Antiestrogenic Potency and Binding Characteristics of the Triphenylethylene H1285 in MCF-7 Human Breast Cancer Cells

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ABSTRACT

The antiestrogenic character and potency of 4-(N,N-diethylaminoethoxy)-4'-methoxy-α-(p-hydroxyphenyl)-α'-ethylstilbene (H1285) and its binding to estrogen receptor and to estrogen-noncompetible antiestrogen binding sites have been studied in MCF-7 human breast cancer cells. H1285 has an affinity for the estrogen receptor (Kd 0.23 nM) which is comparable to that of estradiol (Kd 0.25 nM), and the binding of these two compounds to estrogen receptor is mutually competitive. On high salt sucrose gradients, the sedimentation profiles of nuclear receptor complexes with H1285 and estradiol are different. While the sedimentation profile of the complex with estradiol varies with the buffer composition, being 4.1S in phosphate:thioglycerol:glycerol and predominantly 5.5S in Tris:EDTA buffered gradients, the H1285 receptor complex shows the same sedimentation (5.5S) regardless of the buffer composition. H1285 also binds to estrogen-noncompetible antiestrogen binding sites that are distinct from the estrogen receptor with a low affinity, only 15% that of the antiestrogen tamoxifen.

The biological character and potency of H1285 were examined by determining its effects on cell proliferation, cellular progesterone receptor levels, and plasminogen activator activity. In MCF-7 cells, H1285 was a 30- to 100-fold more potent inhibitor of cell proliferation than was the antiestrogen tamoxifen, and it was approximately equipotent with the higher affinity antiestrogen trans-hydroxytamoxifen. H1285 evoked very minimal increases in cellular progesterone receptor levels, and no increase in plasminogen activator activity over a broad range of concentrations (10^-10 to 10^-6 M), and it suppressed plasminogen activator activity stimulated by estradiol.

Therefore, by the criteria we have used, we conclude that H1285 is a potent and very effective antiestrogen in MCF-7 cells. The ability of estradiol to reverse the suppression of cell proliferation by H1285, and the high affinity of H1285 for estrogen receptor and its low affinity for estrogen-noncompetible antiestrogen binding sites suggest that H1285 exerts its antiestrogenic effects via interaction with the estrogen receptor of these breast cancer cells.

INTRODUCTION

Antiestrogens are compounds that antagonize the action of estrogens in a variety of estrogen target tissues. In particular, these compounds have been demonstrated to inhibit estrogen stimulated uterine growth in many animal species, to alter gonadotropin secretion, to cause the regression of hormone dependent mammary tumors, and to inhibit the growth of estrogen receptor containing human breast cancer cells (1-8). Although they were initially developed as fertility control agents for the human female, they are being used successfully in the treatment of hormone dependent breast cancer, due to their ability to control the growth of these cells.

Antiestrogens, which generally have a triphenylethylene structure, are known to compete with estrogen for binding to estrogen receptor sites, and the antiestrogen-occupied complex becomes localized in the cell nucleus (2, 3). The nuclear antiestrogen receptor complex, however, appears to be only partially active in promoting specific biological responses, and is ineffective in blocking the actions of estrogen (4, 9-11). Previous studies on the molecular mechanism of antiestrogen action, using indirect exchange assays and direct assays with low affinity radiolabeled antiestrogens, have been hindered by the dissociation of the antiestrogen-receptor complex during characterization, due to the low affinity of many antiestrogens for estrogen receptor (2, 3, 12, 13). More recently, the synthesis and study of two radio-labeled antiestrogens having affinities for the estrogen receptor comparable to that of the natural estrogen, estradiol, have allowed more accurate characterization of the direct interactions of antiestrogen with the estrogen receptor (14-16).

H1285, a triphenylethylene derivative, has been reported to have a very high affinity for estrogen receptor in rat uterus and pituitary (relative binding affinity 1100% that of estradiol), and in calf uterus (relative affinity 300% that of estradiol) (17-20). In rat uterus, H1285 has been found to have an antagonistic effect on estrogen stimulated uterine growth (21, 22). To date its effects on breast cancer cells had not been studied, but these few reports suggested that H1285 might prove to be an even more effective antiestrogen than were the triphenylethenes previously examined.

In this study, our aims have been to investigate the biological character and potency of H1285 in MCF-7 breast cancer cells; to examine the binding of H1285 to the estrogen receptor in these cells; and to determine the affinity of interaction of H1285 with estrogen-noncompetible antiestrogen binding sites in these cells that are distinct from the estrogen receptor (23, 24). The MCF-7 cell line, derived from human metastatic breast carci-
noma, was chosen for these studies since these cells contain high levels of estrogen receptor and a progestin binding activity and plasminogen activator that can be modulated by estrogens and antiestrogens; and the growth of these cells is inhibited markedly by antiestrogens (9, 11, 25–31). In addition, these cells contain binding sites, that are distinct from the estrogen receptor, to which antiestrogens bind (23, 24). Our findings indicate that H1285 binds to the estrogen receptor from these cells with high affinity, and that it is very effective in antagonizing the biological effects of estradiol.

MATERIALS AND METHODS

Chemicals and Materials. [3H]H1285 (20 Ci/mmol) was synthesized as described previously (19), and its specific activity was determined by high performance liquid chromatography on a 0.46- x 25-cm Supelco LC8-DB reverse phase column eluted with 60% acetonitrile:40% water containing 2% diethylamine buffered to pH 7.5 with phosphoric acid. The mass associated with the peak of eluted radioactivity was determined by comparison of the area of the UV peak with a standard curve for unlabeled H1285. [3H] Estradiol (106 Ci/mmol) was obtained from Amer- sham (Chicago, IL). The synthetic progestin [3H]R5020 (95 Ci/mmol) was obtained from New England Nuclear (Boston, MA). trans-[3H]Tamoxifen (12.2 Ci/mmol) was synthesized as described previously (32), and non-radioactive trans-tamoxifen and trans-hydroxytamoxifen were from ICI Americas, Inc. (Wilmington, DE). All media, sera, and antibiotics used to culture the MCF-7 cells were obtained from Grand Island Biological Co. (Grand Island, NY). Insulin, hydrocortisone, and estradiol were purchased from Sigma Chemical Co. (St. Louis, MO). The bis(carbobenzoxy-isoleucylpropylarginyl)rhodamine was synthesized and purified according to the procedure of Leytus et al. (33). Plasminogen was purified from fresh dog plasma by modification of the method of Castellino and Sodetz (34), as described in Leytus et al. (35). The toluene-based scintillation fluid was 0.5% PPO and 0.03% POPOP in toluene. The Triton:xylene-based scintillation fluid was 0.3% PPO, 0.02% POPOP, and 25% Triton X-114 in xylene.

Cell Culture. MCF-7 human breast cancer cells were originally obtained from Dr. Charles McGrath of the Michigan Cancer Foundation (Detroit, MI), and were grown in plastic T-150 flasks in Eagle’s MEM supplemented with 10% fetal calf serum, 1% sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 100 mg/ml glucose, 200 µg/ml hydrocortisone, and 5% calf serum that had been treated with dextran-coated charcoal for 45 min at 37°C to remove endogenous hormones (9).

Estrogen Receptor Binding Analysis. Cells from 14 near-confluent T-150 flasks were suspended in 2.0 ml of PTG buffer and homogenized in a Dounce homogenizer using the B-pesette. The homogenate was centrifuged (800 x g for 10 min), and the supernatant was collected. The crude nuclear pellet was washed twice at 0–4°C with buffer, and the nuclear washes were combined with the supernatant fraction. This was centrifuged at 180,000 x g (30 min) to yield the cytosol which was diluted to 15.4 ml with PTG buffer. Aliquots of cytosol (200 µl) were incubated at 0–4°C for 20 h with [3H]estradiol or [3H]H1285 at concentrations ranging from 5 x 10-11 M to 5 x 10-9 M. Parallel tubes contained the radioactive ligand plus a 100-fold excess of radioinert estradiol to assess nonspecific binding. An aliquot of this 180,000 x g supernatant was then incubated with various concentrations of radioactive estradiol or H1285, and 5 x 10-6 M [3H]-estradiol or 1 x 10-4 M [3H]H1285 for 16 h at 0–4°C, and samples were then analyzed exactly as described previously (11).

Sucrose Gradient Analyses. MCF-7 cells from two near confluent T-150 flasks were incubated with 10 nM [3H]estradiol or 10 nM [3H]H1285 for 1 h at 37°C. This time gives maximal localization of antiestrogen and estradiol receptor complexes in the nucleus (9, 15). The cells were then homogenized in 1 ml of PTG buffer. The homogenate was centrifuged at 800 x g for 10 min, and the resulting nuclear pellet was washed twice with 1 ml of ice-cold PTG buffer. The washed pellet was resuspended in 0.125 ml of PTG buffer to which 0.375 ml of 10 mg/ml Tris-HC1:1.5 mM EDTA:10 mM thiglycocer:10% glycerol:0.8 mM KCl (pH 8.5 at 4°C) was added and incubated at 4°C for 1 h with resuspension every 15 min. This procedure reproduced exactly over 90% of the [3H]estradiol or [3H]antiestrogen receptor complexes as determined by ethanol extraction. The suspension was centrifuged for 30 min at 180,000 x g, and the supernatant was treated for 8 min at 0–4°C with charcoal-dextran slurry (1 part slurry:9 parts extract). A 250-µl aliquot was layered on linear 5–20% sucrose gradients (3.6 ml) formed in PTG buffer or 5% TE buffer, both buffers containing 0.4 mM KCl. The gradients were centrifuged at 357,000 x g in an SW60 rotor for 17 h and 2-drop fractions were collected for counting. Cells were also treated with radioactive ligand plus 100-fold excess of radioactive estradiol to assess the level of non-specific binding. Recovery on all gradients was greater than 90%. Sedimentation coefficients were determined according to Martin and Ames (36), relative to the internal markers 14C-labeled ovalbumin (3.5S) and 14C-labeled y-globulin (6.6S) which were included in each gradient (15).

Assays for Binding to Estrogen-noncompetable Binding Sites. The cells from near confluent T-150 flasks were harvested by incubating cells at 37°C for 10 min in Hanks’ balanced salt solution (calcium and magnesium free; Grand Island Biological Co.) with 1 mM EDTA. The cells were washed twice with TEA buffer and homogenized in a Dounce homogenizer (40–50 strokes with a B-pesette).

The binding of H1285 to antiestrogen binding sites was determined in the 12,000 x g 30-min cell-solubilate as described in detail previously (24, 37). Supematant (250 µl) was incubated with 5 µl of 150 nM [3H]tamoxifen in dimethylformamide, 5 µl of ethanol or 10-14 M estradiol in 5 µl ethanol, 20 µl of competitor in dimethylformamide and 220 µl of TEA buffer. Samples were incubated for 1 h at 0–4°C, and then 9 µl of dextran-coated charcoal (5% Norit A and 0.5% dextran in TEA buffer) were added. The charcoal was pelleted by centrifuging at 12,000 x g for 10 min, and an aliquot of the supernatant was removed for determination of bound radioactivity.

Cell Proliferation Experiments. The effect of H1285 on cell proliferation was compared with that of tamoxifen and trans-hydroxytamoxifen, antiestrogens known to suppress the growth of MCF-7 cells (7, 11, 28). MCF-7 cells were seeded into T-25 flasks (2.0 x 105 cells/flask) and grown for 2 days in the MEM medium described in the “Cell Culture” section above. After this time, cells from two flasks were harvested and counted with a Coulter Counter as described by Butler et al. (38)(Day 0). The medium was changed to MEM supplemented as described above, except that it contained 2% charcoal-dextran-treated calf serum and various concentrations of tamoxifen, trans-hydroxytamoxifen or H1285, or estradiol or ethanol vehicle (0.1%). Triplicate flasks of cells were counted at several points throughout the 9- or 10-day growth period.

Hydroxylapatite Assay for Estrogen Receptor. Estrogen receptor content was determined by incubating cytosol or nuclear extracts with 10 nM [3H]estradiol with and without 1 µM radioactive diethylstilbestrol for 3.5 h at 30°C (9). After incubation, samples were cooled to 4°C and assayed for bound radioactivity by an hydroxylapatite slurry assay (9, 11).

Hydroxylapatite Assay for Progesterone Receptor. Cytosol was incubated for 4 h at 0–4°C with 10 nM [3H]R5020 in the presence and absence of 1 µM radioinert R5020, a synthetic progestin. Pretreatment

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of cytosol with 10^{-4} M cortisol, prior to incubation with [3H]R5020, had no effect on the level of progesterin binding, suggesting no contribution from glucocorticoid receptor. After incubation, samples were assayed for bound [3H]R5020 using hydroxyapatite (11).

**Assay for Plasminogen Activator Activity.** The plasminogen activator activity of the cells was measured by a two-step assay using the rhodamine-based compound bis(carbobenzoxyisoleucylpropylarginyl)-rhodamine as a substrate for plasmin (33). The substrate is nonfluorescent but cleavage by plasmin of an amide bond between arginine and rhodamine yields a produce mono(carbobenzoxyisoleucylpropylarginyl)-rhodamine that is highly fluorescent. The two-step assay has been described in detail (11). In brief, cells were incubated with plasminogen for 2 h at 37°C, and the amount of plasmin formed was then measured by incubating an aliquot of the reaction mixture with bis(carbobenzoxyisoleucylpropylarginyl)rhodamine and determining the rate of increase in fluorescence. The rate of activation of plasminogen during the 2-h incubation of plasminogen with cells was found to be constant and, therefore, proportional to the amount of plasminogen activator. Conversion of relative fluorescence units per min to molar concentrations of plasmin was accomplished by using a standard curve as detailed previously (39).

**RESULTS**

**Analysis of the Binding of H1285 to the MCF-7 Estrogen Receptor.** Saturation binding analyses for estradiol and H1285 are shown in Chart 1. These data indicate the presence of a single class of high affinity binding sites in MCF-7 cell cytosol with equilibrium dissociation constants (K_d) of 0.25 nM for estradiol and 0.23 nM for H1285 binding to estrogen receptor. This Scatchard binding analysis (40), therefore, indicates that the affinity of H1285 for receptor is very similar to that of estradiol.

The relative binding affinity of H1285 for the estrogen receptor was also determined indirectly by competitive binding analyses (Chart 2). Comparison of the concentrations of H1285 and estradiol needed to decrease the specific binding of tritiated estradiol by 50% indicates that H1285 has an affinity of 120% compared to that of estradiol (Chart 2A). In addition, the full displacement of [3H]estradiol binding by unlabeled estradiol (Chart 2B) and the full displacement of [3H]estradiol binding by unlabeled H1285 (Chart 2A) indicate that H1285 and estradiol bind in a mutually competitive manner to the MCF-7 estrogen receptor.

**Sedimentation Behavior of Estrogen Receptor Complexes Labeled with Tritiated H1285 or Tritiated Estradiol.** In order to determine whether the physicochemical properties of the estrogen receptor complexed with H1285 differ from those of the estrogen receptor when complexed with estradiol, MCF-7 cells were incubated for 1 h at 37°C with a receptor saturating concentration of radiolabeled estradiol or H1285. The nuclear receptor sites were extracted with 0.6 M salt and analyzed on 5–20% sucrose gradients which were prepared with buffers of two different compositions: PTG buffer or 5x TE buffer. Chart 3 shows that the sedimentation behavior of the nuclear receptor complex is dependent upon the buffer composition of the sucrose gradient. In PTG-buffered sucrose gradients, the receptor complexed with estradiol sedimented as a broad peak at approximately 4.2S. In 5x TE-buffered sucrose gradients, a predominant 5.5S peak existed with a small shoulder at 4.1S. In the case of H1285, the receptor complex always sedimented at 5.5S regardless of whether the sucrose gradient buffer was PTG or 5x TE. This sedimentation profile of the H1285 nuclear receptor complex is similar to that observed for the nuclear receptor complexes of the two other high affinity antiestrogens (trans-hydroxytamoxifen and CI628M) investigated thus far (15, 41).

**Binding of H1285 to Estrogen-noncompetable Binding Sites.** Previous studies have shown that MCF-7 cells contain additional binding sites, distinct from the estrogen receptor, to which antiestrogens bind. These sites appear to be associated with the microsomal fraction and the binding of antiestrogens to these sites is not displaceable by steroidal or nonsteroidal estrogens (23, 24). Although the role of these sites in the actions of antiestrogens is still unknown, we have been interested in examining the biopotencies of different antiestrogens, and trying to correlate their effectiveness as estrogen antagonists with their affinity for estrogen receptor sites and/or these additional binding sites (23, 37). Hence, it was of interest to determine the affinity...
of H1285 for these estrogen-noncomparable binding sites.

Competitive binding assays with the 12,000 x g cell supernatant were used to compare the affinity of H1285 and trans-tamoxifen for these sites. Chart 4 shows the competitive binding curves for the displacement of [3H]tamoxifen by H1285 and tamoxifen, and indicates that H1285 has an affinity for these sites only 15% that of tamoxifen. In a repeat experiment (not shown), H1285 was found to have a similar low affinity, only 11% that of tamoxifen.

Effects of H1285 on MCF-7 Cell Proliferation. The effectiveness and potency of H1285 in inhibiting MCF-7 cell proliferation were compared with that of the antiestrogens tamoxifen and trans-hydroxytamoxifen (Chart 5). These two antiestrogens were selected for comparison because it is known that trans-hydroxytamoxifen is considerably more potent than is tamoxifen itself in inhibiting the proliferation of these breast cancer cells (7, 11, 28). H1285 and trans-hydroxytamoxifen showed very similar dose-response curves for suppression of cell proliferation and, as seen in Chart 5, H1285 and trans-hydroxytamoxifen suppressed cell proliferation at considerably lower concentrations than did tamoxifen. From this, H1285 and trans-hydroxytamoxifen appear to be very potent inhibitors of MCF-7 cell proliferation. That the effectiveness of H1285 is probably due to the high affinity of this compound for the estrogen receptor is suggested by the fact that the cell growth inhibition observed with H1285 is prevented by concomitant administration of estradiol (Chart 6). Estradiol (10^{-7} M) fully blocked the growth suppressive effect of 10^{-8} M or 10^{-9} M tamoxifen or H1285. Estradiol, at 10^{-8} M, also fully prevented growth suppression by 10^{-9} M or 10^{-8} M tamoxifen or 10^{-9} M H1285, but it prevented only partially the growth suppression effected by 10^{-8} M H1285 (data not presented). These findings are consistent with the relative affinity of estradiol, H1285, and tamoxifen for the estrogen receptor (100, 120, and 2%, respectively), and suggest that the inhibitory effect of these concentrations of H1285 and tamoxifen on MCF-7 cell proliferation is probably mediated via the estrogen receptor system.

Effects of H1285 on Cellular Progesterone Receptor Content. Increases in progesterone receptor content have been used as an index of estrogen action in MCF-7 cells (9, 10, 12). In time course studies of progesterone receptor stimulation by different compounds, we found maximal levels of progesterone receptor at 3 to 6 days of exposure to estrogens and antiestrogens (9, 11). Hence, a 5-day time point was selected for evaluation of progesterone receptor stimulation by H1285. Chart 7 summarized data from several experiments demonstrating the effect of H1285 on progesterone receptor levels in MCF-7 cells. In this chart, direct comparisons are made between cytosol estrogen receptor depletion (which reflects the firm localization of receptors in the nuclear compartment after ligand binding) (42, 43) and progesterone receptor induction, a product of estrogen action. Estradiol, as anticipated, was a potent stimulator of progesterone receptor levels, and Chart 7 shows a 6-fold increase in progesterone receptor levels by 10^{-8} M estradiol. Although cytosol estrogen receptor levels were diminished in a concentration-dependent manner by H1285 treatment, to the same low level seen with 10^{-8} M estradiol, H1285 elicited little increase in progesterone receptor levels over a broad range of concentrations.

Effects of H1285 on Plasminogen Activator Activity. H1285, over a wide concentration range, did not stimulate plasminogen activator activity, while plasminogen activator activity was increased markedly by estradiol (Chart 8). The stimulatory effect of estradiol on plasminogen activator activity was suppressed by H1285, and this inhibitory effect of H1285 on estradiol-stimulated plasminogen activator activity was dose dependent.

DISCUSSION

The results of these studies indicate that H1285 is a very potent and effective antiestrogen in MCF-7 cells. Hence, it suppressed the proliferation of MCF-7 cells, suppressed estradiol stimulation of plasminogen activator activity, and was only a weak stimulator of progesterone receptor. In all of these bioassays, H1285 was considerably (at least 30- to 100-fold) more potent than was the antiestrogen tamoxifen, and it showed a potency similar to that of trans-hydroxytamoxifen in its effectiveness in growth suppression. The potency in growth suppression and plasminogen activator suppression by H1285 appear to correlate well with the high affinity of this antiestrogen for the estrogen receptor. Furthermore, as shown in Chart 6, the growth suppression by H1285 and by tamoxifen could be reversed by estradiol, suggesting mediation by an estrogen-competable process, presumably the estrogen receptor.
Chart 3. High salt sucrose gradient analysis of salt-extracted nuclear receptor after incubation of cells at 37°C with radiolabeled estradiol (E2) or H1285. MCF-7 cells were incubated for 1 h at 37°C with 10 nm [3H]estradiol or [3H]H1285, or with radiolabeled compound plus 100-fold excess of radioinert estradiol. A nuclear salt extract was prepared and charcoal-dextran treated as described in "Materials and Methods." A 250-μl aliquot was sedimented in 5-20% sucrose gradients containing 0.4 M KCl and 5 mM phosphate:10 mM thiglycerol:10% glycerol (PTG), pH 7.4, buffer (A) and (B), or 50 mM Tris:7.5 mM EDTA (5x TE), pH 7.4, buffer (C and D) for 17 h at 4°C at 357,000 × g. 14C-Labeled ovalbumin (OV, 3.5S) and 14C-labeled γ-globulin (γ-G, 6.6S) were incubated in all gradients as markers, and their positions are designated by arrows.

Recent studies have revealed that triphenylethylene compounds, such as tamoxifen, bind with high affinity to both the estrogen receptor and to additional sites to which estrogens do not bind. These latter sites have been termed "antiestrogen-specific binding sites." These sites have a subcellular distribution and binding specificity that is quite different from that of the estrogen receptor. In addition, these antiestrogen-specific binding sites are not localized exclusively in estrogen target tissues, and are found in an equal quantity in three human breast cancer cell lines (MCF-7, T47D, and MDA-MB-231) that differ significantly in their sensitivity to growth inhibition by antiestrogens (23, 37). The role of this additional antiestrogen binding component for which the \( K_a \) for tamoxifen is approximately 3 nm (23, 24, 37) is not known at present.

Attempts to determine a possible function for these sites have depended upon correlations between the potency of different antiestrogens and the affinity of these compounds for estrogen receptor versus the antiestrogen specific binder. The studies...
Chart 4. Relative affinity of H1285 for estrogen-noncompetable antiestrogen binding sites. Assays utilized the 12,000 × g × 30-min supernatant fraction of MCF-7 cells which was incubated with 1.5 nM [3H]tamoxifen plus 10^{-9} M estradiol for 30 min at 0°C to fill estrogen receptor sites prior to addition of 10^{-8} to 10^{-6} M concentrations of H1285 or trans-tamoxifen. Incubations were maintained at 0°C for 18 h prior to charcoal-dextran treatment. Data are expressed as [3H]tamoxifen cpm bound per assay. Numbers in parentheses, relative binding affinity for these estrogen-noncompetable antiestrogen binding sites with the affinity of tamoxifen being set at 100.

Chart 5. Effect of H1285 and tamoxifen on the growth of MCF-7 cells. Cells were grown in T-25 flasks in the presence of the indicated concentration of H1285, trans-hydroxytamoxifen, or tamoxifen, and media with compound were changed every 2 days. Data are expressed as the percentage of the control cell number measured after 9 days of growth and represent the mean of triplicate flasks of cells; bars, SE. The control cell doubling time was 2.3 days.

Chart 6. Prevention by estradiol (E2) of the growth suppression by H1285 and tamoxifen (Tam). Cells were grown in T-25 flasks in the presence of 10^{-8} M or 10^{-7} M H1285 or tamoxifen alone (B), or H1285 or tamoxifen plus 10^{-7} M estradiol (+E2), or 10^{-7} M estradiol alone (C). Media and compounds were changed every 2 days. Data are expressed as a percentage of the control cell number measured after 10 days of growth and represent the mean of triplicate flasks of cells; bars, SE. The control cell doubling time was 3.2 days.

Chart 7. Effect of H1285 on cytosol estrogen receptor and progesterone receptor levels in MCF-7 cells. Cells were incubated for 5 days with H1285 or estradiol (E2) at the concentrations indicated. Fresh media and compound were added daily during the 5-day period. The cells were then harvested, fractionated, and the cytosol was assayed for estrogen receptor and for progesterone receptor using hydroxylapatite, as described in "Materials and Methods." The control estrogen receptor and progesterone receptor levels were 1080 ± 43 (SE) fmol/mg DNA and 330 ± 32 fmol/mg DNA, respectively.

Chart 8. Effect of H1285 on cytosol estrogen receptor and progesterone receptor levels in MCF-7 cells. Cells were incubated for 5 days with H1285 or estradiol (E2) at the concentrations indicated. Fresh media and compound were added daily during the 5-day period. The cells were then harvested, fractionated, and the cytosol was assayed for estrogen receptor and for progesterone receptor using hydroxylapatite, as described in "Materials and Methods." The control estrogen receptor and progesterone receptor levels were 1080 ± 43 (SE) fmol/mg DNA and 330 ± 32 fmol/mg DNA, respectively.

The antiestrogen binding sites with an affinity only 10–15% that of tamoxifen. Since our bioassay data show that H1285 is considerably more potent than tamoxifen as a growth antagonist (Chart 5), and comparisons with an earlier study with tamoxifen (11) show it also to be more potent in suppression of estradiol-stimulated plasminogen activator activity, these data suggest that the antiestrogenic potency of H1285 correlates better with its affinity for estrogen receptor than with its affinity for this receptor.
ANTIESTROGEN H1285 IN BREAST CANCER CELLS

![Graph showing the effect of H1285 and estradiol (E₂) on the plasminogen activator activity of MCF-7 cells.](chartimage)

Chart 8. Effect of H1285 and estradiol (E₂) on the plasminogen activator activity of MCF-7 cells. Cells were incubated with the indicated concentration of compound for 4 days, with fresh media and compound renewed every 48 h. Cells were then harvested, distributed to microwells, and assayed for plasminogen activator activity, as described in "Materials and Methods." The initial linear velocity of the reaction was measured by the change in fluorescence (ΔF) per min, representing plasmin formed per min; bars, SE.

additional antiestrogen binding component. It is of note that our previous studies (23, 37) showed three other antiestrogens with high affinity for estrogen receptor (LY117018, CI628M, and trans-hydroxytamoxifen) to also have a low affinity for these antiestrogen sites (2–20% that of tamoxifen). Data with H1285 further strengthen our contention and the research findings of others with tamoxifen derivatives (28), that antiestrogen suppression of breast cancer cell growth is most likely mediated via interaction with the estrogen receptor system of the cells.

Since antiestrogen interacts with the estrogen receptor of breast cancer cells, considerable interest has focused on elucidating the loci in the estrogen receptor-response pathway at which antiestrogen effects may be mediated. These include competition with estradiol for formation of the ligand-estrogen receptor complex, resulting in possible interference with the transformation of receptor to an active form, or interference with the proper localization of receptors in the nucleus (2, 3, 15). The high affinity of H1285 for the estrogen receptor in MCF-7 cells has enabled us to examine in some detail its interaction with the receptor. Recent evidence has demonstrated differences in the physicochemical properties of the nuclear MCF-7 estrogen receptor labeled with estradiol or an antiestrogen (15). Hence, the nuclear antiestrogen receptor complex from MCF-7 cells was found to sediment at 5.5S, while the estradiol receptor complex was found to sediment at 4.1S on high salt sucrose gradients prepared in Tris:EDTA buffers, suggesting a difference in the stability of estradiol and antiestrogens nuclear receptor complexes or in the tendency of the nuclear receptor to be transformed by these different ligands to a dimeric SS form (41, 44, 45). Sucrose gradient analyses with H1285 showed the same characteristic sedimentation profiles on PTG buffered gradients and Tris:EDTA buffered gradients as did the two other high affinity antiestrogens, CI628M and trans-hydroxytamoxifen, studied previously (15), confirming that this difference in the properties of receptors occupied by antiestrogens versus estradiol is apparently characteristic for triphenylethylene antiestrogens in general.

Prior to this study, nothing was known about the biological character of H1285 in breast cancer cells. Indeed, its only biological characterization was that it had antiestrogenic effects on estradiol-stimulated uterine growth in rats, while also being a partial agonist (21, 22). It also was reported to have an extremely high affinity for rat uterine and pituitary estrogen receptor (1100% that of estradiol) and for calf uterine estrogen receptor (300% that of estradiol) (17–20). Our binding data, using both direct binding assays with radiolabeled H1285 and competitive binding assays, have shown that H1285 has an affinity for the estrogen receptor from MCF-7 cells approximately comparable to that of estradiol, and slightly lower than that of the potent antiestrogen trans-hydroxytamoxifen (11, 28); hence the affinity of H1285 for the estrogen receptor of breast cancer cells is high, but not as great as that reported for uterus. In comparative studies, we have verified that the relative affinity of H1285 is indeed greater for rat uterine estrogen receptor than for the human MCF-7 estrogen receptor. While we do not understand the basis for the different relative affinity of H1285 for the estrogen receptor in these different species, this may reflect true differences in the ligand binding specificities of receptor in these different species, or the possible influence of other proteins or factors in these receptor preparations.

It has been demonstrated previously that plasminogen activator activity serves as a useful marker for the biological activity of estrogens in breast cancer cells, and is able to distinguish antiestrogen from estrogen (11, 27, 38, 46). Plasminogen activator activity is stimulated by low, physiological concentrations of estradiol in MCF-7 cells, while antiestrogens such as tamoxifen and trans-hydroxytamoxifen fail to stimulate plasminogen activator activity (11, 27, 46). Plasminogen activator activity is under hormonal control in many tissues, and increased activity is found during rapid tissue growth and remodeling of the uterus and mammary gland (39, 47, 48). Our findings show that H1285 behaved as an antiestrogen in this regard also, in that it had no effect by itself on the activity of plasminogen activator in MCF-7 cells, but it was fully effective in suppressing the estradiol-stimulated increase in plasminogen activator activity.

From the results of this study, it is clearly demonstrated that H1285 is a potent antiestrogen in MCF-7 breast cancer cells. It has an affinity for the estrogen receptor equal to that of estradiol, and it exhibits a biopotency similar to that of the structurally related triphenylethylene trans-hydroxytamoxifen (11, 49–51). Our data suggest that the biological effectiveness of H1285 appears to result from its high affinity of interaction with the estrogen receptor. H1285 hence appears to be an appropriate compound that will be of use in studying the mechanism of antiestrogen action in breast cancer cells.
Antiestrogenic Potency and Binding Characteristics of the Triphenylethylene H1285 in MCF-7 Human Breast Cancer Cells


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