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Structure-Function Relationships of Hydroxylated Metabolites of Tamoxifen that Control the Proliferation of Estrogen-Responsive T47D Breast Cancer Cells *In Vitro*

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SUMMARY

Several hydroxylated derivatives of tamoxifen were tested for their effects on the growth of T47D human breast cancer cells *in vitro*. Compounds containing a fused seven-membered ring were used to prevent isomerization of the triphenyl-ethylenes at the double bond. This stable structure permitted the determination of the activity of the *cis* and *trans* forms of tamoxifen and the true activity of two of its metabolites, 4-hydroxytamoxifen and metabolite E. Estradiol stimulates the growth of T47D cells 3–4-fold over control after 6 days of treatment ($EC_{50} \simeq 3 \times 10^{-12}$ to 3×10^{-11} M, depending upon the particular experiment). The fixed ring form of the *trans* isomer of tamoxifen is an antiestrogen, whereas the *cis* isomer is estrogenic. Fixed ring-*trans*-4-hydroxytamoxifen is a potent antiestrogen, and its *cis* isomer is a weak antiestrogen ($IC_{50} \simeq 4 \times 10^{-8}$ to 2×10^{-7} M). The fixed ring form of *trans*-metabolite E (tamoxifen without the dimethylaminoethane side chain) is only a weak partial estrogen agonist, whereas the fixed ring derivative of its *cis* isomer is a potent estrogen agonist ($EC_{50} \simeq 4 \times 10^{-12}$ to 1×10^{-11} M). These studies have determined the true biological activities of the hydroxylated derivatives of tamoxifen. This information will be valuable for the development of drug receptor models and will be particularly useful when the three-dimensional structure of the receptor complex is determined.

The antiestrogen TAM [(*Z*)-1-[*p*-dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene] is the most widely used antihormonal agent for the treatment of ER-positive breast cancer (1). TAM controls breast cancer cell replication by blocking the binding of estrogen to the ER and preventing estrogen-stimulated growth. Triphenylethylenes like TAM have also proved to be valuable laboratory tools to study the mechanisms of estrogen action *in vivo* and *in vitro* (2).

Progress in understanding estrogen and antiestrogen action has been facilitated by the development of defined cell culture systems to study the direct effects of test compounds without the influence of metabolism, which occurs *in vivo*. Until recently, it had been difficult to detect a growth response of ER-positive breast cancer cells to estrogen *in vitro*. Cells were refractory to stimulation by estrogen but appeared to be sensitive to growth inhibition by antiestrogens (3, 4). Recent studies demonstrate that phenol red, a pH indicator routinely present in cell culture media, contains an estrogenic contaminant that stimulates hormone-responsive cell growth *in vitro* (5–9). Breast cancer cells cultured in phenol red-containing media were already maximally stimulated by the contaminant, which prevented any further growth response to added estrogen. Early structure-activity relationship studies utilized rat pituitary cell prolactin synthesis and uterine cell progesterone receptor induction because, fortuitously, these cellular responses are not as sensitive as cell replication to stimulation by the contaminant in phenol red (10–12). The culture of breast cancer cells in phenol red-free media now provides a sensitive and more clinically relevant assay system with which to study structure-activity relationships of estrogen and antiestrogens. We have previously characterized hormone-responsive T47D breast cancer cells (13, 14) in the presence and absence of estrogen. In the present study, T47D cells are used to study the pharmacological effects of TAM and its metabolites on breast cancer cell growth.

The isomeric instability of hydroxylated triphenylethylenes in tissue culture cast doubt upon the true pharmacological activities of the individual geometric isomers of metabolites of TAM (15–17). The pharmacological activities of individual isomers are presumed but not known. We have synthesized a series of f.r. derivatives of TAM and its metabolites to study their structure-activity relationships (18, 19). These studies,

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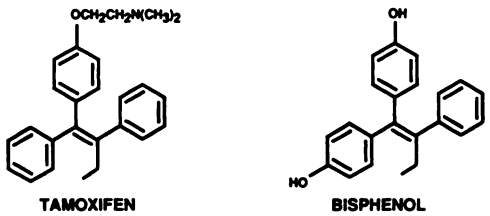
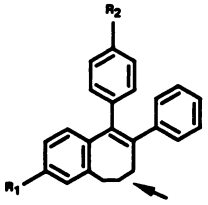
ABBREVIATIONS: TAM, tamoxifen; ER, estrogen receptor; f.r., fixed ring; 4-OHT, 4-hydroxytamoxifen; met E, metabolite E.





TRIVIAL NAME	R_1	R_2
FIXED RING trans TAM	H	$OCH_2CH_2N(CH_3)_2$
FIXED RING cis TAM	$OCH_2CH_2N(CH_3)_2$	H
FIXED RING trans 4-OHT	OH	$OCH_2CH_2N(CH_3)_2$
FIXED RING cis 4-OHT	$OCH_2CH_2N(CH_3)_2$	OH
FIXED RING trans MET E	H	OH
FIXED RING cis MET E	OH	H

Fig. 1. Compounds used in these studies. *Arrow*, area of fused seven-membered ring of test compounds.

along with complementary studies of the ER molecule (20–22), will be important to define the drug-receptor interactions that control breast cancer cell replication.

Materials and Methods

Cell culture. The T47D cell line (13) used in these studies was originally obtained at passage 81 from the American Type Culture Collection (Rockville, MD). Cell stocks were kept in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (heat inactivated), 6 ng/ml bovine insulin (Sigma Chemical Co., St. Louis, MO), 100 units/ml penicillin, and 100 μg/ml streptomycin. All tissue culture reagents were obtained from GIBCO Laboratories (Grand Island, NY) unless otherwise stated. Cells were removed from flasks with 0.25% trypsin/EDTA solution for passage. Cell stocks were kept in T150 flasks (Corning, Park Ridge, IL) in a humidified atmosphere of 95% air/5% CO_2, at 37°. All experiments were conducted on cells between passages 97 and 191.

Hormones. 17β-Estradiol was purchased from Sigma Chemical Co. (St. Louis, MO). TAM, 4-OHT, met E, and bisphenol were obtained from ICI Pharmaceuticals (Macclesfield, England). Nonisomerizable f.r. compounds (Fig. 1) were synthesized as described previously (18, 19). All compounds were prepared in a concentrated form in 100% ethanol and diluted in cell culture medium. Final ethanol concentrations of the media never exceeded 0.2%.

Growth response studies. For growth response studies, cells were plated into T150 flasks and incubated with phenol red indicator-free medium, containing 10% dextran-coated charcoal-stripped serum, for 7–10 days before plating into 24-well dishes. Medium was changed every 3 days. On day 0 of the experiment, cells were plated into 24-well dishes at 2.0–2.5 × 10^5 cells/well. Medium containing compounds was added on day 1 and cells were allowed to grow for 6 days. Medium containing compounds was replaced on day 4. On day 7, cells were washed with complete Hanks' balanced salt solution and were harvested for DNA assays. Cells were treated with 1 ml/well hypotonic calcium/magnesium-free Hanks' balanced salt solution and sonicated for 12 sec

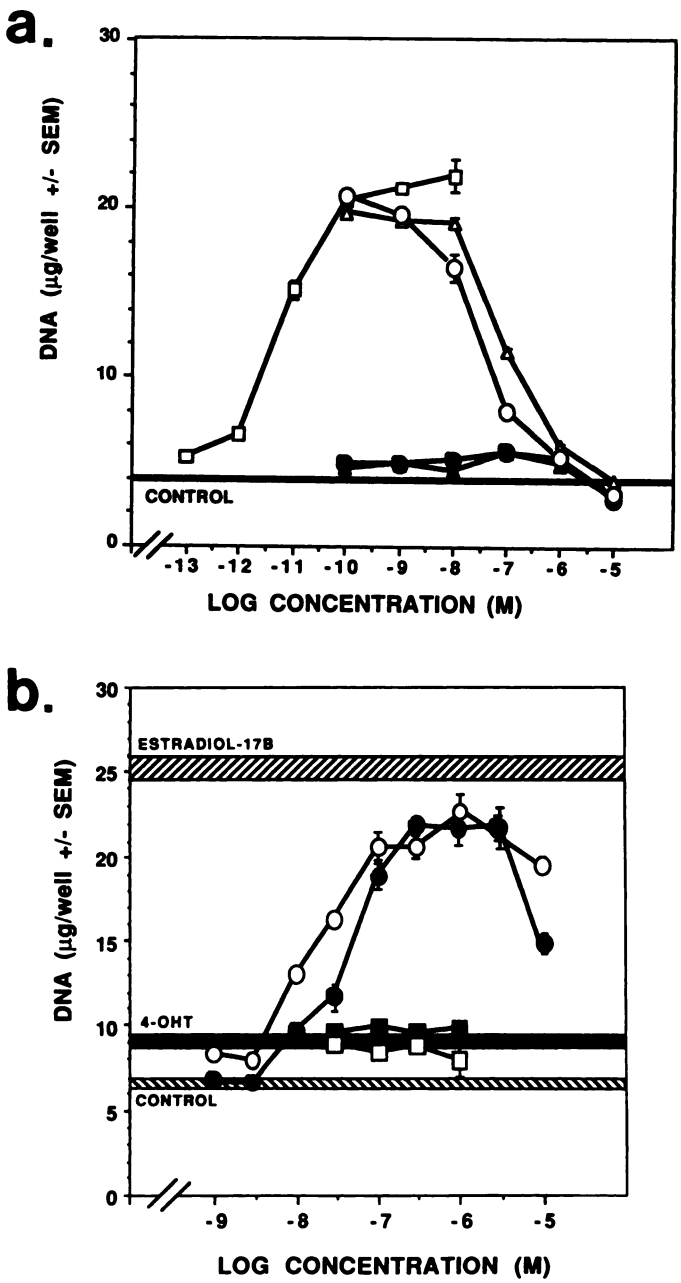


Fig. 2. Growth response of T47D cells to incubation with isomerizable and nonisomerizable forms of *cis*- and *trans*-TAM. Cells were plated at 2.0 × 10^5 cells/well and treated for 6 days with compounds. Each *point* represents three or four assayed wells. a, □, 17β-Estradiol, ▲, *trans*-TAM; ●, *trans*-f.r.-TAM; △, *trans*-TAM plus 17β-estradiol; ○, *trans*-f.r.-TAM plus 17β-estradiol (0.1 nM); ■, control. b, ●, *cis*-TAM; ○, *cis*-f.r.-TAM; ■, *trans*-TAM; □, *trans*-f.r.-TAM; ■, control; ■, 4-OHT (0.1 μM); ■, 17β-estradiol (0.1 nM).

with a Kontes ultrasonic cell disrupter. Samples (50–100 μl) were taken for DNA determinations. Assays for DNA were performed by incubating the samples with Hoechst dye 33258 (Calbiochem-Behring Corp., La Jolla, CA), according to a method described by LaBarca and Paigen (23). Samples were analyzed on a SLM-Aminco Fluoro-Colorimeter III. All points for each DNA measurement represent a mean of three or four sampled wells.

Stability of *cis*-4-OHT. One-milliliter aliquots of culture medium were extracted with 5 ml of hexane/amyl alcohol (98:2), with a 5-min vigorous mixing period. The organic phase was evaporated to dryness under N_2 and reconstituted to 100 μl with a mobile phase (see below). The methodology to determine the *cis* and *trans* isomers of 4-OHT has

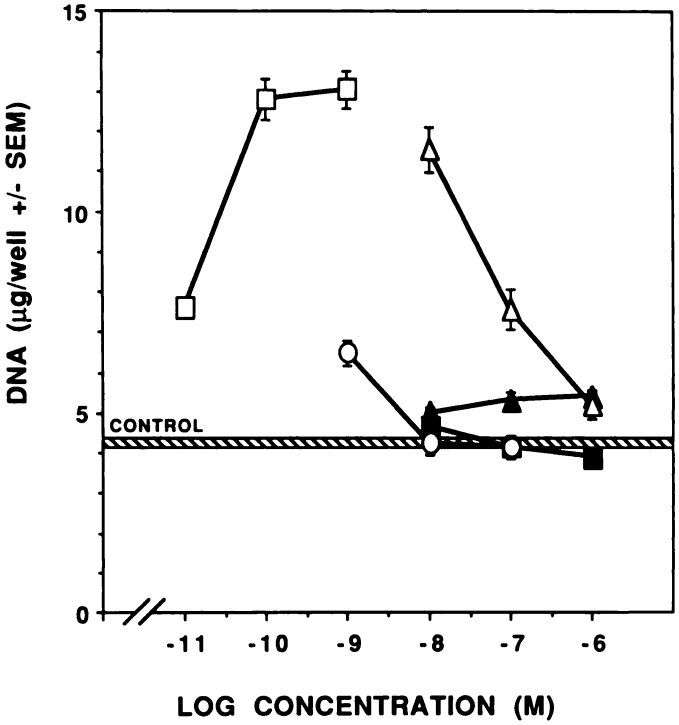


Fig. 3. Effects of isomerizable and nonisomerizable forms of 4-OHT. Cells were plated at 2.5 × 10^5 cells/well and treated for 6 days with compounds. Each *point* represents three or four assayed wells. □, 17β-Estradiol; ○, *trans*-f.r.-4-OHT plus 17β-estradiol (0.1 nM); ■, *cis*-4-OHT plus 17β-estradiol (0.1 nM); ▲, *cis*-f.r.-4-OHT; △, *cis*-f.r.-4-OHT plus 17β-estradiol (0.1 nM); ▨, control.

been described elsewhere (24, 25). Analysis was performed with a silica fast column (100 × 4 mm; Scientific Glass Engineering, Austin, TX). The mobile phase was *iso*-octane/ethanol/acetic acid/diethylamine/isopropanol (75:23.5:0.05:1.5), with a flow rate of 1 ml/min. Fluorescence detection (254-nm excitation; 330-nm reemission) was accomplished by post-column UV activation of triphenylethylenes to phenanthrenes.

Results

We tested the effects of TAM, f.r.-TAM, *cis*-TAM, and *cis*-f.r.-TAM (Fig. 1) on the growth of T47D cells *in vitro* (Fig. 2). *trans*-TAM and *trans*-f.r.-TAM showed little agonist activity alone. These compounds were antiestrogenic and inhibited estradiol-stimulated growth (Fig. 2). *cis*-TAM and *cis*-f.r.-TAM were estrogenic and stimulated cell growth approximately 4-fold (Fig. 2). The activity of *trans*-f.r.-4-OHT was then compared with the activities of *cis*-f.r.-4-OHT and *cis*-4-OHT (Figs. 1 and 3). *trans*-f.r.-4-OHT was antiestrogenic and effectively inhibited estradiol-stimulated (0.1 nM) growth. The isomerizable form of *cis*-4-OHT was also antiestrogenic, displaying antagonistic activity similar to that of the *trans* form. The nonisomerizable f.r. form of *cis*-4-OHT was also antiestrogenic but was significantly less potent than the non-f.r. form. As previously shown (16), it appeared that the non-f.r. form of *cis*-4-OHT was isomerizing to the more active *trans* form of 4-OHT and, therefore, it displayed potent antiestrogenic activity. High performance liquid chromatographic analysis of conditioned cell media detected the conversion of the *cis*-4-OHT form to the *trans* form (Fig. 4a), whereas *cis*-f.r.-4-OHT showed no conversion in media (Fig. 4b). The weak antiestrogenic activity ($IC_{50} \simeq 4 \times 10^{-8}$ to 2×10^{-7} M) of *cis*-f.r.-4-OHT (Fig. 5a) (0.1–1.0 μM) could be reversed by competition with increasing concentrations of estradiol, thereby confirming that its inhibitory effect on cell growth was not nonspecific but was mediated via the ER (Fig. 5b).

We also examined the effects of the isomers of met E (another hydroxylated metabolite of TAM) on the growth of T47D cells. The f.r. forms of met E (Fig. 1) were compared with those of the original triphenylethylenes. The *trans* form of met E appeared to readily isomerize to the more potent estrogenic *cis* form (Fig. 6), analogous to the isomerization of *cis*-4-OHT to its *trans* form in cell culture. As a result of this isomerization, the non-f.r. form of *trans*-met E appeared to display potent full agonist activity. In contrast, the f.r. form of *trans*-met E was a weak partial agonist. The activities of these compounds were compared with that of bisphenol, a known partial agonist (11) (Fig. 1). Bisphenol and the *cis*- and *trans*-f.r. forms of met E all displayed agonist activity (Fig. 7). The *cis* form of f.r.-met E ($EC_{50} \simeq 4 \times 10^{-12}$ to 1×10^{-11} M) was more potent than bisphenol, whereas the partial agonist *trans* form was less potent than bisphenol at stimulating cell growth. Stimulation of cell growth by *cis*-f.r.-met E was effectively decreased by the addition of *trans*-4-OHT (1×10^{-8} M) (Fig. 8). An increased amount of the agonist (100-fold) was needed in order to increase cell growth to the levels produced by incubation with *cis*-f.r.-met E alone. Increasing concentrations of *trans*-f.r.-met E effectively competed with estradiol (1×10^{-10} M) and inhibited the effects of estradiol-stimulated growth to the levels seen with *trans*-f.r.-met E alone (Fig. 8). These studies indicate that this metabolite is a partial agonist and is probably stimulating cell growth through an ER-mediated mechanism.

Discussion

The cloning of the ER (20) and the description of distinct functional domains within the protein (21, 22) have provided important insight into the molecular mechanisms of estrogen action. The antiestrogen *trans*-4-OHT has proved to be a valuable laboratory tool to act as a control substance that binds with high affinity to the ER to inhibit estrogen-regulated genes (26, 27). In fact, it may be feasible to map the receptor binding site to describe facilitatory or inhibitory conformations of the drug-ER complex by studying the structure-activity relationships of triphenylethylenes. However, such studies must pay special attention to the stability of the ligands used in the assay systems. A case in point is the geometric instability of the TAM metabolites 4-OHT and met E (a metabolite of TAM without the dimethylaminoethane side chain). The stable *cis* isomer of TAM is estrogenic, whereas the stable *trans* isomer is an antiestrogen. However, both 4-OHT and met E exist in forms that are unstable and convert to mixtures of isomers in tissue culture. A precise description of the pharmacological properties of each individual isomer (whether it is estrogenic or antiestrogenic) has proved to be difficult.

Earlier reports (16, 17) describe the instability of hydroxylated metabolites of TAM and warn of potential misinterpretation of information obtained from their use in structure-activity relationship studies. Indeed, it may even be important to appreciate this problem when interpreting data from metabolic studies of TAM. A case in point is the finding that only the *cis* form of met E was measured in plasma from TAM-

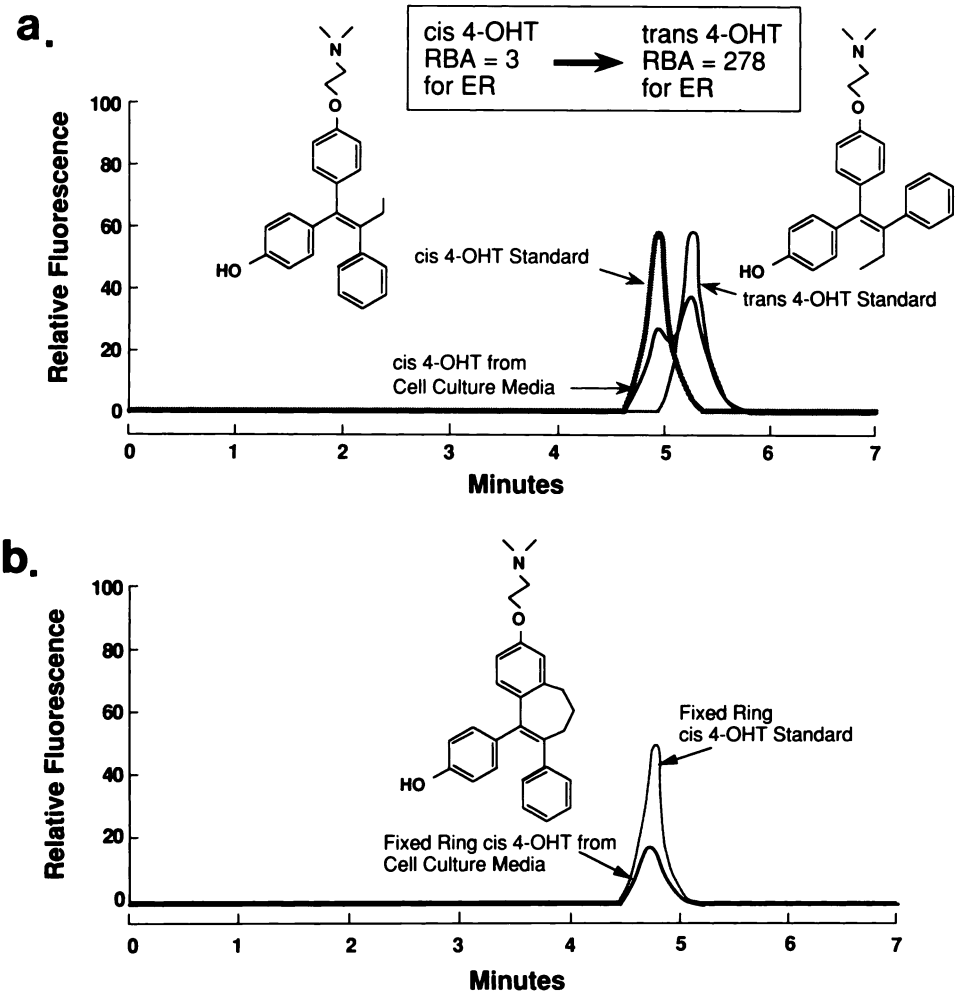
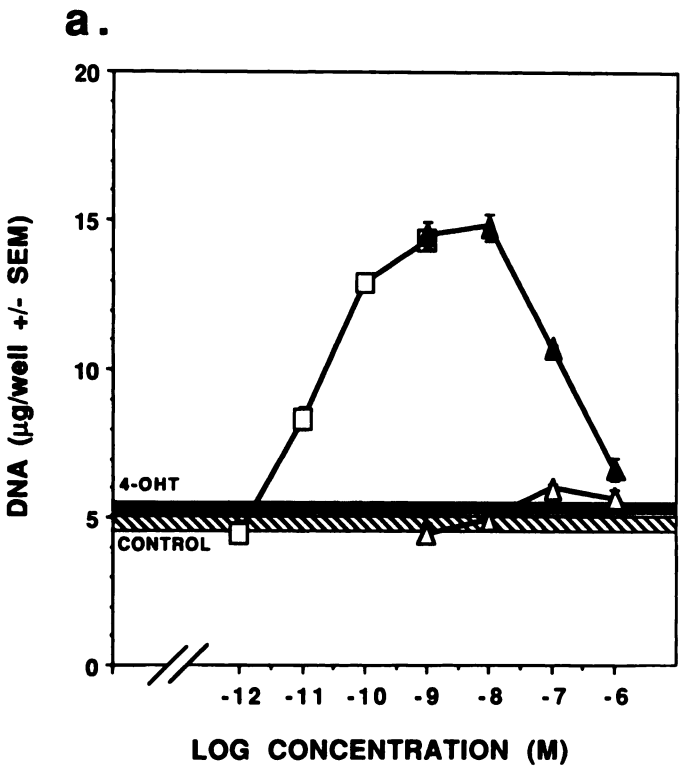


Fig. 4. a, The conversion of *cis*-4-OHT to *trans*-4-OHT in cell culture medium taken from T47D cells incubated (6 days) with 10^{-6} M *cis*-4-OHT. The high performance liquid chromatography methodology is described in Materials and Methods. The relative binding affinity (*RBA*) of each ligand for the ER is from Ref. 10. b, The f.r. derivative of *cis*-4-OHT from culture medium was identified as a single peak on the chromatograph with a retention time that was the same as the standard.

treated patients (28). None of the *trans*-met E form could be detected. Any *trans*-met E that may be present in plasma could readily isomerize to the *cis* form, either *in vivo* or during the extraction and analysis period, making the detection of the *trans* form impossible. Clearly, isomerizaton has an impact on the pharmacological potency of the metabolites and may be important during the treatment of the patient. If, in fact, isomerization occurs in the liver, then this may be responsible for some of the estrogen-like activity noted during TAM therapy (1).

Isomerization should be considered when any structure/function studies involving hydroxylated triphenylethylene-type compounds are completed. For example, Raynaud and colleagues (29) recently evaluated the actions of a series of triphenylacrylonitrile derivatives on MCF-7 cell growth (30). These investigators tested the isomeric purity of the compounds after a 2-day incubation of the compounds at 37°, pH 7.4. Under these conditions, all of the isomers displayed less than 15% conversion to their opposite isomeric form. However, MCF-7 cell growth response assays were performed on cells incubated with test compounds for an 8- to 10-day growth period. It is possible that further isomerization may be occurring over this extended time period, thereby affecting the final growth response of the cells. Furthermore, because the potency of isomers can vary 100-fold, even a slight conversion from the low potency to the high potency isomer will give an inaccurate result. The use of nonisomerizable forms of these compounds would prevent any inconsistencies and allow investigators to study the true structure-function relationships.

Our studies describe the estrogenic and antiestrogenic effects of nonisomerizable derivatives of TAM and its metabolites on the growth of human breast cancer cells *in vitro*. Through the use of these f.r. compounds, we have determined the true biological activity of each isomeric form. However, the precise mechanism through which these compounds exert their biological effects is not known, although several hypothetical drug receptor models have been proposed (2, 10). Besides binding to the ER, it has been suggested that triphenylethylene antiestrogens could exert their effects by binding to "antiestrogen binding sites" (31, 32). These binding sites, present in virtually all tissues, bind antiestrogens [and many other pharmacological agents (33, 34)]; however, estrogenic steroids or compounds without the aminoethoxy side chain do not bind (35). These observations naturally lead to a belief that the antiestrogen binding sites are important for antiestrogen action. To exclude the possibility that 4-OHT and *cis*-f.r.-4-OHT produce their antiestrogenic effects through the antiestrogen binding site, we conducted competition studies. 17β-Estradiol reversed the effects of *cis*-f.r.-4-OHT (Fig. 5), and the effects of *cis*-f.r.-met E were reversibly inhibited by 4-OHT (Fig. 8). These results strongly suggest that antiestrogens exert their antiproliferative effect at these concentrations through the ER and not via the antiestrogen binding site.



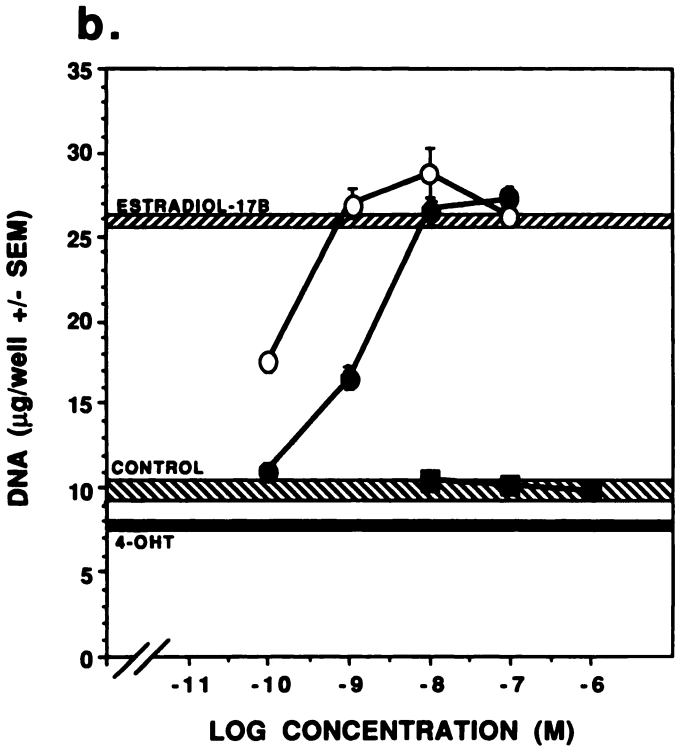


Fig. 5. Effects of *cis*-4-OHT alone and in competition with 17β-estradiol. a, Competition of 17β-estradiol with increasing concentrations of *cis*-f.r.-4-OHT. b, Competition of *cis*-f.r.-4-OHT with increasing concentrations of estradiol. Cells were plated at 2.5×10^5 cells/well, and cells were treated with test compounds for 6 days. Each *point* represents three or four assayed wells. a, □, 17β-Estradiol; △, *cis*-f.r.-4-OHT; ▲, *cis*-f.r.-4-OHT plus 17β-estradiol (0.1 nM); ▩, control; ■ 4-OHT (0.1 μM). b, ○, *cis*-f.r.-4-OHT (0.1 μM) plus 17β-estradiol; ●, *cis*-f.r.-4-OHT (1.0 μM) plus 17β-estradiol; ■, *cis*-f.r.-4-OHT alone; ▩, control; ■, 4-OHT (0.1 μM); ▩, 17β-estradiol (0.1 nM).

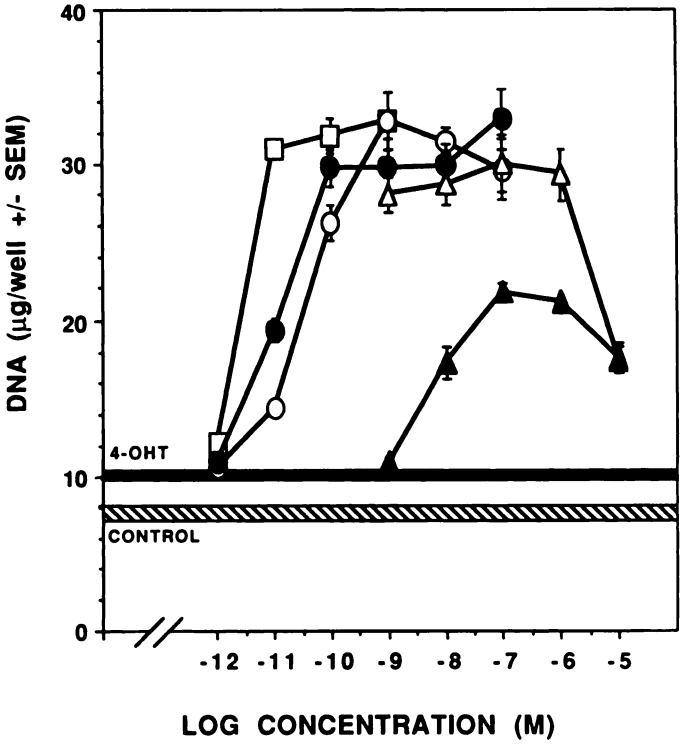


Fig. 6. Comparison of the effects of the isomers of met E versus f.r.-met E nonisomerizable forms. Cells were plated at 2.0×10^5 cells/well and treated for 6 days with compounds. Each *point* represents three or four assayed wells. □, 17β-Estradiol; ○, *cis*-met E; ●, *cis*-f.r.-met E; △, *trans*-met E; ▲, *trans*-f.r.-met E; ▩, control; ■, 4-OHT (0.1 μM).

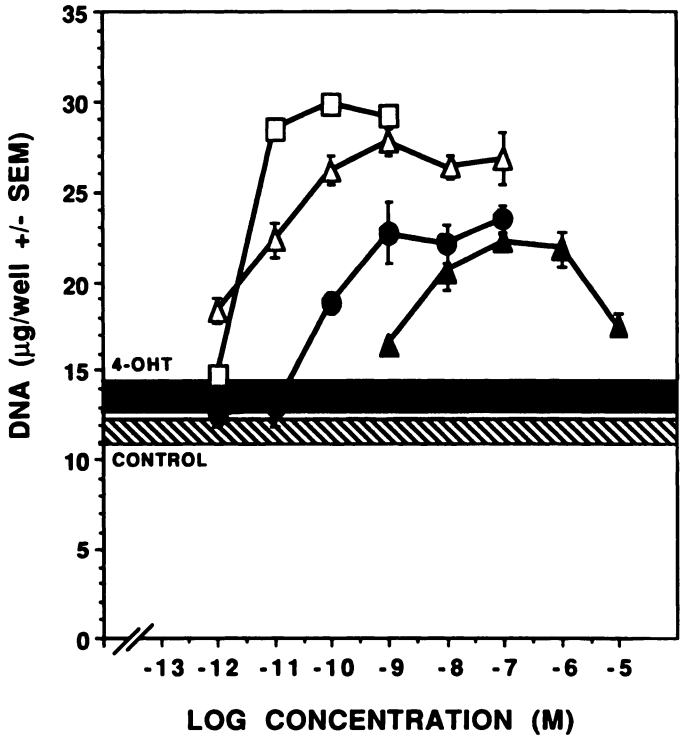


Fig. 7. Comparison of the activity of bisphenol with that of nonisomerizable forms of met E isomers. Cells were plated at 2.0×10^5 cells/well and treated for 6 days with compounds. Each *point* represents three or four assayed wells. □, 17β-Estradiol, △, *cis*-f.r.-met E; ●, bisphenol; ▲, *trans*-f.r.-met E; ▩, control; ■, 4-OHT (0.1 μM).

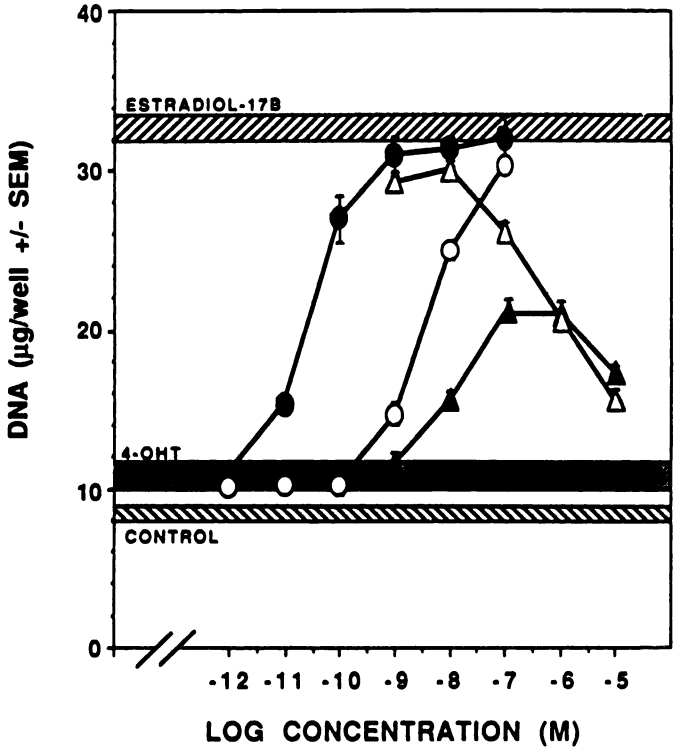


Fig. 8. Inhibition of the estrogenic effect of *cis*-f.r.-met E by 4-OHT and inhibition of estradiol-stimulated growth by partial agonist *trans*-f.r.-met E. Cells were plated at 2.0 × 10^5 cells/well and treated for 6 days with compounds. Each *point* represents three or four assayed wells. ●, *cis*-f.r.-met E; ○, *cis*-f.r.-met E plus 4-OHT (10 nM); ▲, *trans*-f.r.-met E; △, *trans*-f.r.-met E plus 17β-estradiol (0.1 nM); ▩, control; ■, 4-OHT (0.1 µM); ■, 17β-estradiol (0.1 nM).

Stable f.r. compounds have proved to be useful probes for the study of ER-mediated events. Further study with related compounds will provide valuable information about the ligand characteristics that govern receptor-mediated cell replication.

Acknowledgments

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