation system may provide a sensitive model to investigate

the molecular mechanism of action of low concentrations of tamoxifen in ER-negative cells.

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REFERENCES

1. Sutherland RL, Reddel RR and Green MD, Effects of oestrogens on cell proliferation and cell cycle kinetics. A hypothesis on the cell cycle effects of antioestrogens. *Eur J Clin Oncol* **19**: 307–318, 1983.
2. Wakeling AE, Cellular mechanisms in tamoxifen action on tumours. *Rev Endocrine-related Cancer* **30**: 27–33, 1988.
3. Lippman M, Bolan G and Huff K, The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long-term tissue culture. *Cancer Res* **36**: 4595–4601, 1976.
4. Lippman M, Bolan G, Monaco M, Pinkus L and Engel L, Model systems for the study of estrogen action in tissue culture. *J Steroid Biochem* **7**: 1045–1051, 1976.
5. Sutherland RL, Murphy LC, Foo MS, Green MD and Whybourne AM, High-affinity antioestrogen binding site distinct from the oestrogen receptor. *Nature* **288**: 273–275, 1980.
6. Kon OL, An antioestrogen-binding protein in human tissues. *J Biol Chem* **258**: 3173–3177, 1983.
7. Hwang PLH, Unsaturated fatty acids as endogenous inhibitors of tamoxifen binding to antioestrogen-binding sites. *Biochem J* **237**: 749–755, 1986.
8. Hoh YK, Lim EH, Ooi SO and Kon OL, Fatty acid modulation of antioestrogen-binding protein in cultured lymphoid cells. *Experientia* **46**: 1032–1037, 1990.
9. Lam HP, Tamoxifen is a calmodulin antagonist in the activation of cAMP phosphodiesterase. *Biochem Biophys Res Commun* **118**: 27–32, 1984.
10. Kroeger EA and Brandes LJ, Evidence that tamoxifen is a histamine antagonist. *Biochem Biophys Res Commun* **131**: 750–755, 1985.
11. Ritchie GAF, The direct inhibition of prostaglandin synthetase of human breast cancer tissue by tamoxifen. In: *Recent Results in Cancer Research* (Eds. Henningsen B, Linder F and Steichele C), pp. 96–101. C. Springer-Verlag, Berlin, 1980.
12. Tavares IA, Stamford IF and Bennet A, Tamoxifen inhibits 5-lipoxygenase in human polymorphonuclear lymphocytes. *J Pharm Pharmacol* **39**: 323–324, 1987.
13. Wiseman H, Cannon M, Arnstein HRV and Halliwell B, Tamoxifen inhibits lipid peroxidation in cardiac microsomes. *Biochem Pharmacol* **45**: 1851–1855, 1993.
14. O'Brian CA, Liskamp RM, Solomon DH and Weinstein IB, Triphenylethylenes: a new class of protein kinase C inhibitors. *J Natl Cancer Inst* **76**: 1243–1246, 1986.
15. Croxtall JD and Flower RJ, Lipocortin 1 mediates dexamethasone-induced growth arrest of the A549 lung adenocarcinoma cell line. *Proc Natl Acad Sci USA* **89**: 3571–3575, 1992.
16. Croxtall JD, Waheed S, Choudhury Q, Anand R and Flower RJ, N-terminal peptide fragments of lipocortin-1 inhibit A549 cell growth and block EGF-induced stimulation of proliferation. *Int J Cancer* **54**: 153–158, 1993.
17. Danielpour D, Kim K, Winokur TS and Sporn MB, Differential regulation of transforming growth factor-βs 1 and 2 by retinoic acid, epidermal growth factor, and dexamethasone in NRK-49F and A549 cells. *J Cell Physiol* **148**: 235–244, 1991.
18. Jamil A, Croxtall JE and White JO, The effect of antioestrogens on cell growth and progesterone receptor concentration in human endometrial cancer cells (Ishikawa). *J Mol Endocrinol* **6**: 215–221, 1991.
19. Rowlands MG, Parr IB, McCague R, Jarman M and Goddard PM, Variation of the inhibition of calmodulin dependent cyclic AMP phosphodiesterase amongst analogues of tamoxifen; correlations with cytotoxicity. *Biochem Pharmacol* **40**: 283–289, 1990.
20. Lopes MCF, Vale MGP and Carvalho AP, Ca^{2+}-dependent binding of tamoxifen to calmodulin isolated from bovine brain. *Cancer Res* **50**: 2753–2758, 1990.
21. Bignon E, Ogita K, Kishimoto A and Nishizuka Y, Protein kinase C subspecies in estrogen receptor-positive and -negative human human breast cancer cell lines. *Biochem Biophys Res Commun* **171**: 1071–1078, 1990.
22. Edashige K, Sato EF, Akimaru K, Yoshioka T and Utsumi K, Nonsteroidal antioestrogen suppresses protein kinase C—its inhibitory effect on interaction of substrate protein with membrane. *Cell Struct Funct* **16**: 273–281, 1991.
23. Murphy LC and Sutherland RL, Differential effects of tamoxifen and analogs with nonbasic side chains on cell proliferation *in vitro*. *Endocrinology* **116**: 1071–1078, 1985.
24. Sudo K, Monsma FJ and Katzenellenbogen BS, Antiestrogen-binding sites distinct from the estrogen receptor: subcellular localization, ligand specificity, and distribution in tissues of the rat. *Endocrinology* **112**: 425–434, 1983.
25. Faye J, Jozan S, Redeuilh G, Baulieu E and Bayard F, Physicochemical and genetic evidence for specific antiestrogen binding sites. *Proc Natl Acad Sci USA* **80**: 3158–3162, 1983.
26. Sutherland RL, Watts CKW and Ruenitz PC, Definition of two distinct mechanisms of action of antiestrogens on human breast cancer cell proliferation using hydroxytriphenylethylenes with high affinity for the estrogen receptor. *Biochem Biophys Res Commun* **140**: 523–529, 1986.
27. Wakeling AE, The future of new pure antiestrogens in clinical breast cancer. *Breast Cancer Res Treat* **25**: 1–9, 1993.
28. Lippman ME, Dickson RB, Bates S, Knabbe C, Huff K, Swain S, McManaway M, Bronzert D, Kasid A and Gelmann EP, 8th San Antonio Breast Cancer Symposium—Plenary lecture. Autocrine and paracrine growth regulation of human breast cancer. *Breast Cancer Res Treat* **7**: 59–70, 1986.
29. Moses HL, Tucker RF, Leof EB, Coffey RJ, Halper J and Shipley GD, Type β transforming growth factor is a growth stimulator and a growth inhibitor. In: *Cancer Cells* (Eds. Feramisco J, Ozanne B and Stiles C), pp. 65–71. Cold Spring Harbor Laboratory, New York, 1985.
30. Croxtall JD, Jamil A, Ayub M, Colletta AA and White JO, TGF-β stimulation of endometrial and breast-cancer cell growth. *Int J Cancer* **50**: 822–827, 1992.
31. Roberts AB and Sporn MB, Mechanistic interrelationships between two superfamilies: the steroid/retinoid receptors and transforming growth factor-β. In: *Cancer Surveys* (Ed. Parker M), pp. 205–220. Cold Spring Harbor, New York, 1992.
32. Colletta AA, Wakefield LM, Howell FV, Van Roozendal KEP, Danielpour D, Ebbs SR, Sporn MB and Baum M, Antioestrogens induce the secretion of active TFG-β from human fetal fibroblasts. *Br J Cancer* **62**: 405–409, 1990.
33. Butta A, MacLennan K, Flanders KC, Sacks NPM, Smith I, McKinna A, Dowsett M, Wakefield LM, Sporon MB, Baum M and Colletta AA, Induction of transforming growth factor β_1 in human breast cancer *in vivo* following tamoxifen treatment. *Cancer Res* **52**: 4261–4264, 1992.