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Estrogen-Stimulated Prolactin Synthesis *in Vitro*

Classification of Agonist, Partial Agonist, and Antagonist Actions Based on Structure

V. CRAIG JORDAN AND MARA E. LIEBERMAN[1]

Department of Human Oncology, University of Wisconsin, Madison, Wisconsin 53792

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SUMMARY

An *in vitro* assay that depends upon the synthesis of prolactin by primary cultures of dispersed cells from immature rat pituitary cells was used to study the structural requirement for estrogen action. Two categories of estrogens were identified: full estrogens (agonists) and partial estrogens (partial agonists) with antiestrogenic actions against the effects of 0.1 nM estradiol (E_2). All of the agonists [diethylstilbestrol (DES), dimethylstilbestrol (DMS), R2858, and RU16117] produced a dose-related increase in prolactin synthesis equivalent to E_2, although potencies were different: E_2 = DES = R2858 > RU16117 > DMS. Partial agonists [ICI 3188, tri(4-hydroxyphenyl)chloroethylene, and bisphenol] each had bis(4-hydroxyphenol) substitutions at the ethylene double bond and stimulated prolactin synthesis only to about 50% of the maximal response observed with E_2. Trianisylchloroethylene was weakly active as a partial agonist, but at high concentration (10 μM) was able to decrease prolactin synthesis produced by 0.1 nM E_2. Previous studies from these laboratories showed that triphenylethylene derivatives with a strategically located alkyl aminoethoxyside chain are complete E_2 antagonists with no agonist activity. Two series of novel compounds were assayed to determine whether their structures would predict biological activity. LN2299, the *cis* geometric isomer of a triphenylbromethylene, was a full agonist with activity equivalent to zuclomiphene, the *cis* geometric isomer of clomiphene. Cyclofenyl was a partial agonist, but deacetylation to the diphenol increased partial agonist activity and potency. However, introduction of a single pyrrolidinoethylside chain into the deacetylated cyclofenyl increased antiestrogenic potency and completely suppressed the expression of agonist activity. Finally, LN2833, with a novel $C(OH)CH_3$ side chain in the position normally occupied by the alkylaminoethoxyside chain of most antiestrogens, produced antiestrogen activity with no estrogen properties. Antiestrogenic potency was increased in LN2839 by a phenol in the triphenylethylene in a position equivalent to the 3-phenolic hydroxyl of E_2. In general, the potency and biological properties could be predicted by reference to the structure of the molecule. Potent estrogens or antiestrogens have a phenolic hydroxyl in a position that would be equivalent to the 3-phenolic hydroxyl of E_2. Partial agonist action is predicted by a 4-hydroxyphenol attached to the same carbon as the phenyl ring equivalent to the A-ring of E_2. Extending the side chain (acetyl, aminoethoxy) or other substitutions (e.g., ethyl, 1-ethanol) alters the resulting estrogen-receptor complex in such a way as to prevent the expression of prolactin synthesis. These results, with a broad range of compounds, support the general proposals of Belleau's macromolecular perturbation theory.

INTRODUCTION

Estradiol stimulates the synthesis and secretion of prolactin by normal and neoplastic cells of the pituitary gland (1–3), an effect that is believed to be mediated by the ER[2] (4–7). The traditional model for the mechanism of action of E_2 depicts ER binding the steroid in the cytoplasmic compartment of the cell followed by activation and translocation of the complex to the nucleus to

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[1] Present address, Institute of Endocrinology, Sheba Medical Center, Tel Hashamer, Israel.

[2] The abbreviations used are: ER, estrogen receptor; E_2, estradiol;

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initiate estrogen-stimulated events (8, 9). The model has recently been revised based upon the finding that unoccupied ER is associated with the nuclear compartment of GH_3 rat pituitary cells following enucleation with cytochalasin B (10). Furthermore, immunocytochemical techniques have demonstrated ER in the nuclei of breast cancer cells in the absence of estrogen (11). Obviously, these experiments require further confirmation, but the results indicate that the cytosolic ER may be an artifact produced by extraction from the nuclear compartment during tissue homogenization.

Nonsteroidal antiestrogens decrease estradiol-stimulated elevations in circulating prolactin in the rat (12, 13) and competitively inhibit the synthesis of E_2-stimulated prolactin synthesis *in vitro* (14). The antiestrogens are *complete* antagonists of prolactin synthesis *in vitro* (14–16), which contrasts with their well-documented partial estrogenic effects in the uterus *in vivo* (17, 18).

We have established and validated (14) the use of primary cultures of rat pituitary gland cells to assay the structural requirements for a compound to influence prolactin synthesis. The assay has been used to extend our earlier (14, 15, 19) structure-activity relationship studies and to develop further a model for estrogen action based upon the ER binding ligand. The results demonstrate that compounds can be classified by this particular assay *in vitro* into agonists, antagonists, and partial agonists based upon their structure.

EXPERIMENTAL PROCEDURES

Materials. Immature (18-day-old) female rats of the Sprague-Dawley strain were obtained from the Holtzman Company (Madison, Wis.). Tissue culture media and sera were from Grand Island Biological Company (Grand Island, N. Y.). E_2, DES, and other reagents were from Sigma Chemical Company (St. Louis, Mo.). DMS was a gift from Professor C. W. Emmens, University of Sydney (Sydney, Australia). R2858 and RU16117 were from Centre de Recherches Rousell UCLAF (Romainville, France). The structures of these compounds are shown in Table 1. ICI 3188 (Fig. 2) was from ICI PLC, Macclesfield (Cheshire, England), and bisphenol (Fig. 2) was kindly provided by Dr. J. A. Katzenellenbogen, University of Illinois (Urbana, Ill.). TACE and zuclomiphene were from Merrell-Dow (Cincinnati, Ohio). TCE was synthesized by Dr. R. R. Brown, Department of Human Oncology, University of Wisconsin (Madison, Wis.) (Fig. 1). The cyclofenyl derivatives were kindly provided by Dr. R. S. Kapil, Central Drug Research Institute (Lucknow, India). The triphenylbromoethylene derivatives (LN2299, LN2839, and LN2833) were a gift from Dr. H. Pinhas, Recherche Laroche Navarron (Leuville, France) (Table 2). The RBAs of the compounds using immature rat uterine cytosols were determined as previously described (19).

Prolactin assay in primary cultures of pituitary cells. The procedures for the maintenance of primary pituitary cell cultures and analysis of prolactin synthesis have been described in detail (14). Results were calculated as percentage of prolactin synthesis relative to total protein synthesis. All graphs are plotted as percentage prolactin synthesis to be consistent with our previous data (14, 15).

DES, diethylstilbestrol; DMS, dimethylstilbestrol; R2858, 11β-methoxy-17-ethynyl-1,3,5(10)-estratriene-3,17β-diol; RU16117, 11α-methoxy-17-ethynyl-1,3,5(10)-estratriene-3,17β-diol; TACE, trianisylchloroethylene; TCE, 1,1,2-tri(4-hydroxyphenyl)chloroethylene; ICI 3188, 1,1,2-tri(4-hydroxyphenyl)prop-1-ene; LN2299, LN2839, and LN2833, triphenylbromoethylene derivatives; RBA, relative binding affinity.

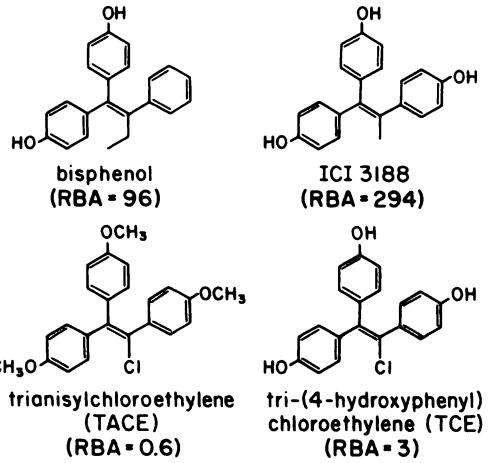
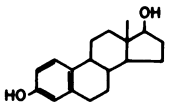
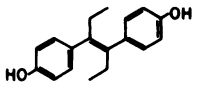
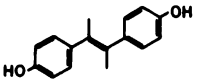
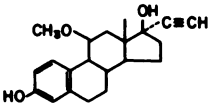
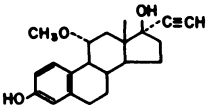


FIG. 1. *Partial estrogenic compounds used in the study* The RBAs are shown in parentheses.

TABLE 1

Summary of the RBA and potency of the estrogenic compounds to stimulate protein synthesis in vitro

Name	Formula	RBA	Potency[a]
			M
estradiol		100 (100)	1×10^{-11}
diethylstilbestrol (DES)		140 (120)	3×10^{-11}
dimethylstilbestrol (DMS)		≈10	3×10^{-9}
Moxestrol R2858		192 (54)	3×10^{-11}
RU16117		4 (3)	4×10^{-10}

[a] Potency is estimated as the concentration of compound required to produce prolactin synthesis that is 50% of maximum. The RBA of DMS is estimated from the data presented by Capony and Rochefort (20) and Terenius (21). The RBAs were determined either at 30° for 30 min or, in parentheses, at 4° for 18 hr.

RESULTS

In the first series of experiments, the estrogenic activity and RBAs of a group of steroids and stilbenes (Table 1) was determined in the prolactin assay. Our primary aim during these studies was to determine whether all of these compounds were fully estrogenic *in vitro*, as several reports have documented the antiestrogenic activity of DMS (22, 23) and RU16117 (24, 25) *in vivo*. Under the assay conditions employed *in vitro*, DES had estrogenic activity equivalent to that of E_2; and while DMS was fully estrogenic, it had approximately 1/30 the potency of DES (Fig. 2). DMS has a lower RBA for the estrogen receptor (20, 21) that would account for a decrease in *X*

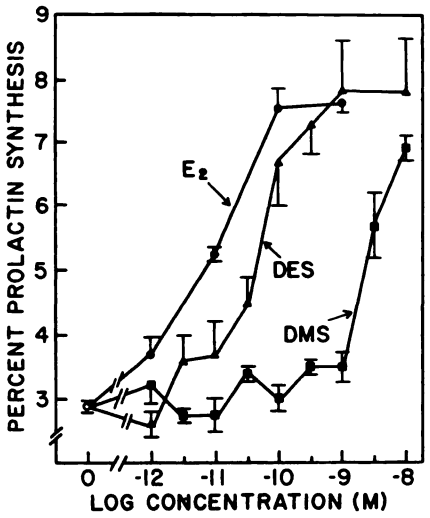


FIG. 2. *Estrogenic effect of E_2, DES, and DMS on prolactin synthesis*
Pituitary cells (2×10^5/dish) were cultured for 8 days in medium containing the indicated concentrations of compounds. Values are means ± standard error for three cultures per point.

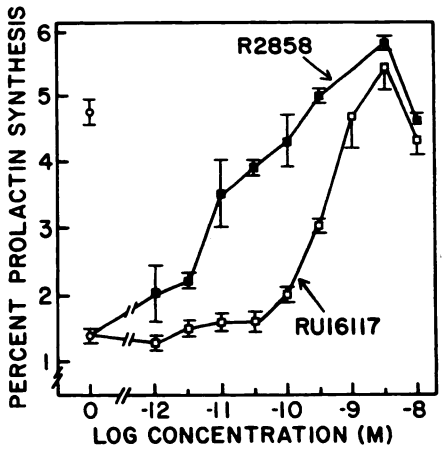


FIG. 3. *Estrogenic effect of R2858 and RU16117 on prolactin synthesis*
A single concentration of E_2 (0.1 nM) was used for comparison purposes of a full agonist response (O). Pituitary cells (2×10^5/dish) were cultured for 6 days in medium containing the indicated concentrations of steroids. Values are means ± standard error for three cultures per point.

potency. The steroid R2858 had estrogenic activity equivalent to that of DES, and its isomer, RU16117, was fully estrogenic with a potency approximately 1/30 that of R2858 (Fig. 3). Again, a decrease in the RBA can account for the decrease in potency of RU16117 compared with R2858.

Bisphenol (Fig. 2) has a high binding affinity for the estrogen receptor (RBA = 96) but partial estrogenic and antiestrogenic activity in the prolactin synthesis assay. The range of concentrations tested was 10^{-11}–10^{-6} M, and the preliminary results are reported in the companion paper (19). The tri-*p*-hydroxylated triphenylpropylene, ICI 3188, was tested for its estrogenic activity over the dose range 10^{-11}–10^{-6} M. Using the standard assay conditions, with medium changes every 3 days, ICI 3188 was able to induce prolactin synthesis only at a rate approximately 50% of that achieved by E_2 assayed over the same concentration range. We were concerned that the compound was unstable *in vitro*, so two further experiments were undertaken with medium/compound changes daily and twice daily. The results illustrated in Fig. 4 were obtained with daily medium changes. ICI 3188 was unable to achieve the full estrogenic response observed with E_2. Partial estrogen agonists, by definition, occupy all of the receptors, but the resulting receptor complexes are unable to elicit full estrogenic responses. Full occupation of receptors by the partial estrogen will deny full estrogens the opportunity to bind and elicit of biological response. ICI 3188 inhibited the fully estrogenic effect of E_2 in a dose-related manner (Fig. 5). This estrogen antagonism was competitive and reversible, as illustrated in Fig. 6. Pretreatment of cell cultures for 2 days with ICI 3188 (10^{-8} M) before addition of increasing

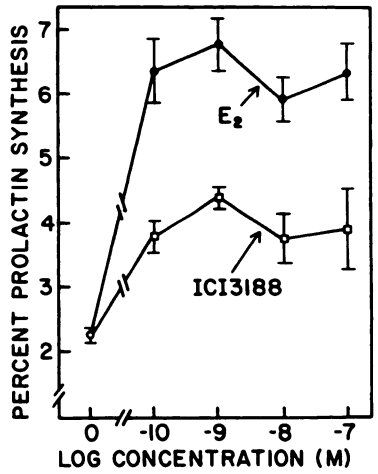


FIG. 4. *Partial estrogenic effect of ICI 3188 compared with E_2 on prolactin synthesis*
Pituitary cells (2×10^5/dish) were cultured for 6 days in medium containing the indicated concentrations of compounds (medium was changed daily). Values are means ± standard error for three cultures per point.

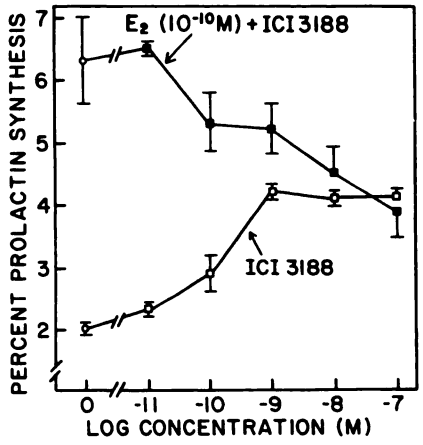


FIG. 5. *Partial estrogenic and antiestrogenic effect of ICI 3188 on prolactin synthesis*
Pituitary cells (2×10^5/dish) were cultured for 6 days with medium containing the indicated concentrations of ICI 3188 alone or with E_2 (0.1 nM). Values are means ± standard error for three cultures per point.

E_2 concentrations did not prevent the expression of the full effects observed by E_2 alone. Treatment of the cells with ICI 3188 (10^{-8} M) throughout the experimental period demonstrated an antagonism of the full effects of E_2. The antiestrogenic action could be reversed by an increase in the concentration of E_2 (Fig. 6).

Similar experiments were conducted using the compounds (Fig. 1) TACE and its demethylated derivative, TCE. Again, a tri-*p*-hydroxylated triphenylethylene (TCE) was a partial estrogen with antiestrogenic properties against E_2 (0.1 nM). The antiestrogenic effect could also be reversed by increasing the concentration of E_2 10-fold (Fig. 7). TACE was less estrogenic and a less potent E_2 antagonist.

At this point in the work, we were able to identify two types of estrogens: those that were full agonists (Table 1) and those that were partial agonists with antagonist properties (Fig. 1). The classification was based upon the structure of the compound, rather than the RBA of the compound (Table 1; Fig. 1). All of the partial estrogens have a *p*-hydroxyphenyl substituted in the stilbestrol nucleus. Previous studies (15, 24) had demonstrated that an aminoethoxy side chain in the position of the phenolic hydroxyl produces a full estrogen antagonist.

We sought to consolidate and confirm these conclusions with two novel series of compounds (Table 2). The derivatives of cyclofenyl (Table 2) were tested first. Cyclofenyl (C), was a partial estrogen and was able to inhibit, in a dose-related manner, E_2 (0.1 nM)-stimulated prolactin synthesis (Fig. 8). Deacetylation to produce the diphenol (COH) increased the estrogenic activity, but the compound was still a partial estrogen agonist. However, introduction of an aminoethoxy side chain to produce compound C_1 completely removed all estrogenic activity and converted the compound to a potent antiestrogen.

The LN series of triphenylethylenes are interesting because they have a *p*-ethyl substitution instead of the

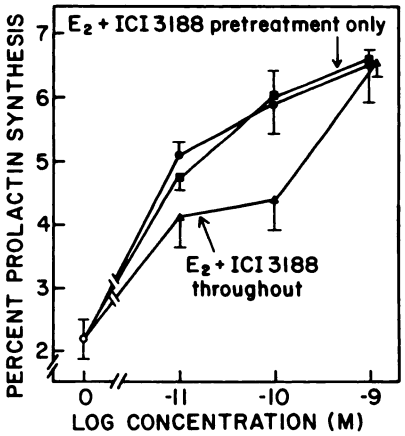


FIG. 6. *Competitive and reversible antiestrogenic effect of ICI 3188 on prolactin synthesis*

Pituitary cells (2 × 10^5/dish) were cultured for 2 days with ICI 3188 (10 nM) or in control medium. New medium was added containing the indicated concentrations of E_2. Three comparisons were made: E_2 alone, pretreatment with ICI 3188 followed by E_2, and E_2 with ICI 3188 throughout. Values are means ± standard error for three cultures per point.

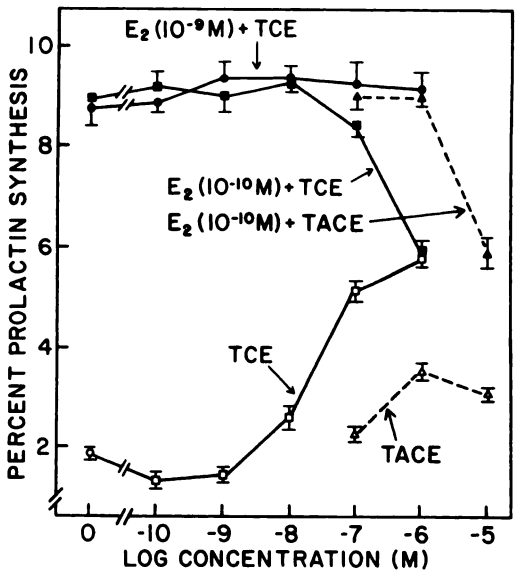


FIG. 7. *Partial estrogenic and antiestrogenic effect of TCE and TACE on prolactin synthesis*

Pituitary cells (2 × 10^5/dish) were cultured for 8 days with medium containing the indicated concentrations of TCE or TACE alone or with E_2 (0.1 or 1 nM). Values are means ± standard error for three cultures per point.

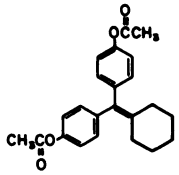
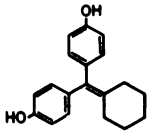
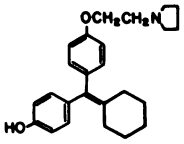
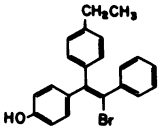
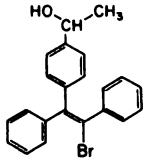
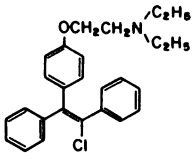
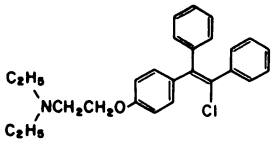
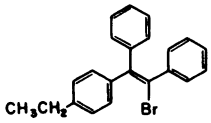
aminoethoxy side chain normally present in antiestrogens. Compound LN2839 was more potent as an antiestrogen than compound LN2833, and neither of the compounds was estrogenic (Fig. 9). In contrast, LN2299, the *cis* geometric isomer of a substituted triphenylethylene, was fully estrogenic. We have previously shown that the *cis* geometric isomer of clomiphene, zuclomiphene, is a full estrogen in the prolactin synthesis assay, whereas the *trans* geometric isomer, enclomiphene, is an antiestrogen (Table 2) (15). The potency of LN2299 was equivalent to that of zuclomiphene (Fig. 10).

DISCUSSION

These studies, which classify estrogens into full agonists, partial agonists, and antagonists, confirm and extend our previous findings using the prolactin synthesis assay *in vitro* (14, 15, 19). This analytical approach to the structure-activity relationships of estrogens *in vitro* avoids the complications of metabolism and pharmacokinetics experienced *in vivo* (26). It is, therefore, possible to dissect the contribution of a particular structural modification of a compound that binds to the estrogen receptor, to affect the biology of an estrogen target tissue. The results from the compounds *in vitro* are particularly interesting when compared with their known pharmacology *in vivo*. Zuclomiphene (27, 28), ICI 3188 (29), and TACE (30) are classified as full agonists in immature rat uterine weight tests. The contrasting pharmacology of TACE *in vivo* and *in vitro* probably involves the demethylation of TACE *in vivo* to phenolic metabolites. Polar metabolites of TACE have been isolated from rat feces; however, these were not specifically identified (30). Recently, the metabolism of TACE *in vitro* has been described by Ruenitz and Toledo (31). Incubation of TACE with male rat microsomes produces a mixture of mono-

TABLE 2

Summary of the RBA and either potency or IC_{50} of groups of compounds tested in the study either to stimulate or inhibit estradiol-stimulated prolactin synthesis in vitro

Name	Formula	RBA	IC_{50}[a] or potency[b]
			M
cyclofenyl (C)		500 (45)	3×10^{-9} a
deacetylcyclofenyl (COH)		500 (40)	PARTIAL AGONIST
Compound C_1		1063 (71)	3×10^{-8} a
LN-2839		83 (40)	8×10^{-8} a
LN-2833		0.5 (0.3)	1×10^{-6} a
enclomiphene		8	3×10^{-7} a
zuclomiphene		0.3	3×10^{-8} b
LN-2299		0.2 (0.1)	1×10^{-8} b

[a] The concentration of compound required to inhibit by 50% the prolactin synthesis produced by 1 nM E_2. The RBA was determined either at 30° for 30 min or, in parentheses, at 4° for 18 hr.

[b] Potency is estimated as the concentration of compound required to produce prolactin synthesis that is 50% of maximum.

phenolic metabolites which consists primarily of a 1:1 mixture of E- and Z-desmethylchlorotrianisene.

It is interesting to observe that the partial estrogenic activity of ICI 3188 *in vitro* is translated to full estrogenic activity in a uterine weight assay *in vivo*. Similarly, the compounds that are complete estrogen antagonists *in*

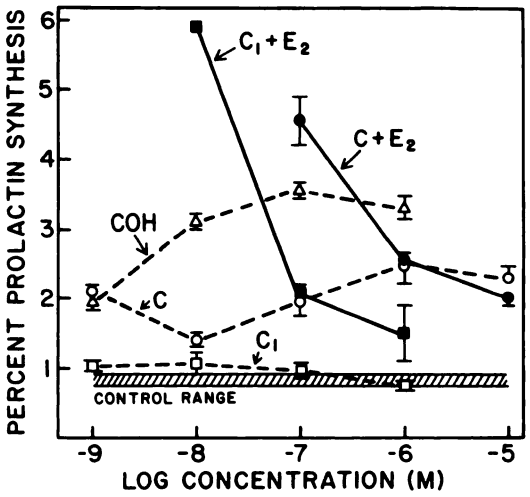


FIG. 8. *Partial estrogenic and antiestrogenic effect of cyclofenyl derivatives (see Table 2 for structures) on prolactin synthesis*

Pituitary cells (2×10^5/dish) were cultured for 6 days with medium containing the indicated concentrations of compounds alone (C_1 and C) or with E_2 (1 nM). E_2 alone gave a value of prolactin synthesis of 6.2 ± 0.1%. Values are means ± standard error for three cultures per point.

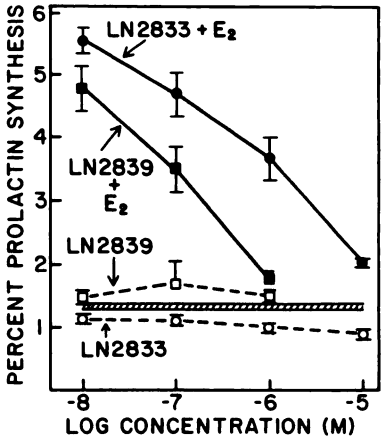


FIG. 9. *Antiestrogenic effect of LN2833 and LN2839 (see Table 2 for structures) on E_2 (1 nM)-stimulated prolactin synthesis*

Pituitary cells (2×10^5/dish) were cultured for 6 days with medium containing the indicated concentrations of compounds alone or with E_2. E_2 alone gave a value of prolactin synthesis of 6.5 ± 0.2%. The *shaded area* indicates the range of control cultures containing medium alone. Values are means ± standard error for three cultures per point.

vitro (14, 15) are partial estrogen agonists in uterine weight assays *in vivo* (19). In the present studies, this is exemplified by the cyclofenyl derivative C_1 (Table 2), which was without estrogenic activity *in vitro* (Fig. 8) but is reported to be a partial estrogen agonist in rat uterine weight tests (32). There is, therefore, a consistent increase in the agonist activity of compounds when they are assayed *in vivo* compared with data derived *in vitro*. The reason for this is at present unknown. Nevertheless, compound C_1, which is related in structure to the triphenylethylene-type of antiestrogens, inhibits estrogen action *in vivo* (32) and *in vitro* (Fig. 8).

The antiestrogenic activity exhibited by the triphenylethylene derivatives *in vivo* and *in vitro* is not consistently observed with the stilbene derivative DMS and the

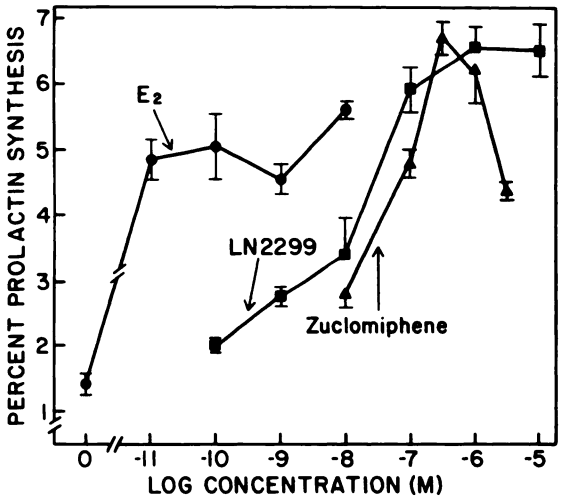


FIG. 10. *Estrogenic effect of E_2, LN2299, and zuclomiphene (see Table 2 for structures) on prolactin synthesis*

Pituitary cells (2×10^5/dish) were cultured for 6 days with medium containing the indicated concentrations of compounds. Values are means ± standard error for three cultures per point.

steroid RU16117. Although both DMS and RU16117 were fully estrogenic in the present studies, DMS exhibits antiestrogenic activity when administered intravaginally in Allen-Doisy assays in ovariectomized mice (20), and RU16117 has antitumor activity against the induction of rat mammary carcinoma with dimethylbenzanthracene (24). Repeated systemic administrations of either DMS or RU16117 produces only estrogenic activity. Martin (23) and Bouton and Raynaud (25) have postulated that the antiestrogenic activity is related to the rapid dissociation rate of the ligand from the estrogen receptor. Thus antiestrogenic activity occurs *in vivo* by RU16117 blocking the binding of E_2 to the estrogen receptor, but the steroid rapidly dissociates from the complex to prevent the expression of full agonist action. Maintenance of ligand concentrations in the area of the target tissue (repeated administration, Silastic implants, or cell culture) would be expected to increase the number of receptor complexes in the target cells and produce a full agonist response. In contrast, the antiestrogenic activity of the triphenylethylene type of antiestrogen is based upon structure (15).

The potency of estrogens and antiestrogens is related to their affinity for the estrogen receptor (15). High-potency compounds have a phenolic hydroxyl in a position on the molecule equivalent to the 3-phenolic hydroxyl of E_2. Estrogenic activity is lost in a ligand with a correctly positioned alkylaminoethoxy side chain, which is a recurrent structural feature of the nonsteroidal antiestrogens. Partial estrogenic activity is returned to the ligand receptor complex by compounds that are anchored at the ligand-binding site by a phenolic group equivalent to the 3-phenolic hydroxyl of E_2 but with another phenolic group projecting away from the stilbene-like structure. Bisphenol, ICI 3188, TCE, and the deacetylated derivative of cyclofenyl typify this category. A short extension of the phenol by either acetylation (cyclofenyl) or methylation (TACE) reduces the estrogenic activity of the ligand.

These findings strongly support the adaptation (19) of Belleau's macromolecular perturbation theory to explain agonist, partial agonist, and antagonist actions (33). However, the alkylaminoethoxy side chain (cf. enclomiphene) is not an *absolute* requirement for antiestrogenic activity, since a side chain as short as an ethyl group is effective (LN2839). Borgna and co-workers (34) have previously shown that the phenolic derivative, LN2839, has biological activity *in vitro* (inhibition of the growth of MCF breast cancer cells) equivalent to that of tamoxifen. Similarly, in the present study, LN2839 was approximately equipotent with our previous results with tamoxifen (14, 15) in the prolactin synthesis assay. The relative potencies of LN2839 and LN2833 as antiestrogens was consistent with their relative binding affinities for the estrogen receptor (34). These findings are particularly significant because it has been suggested (35) that a side chain containing an alcohol or amine (with a lone pair of electrons) is required to hydrogen-bond to the relevant part of the receptor to prevent estrogen action. This conclusion was supported by the loss of antiestrogenic activity by a compound substituted with an allyl oxy side chain (19). However, it is possible that the oxygen atom hinges the alkyl side chain into the wrong position in space. The ethyl side chain in LN2839 remains in the correct position to maintain antiestrogenic properties. The *cis* geometric isomer of the halogenated triphenylethylene that is substituted with an ethyl group (LN2299) instead of an alkylaminoethoxy side chain (zuclomiphene) is fully estrogenic. The result is again consistent with the proposed estrogen receptor model for estrogen action (15).

In summary, we have described categories of ligand that have either agonist, partial agonist, or antagonist actions. The classification is based upon the structure of the ligand; specific substitutions in a particular position in the ligand can control the subsequent biological activity. Future studies to investigate additional substitutions of estrogen receptor binding ligands will enable a precise mapping of the structural restrictions upon biological activity.

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Send reprint requests to: Dr. V. Craig Jordan, Department of Human Oncology, Wisconsin Clinical Cancer Center, University of Wisconsin, 600 Highland Avenue, Madison, Wis. 53792.