Steroid Receptor-mediated Cytotoxicity of an Antiestrogen and an Antiprogestin in Breast Cancer Cells

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ABSTRACT

The antiproliferative and cytotoxic effects of 4-hydroxytamoxifen, an antiestrogen with a high affinity for the estrogen receptor, and of 17β-hydroxy-11β-(4-methylaminophenyl)-17-(1-propynyl)estra-4,9-dien-3-one-6,7 (RU486), an antiprogestin with a high affinity for the progesterin receptor, have been studied on human breast cancer cell lines in culture. The number of dead cells was evaluated by several techniques (trypan blue stain exclusion, DNA cleavage, lactic dehydrogenase activity, morphological changes, and cloning efficiency in soft agar) and found to be increased by both the antiestrogen and the antiprogestin at concentrations correlating with the affinities for their respective receptors. This cytotoxic effect was prevented by the occupation of the respective receptors with estrogen and progesterin and was not found in the estrogen receptor- and progesterin receptor-negative MDA MB 231 and BT20 cell lines. The contrast between the ultrastructural modifications of chromatin and the estrogen and progestin and was not found in the estrogen receptor- and progesterin receptor-negative MDA MB 231 and BT20 cell lines. We conclude that in addition to the receptor-mediated cytostatic activity and the nonspecific cytotoxic activity, antiestrogens trigger a third type of effect that we designate as "receptor-mediated cytotoxic." Similar conclusions can be drawn for the antiprogestin RU486, indicating moreover that the antihormone and antiproliferative activities of this drug are clearly dissociated. The mechanism of these receptor-mediated cytotoxic activities of antiestrogen and antiprogestrone is not known but does not seem to be explained entirely by the antihormone activity of these drugs.

INTRODUCTION

Antiestrogens are synthetic drugs that prevent estrogen from binding to the ER and inhibit the growth of breast cancer cells by a mechanism which is not totally understood (1-5). These estrogen analogues partially activate the ER and trigger incomplete stimulation of estrogen-regulated proteins (6, 7). The mechanism by which they inhibit cell proliferation cannot be exclusively explained by their antiestrogen action. Two series of effects have been described previously: (a) a cytostatic effect by which ER-positive cells are blocked by the drug in early G1 (8, 9) and which might be due to the decreased production of estrogen-induced mitogens (5); (b) a rapid cytotoxic effect characterized by cell death of ER-positive and -negative cells, triggered by much higher concentrations (≥4 μM tamoxifen) that is not reversed by estrogens (10, 11) and may involve antihormone-specific sites (12).

In most studies on cell growth inhibition induced by antiestrogens, total cell number or total DNA was evaluated and no attempt was made to estimate the number of dead cells. In the present work, we have also quantitated the number of altered and dead cells following treatment by low concentrations of the high affinity antiestrogen OH-Tam. We found that this antiestrogen increases the number of dead cells and therefore displays a third type of activity that we defined as "estrogen-receptor mediated cytotoxicity." To support the idea of receptor-mediated cytotoxic drugs, we have also studied the action of an antiprogestin, RU486, on the growth of PR-positive breast cancer cells. RU486 has no progestin-like activities in these cells (13), but surprisingly, like R5020, a pure progesterin, it can inhibit the growth of PR-positive cells (14, 15) giving an example of dissociation between its antagonist activity on progesterin responses and its antiproliferative activity. We now report on a PR-mediated cytotoxic activity of RU486 in the same PR-positive breast cancer cells. These studies together indicate that the antiproliferative activities of these two sex steroid antagonists do not seem to be exclusively explained by their classical antihormone activity, even though they are mediated by their respective steroid hormone receptors.

MATERIALS AND METHODS

Ligands

RU486 and [6,7-3H]RU486 (specific activity, 50.6 Ci/mmol) were provided by the Centre de Recherche Roussel-Uclaf (Drs. E. Sakiz and D. Philibert, Romainville, France). Estradiol and promegestone (R5020) were gifts from Roussel-Uclaf. All steroids were 98% pure as verified by thin layer chromatography.

Tam [α-(4-β-N-dimethylaminoethylphenyl)-α'-ethyl-trans-stilbene], OH-Tam [α-(4-β-N-dimethylaminoethylphenyl)-4-hydroxy-α'-ethyl-trans-stilbene] were donated by ICI Laboratories (England). 4-Hydroxy[ring-3,5-3H]tamoxifen (specific activity, 44 Ci/mmol) was obtained from the Radiochemical Centre (Amersham, England).

Human Breast Cancer Cell Lines

MCF-7 breast cancer cells were obtained from the Michigan Cancer Foundation (16), BT20 and MDA-MB231 breast cancer cells (17, 18) were provided by Dr. V. Piczak (Mason Research Institute, Bethesda, MD), and clone 11, a subline of the T47D breast cancer line (19), was a gift of Dr. I. Keydar (Tel Aviv University, Tel Aviv, Israel).

MCF-7 cells were cultured in a mixture (1/1) of Ham's F12/DEM medium. T47D and BT20 cells were maintained in RPMI 1640 medium and MDA-MB231 cells were grown in DEM medium. All culture media were supplemented with 10% fetal calf serum and 0.6 μg/ml bovine insulin.

Cells growing as monolayers were kept at 37°C in a humid atmosphere in the presence of 5% CO2.

Tests for Cytostatic and Cytotoxic Effects

All tests were performed on cells from which steroids were withdrawn by charcoal as previously described (20). Cells were plated in 35-mm diameter wells (6-well dishes; Linbro-Flow Laboratories SA, Ayrshire, United Kingdom) at a density of 100,000 to 350,000 cells/well depending on cell lines, as indicated in the figure legends. The drugs were added 1 to 2 days after plating, and cells were routinely treated for 5 to 7 days. The cytostatic effect was evaluated by measuring the amount of cellular DNA by dianminobenzoic acid fluorescence assay (21) or...
alternatively by counting cells using a Thoma hemocytometer.

Cytotoxicity was estimated by the following methods.

Trypan Blue Exclusion Test. Following treatment, the medium was recovered and the cells were detached with PBS containing 1 mM EDTA. The medium and the detached cells were pooled and centrifuged at 700 × g for 10 min. Cell viability was estimated by adding an equivalent volume of a 0.25% trypan blue solution to an aliquot of suspended cells. Stained and unstained cells were counted on a hemocytometer (10³ cells were counted for each determination).

DNA Fragmentation Test (22). Cells were detached as in the trypan blue exclusion test. The cell pellet was then lysed in 500 µl of buffer containing 5 mM Tris, pH 7.5, 5 mM EDTA, and 0.5% Triton X-100. The lysates were centrifuged at 12,000 × g for 45 min, and DNA was evaluated by diaminobenzine acid fluorescence assay in the supernatant, the pellet, and total lysate.

Lactic Dehydrogenase Assay (23). Following treatment of the cells, the amount of LDH was evaluated in 0.1 ml of 1 ml culture medium using a LDH-P UV-system kit obtained from Boehringer Mannheim (Meylan, France). Results were expressed in units per liter according to values indicated by the supplier and corrected per µg total DNA.

Cloning in Soft Agar. Cell viability was tested in soft agar using 24-well dishes by adapting the Salmon assay (24) to our cell culture system. The soft agar bilayer was formed by a 0.5% agar underlayer in Ham’s F12/DEM medium supplemented with serum and insulin (as in Table 2) on top of which was poured a 0.3% agar upper layer in the same medium. At day 0, the upper layer contained 3 × 10³ cells/well previously treated or not by the antagonists. Following 2 weeks of growth in agar in quadruplicate wells, colonies were stained with p-iodonitrotetrazolium violet (1.5 mg/ml; Sigma), photographed and counted.

Microscopy

Light Microscopy. Steroid-withdrawn cells were plated on 14-mm diameter glass coverslips and treated with hormones and antihormones for various periods of time. At the end of the treatments, cells were rinsed with PBS, fixed in Carnoy’s fluid, and stained with Mayer’s Hemalum solution.

Transmission Electron Microscopy. After treatment, cells growing in 24-well dishes were rinsed with 0.1 M cacodylate buffer, pH 7.4, and then fixed according to Karnovsky (25) for 0.5 h and postfixed in 1.3% osmium tetroxide in S-collidine buffer for 1 h. Preparations were dehydrated through graded alcohols and embedded in Araldite. Thin sections were stained with lead citrate and viewed in a JEOL JEM 2000 EX transmission electron microscope at 80 kV.

Cellular Uptake

Steroid-withdrawn cells were plated in T75 flasks and incubated for 5 days with [³H]RU486 or [³H]OH-Tam with or without nonradioactive steroid to occupy steroid receptors. Cells were then washed three times in cold PBS and detached in PBS containing 1 mM EDTA. They were homogenized in 500 µl of Tris-HCl buffer, pH 7.4, containing 1.5 mM EDTA, using a glass/glass Dounce homogenizer. An aliquot of the homogenate was counted to determine the whole cellular uptake. Cytosol and nuclear extract were then prepared as described (26), and an aliquot of each fraction was counted for ³H radioactivity.

RESULTS

Cytotoxic Effect of Low Concentrations of the Antiestrogen 4-Hydroxytamoxifen. When MCF7 cells were incubated with 4-hydroxytamoxifen, the rate of cell growth was markedly decreased, as previously described (11, 26). This decrease was not due only to the cytostatic activity of the drug, since cell death was also found to be increased when evaluated by each of the four techniques used: trypan blue dye exclusion, unseminated DNA, LDH activity released into the culture medium, and cloning in soft agar.

Fig. 1 shows that this effect was clearly visible after 4 days of treatment and increased at 5 days but was not significant within the first 2 days. The specific cytotoxic effect of the antiestrogen was concentration dependent, increasing continuously from 0.1 or 1 nm 4-hydroxytamoxifen up to 100 nm (Fig. 2), and was reproducible, although the efficacy of low concentrations varied between the experiments (Table 1).

In contrast with the nonspecific cytotoxic effect (10, 11), cell
Table 1  Cytotoxic effect of OH-Tam in MCF7 cells: protection by estradiol

<table>
<thead>
<tr>
<th>Experiment</th>
<th>% of dead cells</th>
<th>Student's t test (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>OH-Tam (0.1 nM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3 ± 1.0a</td>
<td>10 ± 1.0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>3 ± 1.0</td>
<td>10 ± 1.0</td>
</tr>
</tbody>
</table>

Table 2  Cloning efficiency in soft agar

<table>
<thead>
<tr>
<th></th>
<th>Number of colonies/well</th>
<th>Estradiol (1 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>187 ± 17b</td>
<td>Not done</td>
</tr>
<tr>
<td>OH-Tam</td>
<td>41 ± 9</td>
<td></td>
</tr>
</tbody>
</table>
| One-wk treatment (2-wk rescue)
| Continuous treatment | 263 ± 31          | 46 ± 5           |
|            | 415 ± 40                |                  |

* Mean ± SD of quadruplicate samples.

** Mean ± SD of quadruplicate samples.

Mean ± SD.

Mean of 4 experiments ± SD.

death was inhibited by estradiol binding to the ER (Fig. 2; Table 1), indicating that the effect was ER mediated. After treatment with the antiestrogen for 7 days, the total cell population, compared to control growing cells, decreased from 500,000 to 150,000, while the absolute number of dead cells increased from 50,000 to 100,000 (Fig. 3). This indicates that in vitro, the cytostatic effect is greater than the cytotoxic effect which affects a smaller proportion (10%) of the total cell population. Moreover, a lower concentration of the drug was generally required to induce cytostatic than cytotoxic effect (Fig. 3a).

Both cytotoxic and cytostatic effects were totally inhibited by estradiol (Fig. 3b), and estradiol was consistently mitogenic, the number of cells being increased by 2-fold, confirming previous results (27, 28).

No effect of 4-hydroxytamoxifen could be demonstrated when MDA-MB231 or BT20 cells, which contain no ER sites, were tested on the same parameters (Fig. 4). The cytotoxic effect of the drug was also evaluated by measuring cloning efficiency of MCF7 cells in soft agar. Table 2 shows that 4-hydroxytamoxifen decreased cloning efficiency regardless of whether the MCF7 cells were continuously treated by this drug.
or whether they were transferred into a normal culture medium following 7 days treatment with the drug. Estradiol by contrast was not inhibitory at the same concentration. This indicates that 1 μM 4-hydroxytamoxifen had irreversibly killed 80% of the cell population.

Cytotoxic Effect of Low Concentrations of the Antiprogestin RU486. When the synthetic antiprogestin RU486 was tested on T4D cells, which contain high concentrations of PR sites, similar results were obtained. Fig. 1b shows that the cytotoxic effect of RU486, like that of OH-Tam, increased as a function of time and was significant after 5 or 6 days of culture. At 6 days the effect was concentration dependent and associated with decreased cell number (Fig. 5) as previously reported (14, 15). There was a 2-fold decrease in the total cell number and a 2-fold increase in the number of dead cells, resulting in a 4-fold relative increase in the percentage of dead cells. With RU486 however, the effect on total cell number was partial since 20 to 50% of cells remained resistant to the drug as previously reported (14). In this experiment, the increased number of dead cell also occurred at higher concentrations than with the cyto-static effect (Fig. 5). The significance of the discrepancy between these two responses is unknown, but variable sensitivity of two hormone responsive parameters has previously been observed (29). Two pieces of evidence indicate that the cytotoxic effect of RU486 is mediated by the PR: (a) it was not observed in the PR-negative and glucocorticoid receptor-positive MDA MB231 and BT20 cell lines (Fig. 4, c and d); (b) the cytotoxic effect was slightly inhibited by the progestin R5020 which at higher concentrations also inhibits the growth of these cells (30). A low concentration (1 nm) of the progestin R5020 protected cells against the cytotoxic effect of RU486 whether evaluated by the trypan blue exclusion test or by measuring unsedimented DNA (results not shown).

The effects of RU486 and Tam were additive. At concentrations producing half-maximal cytotoxicity, the two hormones induced a greater LDH release when added together than separately (Fig. 6a). Dose-response experiments confirmed this additivity which was greater at intermediate concentrations (10 to 100 nM) (Fig. 6b). At low concentrations, RU486, which has a higher affinity for its receptor was the most efficient while, at high concentrations, Tam was more active, possibly against a RU486-resistant cell population.

Morphological Effect of the Two Antihormones. The cytotoxic effect of these drugs was also demonstrated by studying the morphology of MCF7 cells monolayer. Optical microscopy showed that hydroxytamoxifen (from 10 nM) (Fig. 7, a and e) and RU486 (from 100 nM) (Fig. 7f) induced cell and chromatin condensation and pyknosis in about 20% of the cells. These alterations were directly observed on attached cells. Cell mitoses were scarce compared to the control (Fig. 7a). At higher concentrations (0.1 to 1 μM) pyknotic nuclei were observed in 30% of the cells. Pyknosis is known to be observed by optical microscopy both in apoptotic and necrotic process (31). Estradiol (100 nm) (Fig. 7f) and R5020 (1 nm) (Fig. 7c) protected the cells from the toxic effects of OH-Tam and RU486, respectively; the number of condensed cells and nuclei decreased to control levels, and the rescued cells could not be distinguished from the control cells. Moreover, in the estradiol-protected cells but not in the R5020 protected cells, numerous mitoses were visible, in agreement with the mitogenic effect of estradiol in these cells (3, 27, 28). Transmission electron microscopy (Fig. 8) also showed the modifications induced by hydroxytamoxifen treatment and characterized by a margination of heterochromatin into fragmented dense masses at the periphery of interphase nuclei (Fig. 8a). The interchromatin region was more diffuse and dispersed than in control cells and contained fibrillar bundles. The nuclear membrane and the mitochondria remained generally intact (Fig. 8b). The cell surface was smooth with few or no microvilli. At a more advanced stage, numerous membrane-bound vacuoles in the cytoplasm (Fig. 8c) and nuclear fragmentation resulted in so-called apoptotic cellular bodies of varying sizes, which were present between structurally normal cells or engulfed in vacuole-like structures (Fig. 8d). These bodies contained either scarce cytoplasm surrounding a nuclear fragment or a poorly digested dense material. These modifications characterize apoptotic cell death (31).

Receptor-mediated Concentration of [3H]OH-Tam and [3H]RU486 in Cells and Nuclei. In an attempt to specify whether receptors were involved in concentrating the drugs in cells, we studied the cellular uptake of [3H]OH-Tam or [3H]RU486 with or without nonradioactive estradiol or R5020 under conditions in which these competitors protect cells against the cytotoxic activity of the antihormones. The cellular uptake of [3H]OH-Tam (10 nm) was decreased 10-fold (from 55,000 to 5,400 cpm/
Fig. 7. Light microscopy of MCF7 cells treated by OH-Tam and RU486. Steroid-withdrawn MCF7 cells were grown on coverslips for 5 days with control medium (a); RU486, 100 nM (b); RU486, 10 nM plus R5020, 1 nM (c); RU486, 10 nM (d); RU486, 100 nM (e); or RU486, 100 nM plus estradiol, 100 nM (f). Cells were then stained for light microscopy as described in “Materials and Methods.” OH-Tam and RU486 induced nuclei pyknosis and chromatin condensation (d, f), but cells were protected by estradiol and R5020 (c and e), respectively. Mitosis can be seen in the control (a) and estradiol-protected cells (f).

These results indicate that in breast cancer cells, free accessible ER and PR sites are necessary to facilitate the cellular uptake and nuclear concentration of the antiestrogen and antiprogestin, respectively.

**DISCUSSION**

In this report we show that low concentrations of an active metabolite of tamoxifen, 4-hydroxytamoxifen, increased the percentage and number of dead MCF7 cells and that this cytolysis is mediated by the ER. Moreover, the antiprogestin RU486 also increases the percentage of dead cells via the PR. Three lines of evidence indicate that the cytotoxic effect of OH-
Tam and RU486 are mediated by the ER and PR, respectively: (a) they are not observed in ER- and PR-negative cells; (b) they are prevented or rescued by the occupation of these receptors by hormones; (c) the drug concentration needed to trigger cytotoxicity is in close agreement with the concentration required to occupy the corresponding hormone receptor. The steroid receptor-mediated cytotoxic activities of low or moderate concentrations (from 0.1 nM to 1 µM) of the two sex steroid antagonists that we report in this study, contrasts with that previously reported at higher concentrations (>4 µM) of antiestrogen (10, 11). To begin with, the latter type of cytotoxicity, which is called nonspecific, was not prevented by estrogen and
did not appear to be ER mediated. Moreover, the nonspecific cytotoxicity was much more rapid than the receptor-mediated cytotoxic effect since all cells were killed within 40 h treatment (10, 11) while there was a 2-day lag until the specific receptor mediated type of cytotoxicity became obvious (Figs. 1 and 6). This delay may indicate a more indirect mechanism eventually related to that of cytoanalysis.

We have recently checked that the removal of the pH indicator phenol red acting as a weak estrogen in the culture medium (32) did not prevent the inhibitory activities of 4-hydroxymethoxytamoxifen in growing cells. This antiestrogen appears therefore to be efficient in the complete absence of estrogen and its mechanism cannot be explained solely by its antihormonal activity. It is paradoxical that both the antiprogestin RU486 (14, 15) and the progestin R5020 (29) inhibit the growth of PR-positive cells. This is explained neither by the agonist activity of RU486 which displays no known progestin effect on breast cancer cells in vitro (13, 14, 33) nor by its antihormone activity, since the drug should be able to prevent the antiestrogenic action of progestin. This PR-mediated antiproliferative effect of RU486 is therefore totally dissociated from its antihormone activity. The similar action of an antiestrogen and an antiprogestin on the growth of breast cancer cells strongly suggest that these antiproliferative agents not only act as hormone antagonists in these cells but also as cytostatic and cytotoxic drugs targeted to the receptor of their corresponding hormone.

The mechanism by which these drugs kill the cells is not known. We have only measured late effects such as increased cell membrane permeability (trypsin blue exclusion and LDH release in medium) and large chromatin and nuclear modification, such as chromatin condensation and DNA fragmentation. One hypothesis is that the first modification occurs in chromatin and DNA where the receptor-bound drugs are concentrated. A decreased production of growth factors can easily explain the major cytostatic effect of these steroid antagonists, since the three types of drug, antiestrogen, progestin, and antiprogestin, elicit a marked decrease in the amount of secreted proteins (13, 14, 20).

The same initial mechanism might also be responsible for the estrogen receptor-mediated cytotoxic effect since we cannot exclude that this cytotoxicity is the result of a prolonged blockade of cells in G0-G1. More active mechanisms such as the induction of growth inhibitory and the activation of endonucleases have also been proposed to explain cell death and apoptosis (31) and the cytotoxicity of glucocorticoids in thymocytes (34).

The clinical significance of this study and the relative importance of the three effects (cytostatic, nonspecific cytotoxic, and receptor-mediated cytotoxicity) when antiestrogens are acting in vivo are unknown. In vitro, however, the cytotoxic effect of a low concentration of the drug affects a lower fraction of the cell population than the cytostatic effect and appears therefore to be less important. The antiprogestin RU486 and the progestin R5020 increase the proportion of PR-positive cells in G0-G1 of the cell cycle, as in the case of antiestrogens and ER-positive cells. It has been debated whether antiestrogens are only cytostatic or also cytolytic drugs in vivo (4, 35), since the plasma concentration is high enough to trigger cytolytic activities (36). Tamoxifen is classically considered as being a cytostatic drug in vivo. This has been shown with human breast cancer cells transplanted in athymic nude mice (35). However, antiestrogen action differs in mice and humans (4) and drug accessibility in tumors transplanted into mice may also differ. That cytotoxic activities may occur during the treatment of breast cancer with antiestrogens is supported by a recent case of tumor lysis syndrome after tamoxifen flare (37). The similar in vitro activities of RU486 and OH-Tam suggest the potential value of the antiprogestin in breast cancer treatment, since both drugs are targeted to different receptors. In a preliminary clinical trial, RU486 was found to be well tolerated and to induce transitory remissions in advanced breast cancers that were resistant to other endocrine therapies (38). The understanding of the molecular mechanism by which these drugs block cell proliferation and induce cell death via physiological receptors may serve as a guide in developing more efficient cytotoxic agents or better combinations to treat breast cancer.
RECEPTOR-MEDIATED ANTIESTROGEN AND ANTIPROGESTIN CYTOTOXICITY

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