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Structural Requirements for the Pharmacological Activity of Nonsteroidal Antiestrogens *in Vitro*

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SUMMARY

The structure-activity relationships of a tamoxifen (TAM) (Z-1-(4β-dimethylaminoethoxyphenyl)1,2-diphenylbut-1-ene) series have been investigated. The tamoxifen derivatives were assayed *in vitro* by their modulation of estradiol (E_2)-stimulated prolactin synthesis in primary cultures of dispersed rat pituitary gland cells. Monohydroxylation of TAM in position 4 of the stilbene ring system was found to be the optimal substitution for binding to the estrogen receptor [relative binding affinity (RBA) = 234] and to inhibit E_2 (1 nM)-stimulated prolactin synthesis (IC_{50} 7 nM) by pituitary cells in primary culture. Substitution in positions 3 and 4 to form a catechol did not decrease affinity for the estrogen receptor (RBA = 252), and potency as an antiestrogen was maintained in the prolactin assay (IC_{50} 20 nM) as long as oxidation of the catechol was prevented. All of the hydroxylated derivatives of tamoxifen tested were estrogen antagonists; however, removal of the alkylaminoethoxy side chain from TAM produced a full estrogen agonist with low potency (20 nM). In contrast, removal of the side chain from 4-hydroxytamoxifen (4-OH TAM) produced a partial agonist. A structural analogue of 4-OH TAM, 3-[β-dimethylaminoethoxy]-11-ethyl-12-(4-hydroxyphenyl)5,6-dihydrodibenzo[a,e]-cyclooctene (7c) had a decreased potency (IC_{50} 16 nM) compared with 4-OH TAM (IC_{50} 4 nM in the same experiment) as an estrogen antagonist. If the side chain was changed from a dimethylaminoethoxy to glyceryl, antagonist activity was reduced (IC_{50} 0.8 μM). An allyl side chain produced a compound with no antiestrogenic activity at concentrations up to 1 μM. An adaptation of Belleau's macromolecular perturbation theory is suggested to explain the interaction of agonists, antagonists, and partial agonists at the ligand binding site of the estrogen receptor.

INTRODUCTION

The discovery that the nonsteroidal compound MER 25 has weak, but effective, antiestrogenic activity (1), initiated an intense search for derivatives with increased potency. Most interest has focused upon substituted derivatives of triphenylethylene, with several compounds—nafoxidine, CI628, and enclomiphene—becoming valuable laboratory tools with which to study estrogen action (2–4). However, the successful introduction of TAM[6] for the treatment of breast cancer has required a better understanding of the molecular pharmacology of the drug (5). Clearly, if the mechanism by which antiestrogens control estrogen-stimulated events were known, then other, more effective, drugs could be designed. Furthermore, drugs that are targeted to different components of the system might be discovered and developed as therapeutic agents.

One approach to understanding the estrogen receptor mechanism is to study the structure-activity relationships of agonists and antagonists to provide precise in-

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[6] The abbreviations used are: TAM, tamoxifen (Z-1-(4β-dimethylaminoethoxyphenyl)1,2-diphenylbut-1-ene); 4-OH TAM, 3,4-diOH TAM, 3-OH TAM, and 3′-OH TAM, 4-hydroxy-, 3,4-dihydroxy-, 3-hydroxy-, and 3′-hydroxytamoxifen; ICI 77,949, TAM without alkyl aminoethyl side chain; 7c, 3-[β-dimethylaminoethoxy]-11-ethyl-12-(4-hydroxyphenyl)5,6-dihydrodibenzo[a,e]-cyclooctene (ring-stabilized derivative of 4-OH TAM); 9c, side chain-altered derivative (allyl) of (ring-stabilized) 4-OH TAM; 11c, side chain-altered derivative (glyceryl) of (ring-stabilized) 4-OH TAM; E_2, estradiol; RBA, relative binding affinity; U-0521, 3′, 4′-dihydroxy-2-methyl prophiophenone.

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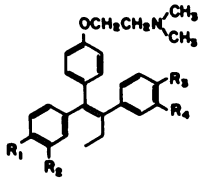
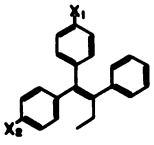
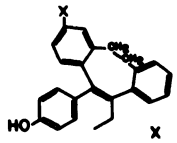
formation about the drug-receptor interactions. Unfortunately, these studies using assay *in vivo* are complicated because of extensive metabolism (6) and differences in pharmacokinetics and pharmacodynamics (7, 8), which can also provide misleading information about the intrinsic potency of a particular drug molecule. To circumvent these problems, we recently validated an assay system *in vitro* to study the structure-activity relationships of estrogenic and antiestrogenic compounds (9, 10). The assay utilizes the regulation of prolactin synthesis in cultures of pituitary gland cells by estrogens (11).

In this study, we describe the effect of hydroxylation, at different positions of the unsubstituted phenyl rings of TAM (12) (Table 1), on the estrogen receptor binding activity and pharmacological potency of the drug as an antiestrogen. Additionally, the effect of removal of the aminoethoxy side chain from TAM and its derivative, 4-OH TAM (13), and alteration of the aminoethoxy side chain in a ring-stabilized derivative of 4-OH TAM (14) is considered. We suggest that the different agonist, partial agonist, and antagonist properties may be described by an adaptation of Belleau's macromolecular perturbation theory (15).

TABLE 1

Formulae RBA and the IC_{50} of compounds used in the study

Group A. Hydroxylated derivatives of tamoxifen. Group B. Hydroxylated triphenylethylenes without an alkylaminoethoxy side chain. Group C. A fixed-ring derivative of 4-OH TAM with different side chains.

Compound					RBA	IC_{50}[a]
						M
A						
	R_1	R_2	R_3	R_4		
TAMOXIFEN (TAM)	H	H	H	H	3	3×10^{-7}
4-HYDROXY TAM	OH	H	H	H	252	7×10^{-9}
3-HYDROXY TAM	H	OH	H	H	21	3×10^{-7}
3,4,-DIHYDROXY TAM	OH	OH	H	H	234	2×10^{-8}
4'-HYDROXY TAM	H	H	OH	H	6	4×10^{-7}
3'-HYDROXY TAM	H	H	H	OH	1	1×10^{-6}
B						
			X_1	X_2		
ICI 77,949			OH	H	4	AGONIST
BISPHENOL			OH	OH	96	PARTIAL AGONIST
C						
		X				
7c	$OCH_2CH_2N(CH_3)_2$				100	1.6×10^{-8}
9c	$OCH_2CH{=}CH_2$				42	AGONIST
11c	$OCH_2CH(OH)CH_2OH$				100	8×10^{-9}

[a] IC_{50} is the concentration of compound required to inhibit by 50% the prolactin synthesis produced by 1 nM E_2.

MATERIALS AND METHODS

Compounds. The formulae of compounds used in the study are illustrated in Fig. 1. TAM, 4-OH TAM, ICI 77,949, 3,4-diOH TAM, the ring-stabilized derivative of 4-OH TAM (7c), and the side chain-altered derivatives 9c (allyl) and 11c (glyceryl) were obtained from ICI PLC (Pharmaceuticals Division) (Macclesfield, England). Bisphenol was a gift from Dr. J. A. Katzenellenbogen, Department of Chemistry, University of Illinois (Champaign, Ill.). The other hydroxylated derivatives of tamoxifen, 3-OH TAM, 4'-OH TAM, and 3'-OH TAM, were synthesized as previously described (12). U-0521 was obtained from the Upjohn Company (Kalamazoo, Mich.).

Prolactin assay in primary cultures of pituitary cells. Immature (18 to 21 days old) female rats of the Sprague-Dawley strain were obtained from the Holtzman Company (Madison, Wisc.). The procedures for the maintenance of primary pituitary cell cultures and analysis of prolactin synthesis have been described in detail (9). In the experiments relating to the stability of 3,4-OH TAM, cells were incubated in medium alone or medium containing ascorbic acid (1 μg/ml) or ascorbic acid and the catechol *O*-methyltransferase inhibitor, U-0521 (1×10^{-5} M).

Results were calculated as percentage of prolactin synthesis relative to total protein synthesis. All groups are plotted as percentage prolactin synthesis to be consistent with our previous data (9, 10).

Inhibition of the binding of [^{3}H]estradiol to the estrogen receptor. Uteri from immature rats were dissected free from adhering tissues, frozen, and stored at −70° until use. Cytosol (100,000 × *g* supernatants) were prepared and the assay was performed as previously described (9). The relative binding affinity was calculated from the following relationship: RBA (molar concentration of E_2 for 50% inhibition/molar concentration of competitor for 50% inhibition) × 100.

RESULTS

In a previous study (9), we showed that E_2, 4-OH TAM, TAM, and 4-methyl TAM had equivalent RBAs in rat pituitary gland and uterine cytosol preparations. Since there is no evidence to suggest that compounds will exhibit unique binding properties in pituitary gland

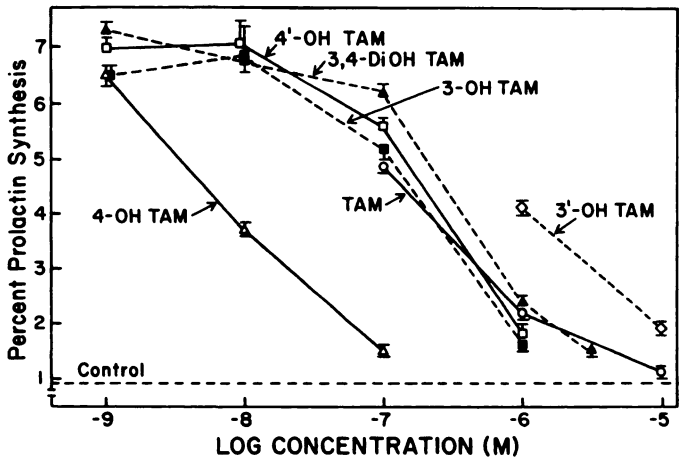


FIG. 1. *Effect of Group A compounds on E_2-stimulated prolactin synthesis in vitro*

Monodispersed pituitary cells (2×10^5/dish) were cultured for 6 days as previously described (10) in medium containing the indicated concentrations of compounds with 1 nM E_2. E_2 alone stimulated prolactin synthesis to a level of 6.8 ± 0.3%. Controls were cultured with medium alone. Prolactin synthesis, expressed as percentage of total protein synthesis, was determined as described previously (10). Values are means ± standard error for three cultures per point.

estrogen receptors, the relative binding affinities of compounds were determined using the rat uterine estrogen receptor. High-affinity binding, relative to E_2, was observed in those compounds with a phenolic group in position 4 of TAM, i.e., 4-OH TAM, 3,4-DiOH TAM, and bisphenol (Table 1). Substitution in position 3 did increase the binding affinity of TAM, but substitution at position 4′ or removal of the side chain did not have a marked effect when compared with tamoxifen. The agonist and antagonist properties of Group A (Table 1) compounds were tested in the prolactin synthesis assay. None of the compounds possessed any agonist properties (Table 2) but did reduce prolactin synthesis below control values. This has been a consistent finding for the nonsteroidal antiestrogens in the assay (9, 10). Each of the compounds was tested for its ability to inhibit E_2 (1 nM)-stimulated prolactin synthesis in a concentration-related manner. 4-OH TAM was the most potent antiestrogen (IC_{50} 7 nM), with a potency approximately 40 times more than that of TAM (IC_{50} 0.3 μM). Substitution of hydroxyls at other positions, 4′-OH TAM or 3-OH TAM, essentially did not improve TAM's potency, and indeed substitution at position 3′ appeared to be deleterious. The anomaly appeared to be 3,4-diOH TAM, with a low antagonist activity in the prolactin synthesis assay (Fig. 1), when compared with its high binding affinity for the estrogen receptor (Table 1). We noticed that fresh alcoholic solutions of the catechol 3,4-diOH TAM discolored within a few days, even if stored in ethanol at 4° with protection from light. It was, therefore, very likely that the catechol was unstable during the cell culture assay. In a separate experiment the prolactin synthesis assay was conducted either in the presence of ascorbic acid (1 μg/ml) as an antioxidant, or ascorbic acid and U-0521, an inhibitor of the enzyme catechol *O*-methyltransferase (EC 2.1.1.6). The results illustrated in Fig. 2 show

TABLE 2

Effect of hydroxylated derivatives of tamoxifen or E_2 alone on basal prolactin synthesis

Pituitary cells (2×10^5/dish) were cultured for 6 days in medium containing 1 nM E_2 or different concentrations of the compounds. Prolactin synthesis was determined previously (9). Values are means ± standard error for three dishes per point.

Compound	Concentration	% Prolactin synthesis
	M	
None		3.6 ± 0.13
E_2	10^{-9}	7.6 ± 0.62
4-OH TAM	10^{-9}	2.6 ± 0.22
	10^{-8}	2.1 ± 0.14
	10^{-7}	1.1 ± 0.04
3-OH TAM	10^{-8}	3.8 ± 0.15
	3×10^{-8}	2.6 ± 0.38
	10^{-7}	1.6 ± 0.31
	3×10^{-7}	1.0 ± 0.08
4′-OH TAM	10^{-7}	1.9 ± 0.38
	3×10^{-7}	1.3 ± 0.05
	10^{-6}	1.3 ± 0.22
	3×10^{-6}	1.3 ± 0.18
3′-OH TAM	10^{-7}	2.3 ± 0.17
	3×10^{-7}	2.0 ± 0.04
	10^{-6}	1.7 ± 0.21

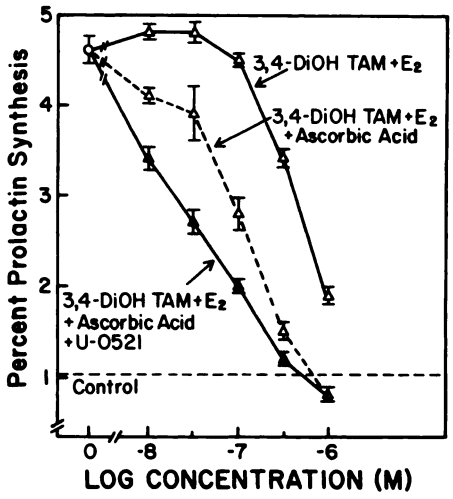


FIG. 2. *Effect of ascorbic acid and U-0521 on the inhibition of E_2-stimulated prolactin synthesis by 3,4-diOH TAM*

Pituitary cells (2×10^5/dish) were cultured for 6 days in three separate types of medium containing increasing concentrations of 3,4-diOH TAM: *1*, 1 nM E_2; *2*, 1 nM E_2 plus ascorbic acid (1 μg/ml); *3*, 1 nM E_2, ascorbic acid, and U-0521 (1×10^{-5} M). Control incubates contained medium or medium supplemented with ascorbic acid or ascorbic acid plus U-0521. The mean value is indicated because each group was not significantly different. The other values are means ± standard error for three cultures per point.

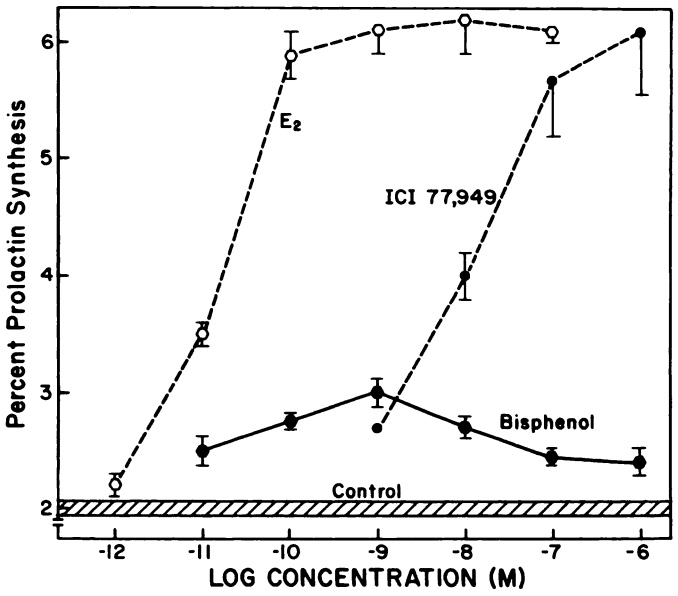


FIG. 3. *Effect of Group B compounds and E_2 on prolactin synthesis in vitro*

Pituitary cells (2×10^5/dish) were cultured for 6 days in medium containing the indicated concentrations of E_2, ICI 77,949, or bisphenol. Control incubates contained medium alone. Values are means ± standard error for three cultures per point.

that the antiestrogenic potency of 3,4-OH TAM (IC_{50} 0.4 μM) was increased 20 times by preventing the deterioration of the catechol (IC_{50} 20 nM).

The Group B compounds, ICI 77,949 and bisphenol (Table 1), were tested in the prolactin synthesis assay as potential agonists (Fig. 3). E_2 was used for comparison. ICI 77,949 was a full estrogen agonist, but, consistent with the affinity for the estrogen receptor (Fig. 2), the

potency was low. The concentration required to produce a 50% maximal response was 2×10^{-8} M, compared with E_2 (3×10^{-11} M). In contrast, bisphenol was a partial agonist over the range of the dose-response curve for E_2. The antiestrogenic activity of bisphenol was demonstrated against 0.1 nM E_2 (Fig. 4), a concentration that produced a maximal increase in prolactin synthesis (Fig. 3). Bisphenol produced a concentration-related inhibition of E_2-stimulated prolactin synthesis.

The Group C compounds (Table 1) were tested as antiestrogens against 1 nM E_2 in the prolactin synthesis assay (Fig. 5). Compound 7c, the fixed-ring structural analogue of 4-OH TAM, was an antiestrogen with an IC_{50} of 16 nM, compared with 4-OH TAM at 4 nM. If the dimethylaminoethoxy side chain was exchanged for a glyceryl (11c) side chain, there was a drop in antiestrogenic potency (IC_{50} 0.6 μM). However, the compound with an allyl side chain (9c) was inactive as an estrogen antagonist but possessed estrogenic activity.

DISCUSSION

The structural requirement for a ligand to bind with high affinity to the estrogen receptor is very specific. The results with the tamoxifen derivatives demonstrate that substitution of a hydroxyl at position 4 in the triphenylethylene system is essential for the correct fit at the ligand-binding site on the estrogen receptor. Even a minor modification in structure, such as the substitution of a phenolic group in position 3 rather than in position 4, results in a reduction in affinity for the receptor (12). Although 3-hydroxytamoxifen has recently been shown to display some increased activity compared with tamoxifen in the control of MCF7 breast cancer cell growth (16), this increased potency was not observed using the inhibition of prolactin synthesis as an assay. However, it should be pointed out that, while the concentrations used to produce antiestrogenic effects with 3-hydroxytamoxifen in the present and previous studies (16) were similar, the comparative activity of tamoxifen against MCF7 cells was much lower (16) than we observed using the prolactin synthesis assay.

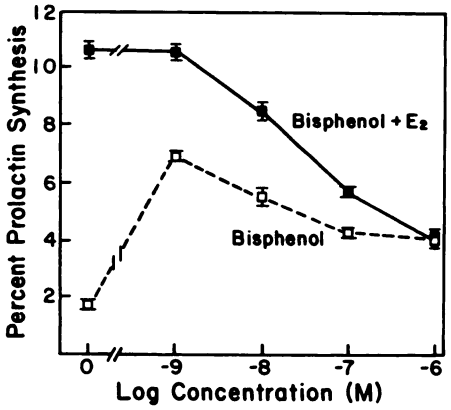


FIG. 4. *Agonist and antagonist properties of bisphenol on prolactin synthesis*

Pituitary cells (2×10^5/dish) were cultured for 8 days in medium containing 0.1 nM E_2 and the indicated concentration of bisphenol or bisphenol alone. Values are means ± standard error for three cultures per point.

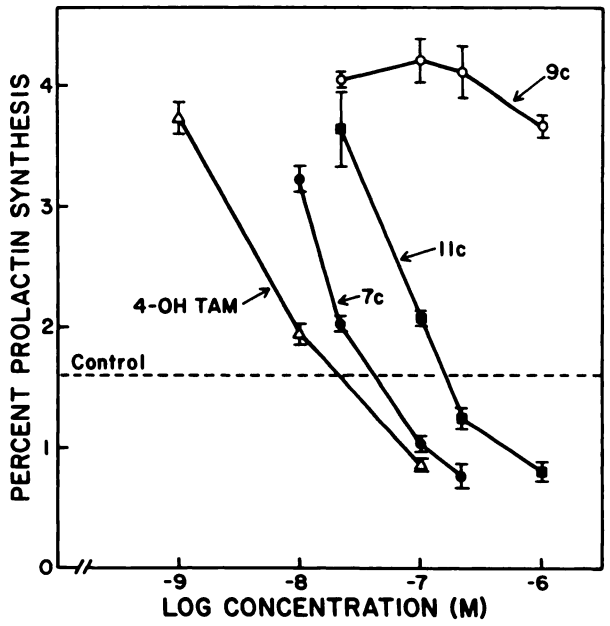


FIG. 5. *Effect of Group C compounds and 4-OH TAM on E_2-stimulated prolactin synthesis in vitro*

Monodisposed pituitary cells (2×10^5/dish) were cultured for 6 days as previously described (10) in medium containing the indicated concentration of compounds with 1 nM E_2. E_2 alone stimulated prolactin synthesis to a level of 3.8 ± 0.2%. Controls were cultured with medium alone. Prolactin synthesis, expressed as percentage of total protein synthesis, was determined as described previously (10). Values are means ± standard error for three cultures per point.

The inconsistency observed between the low biological activity of 3,4-diOH TAM in the prolactin synthesis assay and its high relative binding affinity for the estrogen receptor was reconciled by adding ascorbic acid to the culture medium to prevent oxidation of the catechol to a quinone. A further increase in potency was achieved by addition to the cultures of U-0521, an inhibitor of the enzyme catechol *O*-methyltransferase (17). This inhibitor has previously been used successfully to stabilize steroidal catechol estrogens during incubation with rat uteri *in vitro* (18).

Agonist, partial agonist, or antagonist properties are not predicted from the binding affinities of the compounds for the estrogen receptor. This confirms and extends previous findings (10, 13, 19). Molecular models that describe the interaction of ligands at the estrogen receptor have been proposed (10, 20, 21) which contend that the functions of binding and pharmacological activity are governed by separate areas of the drug. The 4-hydroxyl substituent in a triphenylethylene causes a high-affinity interaction with the receptor and locates the ligand in the correct position so that the phenyl ring with the 4-alkylaminoethoxy side chain is extending away from the binding site. This substituent determines the intrinsic activity of the resulting complex; all antiestrogens with this side chain produce complexes with zero intrinsic efficacy (22, 23) using this assay system (9, 10). This conclusion was further supported by the pharmacological properties of the fixed-ring compounds of Group C (Fig. 1) with different types of side chain. The fixed-ring compounds were used to avoid the potential

isomerization of hydroxylated triphenylethylenes during the assay (24) that might complicate the interpretation of the results. The alkylaminoethoxy side chain was the substitution that produced the highest potency as an antiestrogen, whereas the glyceryl side chain decreased potency and the allyl substitution produced a compound with no antiestrogenic activity. A loss of antiestrogenic activity is noted *in vivo* (14). The present findings indicate that the alteration of the side chain truly reflects an effect at the receptor rather than any change in pharmacokinetics that might be possible *in vivo*. These results appear to support the suggestion (25) that the side chain should contain a lone pair of electrons to hydrogen-bond with the receptor as a prerequisite for antiestrogenic activity.

Bisphenol has a high affinity for the estrogen receptor (26) but was found to be a partial agonist with antiestrogenic activity. The compound is a full agonist *in vivo* (26). The difference in the pharmacology of bisphenol *in vivo* (agonist) and *in vitro* (partial agonist) is particularly interesting because it reflects a similar relative difference in the pharmacology of tamoxifen *in vivo* (partial agonist) and *in vitro* (antagonist). The consistent increase in the estrogenic activity of compounds *in vivo* compared with data *in vitro* cannot be adequately explained at present; however, the results may reflect the metabolic transformation to estrogenic ligands *in vivo* or the involvement of additional factors (not present *in vitro*) that are necessary to express estrogenic activity.

We suggest that partial agonist activity could be explained in terms of our proposed estrogen receptor model (10) by an adaptation of Belleau's macromolecular perturbation theory (15). This hypothesis was originally proposed to explain the agonist, partial agonist, and antagonist activity of drugs at the muscarinic cholinergic receptor. According to Belleau's hypothesis, an agonist binds to the receptor and induces a specific conformational perturbation, and, as a result, the complex has an intrinsic efficacy of 1. An antagonist, on the other hand, binds to the receptor and produces a nonspecific conformational perturbation and a complex with an intrinsic efficacy of zero. Between these extremes, a partial agonist binds to the receptor and produces an equilibrium mixture of agonist and antagonist receptor complexes. Applying these definitions to the estrogen receptor (Fig. 6), E_2 (agonist) binds with high affinity to the resting receptor and induces a specific conformational perturbation which results in the ligand's being locked into the binding site. 4-OH TAM (antagonist) wedges into the resting receptor and only produces a nonspecific conformational perturbation. Bisphenol (partial agonist) interacts at the ligand-binding site, but, although some of the receptors can be induced to lock the ligand into the protein, other ligand interactions are only able to induce a nonspecific conformational perturbation in the complex. While we concede that there may be alternative explanations to explain partial agonist actions at the estrogen receptor, we have proposed this adaptation of Belleau's hypothesis to facilitate a discussion and description of the structure-activity relationships of ligands that can interact with the estrogen receptor binding site.

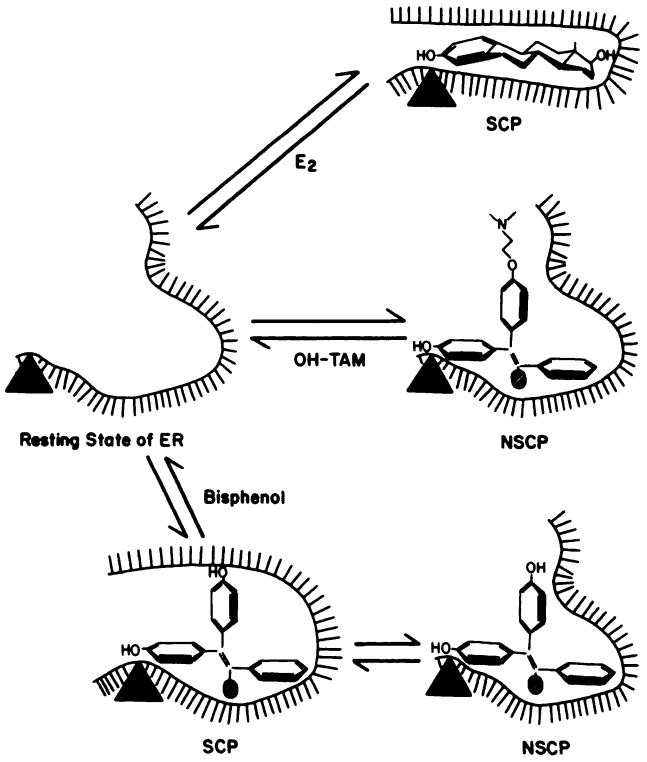


FIG. 6. *Adaptation of Belleau's macromolecular perturbation theory to describe the interaction of agonists, antagonists, and partial agonists with the estrogen receptor (ER)*

The phenol groups on the ligand interact with the phenolic site on the ER (▲) and produce a high-affinity interaction if the geometry of the ligand is correct. E_2 (agonist) induces a specific conformational perturbation (*SCP*), whereas OH-TAM (antagonist) only induces a nonspecific conformational perturbation (*NSCP*). Bisphenol (partial agonist) produces a mixture of SCP and NSCP in the ER.

A consistent finding throughout these and other (24) studies is the exquisite sensitivity of cells in culture to estrogen stimulation. Maximal estrogen-stimulated prolactin synthesis is observed with concentrations of 0.1 nM E_2, which indicates that very few estrogen receptors need to be occupied to induce the response. Although this principle has not been established for the pituitary gland, the rat uterus has been shown to respond maximally with 10% of the nuclear estrogen receptors occupied with E_2 (27). Indeed, the balance of estrogen or antiestrogen action in the rat uterus may be the result of the competitive interaction of agonist or antagonist receptor complexes for acceptor sites within the nucleus (28). The uterus has an excessive pool of estrogen receptors, so that these "spare receptors" are unnecessary for physiological requirements (27, 28). If this concept also applies to the pituitary gland, then limited numbers of agonist or antagonist complexes might compete to modulate prolactin synthesis. Partial agonists may bind to nuclear estrogen receptors to produce an equilibrium mixture of agonist and antagonist receptor complexes. Those complexes will then remain in the same proportion, as the estrogen receptor pool is filled, so that a partial response is maintained.

If the phenolic hydroxyl on the phenyl ring extending away from the binding site is the important structural

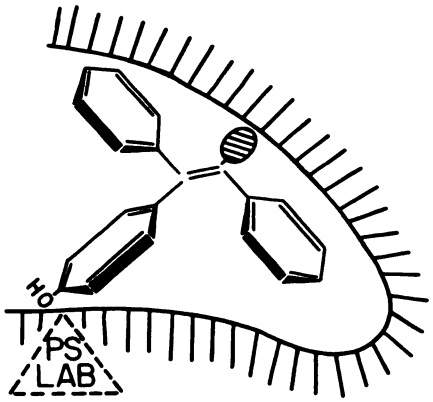


FIG. 7. *Interaction of ICI 77,949 with the ligand-binding site on the estrogen receptor*

The phenol group interacts with the phenolic site (*PS*) on the estrogen receptor, but the geometry of the ligand allows only low-affinity binding (*LAB*). The unsubstituted phenyl ring permits a specific conformational perturbation and the expression of full agonist activity.

feature for partial agonist activity, then ICI 77,949 does not appear to conform to Belleau's hypothesis. Binding at the *trans* stilbene-like part of the molecule would place the phenolic hydroxyl in a position to make the compound a partial agonist rather than the full agonist that is observed. It is believed (29) that the 3-phenolic hydroxyl of E_2 is the most important initial recognition point to draw the steroid toward the binding site before ligand locking. To reconcile the observed pharmacological properties of ICI 77,949 with the ligand binding model, we propose that the dominant site of interaction is via the phenolic hydroxyl to draw the triphenylethylene toward the protein (Fig. 7). However, unlike E_2, ICI 77,949 cannot develop a high-affinity interaction, because the phenyl ring on carbon 2 produces steric inhibition. Nevertheless, a sufficiently high concentration of drug will maintain the ligand at the binding site. In this binding mode, ICI 77,949 has an unsubstituted phenyl ring extending from the binding site which permits a specific conformational perturbation for the complex.

We have previously (10) proposed this type of ligand binding model to describe the interaction of the E isomer of 4-OH TAM. The phenol group interacts at the phenolic site on the receptor, but the ligand has a low affinity for the receptor because the nonplanar structure results in the unsubstituted phenyl ring producing steric inhibition. The remaining phenyl ring that contains the alkylaminoethoxy side chain prevents specific conformational perturbation and induces only nonspecific conformational perturbation. The pharmacology of (E) 4-OH TAM is as an antiestrogen with zero intrinsic activity in this assay (10).

In summary, we have described the high-affinity binding requirements for a hydroxylated triphenylethylene-antiestrogen and suggested that an adaptation of Belleau's macromolecular perturbation theory could be used to explain the partial agonist activity of bisphenol. Also, at present, the proposed models apply only to the pharmacological activity of estrogens and antiestrogens on the modulation of prolactin synthesis *in vitro* and may not be appropriate to describe events *in vivo* in other estrogen target tissues like the rat uterus. Further structure-activity relationship studies are described in the companion paper (30) to probe and challenge this molecular model for estrogen and antiestrogen action *in vitro*.

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