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EVALUATION OF TWO TYPES OF ESTROGEN INHIBITION WITH REGARD TO EFFECTS ON UPTAKE AND BINDING OF [^{3}H]-ESTRADIOL IN THE UTERUS

L. J. BLACK and R. J. KRAAY
Lilly Research Laboratories, Eli Lilly & Company, Indianapolis, Indiana 46206, U.S.A.

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SUMMARY

A variety of anti-estrogenic substances have been examined for their direct and indirect effect on the binding of [^{3}H]-estradiol with uterine cytoplasmic receptors. A distinct separation of the compounds into 2 classes of inhibitors was observed.

The non-steroidal anti-estrogens and the C^{19} steroids, except Norgestrel, interacted with cytoplasmic binding sites. Testosterone, norprogesterone, the C^{21} steroids and Norgestrel did not suppress binding *in vivo* or *in vitro*. These results demonstrate that interference with estradiol binding to uterine cytoplasmic receptor sites is not essential for the inhibition of estrogen responses by some of the most potent anti-estrogenic substances.

[^{3}H]-Estradiol uptake was enhanced by low doses of compounds which at higher doses inhibited uptake. This observation demonstrates the need for a dose response when examining this aspect of competition. Direct interaction with uterine cytoplasmic binding sites in a cell-free system was observed with agents which reduce [^{3}H]-estradiol uptake *in vivo*. After *in vivo* injection of estradiol, uterine cytoplasmic binding capacity for [^{3}H]-estradiol is reduced within the first 5 h, followed by a recovery phase. The dynamics of this process appear to be influenced by the dose of estrogen administered. Interference with the recovery phase is suggested as a possible mechanism of inhibition for compounds having no effect on cytoplasmic binding of estrogen.

INTRODUCTION

MANY compounds have demonstrated the ability to interfere with the action of estrogen on the uterus. These include both natural and synthetic steroids as well as non-steroidal agents. A single mode of action is highly improbable for all of these substances due to the diversity of structure and biological activity.

The bioassays which showed various substances to be anti-estrogenic were based on the reduction of an end-point response to estrogen. This type of information reveals little about the mechanisms involved and a need for additional insight is apparent.

When Jensen and Jacobson[1] observed the selective uptake and retention of [^{3}H]-estradiol by target tissues, the possibility for further study of the early events in the action of estrogen was opened. Since that report, interest in the mechanism of estrogen action has concentrated on binding with specific proteins in target tissue and subsequent intracellular events. Clark and Gorski[2] observed that the estrogen–protein complex of uterine cytoplasm binds to nuclear material of various tissues as well as ground glass. They recognized this as a simple technique for the assay of estrogen–receptor interaction. [^{3}H]-Estradiol was shown to move to the cell nucleus after binding to cytoplasmic receptors[3]. A loss of uterine cytoplasmic binding capacity after exposure of whole tissue to estrogen has also been

described[3–6]. The results of such studies indicate that the methodology is applicable to the assessment and classification of estrogen inhibitors. This report examines the effect of a variety of agents on the uptake and binding of [^{3}H]-estradiol in the uterus.

EXPERIMENTAL

In vivo *uptake*

Immature Cox Standard mice weighing 11–13 g were used in groups of 10. The compounds were dissolved or finely suspended in corn oil and administered subcutaneously in 0·1 ml. Four h later the mice received 1 μCi [^{3}H]-estradiol (6·4 ng) in 0·1 ml 5% ethanol–saline by subcutaneous injection. One h later, uteri were quickly removed at sacrifice, dissected free of extraneous tissue and weighed after uniform blotting. Individual uteri were then dissolved in 1·0 ml NCS solubilizer (Nuclear, Chicago) with gentle warming. Diotol scintillator was added and d.p.m. determined by liquid scintillation spectrometry using an internal standard. The d.p.m./mg for each group and the percent difference from untreated controls were calculated.

In vitro *binding*

Rats (Holtzman strain) 21 days old, weighing 45–55 g were obtained from Harlan Industries. Using the method of Clark and Gorski[2] uterine tissue was homogenized at 4°C in TMK buffer at a concentration of 0·1 ml/5 mg tissue. A pool of cytosol was isolated in an International centrifuge at 2000 rev./min for 15 min at 4°C (850 *g*). Two ml of cytosol was added to tubes containing 2 μCi [^{3}H]-estradiol (12·8 ng) or tracer plus compound. Compounds were prepared in 1% carboxy methyl cellulose–saline solution at a concentration of 100 μg/0·1 ml. After 10 min at 4°C aliquots of 0·25, 0·5 and 1·0 ml of cytosol were transferred to tubes containing 100 mg ground glass 170–200 mesh. Each sample was incubated 30 min at 25°C with shaking. The glass pellets were washed 3 times in 3 ml TMK buffer and centrifuged at 2000 rev./min for 10 min after each wash. Radioactivity remaining bound to the glass was extracted with 3 ml ethanol to which methylcellosolve–toluene scintillator was added. Disintegrations per minute were determined using an internal standard.

Uterine binding capacity

Immature rats in groups of six were given the compounds subcutaneously in 0·1 ml of corn oil. Each group was sacrificed at the designated time and uteri were homogenized in TMK buffer, 0·1 ml/5 mg. The method described for *in vitro* binding was followed with the exception that each pooled group was handled separately and a 1·0 ml aliquot was assayed for each group. Untreated controls represented 100% binding capacity and the percent difference for treated groups was calculated.

Source of compounds

[6,7^{3}H]-Estradiol 17β (42·5 Ci/mmol) was obtained from New England Nuclear Corp. Chlormadinone, Provera, Northindrone and norprogesterone were provided by Syntex Laboratories Inc. Norgestrel was obtained from Wyeth Laboratories, Norethynodrel from Roussel Corp. and Ethynodiol diacetate from G. D. Searle & Co. Richardson–Merrill Inc. supplied Chlomiphene and MER-25.

U-11, 100-A was provided by the Upjohn Co., and CN-55,945-27 by Parke, Davis & Co. Estradiol, progesterone, testosterone and nortestosterone were obtained commercially.

RESULTS

Effects on uptake of [^{3}H]-*estradiol in the mouse uterus* in vivo

Figure 1 illustrates the influence of a range of doses of the compounds on uterine uptake of [^{3}H]-estradiol. Unlabeled estradiol reduced uptake of tracer estrogen at high doses (1-A). However, a significant enhancement in uptake was observed within a certain range resulting in a biphasic dose response curve. The

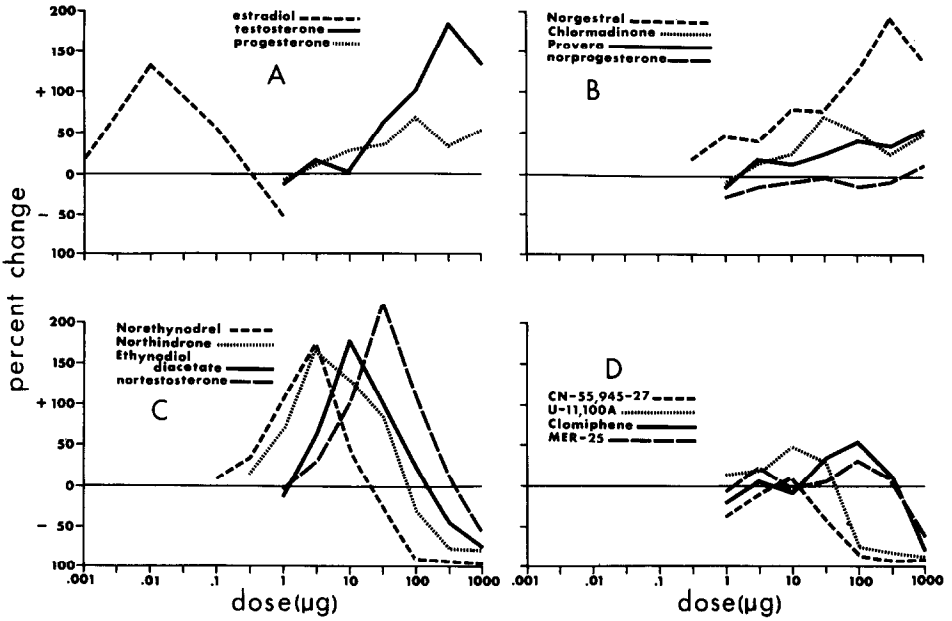

Fig. 1. The effect of a range of doses of various compounds on the uptake of [^{3}H]-estradiol in the mouse uterus. Compounds were administered subcutaneously 5 h before sacrifice in 0·1 ml corn oil and 1 μCi [^{3}H]-estradiol was injected subcutaneously in 0·1 ml ethanol–saline 1 h prior to sacrifice. Each point represents at least 10 animals, and the results are expressed as per cent difference of the mean d.p.m./mg from controls receiving only the tracer.

conditions under which estradiol produces this effect have been described in an earlier report[7]. For each compound there were doses at which a sub-maximal effect on estrogen uptake was observed. These results show that arbitrary selection of dose will not adequately determine if competition is present. Examination of a large range of doses revealed distinct differences among the compounds. Progesterone and testosterone did not reduce estrogen binding but they both significantly increased uterine accumulation of estrogen under these conditions.

Nortestosterone and some closely related synthetic progestins show a striking similarity in the dose response curves to that of estradiol although higher doses were required (1-C). Under these conditions these steroids appeared to compete for estrogen binding sites.

In another group of steroids, only norprogesterone appeared to have little effect on estrogen uptake (1-B). The remainder of these compounds increased uptake, but demonstrated no inhibition. The dose response curves of Chlormadinone and Provera are like that of progesterone while Norgestrel produced the large stimulation of uptake of [^{3}H]-estradiol similar to that seen with testosterone.

The effect of some non-steroidal agents is shown in 1-D. All of these compounds appeared to compete for estrogen binding sites. However, their enhancement of estrogen uptake was much less than that of the steroids.

Effects on interaction of [^{3}H]*-estradiol with uterine cytoplasmic binding sites* in vitro

In order to examine the direct effect of the compounds on the interaction of [^{3}H]-estradiol with cytoplasmic binding sites, a cell-free system was utilized. Binding of [^{3}H]-estradiol is demonstrated by the control group in Fig. 2. Addition of unlabelled estradiol depressed the binding curve proportional to the amount of estradiol added, thus demonstrating competition for the binding sites.

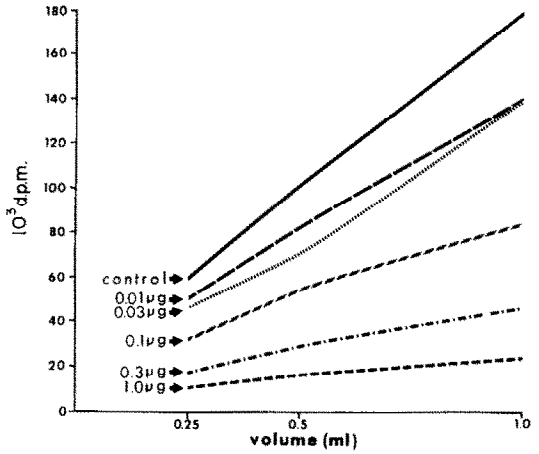

Fig. 2. The effect of various concentrations of unlabeled estradiol on binding of [^{3}H]-estradiol in isolated rat uterine cytosol. 2 ml samples were taken from a pool of cytosol and added to tubes containing 2 μCi [^{3}H]-estradiol plus the indicated amounts of estradiol. Aliquotes were transferred to tubes containing 100 mg ground glass for assay of [^{3}H]-estradiol binding.

All of the compounds were tested in a similar fashion at a concentration of 100 μg/2 ml (Fig. 3). At least 2 separate experiments were performed with each compound. The percentage of reduction from controls was determined at each of the three aliquot volumes and pooled for statistical evaluation. The compounds which produced monophasic dose response curves on [^{3}H]-estradiol uptake all demonstrate less than 15% reduction in [^{3}H]-estradiol binding in this system. MER-25 and Ethynodiol diacetate revealed moderate effects within the 40–60% range of depression while all other agents demonstrate a strong interaction in the 60–90% range of reduction.

Effects of in vivo *treatment on uterine binding capacity for* [^{3}H]*-estradiol*

In the uptake studies the ability of the compounds to reduce accumulation of

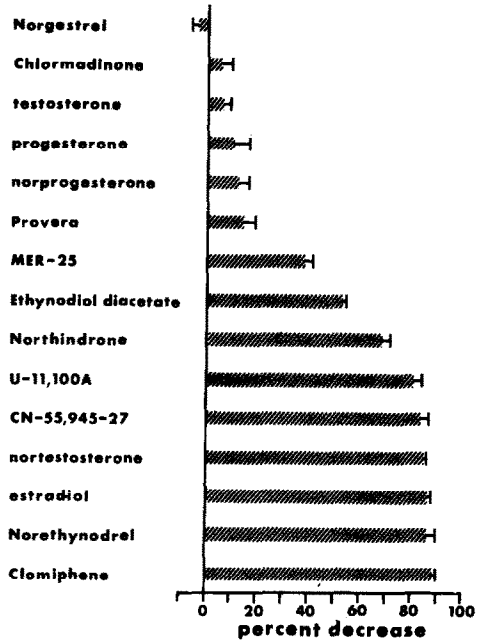

Fig. 3. The binding of [^{3}H]-estradiol in rat cytosol in the presence of anti-estrogens. 2 ml samples of cytosol were taken from a pool and added to tubes containing 2 μCi [^{3}H]-estradiol plus 100 μg of anti-estrogen. Estradiol was tested at 1 μg. Aliquots of 0·25, 0·5 and 1·0 ml were transferred to tubes containing 100 mg ground glass for assay of [^{3}H]-estradiol binding. The per cent reduction was calculated at each aliquot volume and the data were pooled for statistical evaluation. Results are expressed as mean ± S.D. per cent difference from controls receiving only [^{3}H]-estradiol.

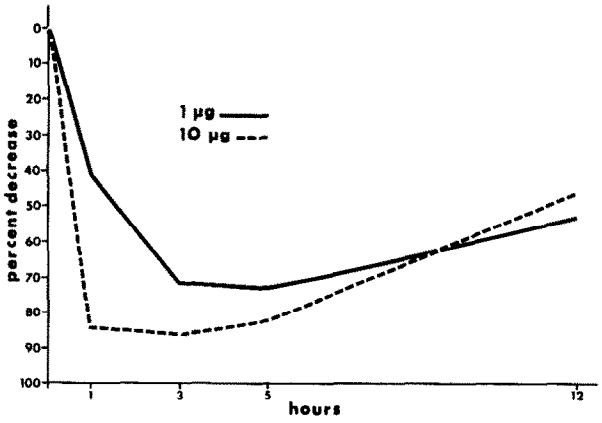

Fig. 4. The effect of *in vivo* subcutaneous administration of estradiol on rat uterine cytoplasmic binding capacity for [^{3}H]-estradiol. Animals were injected at 0 time and groups of six were sacrificed at the indicated times. Two ml of cytosol from each group was added to tubes containing 2 μCi [^{3}H]-estradiol. A 1·0 ml aliquot was transferred to 100 mg ground glass for assay of [^{3}H]-estradiol binding. Results are expressed as per cent reduction from untreated controls.

estrogen was determined, but presumably a small portion of the available sites was occupied by the tracer or a non-competitive process may have been involved. The *in vitro* studies attempted to assess the ability of the compounds to interact directly with cytoplasmic binding sites free of the *in vivo* metabolic factors. In this series of experiments the compounds were administered *in vivo* and then the total

Table 1. The effect of *in vivo* administration on cytoplasmic binding capacity for [^{3}H]-estradiol

Compound	Dose	1 h	3 h	5 h	12 h
Estradiol	1 μg	−41*	−72*	−73*	−54*
	10 μg	−85	−86	−82	−46
Testosterone	100 μg	−11	−5	−13	
	1 mg	+12	+7	−24	
Progesterone	100 μg	−6	+7	+15	
	1 mg	+1	+6	+12	
Nortestosterone	100 μg	+2	−13	−44	
	1 mg	−63	−86	−86	
Norprogesterone	100 μg	−5	−29	−22	
	1 mg	−14	−12	−23	
Norethynodrel	100 μg	−65	−80	−77	
	1 mg	−56	−75	−82	
Northindrone	100 μg	−45	−69	−62	
	1 mg	−78	−82	−85	
Ethynodiol Diacetate	100 μg	−11	−30	−27	
	1 mg	−17	−67	−69	
U-11, 100-A	100 μg	+1	−36	−27	
	1 mg	−28	−71	−74	
CN-55, 945-27	100 μg	−12	−36	−78	
	1 mg	−56	−88	−90	
Clomiphene	100 μg	−9	−21	−24	
	1 mg	−11	−86	−87	
MER-25	100 μg	+17	−6	+15	
	1 mg	−7	−26	−24	
Chlormadinone	100 μg	+18	+12	+20	
	1 mg	+8	+5	−2	
Norgestrel	100 μg	+18	−8	−18	
	1 mg	−14	+2	−21	
Provera	100 μg	−8	−21	−19	
	1 mg	−5	+15	−18	

*Results expressed as per cent difference from untreated controls.

binding capacity for [^{3}H]-estradiol in the cytoplasm was determined by the *in vitro* method. This was attempted in order to establish if under *in vivo* conditions the available number of cytoplasmic sites was reduced due to the treatment.

Figure 4 illustrates the reduction of the *in vitro* cytoplasmic binding of [^{3}H]-estradiol following an injection of unlabeled estradiol. The number of available sites in the cytoplasm was reduced considerably within the first 5 h. After maximum reduction was reached a progressive return toward normal levels was apparent. The amount of estradiol was also significant since the larger dose depletes the uterine cytoplasm of binding capacity more rapidly.

In Table 1 the effect of the compounds at 1, 3 or 5 h after subcutaneous administration of 100 μg or 1 mg is shown. Compounds that previously demonstrated a lack of interaction with binding sites have little effect on the availability of these sites to [^{3}H]-estradiol. Conversely, those agents that have indicated an interaction reduced cytoplasmic binding sites available to [^{3}H]-estradiol.

DISCUSSION

In a discussion of anti-estrogens Emmens and Martin[8] commented on two types of estrogen inhibition. They suggested that synthetic progestins may act in a non-competitive manner similar to progesterone. Eisenfeld and Axelrod observed that Northindrone[9] and Norethynodrel[10] reduced [^{3}H]-estradiol uptake in target tissues, but Chlormadinone[9], progesterone and testosterone[11] did not. In a preliminary report, we demonstrated opposing effects of certain progestins on [^{3}H]-estradiol uptake[12].

The present report established two general classes of anti-estrogenic activity by direct comparison of a variety of substances evaluated under three conditions of estrogen binding in the uterus. A separation of the progestins into two classes of anti-estrogenic activity is confirmed and suggests structural relationships. The C^{21} steroids (type B) had little if any effect on estradiol binding but the C^{19} steroids (type A) Norethynodrel, Northindrone, Ethynodiol Diacetate and nortestosterone demonstrated effects which suggest competition for estrogen receptors. The need to establish the distinction between type A and type B inhibitors is demonstrated by Norgestrel. It is a nortestosterone derivative; yet these studies classify it with progesterone. This places it in sharp contrast with the remaining nortestosterone compounds.

The non-steroidal agents demonstrated an interaction with estrogen binding sites. MER-25 appeared relatively weak in this respect. This is in agreement with other reports demonstrating the competitive nature of these agents to estrogen binding[13–17].

The type A estrogen inhibitors probably contribute to the overall estrogenic effects, since these agents have been shown to produce estrogenic effects themselves[18, 21–23]. The type B inhibitors have generally failed to demonstrate estrogenicity[18]. Results presented here suggest that this is associated with the failure to bind to uterine estrogen receptors, an event considered essential to estrogenic responses. Although both types of compounds reduce estrogenic responses quantitatively, there may be dramatic qualitative differences. The need for insight into the mechanism of type B inhibition warrants greater study and consideration in the development and application of anti-estrogens and progestins. Many possibilities exist as cytoplasmic receptor binding is relatively early in

Table 2. Classification of compounds as type A or type B estrogen inhibitors

Method	Uptake	*In vitro* binding	Binding capacity
	Type A		
Estradiol	**	**	**
Nortestosterone	*	**	**
Norethynodrel	**	**	**
Northindrone	**	**	**
Ethynodiol Diacetate	**	*	**
CN-55,945-27	**	**	**
U-11,100-A	**	**	**
Clomiphene	**	**	**
Mer-25	**	*	*
	Type B		
Progesterone	○	○	○
Norprogesterone	○	○	*
Testosterone	○	○	*
Chlormadinone	○	○	○
Norgestrel	○	○	*
Provera	○	○	*

○ <15%.
* >15% <60%.
** >60%.

the series of events leading to estrogenic responses, and all events subsequent to binding present a potential site of action.

The significance of the increased accumulation of [^{3}H]-estradiol by the uterus is unknown. A dose of estriol (0·03 μg) which enhances estrogen uptake, but has no uterotropic effect, given 5 h prior to various doses of estradiol increased the uterotropic responses in 3 days[21]. Yet 100 μg Chlormadinone, which is also effective in stimulating estradiol uptake, reduces the uterotropic effects of estradiol equally well when given 5 h prior to or simultaneously with the estrogen (unpublished data). Possibly there are qualitative differences in the stimulation of uptake observed here. Progesterone increases the uptake of [^{3}H]-estradiol by stromal tissue[22], and this may explain the increases observed with the type B inhibitors.

Other investigators have demonstrated the loss of uterine cytoplasmic binding capacity within the first few hours after exposure to estrogen[3–6]. Data shown in Fig. 4 indicate that after *in vivo* administration of estrogen the rate of loss and recovery of binding capacity are influenced by the dose of estrogen. The higher dose caused a more rapid onset of receptor depletion and an earlier recovery. These data suggest that both processes occur simultaneously. Competitive interaction at cytoplasmic binding sites clearly is not essential for the inhibition of estrogenic responses. Finn and Martin[26] demonstrated that progesterone and Norgestrel (type B inhibitors) have no effect on uterine response to an initial injection of estrogen, but prevent a further response to subsequent estrogen. This was due to a refractory state of the uterus induced by estrogen and prolonged by the progestins. Perhaps the refractory state induced by estrogen is due to depletion of

cytoplasmic receptors (Fig. 4). Type B progestins may exert their anti-estrogenic activity by preventing the regeneration of binding sites.

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