An Estrogen Receptor Model to Describe the Regulation of Prolactin Synthesis by Antiestrogens in Vitro*

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A hypothetical model of the ligand interaction with the estrogen receptor binding site has been developed to describe the structural features necessary to initiate or to inhibit prolactin synthesis in vitro. The biological potency of the binding ligands is directly related to their relative binding affinity (RBA) for the estrogen receptor. The relative potencies of antiestrogens to inhibit estradiol-stimulated prolactin synthesis was trans-monohydroxytamoxifen = LY117018 > trioxifene > enclomiphene = cis-monohydroxytamoxifen = tamoxifen, consistent with their RBAs for uterine estrogen receptor. Similarly the relative potency of estrogen to stimulate prolactin synthesis was diethylstilbestrol = estradiol > ICI 77,949 > ICI 47,699 = zuclo- miphen, consistent with their RBAs. The compound LY128412 (trioxifene without the aminooxy side chain) did not interact with the estrogen receptor at the concentrations tested (10^{-6}-10^{-8}m) or exhibit estrogenic or antiestrogenic properties using the prolactin synthesis assay. Overall, the ligand-receptor model stresses the structural requirement for high affinity binding and the critical positioning of the alkylaminooxy side chain in space (in relation to the ligand-binding site on the estrogen receptor) to prevent prolactin synthesis.

The monosteroidal antiestrogens inhibit the binding of estradiol to the estrogen receptor (Katzenellenbogen et al., 1979; Sutherland and Jordan, 1981). Viewed superficially, this property can explain the inhibition of estrogen action. However, the precise molecular events that occur to confer low intrinsic activity to the antiestrogen-estrogen receptor complex within target cells are unknown. Based upon the observed estrogenic and antiestrogenic properties of geometric isomers of substituted triphenylethylenes in vivo, a ligand-receptor model has been proposed (Jordan et al., 1981) that describes ligand interaction to produce estrogen (high intrinsic activity) or antiestrogenic (low intrinsic activity)-receptor complexes. A ligand with a high affinity for the estrogen receptor has a correctly positioned phenolic hydroxyl group that is capable of binding at the position of the receptor normally occupied by the 3 phenolic hydroxyl of estradiol. The estrogen diethylstilbestrol (Korenmann, 1969) and the antiestrogen monohydroxytamoxifen (Jordan et al., 1977; Coezy et al., 1982) are good examples of compounds with high affinity for the estrogen receptor, high biological potency, but opposing biological properties. It is apparent from these observations that the antiestrogenic properties are related to the structure of the ligand rather than its affinity.

Several studies in vivo have emphasized the importance of the size and shape of the alkylaminooxy side chain (a recurrent structural feature of antiestrogens) for biological activity (Lednicer et al., 1966; Clark and Jordan, 1976; Jordan et al., 1981). Similarly, in the proposed ligand receptor model there is emphasis on a correctly positioned alkylaminooxy side chain for the ligand to be able to inhibit estrogen action.

In the present study we have used the estrogen responsive pituitary cell culture system (Lieberman et al., 1978, 1983) to challenge the proposed model (Jordan et al., 1981) directly with a broad range of structurally related monosteroidal estrogens and antiestrogens. The results demonstrate the features necessary for a ligand to initiate or to inhibit pituitary prolactin synthesis and relate the biological potency of a ligand (whether it is an estrogen or antiestrogen) with its relative binding affinity for the estrogen receptor.

EXPERIMENTAL PROCEDURES

Materials—Immature (18 to 21-day-old) female rats of the Sprague-Dawley strain were obtained from Holtzman Co. (Madison, WI) and King Animal Laboratories (Oregon, WI). Estradiol-17β, diethylstilbestrol, and other reagents were from Sigma. Tamoxifen (trans-1-(4-hydroxyphenyl)-1,2 diphenylbut-1-ene), trioxifene (3,4-dihydro-2-(4-methoxyphenyl)-1-naphthal- enyl-4,5-(1-pyrrolidinyl)-ethoxy phenyl)methanone methanesulfonic acid salt), LY126412 (3,4-dihydro-2-(4-methoxyphenyl)-1-naphthal- enyl-4-hydroxyphenyl)methanone, and LY117018 (6-hydroxy-2-(4-hydroxyphenyl)-benzo[b]thien-3-yl-4-[2-(1-pyrrolidinyl)ethoxy- phenyl)methanone) were obtained from Lilly.

Methods—Procedures for the maintenance of primary pituitary cell cultures, analysis of prolactin synthesis, and inhibition of [3H]estradiol binding to uterine cytosol have been described in detail (Lieberman et al., 1983). In the experiments relating to the stability of the cis geometric isomer of monohydroxytamoxifen, stock solutions were made in ethanol and stored at 6°C for 1, 2, 3, or 4 weeks or 1
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Summary of the RBA and potency of the estrogenic compounds to stimulate prolactin synthesis in vitro

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>RBA</th>
<th>Potency (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol</td>
<td></td>
<td>100</td>
<td>2 x 10^-11</td>
</tr>
<tr>
<td>DES</td>
<td></td>
<td>141</td>
<td>1.5 x 10^-11</td>
</tr>
<tr>
<td>ICI 77,949</td>
<td></td>
<td>1.2</td>
<td>2 x 10^-8</td>
</tr>
<tr>
<td>ICI 47,699</td>
<td></td>
<td>0.4</td>
<td>3 x 10^-7</td>
</tr>
<tr>
<td>Zuclophene</td>
<td></td>
<td>0.3</td>
<td>1 x 10^-7</td>
</tr>
<tr>
<td>LY126412</td>
<td></td>
<td>inactive</td>
<td>inactive</td>
</tr>
</tbody>
</table>

* Potency is calculated as the concentration (M) of compound required to produce prolactin synthesis that is 50% of maximum.

Table II

Summary of the RBA and the 50% inhibitory concentration (IC50) of the antiestrogenic compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>RBA</th>
<th>IC50 (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY117018</td>
<td></td>
<td>167</td>
<td>7 x 10^-9</td>
</tr>
<tr>
<td>monohydroxytamoxifen (trans)</td>
<td></td>
<td>300</td>
<td>3 x 10^-9</td>
</tr>
<tr>
<td>monohydroxytamoxifen (cis)</td>
<td></td>
<td>13</td>
<td>1 x 10^-7</td>
</tr>
<tr>
<td>Trioxifene</td>
<td></td>
<td>8</td>
<td>3 x 10^-7</td>
</tr>
<tr>
<td>Enclomiphene</td>
<td></td>
<td>2</td>
<td>1 x 10^-4</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td></td>
<td>6</td>
<td>1 x 10^-6</td>
</tr>
</tbody>
</table>

* The IC50 is the concentration (M) of compound required to inhibit by 50% the prolactin synthesis produced by 1 nM estradiol.

RESULTS

Each of the test compounds was examined for its ability to inhibit the binding of [3H]estradiol to uterine estrogen receptor and the RBA was calculated. The RBAs for all the compounds are shown in Tables I and II but an example of the binding curves is shown in Fig. 1. The new compounds LY117018 and trioxifene were more potent inhibitors of [3H] estradiol binding than tamoxifen. The RBA of LY117018 was the same as that of monohydroxytamoxifen. The cis isomers of tamoxifen (ICI 47,699) and clomiphene (zuclophene) had approximately 10-fold lower binding affinities than their respective trans isomers, whereas cis-monohydroxytamoxifen had a 100-fold lower binding affinity than its trans form. The effect of removing the aminoethoxy side chain from tamoxifen and trioxifene on the relative binding affinity was examined and compared with that of estradiol and diethylstilbestrol (Tables I and II). Removal of the side chain from tamoxifen to produce the compound ICI 77,949 did not affect its binding to the receptor. However, removal of the side chain from trioxifene to produce the compound LY126412 essentially destroyed its affinity for the receptors at the concentrations tested (up to 10^-6 M).

The biological activity and relative potency of each of the above compounds was assessed in the estrogen-responsive pituitary cell system. Consistent with the receptor-binding data, the compound LY117018 had a potency similar to that of trans-monohydroxytamoxifen at inhibiting estradiol-stimulated prolactin synthesis; trioxifene was 15-30 times less active (Fig. 2). The importance of the geometric shape of the ligand on its biological activity was investigated. Tamoxifen and enclomiphene inhibited estradiol-stimulated prolactin synthesis over a similar concentration range, whereas their respective cis isomers, ICI 47,699 and zuclophene, were estrogenic, i.e. both compounds stimulated prolactin synthesis and neither displayed antagonistic properties (Table I). Only the experi-

1 The abbreviation used is: RBA, relative binding affinity.

Fig. 1. Inhibition of the binding of [3H]estradiol-17β to estrogen receptors derived from immature rat uterine cytosol by estradiol (E2) (A); diethylstilbestrol (DES) (C); monohydroxytamoxifen (OH-TAM) (X); LY117018 (E) trioxifene (O); and tamoxifen (TAM) (O). Specific binding was determined as previously described (Lieberman et al., 1983). Points are means of duplicate incubates.
Fig. 2. Effect of antiestrogens on basal and estradiol-stimulated rates of prolactin synthesis. Monodispersed pituitary cells (2 × 10^6/dish) were cultured for 6 days as described previously (Lieberman et al., 1983) in medium containing the indicated concentrations of monohydroxytamoxifen (OH-TAM) (ΔΔ), LY17018 (ΔΔΔΔ), and trioxifene (O---O) alone or with 1 nM estradiol (closed symbols with solid line, respectively). Prolactin synthesis, expressed as per cent of total protein synthesis, was determined as described (Lieberman et al., 1983). Values are means ± S.E. for 3 cultures/point.

Fig. 3. Differential effects of cis and trans isomers of antiestrogens on prolactin synthesis. Pituitary cells (2 × 10^6/dish) were cultured for 6 days in media containing the indicated concentrations of the compounds. A, tamoxifen (TAM) (trans, □); tamoxifen + 1 nM estradiol (●); ICI 47,699 (cis-tamoxifen, ○); ICI 47,699 + 1 nM estradiol (●●●●). B, trans-monohydroxytamoxifen (OH-TAM) (ΔΔ); cis-monohydroxytamoxifen (OH-TAM) (ΔΔΔΔ); cis-monohydroxytamoxifen + 1 nM estradiol (●●●●); cis-monohydroxytamoxifen (stored at 25 °C for 7 days) + 1 nM estradiol (●●●●). Values are means ± S.E. for 3 cultures/point.

ments with tamoxifen and ICI 47,699 are shown in Fig. 3A but enclomiphene and zuclophene produced a similar result. A comparison between the geometric isomers of monohydroxytamoxifen demonstrated that both isomers inhibited estradiol-stimulated prolactin synthesis, although the cis isomer was approximately 100-fold less potent (Fig. 3B).

We considered the possibility that cis-monohydroxytamoxifen might be sufficiently unstable in solution to convert fractionally to the more potent trans form. We, therefore, investigated the effects of storage of solutions on receptor-binding activity. This method has great sensitivity, since conversion of 50% of the cis to the trans isomer would result in 100-fold increase in the total binding affinity (Table II). Storage of cis-monohydroxytamoxifen for up to 4 weeks did not alter its binding affinity. Similarly, there was no detectable difference in antiestrogenic potency between a week old and a freshly prepared solution of the compound; both preparations inhibited prolactin synthesis to a similar extent (Fig. 3B).

The importance of the aminoethoxy side chain for the antiestrogenic activity of tamoxifen and trioxifene was investigated. Removal of the side chain from tamoxifen, yielding the compound ICI 77,949, converted it to a fully estrogenic ligand (Fig. 4) having a potency approximately 0.1% of estradiol or diethylstilbestrol (Table I). In contrast, removal of the side chain from trioxifene, yielding the compound LY126412, resulted in a molecule which possessed neither estrogenic (Fig. 4) nor antiestrogenic properties at the concentrations tested (up to 10^-5 M) (data not shown). This finding is consistent with the lack of affinity of LY126412 for the estrogen receptor (Table I).

DISCUSSION

This study demonstrates that the modulation of prolactin synthesis in cultured pituitary cells is influenced by specific changes in the structure of the ligand that binds to the estrogen receptor. Overall, the potency of a compound is dependent upon its relative binding affinity, but as previously noted (Ferguson and Katzenellenbogen, 1977; Jordan et al., 1977), a structural change that increases the affinity of an antiestrogen for the estrogen receptor does not change the potential intrinsic activity of the drug. Structural modifications of the ligand that determine affinity for the receptor and the subsequent intrinsic activity of the ligand-receptor complex are separate.

The simple interaction of estradiol-17β with a hypothetical binding site on the estrogen receptor has been taken as a starting point to evolve a model for drug-receptor interaction that results in the stimulation or inhibition of prolactin synthesis (Fig. 5). It should be noted that we perceive the ligand-binding site to be a small part of the whole receptor protein. Estradiol has a high affinity for the receptor, which is dependent upon a free phenolic group. Substitution of the phenolic hydroxyl reduces the affinity for the receptor and reduces estrogenic potency (Eisenfeld, 1974). To expand the model to consider estrogenic and antiestrogenic action, two factors appear to be important: interaction with a phenolic site on the receptor to impart high affinity binding and the potential interaction at a second site, a hypothetical antiestrogenic region, to prevent the expression of estrogen action.
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To address directly the role of side chain interaction at a hypothetical antiestrogenic region, we compared the biological activity of tamoxifen and trioxifene with their respective derivatives without this substitution. Removal of the side chain from tamoxifen to produce IC1 77,949 essentially did not alter the estrogenic activity, since the aminoethoxy side chain without this substitution. Removal of the side chain resembles the estrogenic triphenylethylenes but LY126412 is inactive. The inability of LY126412 to bind to the estrogen receptor at the concentrations tested can be viewed from two perspectives. Binding at the 3,4-dihydronaphthalene ring system would be very weak without the presence of a 6-phenolic hydroxyl (Mueller and Kim, 1978). Alternatively, attempts by the free phenol of LY126412 to bind at the phenolic side would be complicated by the flexed nature of the molecule at the ketone group (Table I). Mueller and Kim (1978) have investigated the ability of various alkyl phenols to compete with [\(^{3}H\)]estradiol at the estrogen-binding site and concluded that any para substitution of a simple phenol should be hydrophobic to mimic the B ring of estradiol-17\(\beta\). Using this criterion, LY126412 would not be expected to bind to the receptor.

Among the triphenylethylenes, compounds that have cis and trans geometric isomers are extremely important for the development of a ligand-receptor model because the isomeric molecules encompass both estrogenic and antiestrogenic activity. Several reports have described the contrasting biological properties of the geometric isomers of tamoxifen (Harper and Walpole, 1966; Jordan et al., 1981) and enclomiphene (Clark and Guthrie, 1981; Jordan et al., 1981) in vivo. The isomers have low relative binding affinities (Skidmore et al., 1972) for estrogen receptors from the uterus and the pituitary gland, but the cis isomers (zuclomiphene and ICI 47,699) are estrogenic in the rat. Although a geometric requirement for estrogenic activity has been stressed previously, the data derived from observations in vivo could not exclude the possibility that the cis isomers are preferentially metabolized to estrogens in the liver before binding in the target tissue. This is clearly not the case, as both zuclomiphene and ICI 47,699 (cis tamoxifen) are directly estrogenic in cultured pituitary cells. It has recently been confirmed that there is very little metabolism of ICI 47,699 to hydroxylated metabolites with high affinity for the estrogen receptor (Robertson et al., 1982).

To describe the interaction of the geometric isomers with the estrogen receptor, the trans stilbene-like structure of tamoxifen and enclomiphene could sit loosely at the binding site, with low affinity binding, so that the phenyl ring substituted with the \(\alpha\)-alkylaminoethoxy side chain is projected out of the plane of the stilbene-like system (Fig. 5A). The estrogenic ligands zuclomiphene and ICI 47,699, with their low affinity for the estrogen receptor, can create a trans stilbene-like structure with the para substituted phenyl ring. In this binding state, the aminoethoxy side chain would lie next to the phenolic site with a weak interaction with the ether oxygen (Fig. 5B). There would be no interaction of the side chain with the antiestrogenic region and, as a result, no inhibition of estrogen action.

Introduction of a phenolic hydroxyl into tamoxifen to produce monohydroxytamoxifen increases both the affinity for the estrogen receptor and antiestrogenic activity (Jordan et al., 1977; Coezy et al., 1982). A similar high affinity for the estrogen receptor and potent antiestrogenic activity was observed with the fixed ring phenolic compound LY117018 (Table II and Black et al., 1981). Both monohydroxytamoxifen and LY117018 can bind with high affinity at the phenolic site on the receptor with a weak interaction with the ether oxygen (Fig. 5B). There would be no interaction of the side chain with the antiestrogenic region and, as a result, no inhibition of estrogen action.
droxytamoxifen essentially did not alter the relative binding affinity (Tables I and II).

The antiestrogenic properties of the cis isomer of monohydroxytamoxifen would appear to be inconsistent with the ligand-binding model for cis isomers unless the interaction of the free phenolic group with the complimentary phenolic site on the receptor is preferable to the loose fit of the trans stilbene-like structure incorporating the phenyl ring with the side chain at the ligand-binding site. Compared with the exact fit of trans-monohydroxytamoxifen at the binding site, the steric inhibition implicit in the cis-monohydroxytamoxifen molecule would explain the low affinity interaction. Nevertheless, in this binding state, the phenyl ring substituted with the alkylaminoethoxy side chain would be in a position in space analogous to the other antiestrogenic compounds for interaction with the antiestrogenic region (Fig. 5).

Overall this direct study of the structure activity relationships of nonsteroidal estrogens and antiestrogens strongly supports a model for antiestrogen action that requires the correct positioning in space of the aminoethoxy side chain relative to the main ligand-binding site.

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