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EFFECT OF ESTROGEN AND ANTIESTROGENS ON CELL PROLIFERATION AND SYNTHESIS OF SECRETED PROTEINS IN THE HUMAN BREAST CANCER CELL LINE MCF-7 AND A TAMOXIFEN RESISTANT VARIANT SUBLINE, AL-1

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The human breast cancer cell line MCF-7 contains estrogen receptors and responds to estrogens with an increase in growth rate and to antiestrogens with a decrease in growth rate. Estrogen stimulation of cell proliferation is concomitant with an increase in the synthesis and secretion of three proteins with mol.wt 52 kDa, 61 kDa and 66 kDa and a decrease in the synthesis and secretion of a 42 kDa protein. The antiestrogen ICI 164,384 has a complete estrogen antagonistic effect on the synthesis of these secreted proteins, whereas the antiestrogen tamoxifen has an agonistic effect on the synthesis and secretion of the 52 kDa protein. We believe that the above mentioned estrogen regulated secreted proteins are either directly or indirectly involved in control of cell proliferation, and the less pronounced inhibitory effect of tamoxifen on cell proliferation compared to ICI 164,384 may be due to agonistic effects of tamoxifen. A tamoxifen resistant variant of the MCF-7 cell line, the AL-1 subline, can be growth inhibited by ICI 164,384, although a higher concentration is needed to inhibit the AL-1 cells compared to the parent MCF-7 cells. Tamoxifen has no effect on secreted proteins from the AL-1 cells, whereas ICI 164,384 has a complete estrogen antagonistic effect on secreted proteins, indicating that the mechanisms by which estrogens and antiestrogens influence cell proliferation may be via up and down regulation of secreted proteins with growth regulatory functions.

Key words: Breast cancer cells, estrogen, antiestrogens, tamoxifen resistance, secreted proteins.

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Many human breast tumors grow as estrogen dependent tumors and much research has tried to elucidate the mechanism for estrogen stimulated cell proliferation of human breast cancer. The ability of cancer cells to produce and secrete proteins with growth factor activity was shown already in 1978 (1), and Sirbasku (2) proposed in 1981 that estradiol stimulates cell proliferation in estrogen receptor positive human breast cancer cells by inducing the synthesis and secretion of proteins with mitogenic activity. The first step in the mechanism for estrogen stimulation is binding of estradiol to the estrogen receptors. Bound estrogen receptors interact with estrogen-responsive elements on the chromatin and induce alterations of specific gene transcription and protein synthesis. Growth factors as TGF-α, IGI-I. IGF-II, TGF-β and 52 kDa cathepsin D have been found to be regulated by estradiol in breast

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cancer cells (3–5), but the mitogenic effect of the individual factor seems to be very dependent on the conditions under which the growth experiments are performed (6–8). The importance of secreted growth factors for stimulation of tumor growth in vivo has been studied in the nude mouse model, and antibodies to the type I somatomedin receptors were found to inhibit growth of the estrogen independent MDA-231 human breast cancer cell line, but not growth of the MCF-7 cell line (9), although the antibodies inhibited in vitro growth of estrogen stimulated MCF-7 cells (8). These results do not support the idea of the importance of estrogen stimulated secreted IGF-I for tumor growth in vivo, however, they do not rule out that other estrogen regulated proteins may have vital functions for tumor growth in vivo. The mechanism for estradiol stimulation of breast cancer cell proliferation probably involves several factors with direct or indirect regulatory functions and estradiol may upregulate the synthesis of factors with positive regulatory functions and down regulate the synthesis of factors with negative regulatory functions (10–15).

Patients with estrogen dependent breast tumors respond to treatment with the antiestrogen tamoxifen (16, 17). Tamoxifen binds to estrogen receptors and the inhibitory effect on cell proliferation is at least partly supposed to be due to the antagonistic effect on estradiol regulated proteins with growth regulatory functions (11, 18, 19). Tamoxifen has, however, several agonistic effects on breast cancer cells (20–22) and new antiestrogens with complete estrogen antagonistic effects have been produced in order to find a compound with a therapeutic advantage over the partial agonist tamoxifen. The steroidal antiestrogen ICI 164,384 has been found to be such a complete estrogen antagonist (23–25), and in the present work we have compared the relative potencies and the maximum inhibitory efficacies of tamoxifen and ICI 164,384 for inhibition of cell proliferation of the estrogen receptor positive human breast cancer cell line MCF-7. The effect of the two antiestrogens on synthesis of specific estradiol regulated secreted proteins have been studied and the lower maximum inhibitory efficacy of tamoxifen compared to ICI 164,384 may be explained by the partial agonistic effect of tamoxifen on the synthesis of some of the secreted proteins. The tamoxifen resistant variant of the MCF-7 cell line, the AL-1 subline (26), responds to ICI 164,384 although a hundred-fold higher concentration is needed to obtain maximum growth inhibition compared to MCF-7 cells. Tamoxifen has no effect on the synthesis of secreted proteins in the AL-1 cells, whereas ICI 164,384 has a complete antagonistic effect on the synthesis of the studied proteins, indicating that the inhibitory effect of ICI 164,384 on cell proliferation of the tamoxifen resistant cell line could be due to the antagonistic effect on the synthesis of proteins with growth regulatory function.

Material and Methods

Cells and cell cultures. The human breast cancer cell line MCF-7 was obtained from the Human Cell Culture Bank, Mason Research Institute, Rockvill, MD, USA. The cell line has been adapted to grow in medium with low serum content (27), and for these experiments the cells were propagated in phenol red free Dulbecco's minimal essential/Ham's F12 medium (DME/F12 1:1) supplemented with 1% fetal calf serum (FCS), 2 mM glutamine and 6 ng/ml insulin (porcine, Novo-Nordisk, Denmark). The AL-1 cell line is derived from MCF-7 cells after long-term treatment with tamoxifen (26), and the cells were maintained in the same growth medium as the parent cell line MCF-7 plus 10^{-6} M tamoxifen. Both cell lines were propagated in plastic T-flasks (Nunc, Denmark), split every weak by trypsinization and seeded with 5×10^3 cells/cm^2. Medium was changed every second or third day.

Growth and labelling experiments. Multiwell dishes (2 cm^2, Nunc, Denmark) were seeded with MCF-7 or Al-1 cells, 5×10^3/cm^2 in growth medium. Two days after seeding medium was changed to experimental medium (day 0) consisting of growth medium supplemented with 10% newborn calf serum (NCS), 10% NCS plus 10^{-8} M estradiol, tamoxifen, ICI 164,384, or antiestrogen and estradiol in the indicated concentrations. Cell numbers were determined by counting in an automatic cell counter (Analys Instrument AB/VDA 140) of nuclei after cell lysis with 1% Triton X100 in PBS with 1.5 mM $MgCl_2$ and 2 mM EGTA (Sigma). Results appear as the mean of the cell numbers in four wells with SD. Labelling for 6 h with ^{35}S-methionine (NEG-009H, NEN Res. Products, spec act. 1151 Ci/mM) were performed after 6 days in experimental medium, 100 μCi/ml in serum-free DME-medium with 1/10 the normal concentration of methionine. Treatment of labelled conditioned medium, SDS-PAGE analysis on 15% acryl amide gels under denaturation conditions and autoradiography were performed as described previously (13).

Competition experiments. Cytosol of MCF-7 cells were prepared as described in detail in ref. 27. 1.5 nM ^{3}H-estradiol (Amersham, spec. act. 96 Ci/mM) was used as a saturating concentration for binding of estrogen receptors. Unlabelled estradiol, tamoxifen and ICI 164,384 at the indicated concentrations were added together with labelled estradiol to the cytosol preparations and incubations were performed at 4°C for 16 h. Unbound hormone was precipitated with dextran coated charcoal (DCC) (28). The result is expressed as percentage of the maximal binding obtained with ^{3}H-estradiol alone and corrected for non-specific binding by subtraction of the counts bound in the presence of a 100-fold excess of non-radioactive estradiol.

Results

The human breast cancer cell line MCF-7 exhibits estrogen responsive growth provided the culture conditions do

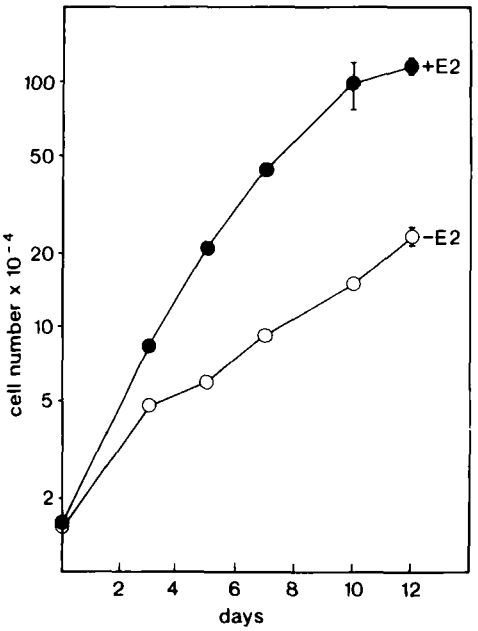

Fig. 1. Growth curves for MCF-7 cultures in medium with and without estradiol. MCF-7 cells were seeded in multidishes in growth medium and two days after seeding (day 0) medium with 10% NCS (− E2) or with 10% NCS plus 10^{-8} M estradiol (+ E2) was added. Cell numbers of four wells were determined at the indicated days after addition of experimental medium. Mean cell number with SD is shown, when large enough for drawing.

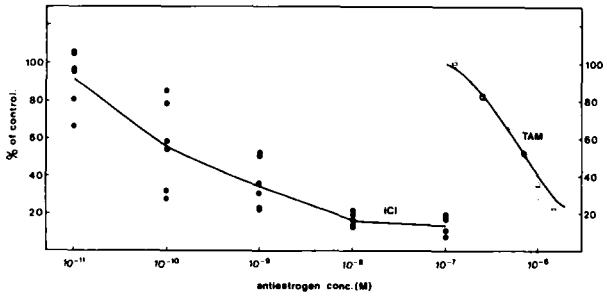

Fig. 2. Dose-response curves for tamoxifen and ICI 164,384 on cell proliferation of MCF-7 cells. MCF-7 cells were seeded in multidishes in growth medium and two days later, medium was changed to experimental medium containing the indicated concentrations of ICI 164,384 (ICI) or tamoxifen (TAM). Experimental media were renewed at day 3 and 5, and cell numbers in 4 wells determined at day 6. Mean cell numbers are expressed as percentage of cell number in the corresponding control culture (100%). The results of 6 experiments with ICI 164,384 and 5 experiments with tamoxifen are shown. Smooth curves are drawn between the average values for each tested concentration.

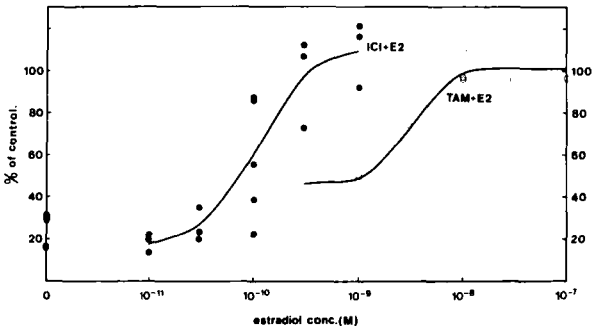

Fig. 3. Abrogation of antiestrogen inhibition of cell proliferation of MCF-7 cells with estradiol. MCF-7 cells were seeded in multidishes in growth medium. Two days later medium was changed to experimental medium with 10^{-8} M ICI 164,384 (ICI) plus the indicated concentrations of estradiol (E2) or 10^{-6} M tamoxifen and the indicated concentrations of estradiol (E2). Experimental media were renewed at day 3 and 5 and cell numbers in four wells determined at day 6. Mean cell numbers are expressed as percentage of cell number in the control culture (100%). The results of five experiments with ICI 164,384 and estradiol and the results of four experiments with tamoxifen and estradiol are shown. Smooth curves are drawn between the average values for each tested concentration.

not allow maximal cell proliferation rate. In our conventional phenol red free growth medium with 1% FCS, estradiol stimulates cell proliferation with an increase in cell number of about 50% after 6 days in culture. Under growth retarded conditions, which may be medium with antiestrogens, medium with steroid stripped serum, growth factor depleted medium, medium with high concentration of newborn calf serum or bovine serum albumin, estradiol exerts a significant growth stimulation (26, 29–32). Fig. 1 shows the result of a growth experiment with MCF-7 cells grown in medium with 10% NCS in presence and absence of estradiol. The estradiol stimulation is about 4-fold after 6 days treatment and the doubling time is 36 h and 65 h in medium with and without estradiol respectively.

The non-steroidal antiestrogen tamoxifen and the steroidal antiestrogen ICI 164,384 inhibit growth on MCF-7 cells propagated in medium with 1% FCS. Dose response curves with these two antiestrogens are shown in Fig. 2. The ICI 164,384 compound inhibits growth at very low concentrations and half maximal inhibition is obtained at a concentration of 10^{-10} M. Half maximal inhibition with tamoxifen is found with a 4×10^{-7} M concentration. Maximal inhibition (85%, i.e. 15% of the cell number in the control culture) with ICI 164,384 is obtained at 10^{-8} M, and the same level of inhibition is seen with 10^{-7} M and 10^{-6} M concentrations (not shown). Tamoxifen exerts a maximal inhibition of about 60% in a concentration of 10^{-6} M, at concentrations of tamoxifen above 2 to 3×10^{-6} M an estrogen irreversible cytotoxic effect is obtained (27). The effect of 10^{-7} M ICI 164,384 on cell proliferation is estrogen-reversible, whereas the effect of 10^{-6} M ICI 164,384 is estrogen-irreversible.

Estradiol is able to completely abrogate the effect of 10^{-8} M ICI 164,384 and 10^{-6} M tamoxifen. Fig. 3 shows that the effect of tamoxifen is abolished at a concentration of 10^{-8} M estradiol, and half maximal inhibition (or abrogation) is seen with a concentration of estradiol of 3×10^{-9} M (E2/TAM ratio 1/333). The effect of 10^{-8} M ICI 164,384 is completely abrogated with addition of

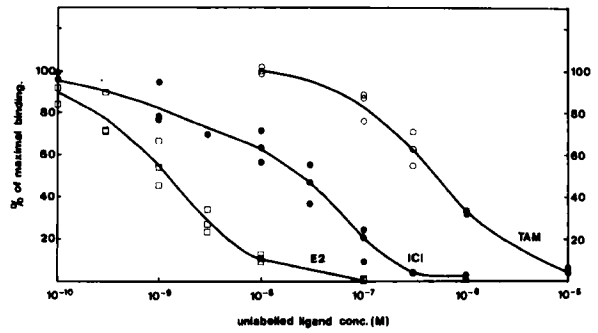

Fig. 4. Competition for estrogen binding sites. Samples of cytosol from MCF-7 cells were incubated with 1.5 nM ^{3}H-estradiol and the indicated concentrations of unlabelled estradiol (E2, □), ICI 164,384 (ICI, ●) or tamoxifen (TAM, ○) at 4°C for 16 hours. Free steroids were removed by dextran coated charcoal adsorption. Binding is expressed as percentage of the binding obtained with radiolabelled estradiol alone (corrected for non-specific binding). Smooth curves are drawn between the average values for each tested concentration.

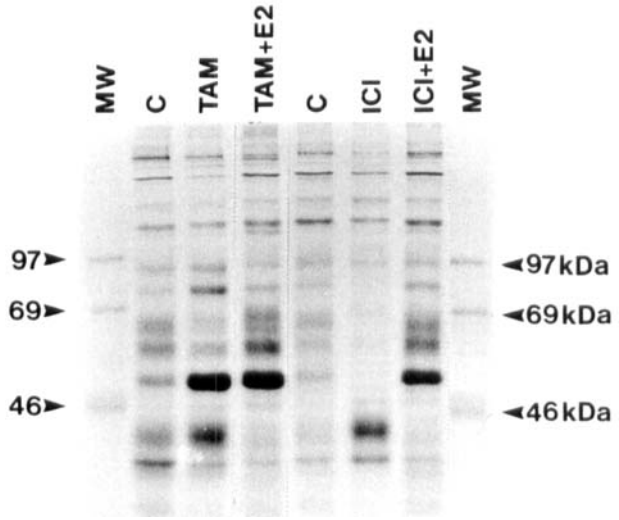

Fig. 5. Autoradiogram of ^{35}S-methionine labelled proteins secreted from MCF-7 cells analyzed by SDS-PAGE. ^{35}S-methionine in serum free growth medium was added to the cultures after 6 days in experimental medium, and conditioned media collected after 6 hours. Equal amounts of TCA-precipitable cpm from the respective conditioned media were applied on each lane, conditioned media from control culture (C), culture with 10^{-6} M tamoxifen (TAM), culture with 10^{-6} M tamoxifen and 10^{-8} M estradiol (TAM + E2), culture with 10^{-8} M ICI 164,384 (ICI) and culture with 10^{-8} M ICI 164,384 and 10^{-9} M estradiol (ICI + E2). Lanes with molecular weight markers are indicated MW.

10^{-9} M estradiol, and 10^{-10} M estradiol (E2/ICI ratio 1/100) reduces the inhibition of ICI 164,384 to half maximal inhibition of cell proliferation.

Fig. 4 shows the result of competition experiments for binding of estradiol, tamoxifen and ICI 164,384 to cytosolic estrogen receptors from MCF-7 cells in presence of 1.5×10^{-9} M ^{3}H-estradiol. Unlabelled estradiol gives half maximal competition at a concentration of about 10^{-9} M as expected. Tamoxifen at 5×10^{-7} M (E2/TAM ratio 1/333) exerts half maximal competition, whereas a concentration of 2.5×10^{-8} M ICI 164,384 produces half maximal competition (E2/ICI ratio 1/17).

Fig. 5 shows ^{35}S-methionine labelled proteins secreted from MCF-7 cells grown in control medium, in medium with concentrations of tamoxifen or ICI 164,384, which gives maximal inhibition, and in medium with antiestrogen plus estradiol in a ratio, which is sufficient for complete abrogation of the inhibitory effect of the antiestrogen on cell proliferation. It can be seen that tamoxifen inhibits the synthesis and secretion of a 66 kDa and a 61 kDa protein and stimulates the synthesis of a 52 kDa and a 42 kDa protein. Cells grown with estradiol added in combination with tamoxifen show increased synthesis of 66 kDa, 61 kDa and 52 kDa proteins and inhibition of a 42 kDa protein compared to control cells. ICI 164,384 inhibits the synthesis and secretion of the three proteins with mol. wt 66 kDa, 61 kDa and 52 kDa and stimulates the synthesis of the 42 kDa protein. Estradiol added to cultures with ICI 164,384 stimulates the synthesis of the 66 kDa, the 61 kDa and the 52 kDa proteins and inhibits the synthesis of a 42 kDa. Cultures treated with estradiol alone secrete the four proteins in a pattern similar to the cultures with antiestrogen plus estrogen (13).

We have developed a tamoxifen resistant variant of the MCF-7 cells (AL-1 subline) by long-term treatment with tamoxifen (26). Fig. 6 compares the effect of the antiestrogens on cell proliferation of the parent cell line, MCF-7 and the AL-1 subline. As shown previously 10^{-6} M TAM significantly inhibits growth of MCF-7 cells, whereas the AL-1 cell line is only slightly affected by addition of 10^{-6} M TAM to the culture medium. MCF-7 is very sensitive to the inhibitory effect of the ICI 164,384 compound, whereas the AL-1 cell line shows very little effect even at the concentration of 10^{-8} M, which gives maximal inhibition of the MCF-7 cells. The tamoxifen resistant cell line is, however, not resistant to higher concentrations of ICI 164,384, as 10^{-7} M and 10^{-6} M concentrations inhibit cell proliferation to 23% and 6% respectively of the cell number in the untreated control culture. The inhibitory effect of 10^{-7} M ICI 164,384 on AL-1 cells can be completely abrogated by addition of 10^{-7} M estradiol.

The effects of tamoxifen and estradiol on secreted proteins from MCF-7 and AL-1 cells are compared in Fig. 7. Tamoxifen at concentrations of 4×10^{-7} M and 10^{-6} M has no effect on secreted proteins in AL-1 cells, and estradiol stimulates the synthesis of the 61 and the 52 kDa proteins and inhibits the synthesis of the 42 kDa protein. No stimulatory effect of estradiol on the 66 kDa protein synthesis is observed in AL-1 cells and the control culture of AL-1 cells also secretes very small amounts of 66 kDa protein compared to MCF-7 cells, indicating variation in specific protein induction by estradiol in the two cell lines.

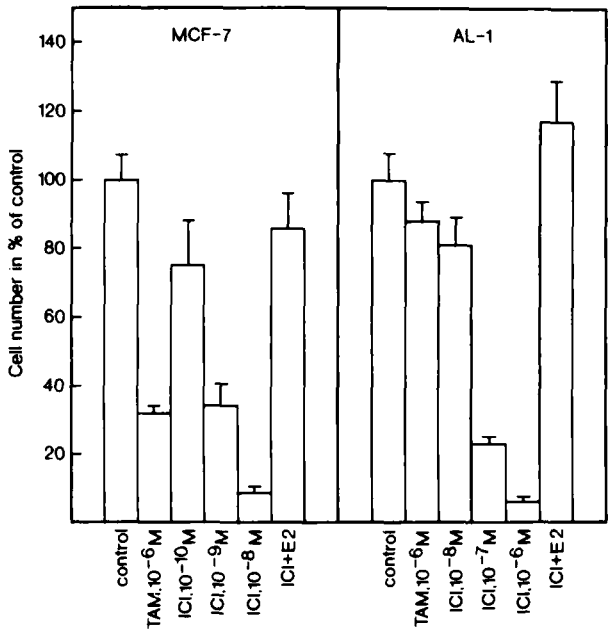

Fig. 6. Effect of antiestrogens and antiestrogens plus estrogen on cell proliferation of MCF-7 and AL-1 cells. The cells were seeded in multidishes in growth medium and two days after seeding experimental media with the indicated concentrations of antiestrogen or antiestrogen plus estradiol were added. Experimental media were renewed at days 3 and 5, and cell numbers of four wells in each group determined at day 6. Cell numbers are expressed in percentage of the cell number in the corresponding control culture. The concentration of antiestrogen and estradiol in the experiment with MCF-7 cells were 10^{-8} M ICI 164,384 and 10^{-8} M estradiol, with AL-1 cells the concentrations were 10^{-7} M ICI 164,384 and 10^{-7} M estradiol.

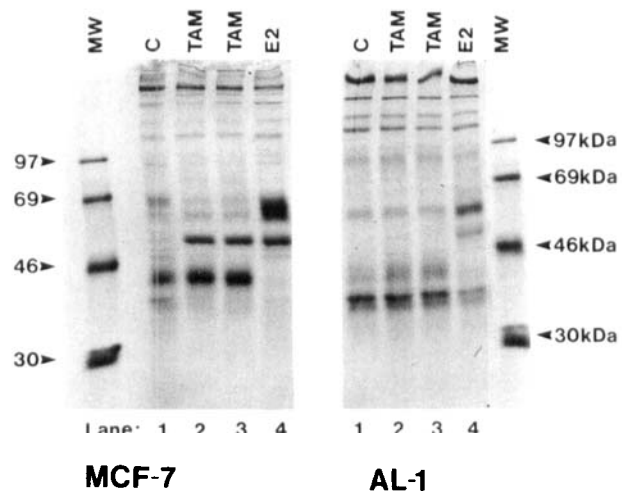

Fig. 7. Autoradiogram of ^{35}S-methionine labelled proteins secreted from MCF-7 cells and from AL-1 cells analyzed by SDS-PAGE. The cells were grown as described in Fig. 6. ^{35}S-methionine in serum free growth medium was added to the cultures after 6 days in experimental medium, and conditioned media collected after 6 h. Equal amounts of TCA-precipitable cpm from the conditioned media were applied on each lane. The concentrations of tamoxifen (TAM) used were 4×10^{-7} M and 10^{-6} M, the concentration of estradiol (E2) 10^{-9} M. Medium from control cultures are indicated with a C and the lanes with molecular weight markers are indicated MW.

Studies of the effect of ICI 164,384 on proteins secreted from AL-1 cells revealed that ICI 164,384 has an estrogen antagonistic effect by inhibiting the synthesis of the 61 kDa and the 52 kDa proteins and stimulating the 42 kDa protein (results not shown).

Discussion

The steroidal antiestrogen ICI 164,384 has been found to have pure antiestrogenic activities on breast cancer cells both in vivo and in vitro (23–25), and the complete blockade of the estrogen effects makes this and other related pure antiestrogens potential new compounds in the treatment of breast cancer patients (33). Several papers have decribed the specific inhibitory effects of the ICI 164,384 compound on cell proliferation of experimental animal breast tumors and breast cancer cell lines (23, 34, 35), on synthesis of estrogen-induced mRNAs (36, 37) and on synthesis of the estrogen-induced proteins 52 kDa cathepsin D and progesterone receptor (36, 38). In the present paper we have tried to elucidate whether the observed inhibitory effect of the two antiestrogens ICI 164,384 and tamoxifen on cell proliferation of the estrogen receptor positive human breast cancer cell line MCF-7 and of a tamoxifen resistant variant of this cell line, the AL-1 cell line, can be correlated to the effects of these antiestrogens on the estrogen regulated secreted proteins 66 kDa, 61 kDa, 52 kDa and 42 kDa, which are directly or indirectly involved in growth regulation.

Our MCF-7 cells are estrogen responsive with respect to cell proliferation rate provided the cells are propagated under growth-retarded conditions, which can be obtained by addition of e.g. 10% NCS, as shown in Fig. 1. In our conventional phenol red free growth medium with 1% FCS the supply of growth factors and estrogen compounds by the serum supplementation is close to being optimal for rapid cell proliferation rate, and this culture condition is well suited for the study of the effects of antiestrogens on cell proliferation and regulation of secreted proteins (13, 39). Our growth experiments have shown that the relative potency for maximum inhibitory effect of ICI 164,384 is more than 100-fold higher than for tamoxifen, and this is in agreement with the observations of others (34, 35), and may be explained by the higher binding affinity of ICI 164,384 to the estrogen receptor compared to tamoxifen. The equilibrium dissociation constants, K_D, for binding of estradiol and ICI 164,384 to human estrogen receptor are in the same order of magnitude (0.44 nM and 0.69 nM respectively (40)), whereas the K_D for binding of tamoxifen to human estrogen receptors is about 30-fold the K_D for estradiol (3.70 nM and 0.12 nM respectively (41)),

indicating that tamoxifen binds with an affinity of less than 5% the binding affinity of ICI 164,384.

Inhibition of cell proliferation by antiestrogens can be abolished by simultaneous addition of estradiol. In the presented experiments, the FCS in the culture media adds a background level of estrogenic activity. We have determined the content of estradiol, estrone and estrone sulfate in FCS (unpublished) and have calculated the amount of estrogenic activity in the medium with 1% FCS to correspond to an estradiol concentration in the order of about 1 pM. This concentration of estradiol is low compared to the concentrations needed to reduce the growth inhibitory effect of the antiestrogens and are therefore not included in the calculations of the ratio estradiol/antiestrogen for abrogation of cell proliferation. The low estradiol/tamoxifen ratio (1/333) required to obtain 50% abrogation of the inhibitory effect of tamoxifen on cell proliferation can only partly be explained by the higher K_D-value for binding of tamoxifen to the estrogen receptors. The relative binding affinities of the two components are of importance for the effect on cell proliferation, and competition assays for binding to cytosolic estrogen receptors from MCF-7 cells revealed that the relative binding affinity of tamoxifen is 0.3% the binding of estradiol, strongly supporting that the difference in relative binding to the estrogen receptors may explain the observed effect on cell proliferation. Relative binding affinity of tamoxifen compared to estradiol is described in the literature to be in the order of about 0.3% (41, 42), which is in accordance with our results. As mentioned above ICI 164,384 and estradiol have K_D-values in the same order of magnitude, and the observation that estradiol in a concentration as low as 1% of the ICI 164,384 concentration can reduce the growth inhibition with 50%, indicates that the relative binding of ICI 164,384 to estrogen receptors is low compared to estradiol. We find that the relative binding affinity of ICI 164,384 to cytosolic estrogen receptors is 6% of the binding affinity of estradiol, and this observation is in good agreement with previously published relative binding affinities determined at 0°C (33, 43), and the finding that a large molar excess (50-150-fold) of ICI 164,384 is required to antagonize the effect of estradiol on synthesis of estrogen inducible mRNAs (36). The lower relative binding affinity of ICI 164,384 to estrogen receptors can, to some extent, be explained by the high non-specific binding of the compound to other components in the cells or in the cytosol (40), and a reduced association rate for binding to the estrogen receptors compared to estradiol (43).

Estradiol regulates the synthesis of several proteins in human breast cancer cells (11–13, 18, 21, 44), and many of these proteins are assumed to have a direct or indirect growth regulatory function (5, 11, 13, 14, 44). In our MCF-7 cells estradiol stimulates the synthesis and secretion of three proteins with mol.wt 66 kDa, 61 kDa and 52 kDa and inhibits the synthesis of one protein with mol.wt 42 kDa (13, 14, 45). The 66 kDa and the 61 kDa proteins, which are proteins homologous to the serum antiproteases α_1-antichymotrypsin and α_1-antitrypsin respectively have been found to have indirect growth stimulatory activities ((14) and Inga Laursen, Biochim Biophys Acta, in press, 1992). The 52 kDa protein is identified as procathepsin D, which has been described to have a weak direct growth stimulatory function on growth of estrogen deprived MCF-7 cells (5, 14). The 42 kDa protein is still unidentified, and the function of the pure protein is unknown. However, results of growth experiments with partially purified 42 kDa protein indicate that this protein has a negative growth regulatory function (14). We show in this paper that the antiestrogen tamoxifen has an agonistic effect on the synthesis of the 52 kDa protein and antagonistic effect on the synthesis of the three other estrogen regulated proteins mentioned above. The ICI 164,384 is a complete estrogen antagonist with respect to synthesis of the four estrogen regulated proteins. In the first paper, which described estrogen stimulation of the 52 kDa protein (originally called 46 kDa protein), tamoxifen had an inhibitory effect on the synthesis of this protein (18). It has later been shown, however, that tamoxifen upregulates both the synthesis of the pNR-100 RNA, which is the transcript of the 52 kDa cathepsin D gene, and the 52 kDa cathepsin D synthesis (46). Similar estrogenic activity of tamoxifen has been described for induction of the progesterone receptor (21) and for other estrogen inducible mRNAs (22, 46). We suggest that our observation of the higher maximum inhibitory efficacy of ICI 164,384 than of tamoxifen on cell proliferation is due to the complete estrogen antagonistic effect of ICI 164,384 on synthesis of proteins with growth regulatory function. The lower maximum inhibitory efficacy of tamoxifen on cell proliferation may be explained by the partial agonistic/antagonistic effects of tamoxifen on proteins with growth regulatory function. Whether the agonistic effect of tamoxifen on the synthesis of the 52 kDa protein alone can ascribe for the lower inhibitory efficacy of tamoxifen on cell proliferation is unknown. It appears likely that tamoxifen may have a similar agonistic effect on other proteins involved in estrogen stimulation and that the reduced inhibitory effect on cell proliferation compared to the effect of the pure antiestrogen ICI 164,384 results from agonistic effect of tamoxifen on several growth regulatory factors.

Development of resistance to tamoxifen treatment is a great problem in treatment of breast cancer patients, and it has been suggested that 'pure antiestrogens may have a therapeutic advantage over tamoxifen in reducing the probability of treatment failure due to the regrowth of tumors from resistant cells' (25). Studies on development of resistance to pure antiestrogens have not yet been published, but we are at present trying to produce ICI 164,384 resistant cell lines by long-term treatment of MCF-7 cells with the pure antiestrogen. Another interesting aspect is whether breast

cancer cells which have developed resistance to tamoxifen respond to treatment with the pure antiestrogen. We have used our tamoxifen resistant cell line AL-1 derived from MCF-7 cells (26) to address this question. We find that the AL-1 cells are growth-inhibited by ICI 164,384, although a 100-fold higher concentration of ICI 164,384 is required to obtain the same degree of inhibition as seen with the parent cell line MCF-7. The AL-1 cells contain estrogen receptors, but at a reduced level compared to MCF-7 cells, and differ from the parent cell line in exhibiting no estrogen stimulation of progesterone receptor synthesis (26). In this paper we show that estradiol exerts very little induction of 66 kDa protein synthesis in these cells compared to MCF-7 cells, although the effect on 61 kDa, 52 kDa, and 42 kDa protein synthesis is similar to that of MCF-7. In spite of the observations of several altered estrogen receptor functions in the AL-1 cells, estradiol can completely abrogate the inhibitory effect of ICI 164,384 on cell proliferation. Tamoxifen has no effect on the synthesis and secretion of the 61 kDa, the 52 kDa and the 42 kDA protein, which are estrogen-regulated in AL-1 cells as in MCF-7 cells, whereas ICI 164,384 has a complete estrogen antagonistic effect on the synthesis of these three proteins, indicating that the lack of tamoxifen inhibition on growth of AL-1 cells may be due to the lack of antagonistic effect on estrogen-regulated proteins with growth regulatory function. The growth inhibitory effect of ICI 164,384 on AL-1 cells may be due to the observed antagonistic effect of ICI 164,384 on estrogen regulated proteins. The fact that estradiol can abrogate the inhibitory effect of ICI 164,384 on cell proliferation may be due to the normal function of the estrogen receptor when bound to estradiol in controlling the estradiol regulated proteins. The observed complete lack of effect of tamoxifen on estrogen regulated proteins could be assumed to be due to lack of uptake of tamoxifen in this resistant cell line. This is, however, not the case as we can measure that AL-1 cells grown in presence of 10^{-6} M tamoxifen contain bound nuclear estrogen receptors, whereas the AL-1 cells grown without tamoxifen contain primarily free estrogen receptors (unpublished). We therefore suggest that the transcription activation functions in the estrogen receptor mechanism in AL-1 cells are changed, either due to presence of variant estrogen receptors or to changes in other transcription factors involved in expression of estrogen regulated genes (47).

In summary, this paper shows that the effect of the two antiestrogens tamoxifen and ICI 164,384 on cell proliferation can be correlated to the effect of these antihormones on synthesis of four secreted proteins which have been found to have growth regulatory functions (14). Several other proteins, besides the mentioned four ones, are estrogen-regulated in breast cancer cells and we suppose that estrogen stimulation and antiestrogen inhibition occur via a complex interaction between factors, some of which are estrogen-regulated. Finally, we have shown that a tamoxifen resistant variant subline of the human breast cancer cell line MCF-7 can be growth-inhibited by the pure antiestrogen ICI 164,384, suggesting that breast cancer patients who develop resistance to tamoxifen treatment may benefit from treatment with a pure antiestrogen.

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