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Antiestrogen Interaction with Estrogen Receptors and Additional Antiestrogen Binding Sites in Human Breast Cancer MCF-7 Cells

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To gain further insight into the mechanism of action of antiestrogens, we examined the interaction of antiestrogen with the estrogen receptor system and with estrogen- noncompetable antiestrogen binding sites. In addition to binding directly to the estrogen receptor, antiestrogens can be found associated with binding sites that are distinct from the estrogen receptor. In contrast to the restriction of estrogen receptors to estrogen target cells, such as those of uterus and mammary glands, antiestrogen binding sites are present in equal amounts in estrogen receptor-positive and -negative human breast cancer cell lines, such as MCF-7, T47D, and MDA-MB-231 that differ markedly in their sensitivity to antiestrogens. In order to gain greater insight into the role of these antiestrogen binding sites in the action of antiestrogens, we have examined the biopotency of different antiestrogens for the antiestrogen binding sites and that is CI628 > tamoxifen > trans-hydroxy tamoxifen > CI628M > H1285 > LY117018. This order of affinities does not parallel the affinity of these compounds for the estrogen receptor nor the potency of these compounds as antiestrogens. Indeed, compounds with high affinity for the estrogen receptor and greatest antiestrogenic potency have low affinities for these antiestrogen binding sites. Antiestrogenic potency correlates best with estrogen receptor affinity and not with affinity for antiestrogen binding sites. In summary, our findings suggested that interaction with the estrogen receptor is most likely the mechanism through which antiestrogens evoke their growth inhibitory effects.

Key words : *trans*-Tamoxifen, Estrogen receptor, MCF-7, T47D, MDA-MB-231, Antiestrogen binding sites

INTRODUCTION

Antiestrogens are intriguing compounds that are able to antagonize many effects of estrogens. Although these nonsteroidal triphenylethylene-type compounds were developed initially by pharmaceutical companies as fertility regulating agents, they are of particular interest and importance today because of their clinical efficacy in controlling the growth and spread of hormone responsive breast cancers. With these agents, it appears possible to achieve non-invasively the same hormonal effects and tumor suppression that normally follow the more devastating endocrine ablative surgeries (ovariectomy, adrenalectomy, and hypophysectomy). Over past 25 years, clinical trials employing antiestrogen have documented that antiestrogen treatment is an effective endocrine therapy for breast cancer, with fewer side effects than are associated with pharmacologic hormone therapy (Nandi and McGrath, 1973; Katzenellenbogen *et al.*, 1985). The determination of the estrogen receptor content in human breast cancer tissue has gained an important role in the prediction of the success of an endocrine treatment (Dickson *et al.*, 1993). However, the receptor status of the tumor only allows a general evaluation; patients with a receptor-negative tumor respond rarely to an endocrine treatment; patients with receptor-positive tumors respond more frequently. An individual prediction in a single patient is not possible (Nandi and McGrath, 1973). While the precise mechanism by which antiestrogens evoke their antitumor effects is still incompletely understood, considerable experimental data are consistent with the hypothesis that anitestrogens exert their effects through the estrogen receptor system of target cells (Katzellenbogen *et al.*, 1984) 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 (Horwitz and McGuire, 1978). The nuclear antiestrogen receptor complex, however, appears to be only partially active in promoting specific biological responses, and is effective in blocking the actions of estrogen (King and

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Green, 1984). Furthermore, the affinity of different antiestrogens for the estrogen receptor correlates well with their potency in inhibiting tumor cell growth (Henderson, 1993; Bonadonna, 1993). Recently, it has been found that these triphenylethylene compounds also bind to additional saturable sites present in human breast cancers and in many other tissues (Knabbe, 1991). These sites are distinct from the estrogen receptor and are most readily distinguishable by the fact that they bind triphenylethylene antiestrogens, but in contrast to the estrogen receptor, they do not bind steroidal or nonsteroidal etrogens. these sites have been termed "antiestrogen-specific" or "estrogen-noncompetable" binding sites. In this paper, we describe the effects of antiestrogen on the properties of established human breast cancer cell lines, as guides to understanding the action of antiestrogens in human breast cancer *in vivo*. We examined the interaction of antiestrogen with the estrogen receptor system and with these estrogen-noncompetable antiestrogens binding sites. Our data indicate that antiestrogens exert most of their effects through the estrogen receptor system.

MATERIALS AND METHODS

Chemicals and Materials

[^{3}H]Estradiol (106 Ci/mmol) and [^{3}H]trans-tamoxifen (60 Ci/mmol) were obtained from Amersham (Arlington Heights, IL, USA). The synthetic progestin [^{3}H]R5020 (17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione) (89 Ci/mmol) was obtained from New England Nuclear (Boston, MA, USA). All media, sera and antibiotics used to culture the MCF-7 cells were obtained from Grand Island Biological Co. (Grand Island, NY, USA). Insulin, hydrocortisone and trans-tamoxifen were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bis (Cbz-Iso-Pro-Arg)-rhodamine was synthesized and purified according to the procedure of Leytus *et al.* (1984). Plasminogen was purified from fresh dog plasma by modification of the method of Castellino and Sodetz as described in Leytus *et al.* (1984). The toluene-based scintillation fluid was 0.5% 2,5-diphenyloxazole and 0.03% p-bis-[2-(5-phenyloxazoyl)]-benzene in toluene. The Triton-xylene-based scintillation fluid was 0.3% 2,5-diphenyloxazole, 0.02% p-bis[2-(5-phenyloxazoyl)]-benzene, and 25% Triton X-114 in xylene. CI628, tamoxifen, trans-hydroxy tamoxifen, CI628M, H1285, LY117018 were kindly gifted from Dr. Katzellenbogen at University of Illinois at Urbana-Chanpaign.

Cell Culture

MCF-7, T47D, MDA-MB-231 human breast cancer cells were originally obtained from Dr. Charles McGrath of the Michigan Cancer Foundation (Detroit, MI, USA) and were grown in 60 mm plastic culture dishes in Eagles Minimal Essential Medium (MEM) without phenol red supplemented with 10 mM HEPES buffer, gentamycin (50 μg/ml), penicillin (100 U/ml), streptomycin (1 mg/ml), bovine insulin (6 ng/ml), hydrocortisone (3.75 ng/ml), and 5% calf serum that had been treated with dextran-coated charcoal at 55°C for 45 minutes to remove endogenous hormones.

Estrogen Receptor Binding Analysis

Cells from 20 near-confluent 100 mm culture dishes were suspended in 2.0 mL of PTG buffer (5 mM sodium phosphate, pH 7.4 at 4°C, 10 mM thioglycerol, and 10% glycerol) and homogenized in a Dounce homogenizer using the B-pestle. The homogenate was centrifuged (800×g, 10 minutes) and the supernatant was collected. The crude nuclear pellet was washed twice at 0-4°C with buffer and the nuclear washes combined with the supernatant fraction. This was centrifuged at 180,000×g for 30 minutes 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 [^{3}H]estradiol at concentrations ranging from 5×10^{-11} M to 5×10^{-9} M. Parallel tubes contained the radioactive ligand plus a 100-fold excess of radioinert estradiol to assess non-specific binding. An aliquot was withdrawn for determination of total radioactivity, and unbound ligand was then removed by incubating one part charcoal-dextran slurry (5% Norit A, 0.5% dextran in buffer) with nine parts extract at 0~4°C for 8 minutes . The charcoal was pelleted by a 3 minute centrifugation at 12,800×g, and an aliquot of the supernatant was withdrawn for counting.

Competitive Binding Assays with Estrogen Receptor

Cells were harvested from 10 cm culture dishes and cytosol was prepared at a protein concentration of 1.5 mg/ml in PTG buffer. An aliquot of this 180,000×g supernatant was then incubated with various concentrations of radioinert estradiol or trans-tamoxifen, or CI628 or trans-hydroxy tamoxifen or CI628M or H1285 or LY117018 and 5×10^{-9} M [^{3}H]estradiol at 0~4°C for 16 h and samples were then analyzed.

Assays for Binding to Estrogen-Noncompetable Binding Sites

The cells from near confluent 100mm culture dishes were harvested by incubating cells at 37°C for 10 minutes in Hank's Balanced Salt Solution (calcium and magnesium free; GIBCO, Grand Island, N.Y., USA) with 1 mM EDTA. The cells were washed twice with 10 mM Tris, 1.5 mM EDTA, 0.2% sodium azide, pH 7.4 buffer (TEA buffer) and homogenized in a Dounce

homogenizer (40~50 strokes with a B-pestle). The binding of [^{3}H]trans-tamoxifen to antiestrogen binding sites was determined in the 12,000 g×30 minutes cell supernatant. Supernatant (250 μL) was incubated with 5 μL of ethanol or 10^{-4} M estradiol in 5 μL ethanol, 20 μL of competitor (CI628 or trans-hydroxy tamoxifen or CI628M or H1285 or LY117018) in dimethylformamide and 220 μL of TEA buffer. Samples were incubated at 0~4°C for 16 h and then 88 μL of dextran-coated charcoal (5% Norit A and 0.5% dextran in TEA buffer) was added. The charcoal was pelleted by centrifuging at 12,800×g for 10 minutes and an aliquot of the supernatant was removed for determination of bound radioactivity.

Cell Proliferation Experiments

The effect of estradiol on cell proliferation was studied in MCF-7, T47D and MDA-MB-231 cells. MCF-7, T47D and MDA-MB-231 cells were seeded into 35 mm culture dishes (20×10^3 cells/dish) and grown for two 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 (Day 0). The medium was changed to MEM supplemented as described above except containing 2% charcoal-dextran treated calf serum and various concentrations of trans-tamoxifen or ethanol vehicle (0.1%). Triplicate dishes of cells were counted at several points throughout the 13 day growth period.

RESULTS AND DISCUSSION

Effects of trans-tamoxifen on proliferation of breast cancer cells *in vitro*

Effects of trans-tamoxifen on the proliferation of three human breast cancer cell lines that differed in their estrogen receptor contents were examined. As shown in Fig. 1, 1 μM trans-tamoxifen markedly inhibited the estrogen stimulated proliferation of MCF-7 human breast cancer cell that contained high levels of estrogen receptor over that of control when trans-tamoxifen was administered into cell concomitantly with inhibited estrogen. However, trans-tamoxifen alone treatment did not change the cell numbers compared to that of control. In T47D cells that contained low levels of estrogen receptor, 1 μM trans-tamoxifen showed minimal inhibitory effect on the estrogen stimulated cell proliferation over that of control (Fig. 2) (Lee and Sheen, 1997). MDA-MB-231 cells, that contained no detectable levels of estrogen receptors, had their growth unaffected by trans-tamoxifen (Fig. 3). These results showed their sensitivity to growth inhibition by antiestrogen correlated well with their estrogen receptor contents. These findings are mirrored by the results with human breast cancer patients indicating

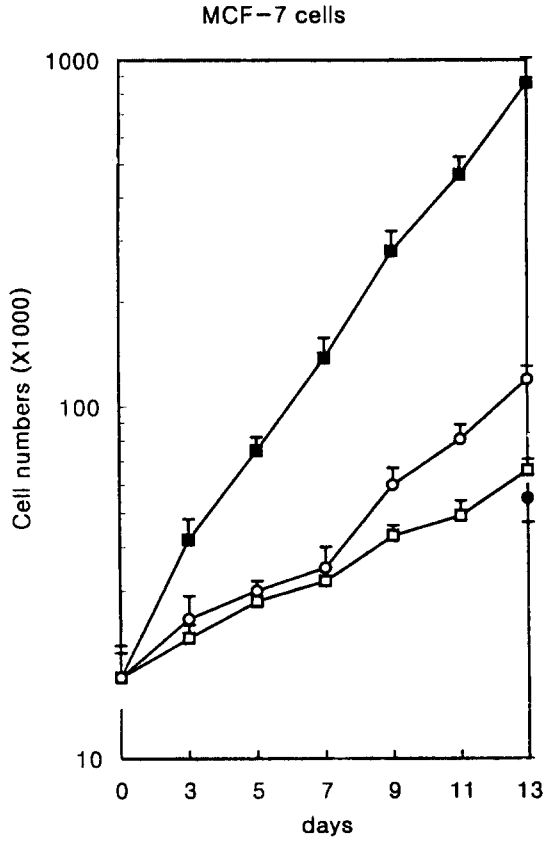

Fig. 1. Effect of trans-tamoxifen on the growth of MCF-7 cells. Cells were grown in the continuous presence of 1 μM trans-tamoxifen in the presence or absenece of 10 nM estradiol, and media with fresh trans-tamoxifen were renewed every other day. On the days indicated, triplicate dishes of cells were counted. Values are the means of the triplicate determinations. Bars represent S.E. ○: estradiol+trans-tamoxifen, ●: trans-tamoxifen, □: estradiol.

that estrogen receptor-containing breast cancers are most sensitive to antiestrogen treatment (McGuire, 1979). Studies with antiestrogen in human breast cancer cells in culture indicate that antiestrogen selectively inhibits the proliferation of estrogen receptor-containing breast cancer cells.

Analysis of the binding to MCF-7 estrogen receptor

Based on data of saturation binding analysis for estradiol in MCF-7 cells (Lee and Sheen, 1997) the presence of a single class of high affinity binding site in MCF-7 cell cytosol with equilibrium dissociation constants (Kd) of 0.25 nM for estradiol was observed. Estrogen receptor levels in human breast cancer cell lines were compared (Lee and Sheen, 1997). MCF-7 cell contained high level of estrogen receptor and T 47D cell contained low level of estrogen receptor. However, MDA-MB-231 cell contained no detectable estrogen receptor. The relative binding affinity of trans-tamoxifen for the estrogen receptor was also det-

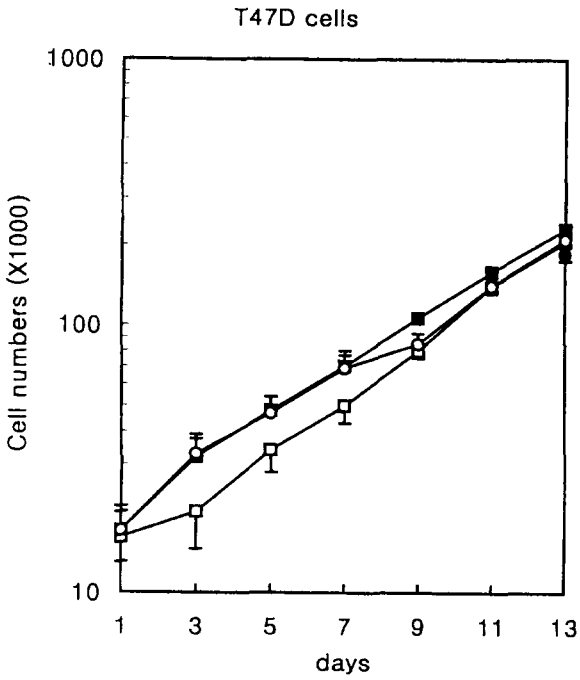

Fig. 2. Effect of trans-tamoxifen on the growth of T47D cells. Cells were grown in the continuous presence of 1 μM trans-tamoxifen in the presence or absenece of 10 nM estradiol, and media with fresh trans-tamoxifen were renewed every other day. On the days indicated, triplicate dishes of cells were counted. Values are the means of the triplicate determinations. Bars represent S.E. ○: estradiol+trans-tamoxifen, ●: trans-tamoxifen, □: estradiol.

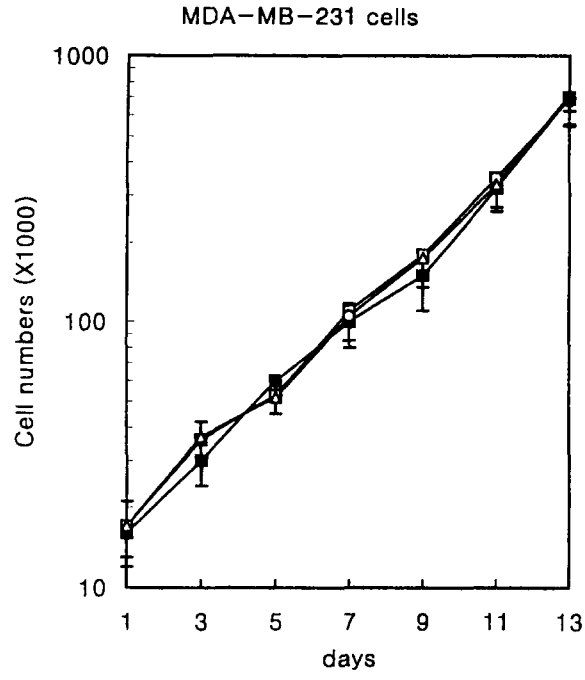

Fig. 3. Effect of trans-tamoxifen on the growth of MDA-MB-231 cells. Cells were grown in the continuous presence of 1 μM trans-tamoxifen in the presence or absenece of 10 nM estradiol, and media with fresh trans-tamoxifen were renewed every other day. On the days indicated, triplicate dishes of cells were counted. Values are the means of the triplicate determinations. Bars represent S.E. ○: estradiol+trans-tamoxifen, ●: trans-tamoxifen, □: estradiol.

ermined indirectly by competitive binding analyses (Lee and Sheen, 1997). Comparison of the concentrations of tamoxifen, CI628, trans-hydroxy tamoxifen, CI628M, H1285, LY117018 and estradiol needed to decrease the specific binding of tritiated estradiol by 50% indicates that tamoxifen, CI628, trans-hydroxy tamoxifen, CI628M, H1285, LY117018, and t-butylphenoxyethyl diethylamine (BPEA) had an affinity of 6%, 3%, 185%, 90%, 120%, and 160%, respectively, compared to that of estradiol (Table I). In addition, the full displacement of [^{3}H]estradiol binding by unlabeled tamoxifen, CI628, trans-hydroxy tamoxifen, CI628M, H1285, LY117018, BPEA and the full displacement of [^{3}H]trans-tamoxifen binding by unlabeled estradiol indicated that the trans-tamoxifen and estradiol bind in a mutually competitive manner to the MCF-7 estrogen receptor.

Table I. Affinity of compounds for estrogen receptor. Measurement was carried out as described in materials and methods

Compunds	Binding affinity*
LY117018	160
H1285	120
CIM628M	90
trans-OH-Tam	185
trans-tamoxifen	6
CI628	3
BPEA	0

*Affinity for estrogen receptor where the affinity of estradiol is set at 100.

Interaction of antiestrogen with antiestrogen binding sites distinct from the estrogen receptor

In addition to binding directly to the estrogen receptor, antiestrogens can be found associated with binding sites that are distinct from the estrogen receptor. Since this initial observation, made in Robert Sutherland's laboratory (Sutherland *et al.*, 1980), there has been considerable interest in elucidating the properties and nature of these binders because of desire to know whether these additional sites may be involved in mediating or modulating some of the actions of antiestrogens. In contrast to the restriction of estrogen receptors to estrogen target cells, such as those of uterus and mammary glands, antiestrogen binding sites are present in a wide variety of estrogen target and non-target tissues (Sudo *et al.*, 1983) and are present in equal amounts in estrogen receptor-positive and -negative human breast cancer cell lines (MCF-7, T47D, and MDA-MB-231; Table II) that diff-

Table II. Levels of antiestrogen binding sites and their affinity for tamoxifen in three different human breast cancer cells. Measurement was carried out as described in materials and methods

Cells	Kd (nM)	[^{3}H]Tamoxifen bound (fmol/mg protein)
MCF-7	2.0	310
T47D	3.1	260
MDA-MB-231	4.4	230

Data on antiestrogen binding sites are analyzed by Scatchard plot analysis from which Kd and number of sites are calculated.

Table III. Affinity of compounds for antiestrogen binding sites. Measurement was carried out as described in materials and methods

Compunds	Binding affinity*
LY117018	5
H1285	13
CIM628M	18
trans-OH-Tam	38
trans-tamoxifen	100
CI628	156
BPEA	6

*Affinity for antiestrogen binding sites where the affinity of tamoxifen is set at 100.

er markedly in their sensitivity to antiestrogens.

Differential centrifugation studies and studies with enzyme markers for different subcellular fractions (Sudo *et al.*, 1983) indicated that these estrogen-noncompetable antiestrogen binding sites were associated with microsomal fraction. These sites were highest in concentration in the liver, which showed levels of approximately 10-fold higher than that found in uterus, esophagus, ovary, brain, or kidney (Sudo *et al.*, 1983). In further fraction studies (Sudo *et al.*, 1983), they have used the method of Adelman *et al.* (Adelman *el al.*, 1974) to study in detail the microsomal association of these antiestrogen binding sites, they have found that more than half of binding sites are associated with rough endoplasmic reticulumn fraction (which is comtaminated to some extent with rough endoplasmic reticulumn), with very little of these binding sites being associated with ribosome. Hence, the majority of the microsomal antiestrogen binding sites appear to be associated with the membrane component of the rough endoplasmic reticulum, a finding in agreement with other study (Watt and Sutherland, 1984) which used slightly different microsomal fractionation procedure. Hence, our findings and those of Watts and Sutherland (Watt and Sutherland, 1984), suggest that almost all of the antiestrogen binding sites in cells are associated with microsome, whereas the estrogen receptor is largely nuclear after hormone interaction. The antiestrogen binding sites are destroyed upon treatment with protease (Sudo, 1983) and their rapid sedimentation on sucrose gradients. Although the role of these antiestrogen binding sites still remains to be determined, these sites are present in equal amounts (Table II) in three breast cancer cell lines (MCF-7, T47D, and MDA-MB-231) that differ markedly in their estrogen receptor content and in their senstivity to growth supression by antiestrogens (Fig. 1-3). In addition, these antiestrogen binding sites are present in equal amounts in MCF-7 cells and in two variant MCF-7 clones designated R27 and R3-98 which are no longer growth inhibited by antiestrogens (Miller *et al.*, 1984). However, others reported on tamoxifen resistant MCF-7 variant cell that is no longer sensitive to antiestrogens and is reported to have the antiestrogen binding sites (Faye *et al.*, 1983). In order to gain greater insight into the role of these antiestrogen binding sites in the action of antiestrogens, we and others have been interested in examining the biopotency of different antiestrogens for the antiestrogen binding sites is CI628 > tamoxifen > trans-hydroxy tamoxifen > CI628M > H1285 > LY117018. As shown in Table III, this order of affinities does not parallel the affinity of these compounds for the estrogen receptor nor the potency of these compounds as antiestrogens. Indeed, compounds with high affinity for the estrogen receptor and greatest antiestrogenic potency have low affinities for these antiestrogen binding sites. Antiestrogenic potency correlates best with estrogen receptor affinity and not with affinity for antiestrogen binding sites (i.e., high ER/ABS ratio, Table IV). It is worth noting, as well, that cis-tamoxifen has an affinity for the antiestrogen binding sites equal to that of trans-tamoxifen and that cis-tamoxifen behaves as an estrogen in MCF-7 cells and rat uterus (Sudo *et al.*, 1983; Katzellenbogen *et al.*, 1984; Eckert and Katzellenbogen, 1983; Davison, 1986). Studies with BPEA that was designed to incorporate the features important in antiestrogen binding to antiestrogen binding sites, namely an aromatic ring system and an amine side chain, but lacking the features required for binding to the es-

Table IV. Affinity of compounds for estrogen receptor (ER) vs antiestrogen binding sites (ABS) and their potencies in growth inhibition. Measurement was carried out as described in materials and methods.

Compunds	Binding affinity (ER/ABS)	Growh inhibitin (potency*)
LY117018	5	32.0
H1285	13	9.2
CIM628M	18	5.0
trans-OH-Tam	38	4.9
trans-tamoxifen	100	0.06
CI628	156	0.02
BPEA	6	0

*Potency in inhibiting the growth of MCF-7 cells *in vitro*.

trogen receptor showed affinity for estrogen receptor was important for the antiestrogen action (Sheen, *et al.*, 1985). As shown in Table I, BPEA has an affinity for antiestrogen binding sites 6% that of tamoxifen, but has no affinity for estrogen receptor (less than 0.0003% that of estradiol) for the estrogen receptor (Table I). This compound appeared to have no effect, either stimulatory or inhibitory, on proliferation of MCF-7 cells over a wide range of concentrations, we would expect this compound to fully occupy the antiestrogen binding sites. In addition, this compound exhibits no uterotrophic activity when assayed in immature female rats (Sheen *et al.*, 1985). Therefore, we could conclude that occupancy exclusively of antiestrogen binding sites, at least by BPEA did not result in growth supression. Our findings raised serious doubts about the role of the antiestrogen binding sites in mediating directly the estrogen antagonism of antiestrogens, and suggested that affinity for the estrogen receptor most closely corresponded with the potency of these compounds as antiestrogens. It is possible that the antiestrogen binding sites might influence the distribution of antiestrogens and hence, their accessability to estrogen receptor in estrogen receptor-positive cells, or they might mediate actions of antiestrogens that are unrelated to estrogen antagonism. These sites might, for example, also be involved in antiestrogen metabolism which was known to occur in liver microsomes and to be associated with the cytochrome P450 system (Ruenitz *et al.*, 1984). In this regards, the roles of potential endogenous ligands for the antiestrogen binding sites are also compeletely unknown at present. Studies with BPEA, which binds exclusively to the antiestrogen binding sites, not at all to estrogen receptor, should be particular instructive in determining the effects of the antiestrogen binding and their biopotency. In summary, our findings suggested that interaction with the estrogen receptor is most likely the mechanism through which antiestrogens evoke their growth inhibitory effects. Continuing studies aimed at elucidating physicochemical and conformational differences in antiestrogen- vs estrogen receptor complexes that underlie their differing nuclear interactions, should provide valuable information on the mechanism of action of antiestrogens.

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