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Growth Inhibition of Oestrogen Receptor-positive Human Ovarian Carcinoma by Anti-oestrogens *In Vitro* and in a Xenograft Model

S.P. Langdon, A.J. Crew, A.A. Ritchie, M. Muir, A. Wakeling, J.F. Smyth and W.R. Miller

This paper presents results of the *in vitro* and *in vivo* effects of anti-oestrogens on the growth of human ovarian cancer cells. Tamoxifen and the "pure" anti-oestrogens, ICI 164,384 and ICI 182,780, inhibited the oestrogen-stimulated growth of the oestrogen receptor (ER)-positive PE04 and PE01 cell lines grown in culture, the latter two compounds being more potent than tamoxifen. In the absence of 17β-oestradiol (E_2), tamoxifen, but not the pure anti-oestrogens, produced a small degree of growth stimulation in the PE01 and PE04 lines at concentrations between 10^{-7} and 10^{-9} M. In contrast, growth of the ER-negative PE014 line was unaffected by E_2 and all three anti-oestrogens. The effects of tamoxifen and ICI 182,780 on PE04 cells grown as xenografts in nude mice were also studied. Both anti-oestrogens produce significant growth inhibitory effects. These results indicate that ovarian carcinoma cells may be sensitive to anti-oestrogens *in vitro* and *in vivo*, and support the view that anti-oestrogens merit further clinical studies in patients with ER-positive tumours.
Eur J Cancer, Vol. 30A, No. 5, pp. 682–686, 1994

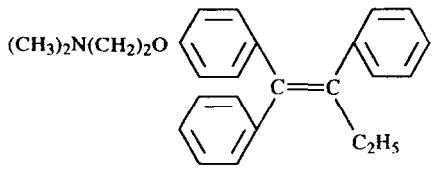
INTRODUCTION

THE ROLE of anti-oestrogens in the treatment of ovarian cancer remains controversial. The presence of oestrogen receptors (ER) in 67% of ovarian adenocarcinomas (reviewed in [1]) suggests that this disease might be responsive to anti-oestrogen therapy, and several small clinical trials with tamoxifen have reported response rates of between 6 and 36% [2–7], although others have not observed any benefit [8–11]. Many of these studies concluded that, although the response rate to tamoxifen is low, there is a group of patients who could benefit from anti-oestrogen therapy. In this respect, it is interesting that an association between the presence of ER and tumour response has been

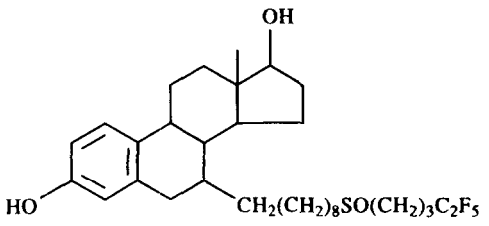
Table 1. *Characteristics of the cell lines and xenografts*

Cell line/ xenograft	Origin	Previous treatment	Oestrogen receptor concentration* (fmol/mg protein)
PE04 cell line	Ovarian serous adenocarcinoma	CDDP, 5-FU and chlorambucil	112 (60–203)†
PE01 cell line	Ovarian serous adenocarcinoma	CDDP, 5-FU and chlorambucil	96 (73–145)
PE014 cell line	Ovarian serous adenocarcinoma	None	0
PE04 xenograft	Ovarian serous adenocarcinoma	CDDP, 5-FU and chlorambucil	242 (125–362)

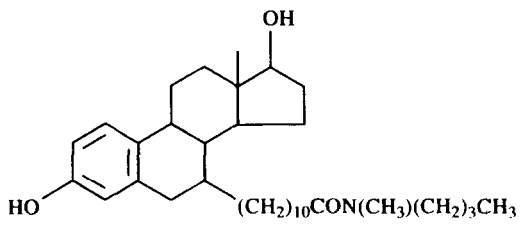
CDDP, cisplatinum; 5-FU, 5-fluorouracil. See [14, 15]. †Range of values for measurements on three to six samples (see [14, 15]).



Tamoxifen



ICI 182,780



ICI 164,384

Figure 1. Structures of the anti-oestrogens.

noted [3, 12], and in one study [6], eight of the nine tumours displaying a complete response to tamoxifen possessed elevated ER levels.

In order to further investigate the efficacy of anti-oestrogens against ovarian cancer, we have examined the effect of three anti-oestrogens, namely tamoxifen (ICI 46,474), ICI 164,384 and ICI 182,780, against ER-positive and ER-negative human ovarian carcinoma systems *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell lines and xenografts

The human ovarian adenocarcinoma cell lines PE04, PE01 and PE014 (Table 1) were established as described previously [13, 14]. These were maintained routinely at 37°C in a humidified atmosphere at 5% CO_2/95% air in RPMI 1640 (Gibco, Paisley, UK) containing 10% heat-inactivated fetal calf serum (FCS) and supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml) and glutamine (2 mM).

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The PE04 xenograft was initiated from the cell line by subcutaneous injection of 10^7 cells into the flanks of female nude mice [15]. Once established, the xenograft was maintained as a subcutaneous tumour in the flanks of the mice, and used at passages 3–10 for these experiments.

Tamoxifen, ICI 164,384 and ICI 182,780 (Figure 1) were gifts from Zeneca Pharmaceuticals (U.K.). These were dissolved in absolute ethanol to give stock solutions of 10^{-2} M and kept at 4°C until use.

In vitro *growth experiments*

Exponentially growing cells were harvested by trypsinisation and plated in 24-well plates (Falcon, Oxford, U.K.) at densities of 5×10^4 cells/well (four wells/experimental condition) in RPMI 1640 containing phenol red and 10% FCS. After 24 h, to allow for attachment, the medium was removed and cells washed twice with phosphate-buffered saline (PBS) before addition of RPMI 1640 without phenol red, but containing 5% charcoal-stripped FCS [16]. The cells were incubated for a further 24 h and then washed with PBS. RPMI 1640 containing 5% charcoal-stripped FCS, with or without either anti-oestrogen (10^{-9} to 10^{-5} M) and/or 17β-oestradiol (E_2) (10^{-10} M) added. This time point was designated as day 0. Medium was replenished on day 3. Cells were harvested from wells on days 0 and 6 by trypsinisation, and counted using a Coulter counter.

In vivo *growth experiments*

Female nude (nu/nu) mice (originally bred at ICRF Laboratories, London, U.K.) were obtained from OLAC Ltd, (La Calhen, Cambridge, U.K.) and maintained in negative pressure isolators. Mice were at least 8 weeks old at the time of experimentation. Fragments of the stock PE04 xenograft were implanted subcutaneously into the flanks of animals. After approximately 1 month, when tumours reached a mean volume of 32 mm^3, animals were randomly allocated to treatment or control groups (each of four to six animals) and treatment commenced (defined as day 0). Tamoxifen was administered via slow-release pellets obtained from Innovative Research of America (Ohio, U.S.A.). The doses used were 0.01 mg/pellet and 1.5 mg/pellet (30-day release) in the first experiment, and 5 mg/pellet (60-day release) for the second experiment. A previous study had demonstrated that breast and ovarian carcinoma xenografts might have very differing sensitivities to E_2, therefore a low dose (0.01 mg) and a moderate dose of tamoxifen (1.5 mg) were selected for the first experiment to try and ensure that an effect, if present, might be observed. The pellets were given subcutaneously in the flank distant from the xenograft with the aid of a trochar. ICI 182,780 (5 mg) was suspended in arachis oil (0.1 ml) and injected subcutaneously in the flank opposite to the xenograft. Tumours were measured at least once each week, and the volume, measured in mm^3, was estimated using the formula: volume (V) = $\pi/6 \times I \times w^2$ where I is the longest diameter and w is the diameter perpendicular to this.

The relative tumour volume, V_t/V_o (where V_o is the tumour volume and V_t is the tumour volume at any given time) was calculated for each individual tumour at each time point.

RESULTS

Anti-oestrogen growth effects in vitro

The effects of adding tamoxifen, ICI 164,384 and ICI 182,780, either alone or in the presence of E_2, are shown in Figure 2 (for PE04), Figure 3 (for PE01) and Figure 4 (for PE014). When added alone, E_2 produced a growth stimulation in both the PE04 and PE01 lines but not in the PE014 lines.

The effect of tamoxifen on oestrogen-stimulated growth in the PE04 and PE01 lines was concentration-dependent. Thus concentrations between 10^{-9} and 10^{-7} M had little effect, but 10^{-6} M almost completely abolished E_2 stimulation in both cell lines. Tamoxifen alone at 10^{-7} and 10^{-8} M produced a growth stimulation in the PE04 line and to a lesser extent in the PE01 line.

In the PE014 line, tamoxifen had no effect on growth at concentrations of 10^{-9} to 10^{-6} M.

ICI 164,384 and ICI 182,780 were also capable of blocking E_2-induced growth in the PE04 and PE01 lines, but the concentrations required were less than those for tamoxifen. Thus, both pure anti-oestrogens produce an almost complete inhibition of oestrogen-stimulated growth in these lines at 10^{-7} M and significant inhibition at 10^{-8} M. In contrast to tamoxifen, when added alone, ICI 164,384 and ICI 182,780 had no stimulatory effect on the cells and indeed produced a small degree of growth inhibition at concentrations greater than 10^{-8} M. As with tamoxifen, ICI 182,780 and ICI 164,384 produced no significant effects on the growth of PE014 cells.

Anti-oestrogen growth effects in vivo

The effects of tamoxifen and ICI 182,780 were also tested *in vivo* against PE04 cells grown as xenografts in nude mice. These tumours grow in the absence of oestrogen supplementation.

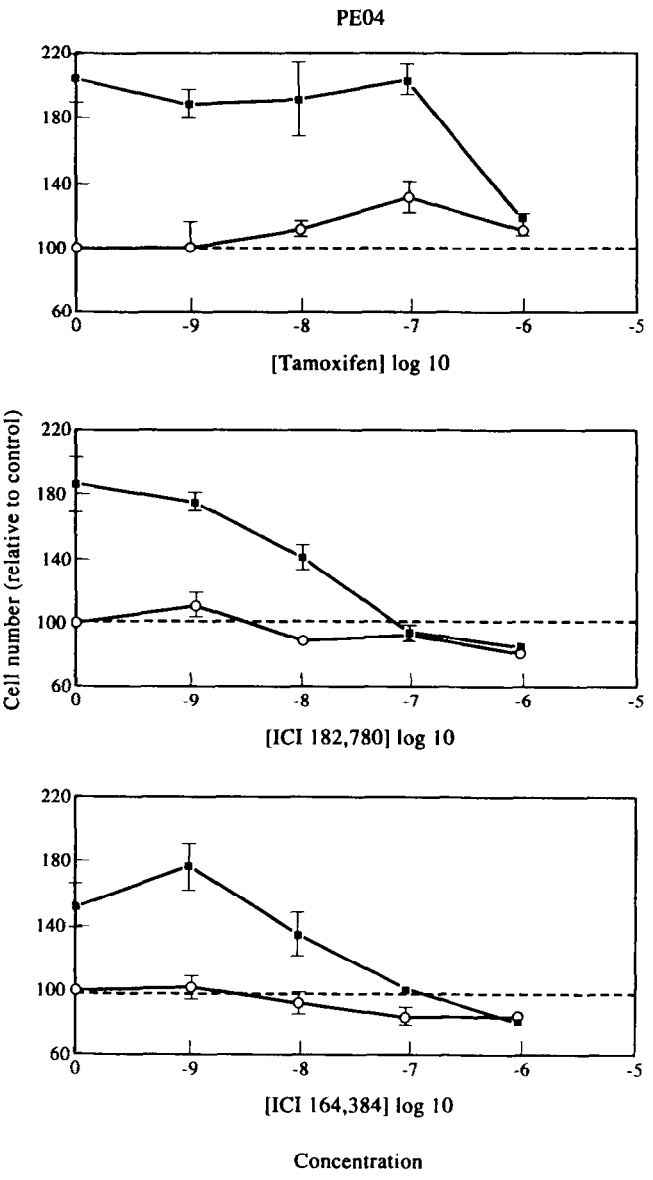

Figure 2. Effects of anti-oestrogens on the growth of PE04 cells in the absence and presence of 17β-oestradiol (E_2). Cells were treated with anti-oestrogens with (■) or without (○) 10^{-10} M E_2 for 6 days as described in Materials and Methods. Each point is the mean value of quadruplicate observations. Error bars represent standard deviation. The experiment shown is representative of three identical experiments.

Tamoxifen administered subcutaneously, at a site distant from the xenograft, produced a dose-related inhibition of growth as compared with untreated animals (Figure 5). In a further study, the effects of tamoxifen and ICI 182,780 were compared. Both produced marked and equivalent inhibitory effects (Figure 6). In this second experiment, while tamoxifen produced a statistically significant inhibition of growth, the effect was less pronounced than that obtained in the first experiment (Figure 5).

DISCUSSION

We have previously shown that, *in vitro*, the growth of an ovarian carcinoma cell line (PE04), which has high levels of ER (100 fmol/mg protein—a level found in 20–25% of ovarian tumours [17, 18]), can be stimulated by E_2, and this response can be antagonised by tamoxifen [14]. In the present study, we

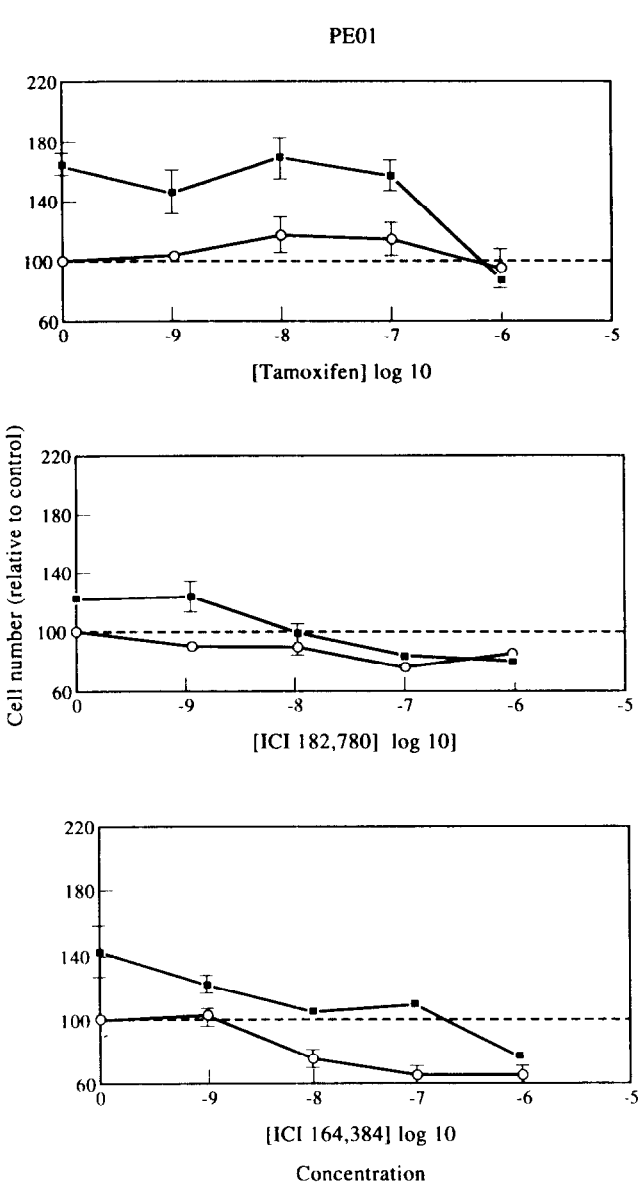

Figure 3. Effects of anti-oestrogens on the growth of PE01 cells in the absence and presence of 17β-oestradiol (E_2). Cells were treated with anti-oestrogens with (■) or without (○) 10^{-10} M E_2 for 6 days as described in Materials and Methods. Each point is the mean value of quadruplicate observations. Error bars represent standard deviation. The experiment shown is representative of three identical experiments.

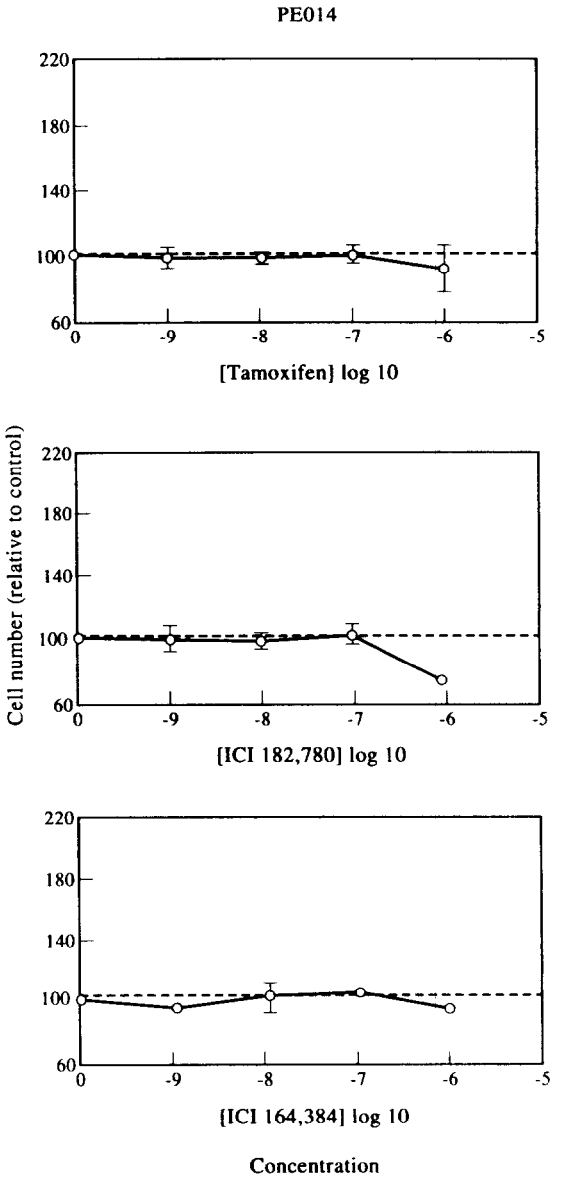

Figure 4. Effects of anti-oestrogens on the growth of PE014 cells in the absence and presence of 17β-oestradiol (E_2). Cells were treated with anti-oestrogens for 6 days as described in Materials and Methods. Each point is the mean value of quadruplicate observations. Error bars represent standard deviation.

have extended these investigations by showing that (i) the growth of another ER-positive ovarian carcinoma cell line, PE01, is also stimulated by E_2, an effect which is again antagonised by tamoxifen; (ii) the growth of the ER-negative PE014 line is unaffected by the addition of either E_2 or tamoxifen; (iii) the growth of the PE04 and PE01 lines but not the PE014 line is affected by other anti-oestrogens; and (iv) both tamoxifen and ICI 182,780 inhibit the growth of PE04 cells *in vivo*.

Although tamoxifen antagonised the oestrogen-stimulated growth of the ER-positive cell lines *in vitro*, when added to the cells in the absence of E_2 at concentrations of 10^{-7} or 10^{-8} M, there was a small growth stimulation detected in the PE04 and PE01 lines but not in the ER-negative PE014 line. This is consistent with the known partial oestrogen agonist activity of tamoxifen which has been demonstrated in ER-positive breast carcinoma cell lines [19, 20] and normal uterus [21]. Because such agonist activity may compromise the antitumour effect of tamoxifen, anti-oestrogens without this activity have been sought. ICI 164,384 and ICI 182,780 are representatives of a new class of anti-oestrogens which bind ER with high affinity without activating any of the normal transcriptional responses of oestrogen [22, 23]. Both of these agents are also more potent than tamoxifen on a molar basis against ER-positive breast cancer cell lines *in vitro* [23, 24]. These two properties are also demonstrated against the ER-positive ovarian cancer cell lines tested in this study. Thus, concentrations of 10^{-7} M of these agents have equivalent or greater potency than 10^{-6} M tamoxifen as antagonists of oestrogen-stimulated growth *in vitro*. Furthermore, in the range of concentrations tested here, there was no evidence of growth stimulation by the anti-oestrogen in the absence of oestrogen. In these cell lines, both the anti-oestrogens demonstrate comparable effects on growth, while in previous studies, ICI 182,780 demonstrated an approximately 5-fold increase of potency over ICI 164,384 [23].

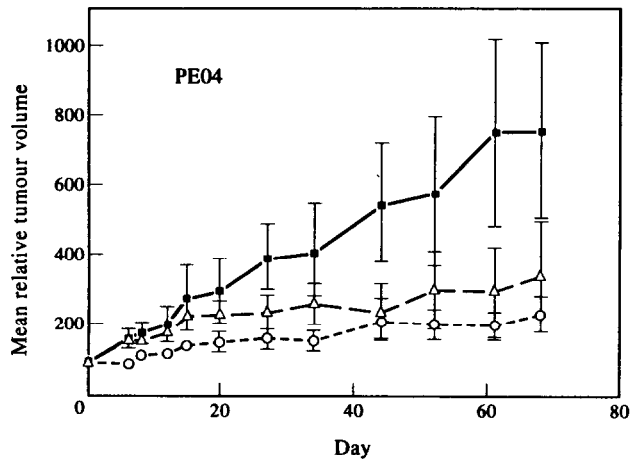

Figure 5. Effect of tamoxifen on the growth of established PE04 xenografts in nude mice. Groups of nude mice received either no treatment (■) or tamoxifen pellets (30-day slow release pellets) containing either 0.01 mg/pellet (△) or 1.5 mg/pellet (○). New pellets were implanted after 30 days. Points shown are mean values for groups of four to six animals. Error bars, standard errors.

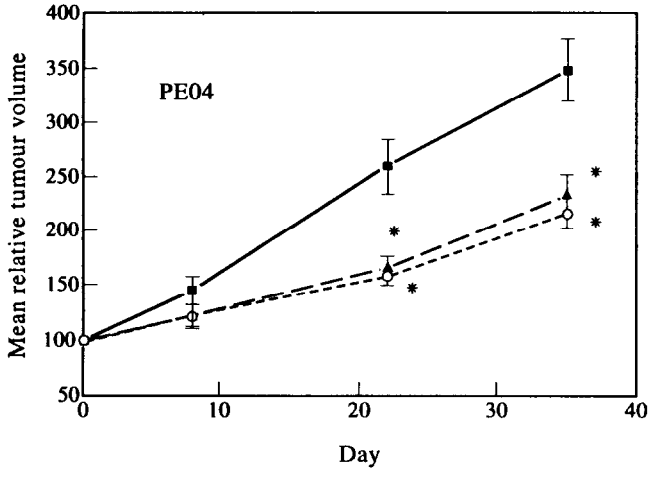

Figure 6. Effect of ICI 182,780 on the growth of established PE04 xenografts in nude mice. Groups of nude mice received either no treatment (■), a single subcutaneous injection of ICI 182,780 (5 mg in arachis oil) (○) or tamoxifen (5 mg slow-release pellet: 60-day pellet) (▲). Points shown are mean values for groups of five animals. Error bars = standard errors.

ICI 182,780 was also active *in vivo* against the PE04 xenograft, at a dose which has previously been shown to be effective at inhibiting ER-positive breast cancer xenografts [23]. As in this previous study, activity with the steroidal anti-oestrogen was comparable, rather than superior, to the *in vivo* activity of tamoxifen. ICI 182,780 is currently undergoing clinical trials in breast cancer. We suggest that this group of anti-oestrogens may have utility in the therapy of patients with ovarian cancer.

In conclusion, this is the first report of anti-oestrogens producing activity against ER-positive ovarian cancer cells in an *in vivo* model, and also the first study to demonstrate activity of the novel "pure anti-oestrogens" against ER-positive ovarian carcinoma both *in vitro* and *in vivo*. These results support the view that anti-oestrogens merit further clinical studies in patients with ER-positive tumours.

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