Partial Antagonism between Steroidal and Nonsteroidal Antiestrogens in Human Breast Cancer Cell Lines

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ABSTRACT

Nonsteroidal antiestrogens, such as tamoxifen, are well established in the treatment of breast cancer. The development of new steroidal compounds without partial agonist activity allows deeper insights into the mechanism of antiestrogen action, and thus far, the combined use of steroidal and nonsteroidal antiestrogens has not been studied extensively. We compared the nonsteroidal 4-trans-hydroxytamoxifen (OHT) with the two steroidal antiestrogens, ICI 182780 and RU 58668, in the estrogen receptor-positive human breast cancer cell lines MCF-7 and T47D. The effect of each compound alone or of OHT in combination with one of the steroidal antiestrogens was studied in regard to cell proliferation, expression of estrogen receptors (ERs) and progesterone receptors, and secretion of transforming growth factor beta 2 (TGF-β2). All antiestrogens examined led to enhanced secretion of TGF-β2, which is correlated with their individual growth-inhibitory potential. OHT partially counteracts the larger growth inhibition of human breast cancer cells exerted by the steroidal antiestrogens ICI 182780 and RU 58668. Also, OHT antagonizes the higher induction of TGF-β2 seen after treatment of MCF-7 cells with steroidal antiestrogens. The loss of ER and down-regulation of progesterone receptor under treatment with the steroidal antiestrogens is prevented by OHT, whereas the steroidal antiestrogens prevent the ability of hydroxytamoxifen to increase the ER content. These results indicate that TGF-β2 is a marker of action for both types of compounds, but steroidal and nonsteroidal antiestrogens partially antagonize each other in blocking ER-mediated cellular events. It would appear that no additive or synergistic effect of the two types of antiestrogens can be expected in the treatment of breast cancer.

INTRODUCTION

Breast cancer is the most common malignant disease of women in the western world. Since the end of the last century, it has been known that ovarian secretions, later shown to be estrogens, play an important role in the pathogenesis and progression of the disease (1, 2). This role has been studied extensively in the last decades.

In the 1960s, the concept of receptor-mediated estrogen action was developed (3, 4). In the 1970s, the use of estrogen receptor assays to identify breast cancers became part of clinical diagnosis (5, 6). Better understanding of estrogen action made it possible to search for compounds that block the stimulating action of estrogens on tumor tissue. Since the late 1970s, the triphenylethylene-related antiestrogen, tamoxifen, has become well established in the endocrine treatment of breast cancer. Like other antiestrogens with a nonsteroidal structure, tamoxifen is a partial estrogen agonist as well as antagonist (7–10). The proportion of patients with advanced breast cancer who respond to tamoxifen and the average duration of response are comparable to that seen with other endocrine treatments (11), but tamoxifen therapy is limited by the eventual development of resistance to the treatment (12, 13). These limitations of therapeutic effectiveness have been attributed in part to the estrogen-like effects of tamoxifen. Complete ablation of estrogen-mediated tumor growth is a desirable aim because it might provide more rapid or longer lasting tumor responses.

The search for new antiestrogens has led to the development of compounds without partial agonistic properties (14–16). These "pure" antiestrogens have a steroidal structure and have been called type 2 antiestrogens to distinguish them from type 1 agents, such as tamoxifen, that depending on the species, tissue and concentration, can show either agonist or antagonist actions. Both types of antiestrogens compete with estradiol for its binding to the receptor, but thereafter their action mechanisms have been thought to differ (17–19). Several steroidal antiestrogens have been described, one of these compounds, ICI 182780, a 7α-substituted derivative of estradiol, appears to have clinical potential and is presently under clinical study (16, 20, 21). Both ICI 182780 and ICI 164384 are able to inhibit the growth of tamoxifen-resistant tumors (22, 23). Another substance, RU 58668, was described more recently and has a 11β-substituted estradiol structure (24). This antiestrogen is also free of estrogenic properties and, unlike tamoxifen or ICI 182780, is able to induce long-term regression of estradiol-stimulated tumor growth in nude mice bearing xenografts of MCF-7 human mammary cancer.

The complete blockade of estrogen effects by treatment with pure antiestrogens alone could cause problems with osteoporosis and atherosclerosis where tamoxifen, probably due to its agonist activity, has been shown to have a protective effect (25–27). Moreover, if the two classes of antiestrogens show different mechanisms of action, there could be a possibility that combined use of the compounds would offer an advantage by showing an additive or even synergistic effect. Also, it might be possible to minimize clinical side effects by reducing the required doses. To compare the antagonistic abilities of the two types of antiestrogens and the potential use of a combined application, we have studied the effects of 4-hydroxytamoxifen, an active metabolite of tamoxifen (28), as a nonsteroidal antiestrogen and of ICI 182780 and RU 58668 as steroidal antiestrogens, alone and in combination, in respect to different aspects of estrogen-inhibitory action.

Antiestrogens are thought to exert their antagonistic effects in part through the enhancement of the secretion of inhibitory growth factors, for example TGF-β2, that has been shown to play a role in autocrine and paracrine growth regulation (29, 30) and to represent a marker of estrogen-inhibitory action (31). Thus far, no quantitative comparison between the antagonistic properties of antiestrogens and their ability to induce the inhibitory TGF-β proteins has been made, and it is not clear whether there is a difference in the extent of induction of TGF-β proteins between different classes of antiestrogens.

We have looked at the growth inhibition of estrogen-dependent MCF-7 breast cancer cells as an end point of inhibitory action. Furthermore, we have examined the regulation of estrogen and progesterone receptors as two estrogen-regulated proteins. Finally, the potential of the antiestrogens to enhance the secretion of TGF-β2 proteins was evaluated.

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MATERIALS AND METHODS

Chemicals. The antiestrogen ICI 182780 was a gift of A. Wakeling (Zeneca Pharmaceuticals, Macclesfield, United Kingdom). The antitumor 4-hydroxytamoxifen was a gift of V. C. Jordan (Northwestern University, Chicago, IL). RU 58668 was a gift of Roussel-UCLAF (Romainville, France). Chemicals were obtained from Sigma Chemical Co., Deisenhofen, Germany, if not otherwise indicated. Materials for cell culture were from Life Technologies, Inc., Eggenstein, Germany.

MCF-7 Cell Culture. MCF-7 cells were obtained from M. Lippman (Georgetown University, Washington, DC). Cells were maintained in DMEM (DMEM) containing 4.5 g of glucose/l supplemented with 25 μg/ml gentamicin, 5 mg/ml insulin, 200 mEq glutamine, and 10% FCS. The medium was changed every third day, and cells were passaged once per week. Before their use in experiments, cells were maintained for 1 week in the same medium as described above but with 5% CDFCS instead of FCS. All media contained phenol red that is known to have a weak estrogenic effect (32). Ligands were added in ethanol, giving a final ethanol concentration of 0.1%.

T47D Cell Culture. T47D cells were cultured under similar conditions to the MCF-7 cells.

Regulation of Estrogen Receptor Content in the Presence of Antiestrogens. MCF-7 cells were cultured for 1 week in medium containing 5% CDFCS and then plated at a density of 10 6 cells per 140-mm cell culture dish. Ligands and control vehicle were added after 4 days, and cells were harvested after 24 h of treatment. Cells were homogenized with a Dounce homogenizer in buffer containing 10 mM Tris, 1.5 mM EDTA, 5 mM sodium molybdate, 1 mM monothioglycerol, and 400 mM KCl. After centrifugation, the estrogen receptor content of the cytosol fraction was determined by enzyme immunoassay (Abbott, Delkenheim, Germany) according to the manufacturer's instructions. Receptor content was normalized to the protein content of the cytosol.

Regulation of Progesterone Receptor Content in the Presence of Antiestrogens. Progesterone receptor content was determined under similar conditions like estrogen receptor, except that ligands were added 2 days after plating of the cells, and cells were harvested 4 days later. Receptor content of the cytosol was measured with a progesterone receptor enzyme immunoassay from Abbott. Receptor content was normalized to the protein content of the cytosol.

Proliferation Assays for MCF-7 Cells. Cells were cultured for 1 week in medium with 5% CDFCS and then plated in triplicate into 24-well cell culture dishes at a density of 1.5 × 10 4 cells/well. On the next day, the medium was changed, and fresh medium and ligand or control vehicle was added. Medium and ligands were changed every second day. Cells were harvested with PBS containing 1.5 mM EDTA, transferred to Isotone solution (Coulter Instruments), and counted using a Coulter particle counter.

Regulation of TGF-β Secretion by Antiestrogens. MCF-7 cells were cultured 1 week in medium containing 5% CDFCS and then plated into 140-mm cell culture dishes at a density of 1 million cells/plate. Twenty-four h later, ligands and control vehicle were added. Five days later, the cell culture medium was changed to a medium without serum but with 1 μg/ml of transferrin; 24 h later, the medium was changed again, and after 48 h, the medium was collected, and protease inhibitors were added (10 μg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml pepstatin A). The secreting cell layer was harvested, and cells were collected. TGF-β secretion was normalized for the cell number. The medium was concentrated 30-fold with a Centricon filter system (Amicon, Witten, Germany) with a cutoff molecular weight of M, 10,000 and stored at −70°C. For the determination of TGF-β protein, the medium was further concentrated 2.5-fold with a vacuum centrifuge. A 100-μl portion of the protein suspension was mixed with 500 μl of HCl-ethanol and centrifuged at 12,000 rpm for 30 min at 4°C in an Eppendorf centrifuge. The supernatant was decanted and neutralized with 200 μl of Tris (0.855 M). The liquid was evaporated in a vacuum centrifuge, and the resulting pellet was resuspended in a binding buffer (1 mg/ml BSA, 150 mM NaCl, 0.02% NaN 3, and 100 mM Tris-HCl, pH 7.4). All materials for handling of the conditioned media were coated with buffer containing 1 mg/ml BSA.

Enzyme Immunoassay for Determination of TGF-β. The enzyme immunoassay for determination of TGF-β was performed as described recently (33). In brief, the protein concentrated from the conditioned media was added to 96-well microtiter plates that had been coated previously with a mouse polyclonal anti-TGF-β-antibody (anti-TGF-β1, anti-TGF-β2, and anti-TGF-β3; Genzyme, Cambridge, MA). After incubation at room temperature for 1 h and careful washing with PBS, the second antibody was added (anti-TGF-β from rabbit; R&D Systems, Minneapolis, MN). After another 1 h incubation and careful washing, the third antibody was added (phosphatase-labeled anti-rabbit; Kirkegaard & Perry, Gaithersburg, MD), followed by several washings with PBS and the addition of phosphatase substrate. After further incubation for 1 h, absorption was measured on an ELISA reader at 405 nm. Porcine TGF-β2 (R&D Systems) was used as standard. Cross-reactivity to TGF-β1 was less than 0.01%; the detection limit was 25 pg/ml. All TGF-β2 determinations were done in duplicate.

RESULTS

Growth Inhibition of MCF-7 Cells. MCF-7 breast cancer cells contain estrogen receptors, and their growth is inhibited by antiestrogens. They provide a well-established model system to study the effects of antiestrogens on estrogen-dependent tumor growth. To examine the growth-inhibitory action of the estrogen antagonists in our system, the cell number after 5 days was used as parameter for cell growth. All compounds caused growth inhibition; the steroidal ICI 182780 and RU 58668 showed a higher antagonistic potential than the nonsteroidal hydroxytamoxifen. ICI 182780 reduced the cell number to 7% of the untreated control, and RU 58668 induced a decrease to 8% of the control, as compared with hydroxytamoxifen, which gave an inhibition to 44% (Fig. 1). IC 50 s were 0.2 nM for ICI 182780, 0.09 nM for RU 58668, and 0.8 nM for hydroxytamoxifen.

The steroidal antiestrogens also showed a stronger growth inhibition at lower concentrations than hydroxytamoxifen; ICI 182780 and RU 58668 showed reductions to 11 and 9%, respectively, of the control already at 1 nM, whereas hydroxytamoxifen gave a reduction only to 68% at this concentration.

Growth Inhibition by a Combination of Steroidal Antiestrogens with Hydroxytamoxifen. A combination of hydroxytamoxifen with the steroidal compounds was examined to determine whether this shows more inhibition than either compound alone. When used with type 2 antiestrogens at their maximal growth-inhibitory concentration (10 nM), increasing concentrations of hydroxytamoxifen counteracted the stronger growth-inhibitory effect of ICI 182780 and RU 58668 (Fig. 2). The combination of 100 nM hydroxytamoxifen with RU 58668 or ICI 182780 resulted in respective decreases of the cell number to 45 and 42% of the control, as compared with 8% for ICI 182780 and 7% for RU 58668 when the steroidal antiestrogens were used alone.
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Cellular Estrogen Receptor Content, Apparently by Increasing Its Turnover (34, 35). After 24 h of treatment, the estrogen receptor content of MCF-7 cells was measured by immunoassay. Both steroidal antiestrogens led to a dramatic, dose-dependent loss of estrogen receptor of the control (Fig. 5). IC50s were 0.4 nM for ICI 182780 and 0.04 nM for RU 58668. ICI 182780 reduced receptor content to 32% and RU 58668 to 26% of the control, with RU 58668 showing an effect already at 0.1 nM. In contrast, hydroxytamoxifen increased the receptor content to 152% of the control value at 100 nM. At their maximum effective concentration (1 nM), the effects of ICI 182780 and RU 58668 were completely counteracted by hydroxytamoxifen, bringing the receptor content back to values seen when the latter compound was used alone (Fig. 6). Hydroxytamoxifen partially reduced the effectiveness of the steroidal antiestrogens when these were used at a higher concentration (10 nM). In turn, a 10 nM concentration of either steroidal antiestrogen prevented the stimulatory effect of hydroxytamoxifen on the estrogen receptor content.

Regulation of Progesterone Receptor Levels in T47D Cells. One of the proteins that is regulated by estrogens in many cells is the progesterone receptor. In T47D breast cancer cells, which have a relatively high content of progesterone receptor (165 fmol/mg of cytosol protein in the untreated control), hydroxytamoxifen showed...
no significant effect on the progesterone receptor content after 4 days of treatment, even at 100 nM concentrations (Fig. 7) or higher. At a concentration that produced a maximal growth-inhibitory effect (10 nM), the steroidal antiestrogens ICI 182780 and RU 58668 led to a decrease in progesterone receptor content to 39% (ICI 182780) and 36% (RU 58668) of the control. This effect of the steroidal antiestrogens on the progesterone receptor content was counteracted by hydroxytamoxifen when used in combination with the steroidal compounds.

**DISCUSSION**

An end point of antiestrogen action is obviously the growth inhibition of breast cancer cells. Still, the exact mechanism of growth-inhibitory action exerted by antiestrogens is not clear (18). It is remarkable that the new steroidal antiestrogens show not only a substantial effect at very low concentrations but the maximal inhibition of growth is greater than that achieved with hydroxytamoxifen (Fig. 1). This growth-inhibitory effect is completely blocked when the steroidal antiestrogens were used in combination with hydroxytamoxifen (Fig. 2), in contrast to a much smaller effect reported previously with related compound ICI 164384 (36).

It has been suggested that the rapid decrease of estrogen receptor content seen after treatment with ICI 164384 might contribute to the pure antagonistic properties of this compound by suppressing a constitutive activity of the unoccupied estrogen receptor (34). Also, a decrease in estrogen receptor was seen after short-term treatment of breast cancer patients with ICI 182780 (37) as well as in experimental systems. We show that the steroidal antiestrogens, ICI 182780 and RU 58668, also induce a decrease in receptor content of cultured cells in a dose-dependent manner (Fig. 5), supporting the concept that this ability is a common feature of steroidal antiestrogens and may be necessary for “pure” antagonistic action.

Growth inhibition by antiestrogens seems to involve not only the inhibition of overall protein synthesis but also the enhancement of the secretion of certain proteins, such as the inhibitory growth factor TGF-β2 (29). TGF-β proteins are multifunctional growth factors that belong to a polypeptide family consisting of three known isoforms in humans (38). Antiestrogens lead to an enhanced secretion of biologically active TGF-β proteins in most breast cancer cell lines, which can be inhibited by these compounds (31). Breast cancer cells resistant to treatment with antiestrogens still can be inhibited by TGF-β in vitro, suggesting a role of TGF-β protein as paracrine inhibitor in breast cancer growth (29, 38). It has been proposed that TGF-β2 is a marker of antiestrogen action, and a correlation was shown between a rapid increase of TGF-β serum protein levels and clinical remission of breast cancer patients with advanced disease (33). The higher induction of TGF-β2 protein by the steroidal antiestrogens ICI 182780 and RU 58668 compared with hydroxytamoxifen (Fig. 3) might reflect the higher potential of the steroidal compounds to activate an autoinhibitory growth control loop, with the higher growth inhibition of these compounds also being due to this fact. Our results also suggest that TGF-β2 represents not only a useful marker for clinical response to treatment with nonsteroidal antiestrogens but it also might serve as parameter for controlling the response to endocrine therapy with new steroidal antiestrogens in the future.

The progesterone receptor is one of the proteins regulated by estrogens and antiestrogens (39). We have examined T47D cells because they contain substantial amounts of both progesterone and estrogen receptors. It is interesting that, in this system, hydroxytamoxifen has no significant effect on progesterone receptor content, whereas the steroidal antiestrogens induce a reduction in receptor content consistent with their stronger inhibitory potential. Again, this effect is counteracted by hydroxytamoxifen when used in combination with the steroidal compounds (Fig. 7).

The properties of type 2 antiestrogens make them attractive for second-line treatment of breast cancer patients (14, 21). Still, little is known about the side effects of the new antiestrogens in humans, with complete blockage of estrogen receptors possibly causing problems in the bone and vascular system, where tamoxifen has been shown to have beneficial effects (25–27). Therefore, it is important to examine the combined use of the steroidal antiestrogens with tamoxifen, which shows some estrogenic effects and can reduce the side effects of complete estrogen ablation. In spite of their probably partly different mechanisms of action (17–19), the two classes of antiestrogens show no additive or synergistic effects in respect to all aspects of antiestrogen action we have examined.

Prevention of the effects of the steroidal antiestrogens could be due to their displacement from the receptor by hydroxytamoxifen, which has a considerably higher binding affinity (16, 24, 40), so that essentially all of the receptor is associated with hydroxytamoxifen. However, this does not explain the ability of 10 nM steroidal antiestrogens to prevent the increase in estrogen receptor content induced by 100 nM hydroxytamoxifen (Fig. 6). Therefore, one should consider differences between steroidal and nonsteroidal antiestrogens distinct from receptor binding. These might include a different potential of these compounds in the activation of TGF-β secretion or in their ability to influence estrogen receptor content (Figs. 4 and 6).

The observation that the combination of nonsteroidal and steroidal antiestrogens does not show additive or synergistic effects in our experimental systems suggests that one may not expect any clinical advantage from their combined use in treating breast cancer patients.
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