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POSSIBLE MECHANISMS FOR THE AGONIST ACTIONS OF TAMOXIFEN AND THE ANTAGONIST ACTIONS OF MER-25 (ETHAMOXYTRIPHETOL) IN THE MOUSE UTERUS*

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Abstract—Experiments were conducted to determine why tamoxifen, a non-steroidal antiestrogen, is uterotrophic in mice, whereas MER-25 (ethamoxytriphetol), a structurally related compound, is antiuterotrophic. Initial experiments indicated that the pituitary was not required for a uterotrophic response in mice to either estradiol (E_2), tamoxifen (TAM), or 4-hydroxytamoxifen (4-OH-TAM) MER-25 was not uterotrophic in mice but was capable of completely inhibiting the uterotrophic responses of mice to estrogens (E_2) as well as antiestrogens (TAM and 4-OH-TAM); this inhibition was reversible by increasing the dose of the antiestrogen (TAM). The relative binding affinities (RBA) of TAM, 4-OH-TAM, and MER-25 to mouse uterus estrogen receptor (ER) and mouse liver antiestrogen binding sites (AEBS) were compared to determine whether either (or both) of these sites mediate the biological effects of these compounds. E_2 is arbitrarily assigned an RBA of 100 for ER; similarly, TAM is assigned an RBA of 100 for AEBS. MER-25 bound to AEBS with an RBA of 8.9 and to ER with an RBA of < 0.06; in contrast, TAM and 4-OH-TAM bound to AEBS with RBAs of 100 and 53, respectively, and to ER with RBAs of 2 and 131, respectively. Five other compounds that had similar RBAs as MER-25 for AEBs (RBAs in the range 4–9) and for ER (RBAs < 0.06) were tested for their antiuterotrophic activities *in vivo* against both estrogen (E_2) and antiestrogen (TAM) in ovariectomized mice. None of these compounds were antiuterotrophic against either estradiol or tamoxifen ($P < 0.01$), nor were any of the compounds uterotrophic in mice. These data suggest that differences in the biological actions of tamoxifen and MER-25 in mice are not mediated through AEBS and are most likely due to differences in their interactions with ER.

One of the major unresolved questions about the mechanism of action of antiestrogens is why these drugs have different pharmacological effects in different species. Tamoxifen, a non-steroidal antiestrogen used in the treatment of breast cancer, is a pure estrogen antagonist in the chicken oviduct [1], a partial estrogen agonist/antagonist in the rat uterus [2], and a complete estrogen agonist in the mouse uterus [3]. One possible explanation of these data would be the formation *in vivo* of estrogenic metabolites of tamoxifen in those species in which tamoxifen is uterotrophic. However, we were unable to find any major differences in the metabolism of tamoxifen in these species that would account for its disparate biological effects *in vivo* [4]. Therefore, we decided to concentrate our biological studies in the mouse in order to determine if the uterotrophic effects of tamoxifen and estradiol are mediated through a common mechanism or through separate pathways.

The trophic activity of estradiol in the uterus is thought to be mediated by binding of this drug to estrogen receptors [5]. Similarly, since tamoxifen and its major metabolite, 4-hydroxytamoxifen, are also capable of binding with high affinity to uterine estrogen receptors [6], it is assumed that the uterotrophic properties of these compounds are also mediated through these receptors. However, with the advent of radiolabeled antiestrogens, a new class of binding sites was found in uterine cells that bound antiestrogens with high affinity and specificity, but did not bind estradiol [7, 8]. Unlike estrogen receptors, which are found in estrogen target tissues [5], antiestrogen binding sites are present in virtually all body tissues that have been examined [8, 9]. The function, if any, of these antiestrogen binding sites (AEBS) is unknown, although it has been suggested that these sites, either alone or in combination with the estrogen receptor, mediate the biological effects of antiestrogens. To date, however, very little evidence [10] has been presented to support the hypothesis that antiestrogen binding sites play a role in mediating the activity of antiestrogens.

Virtually all antiestrogens that are structurally related to tamoxifen (e.g. enclomiphene [3], nafoxidine [3], LY 117018 [11]) are uterotrophic in mice. One notable exception is MER-25 (ethamoxytriphetol), which not only is devoid of uterotrophic activity in the mouse, but also is capable of completely inhibiting the uterotrophic response to estradiol [12]. Unlike tamoxifen, which has different bio-

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logical effects in mice, rats and chickens, MER-25 has antiuterotrophic properties in all three of these species. Given the structural similarities between MER-25 and tamoxifen, it is not apparent why these compounds have opposite biological effects in the mouse. Therefore, we decided to compare the biological effects of estradiol, tamoxifen, 4-hydroxytamoxifen, and MER-25 in mice with the relative binding affinities of these compounds for estrogen receptors and for antiestrogen binding sites. We now report that (1) MER-25 completely inhibited the uterotrophic response of mice to two antiestrogens, tamoxifen and 4-hydroxytamoxifen, as well as estradiol, and (2) binding of these compounds (and others) to antiestrogen binding sites did not correlate with their biological properties *in vivo*.

MATERIALS AND METHODS

Materials. *trans*-[ring-^{3}H]Tamoxifen (sp. act. 19.9 Ci/mmole; 97% radiochemically pure) and Z-4-hydroxy[ring-3,5-^{3}H]tamoxifen (sp. act. 42 Ci/mmole; 98% radiochemically pure) were gifts of ICI, plc, Macclesfield, England, and Amersham, England, as were the *trans* isomers of tamoxifen and 4-hydroxytamoxifen. [6,7-^{3}H]β-Estradiol (sp. act. 52 Ci/mmole; 95% radiochemically pure) was purchased from Amersham, Arlington Heights, IL. SKF-525A was a gift of Smith, Kline & French Laboratories, Philadelphia, PA; metyrapone was a gift of CIBA–GEIGY Corp., Summit, NJ; MER-25 was a gift of Merrell Dow Pharmaceuticals. Cincinnati, OH; nafoxidine (U11,000A) was a gift of the Upjohn Co., Kalamazoo, MI; keoxifene (Lilly 156758), Lilly 18947, and 2,4-dichloro-6-phenylphenoxyethyl-diethylamine (DPEA) were gifts of Lilly Research Laboratories, Indianapolis, IN; and ellipticine (synthesized by Dr. Gordon Gribble, Dartmouth College, Hanover, NH) was a gift of Dr. Peter Sinclair, VA Medical Center, White River Junction, VT. Adiphenine, benactyzine, 17β-estradiol, imipramine, and phenyltoloxamine citrate were purchased from the Sigma Chemical Co., St. Louis, MO. The following compounds were purchased from the Alfred Bader Catalog of Rare Chemicals, Aldrich Chemical Co., Milwaukee, WI: 1-(4-chloro-α-phenylbenzyl)hexahydro-4-methyl-1*H*-1,4-diazepine; 4-chloro-α-[2-(*p*-diethylaminophenyl)-ethoxy]-α(*p*-tolyl)-phenethyl alcohol; 1-(4-chloro-4′-methoxybenzhydryl)-piperazine; and α-(2dimethylaminoethyl)-α-phenylhydrocinnamonitrile; the structures of these compounds are given in the figures.

Animals. Female mice (outbred ICR strain) were purchased from Sasco/King Animal Laboratories, Oregon, WI. Female outbred CD-1 mice that had been ovariectomized or ovariectomized and hypophysectomized were purchased from the Charles River Breeding Laboratories, Inc., Wilmington, MA. All animals were given food and water *ad lib.*, unless otherwise noted.

Uterine weight tests. Adult female ICR mice (10 weeks old) were ovariectomized under ether anesthesia and used 8 days later. Test compounds were dissolved in peanut oil as previously described [13]; compounds not soluble in peanut oil were dissolved in normal saline (0.85% NaCl). All injections were given subcutaneously (s.c.) in a volume of 0.1 ml. When two compounds were given in the same experiment, they were injected separately into different sites. Mice were injected with the test compounds for 3 days; on day 4, the mice were killed, their uteri were removed, intraluminal fluid was pressed out of the tissue, and the uteri were weighed. Mice were also weighed to monitor for possible toxicity of the test compounds.

Buffers. The buffers used were TEA [10 mM Tris, pH 7.4 (22°), 1.5 mM Na_2EDTA, 0.2% sodium azide] and TEM [10 mM Tris pH 7.4 (4°), 1.5 mM Na_2EDTA, 5 mM monothioglycerol].

Tissue preparing for antiestrogen binding site analysis. Mice were killed by cervical dislocation. Livers were homogenized with a glass–Teflon homogenizer, and uteri with a Polytron tissue homogenizer, in TEA buffer at concentration of 40 and 80 mg wet weight tissue/ml TEA respectively. Homogenates were centrifuged at 12,000 *g* for 30 min (4°), and the supernatant fraction were used for AEBS determination. In some experiments, the supernatant fraction was then spun for 1 hr at 100,000 *g* (4°), and the precipitated fraction (microsomes) was used for determining the affinity of ligands for AEBS. Pooled tissues from two or three mice were used in each experiment.

Tissue preparation for estrogen receptor assays. Adult female ICR mice were ovariectomized under ether anesthesia. Forty-eight hours later their uteri were removed and homogenized with a Polytron tissue homogenizer in TEM buffer at a concentration of two uteri/ml TEM. The homogenate was centrifuged at 12,000 *g* for 30 min (4°) and the resulting supernatant fraction was then spun at 100,000 *g* for 1 hr (4°). The supernatant (cytosol) was then used for determining the affinity of ligands for the estrogen receptor. The pooled uteri from twenty-five mice were used in each experiment.

Determination of ligand affinity for estrogen receptor. Our assay was a modification of the procedure of Jordan and Gosden [13]. The 310 μl reaction mixture comprised 5 nM [^{3}H]17β-estradiol (added in 5 μl ethanol, 1.75×10^5 dpm/assay), 10^{-10} to 10^{-5} M competing ligand (in 5 μl ethanol), and 300 μl of uterine cytosol in TEM buffer. The cytosol was incubated with [^{3}H]estradiol and the competing ligand for 18 hr at 4°, and then 0.5 ml of dextran-coated charcoal slurry (0.25% acid washed charcoal and 0.025% dextran in TEM buffer) was added to the tubes, and the contents were mixed. The tubes were incubated for 20 min at 4° and then centrifuged (4°) at 2000 *g* for 10 min to pellet the charcoal. An aliquot of the supernatant fraction was removed to quantify the radioactivity by liquid scintillation spectrometry. Non-specific binding was calculated using 1 μM β-estradiol as the competing ligand. Radioactivity in the supernatant fraction was plotted as a function of the log concentration of competing ligand in the assay, and the relative binding affinity (RBA) of the test compound was calculated as previously described [13].

Determination of ligand affinity for antiestrogen binding sites. Our method was modified from the procedure of Sudo *et al.* [8]. The 0.5 ml reaction

mixture comprised 1.5 nM [^{3}H]tamoxifen (added in 5 μl ethanol, 3.8×10^4 dpm/assay), 1 μM 17β-estradiol (in 5 μl ethanol), 10^{-10} to 10^{-4} M competing ligand (in 5 μl ethanol of dimethylformamide), and 485 μl of either 12,000 g liver supernatant fraction or microsomes in TEA buffer. The 12,000 g liver supernatant fraction was preincubated with estradiol for 30 min (4°) to fill estrogen receptor sites; this step was omitted in experiments in which microsomes were used in place of 12,000 g supernatant fraction. This tissue preparation was then added to tubes containing [^{3}H]tamoxifen and the competing ligand, and the tubes were incubated at 4° for 18 hr. The reaction was ended by pipetting 60 μl of charcoal–dextran slurry (5% acid washed charcoal, 0.5% dextran in 10 mM Tris buffer, pH 7.4, containing 0.02% sodium azide) into the tubes. The contents of the tubes were mixed and then incubated at 4° for 10 min. The tubes were then spun for 10 min at 2000 g (4°) to pellet the charcoal, and an aliquot of the supernatant fraction was removed and the radioactivity in it was quantified by liquid scintillation spectrometry. Nonspecific binding was determined by using 1 μM unlabeled tamoxifen as the competing ligand. The RBAs of the test compounds were calculated as described above for the estrogen receptor.

Effect of test compounds on [^{3}H]*estradiol and* [^{3}H]-4-*hydroxytamoxifen uptake* in vivo. Test compounds (1 mg) were administered by subcutaneous injection in saline or peanut oil to 7- or 10-week-old ovariectomized mice. In one experiment, mice were administered 1 mg of 17β-estradiol in addition to the test compound. Two hours after the initial injection(s), each mouse was given an injection (s.c.) of either 10 μCi (53 ng) [^{3}H]estradiol or 10 μCi (92 ng) [^{3}H]4-hydroxytamoxifen in 0.1 ml peanut oil. The mice were killed 4 hr af.er injection of the radioactivity, and their hearts, uteri, and a portion of liver were removed and weighed. The tissues were dried (22°) for 7 days, weighed and burned in a tissue oxidizer; the resulting ^3H_2O was then quantitated by liquid scintillation spectrometry.

Protein measurements. Proteins were measured by the method of Lowry *et al.* [14] with bovine serum albumin as a standard.

Statistical analysis. Student's *t*-test was used, and the level of significance was set at $P < 0.01$.

RESULTS

Role of the pituitary in the uterotrophic response to estrogens and antiestrogens. One of the basic assumptions about the uterotrophic activity of estrogens (and antiestrogens) in mice is that these drugs act directly on the uterus. Several workers, however, have suggested that a pituitary factor is required in rodents for a complete uterotrophic response to estradiol [15, 16], whereas several other workers [17, 18] indicate it is not. Therefore, we compared the uterotrophic activity of estradiol, tamoxifen and 4-hydroxytamoxifen in ovariectomized mice and in ovariectomized–hypophysectomized mice to determine if the pituitary is required for a full uterotrophic response to either estrogens or antiestrogens (Fig. 1). No differences were seen in the uterine weights of control versus hypophysectomized mice, indicating

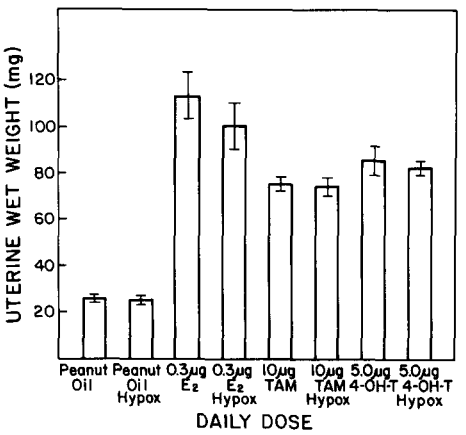


Fig. 1. Uterotrophic activities of estradiol (E_2), tamoxifen (TAM), and 4-hydroxytamoxifen (4-OH-T) in ovariectomized and ovariectomized–hypophysectomized adult CD-1 mice. The experiment was started 11 days after surgery. Mice were given injections of the indicated compounds once daily for 3 days and were killed on day 4, and their uteri were removed and weighed. All mice were given 5% glucose to drink. There were five or six mice per group; values shown are mean ± S.E.M.

that the pituitary is not required in the mouse for a uterotrophic response to either estrogens or antiestrogens.

Effect of MER-25 on the uterine response to estrogens and antiestrogens. MER-25 is a non-steroidal antiestrogen that has been shown to be non-uterotrophic in immature ovariectomized mice [12]. This compound has also been shown [12] to block the uterotrophic response of mice to estradiol (in a dose-related manner), to bind to estrogen receptors [19], and to inhibit the uptake of tritiated estradiol into estrogen target tissues [20, 21]. If estrogens and antiestrogens exert their uterotrophic effects in mice via a common mechanism, then MER-25 should be capable of blocking the uterotrophic response of mice to both of these agents. We found that MER-25 was capable of inhibiting the uterotrophic response of mice to estradiol, tamoxifen, and 4-hydroxytamoxifen, in a dose-dependent manner (Fig. 2), and confirmed that MER-25 is not uterotrophic in mice (Fig. 2). MER-25 is not a very potent antiestrogen and the dose of this compound required for a full antiuterotrophic effect was at least 3 orders of magnitude greater than the doses of those compounds whose action was inhibited. Increasing doses of tamoxifen were capable of completely reversing the antiuterotrophic effect of a fixed dose of MER-25 (Fig. 3), indicating that the MER-25 inhibition was not a result of non-specific toxic effects on the uterus.

These data suggest that the activities of MER-25, estradiol, tamoxifen, and 4-hydroxytamoxifen are all mediated through the estrogen receptor. If this is true, then it is not clear why MER-25 and tamoxifen, given their structural similarity, have opposite biological activities in mice. It has been suggested [7] that some of the biological effects of antiestrogens may be mediated through their binding to antiestrogen binding sites. Therefore, we decided to

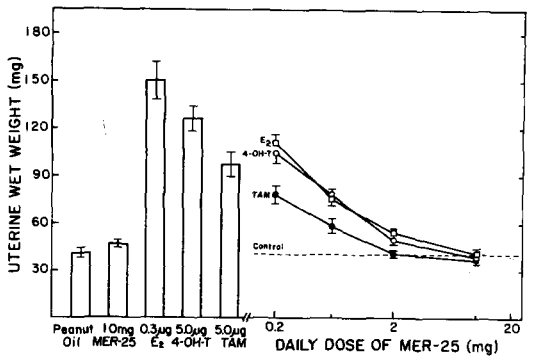


Fig. 2 Antiuterotrophic activity of MER-25 in ovariectomized adult ICR mice. Increasing doses of MER-25 were administered with either estradiol (E_2), tamoxifen (TAM), or 4-hydroxytamoxifen (4-OH-T); uterine weight tests were performed as described in Materials and Methods. The MER-25 was administered as two sub-doses at 8:00 a.m. and 8:00 p.m.; all other compounds were given only at 8:00 a.m. There were six or seven mice per group; values shown are mean ± S.E.M.

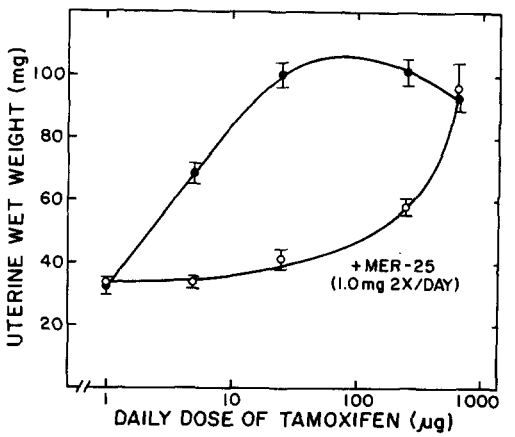


Fig. 3. Effect of increasing doses of tamoxifen on the antiuterotrophic activity of MER-25 in ovariectomized adult ICR mice. MER-25 was administered at 8:00 a.m. and 8:00 p.m.; tamoxifen was given at 8:00 a.m. only. Uterine weight tests were performed as described in Materials and Methods. Values shown are the mean ± S.E.M.; there were seven mice per group.

compare the binding of tamoxifen, 4-hydroxytamoxifen, and MER-25 to estrogen receptors and to antiestrogen binding sites to determine if the differences in their biological activity could be correlated with binding to these two sites.

Comparative binding to estrogen receptor and to antiestrogen binding sites. The relative binding affinity of compounds for ER was determined using mouse uterus 100,000 *g* supernatant (cytosol), and the affinity of compounds for antiestrogen binding sites (AEBS) was measured using mouse liver 12,000 *g* supernatant fraction. Liver was chosen for antiestrogen binding site measurements because (1) the total number of these sites is much greater in liver than in uterus [8], and (2) because preliminary

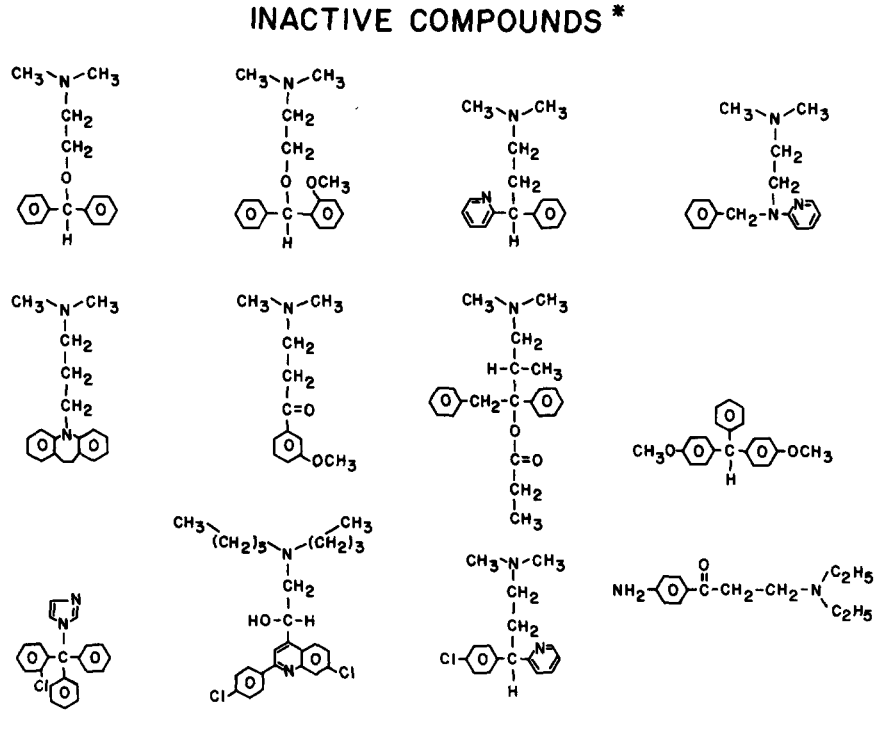
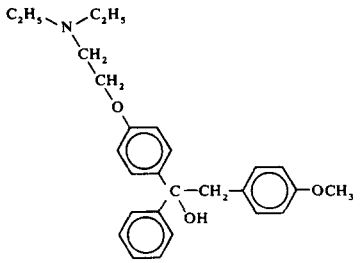
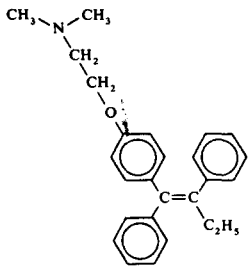
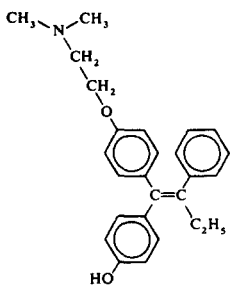
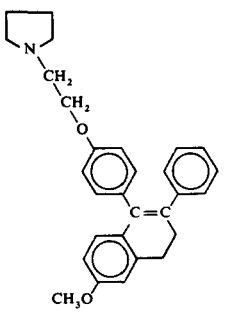
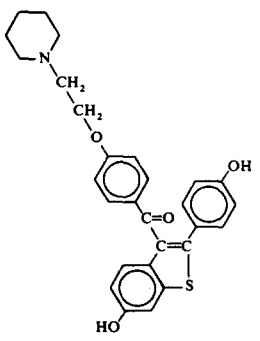


Fig. 4. Compounds that did not bind to mouse liver antiestrogen binding sites. Most of the chemicals were selected from the Alfred Bader Catalog of Rare Chemicals as a result of their structural similarities to the side chain of tamoxifen.

Table 1. Relative binding affinities (RBA) of a series of triphenylethylene derivatives for mouse liver antiestrogen binding sites (AEBS) and for mouse uterus estrogen receptor (ER)*

Compound		Mouse liver AEBS RBA (TAM = 100)	Mouse uterus ER RBA (E_2 = 100)
	Mer-25	8.9	0.06
	Tamoxifen	100	2.5
	4-Hydroxytamoxifen	53	131
	Nafoxidine	124	13
	Keoxifene	10.0	118

* Binding assay were performed as described in Materials and Methods.

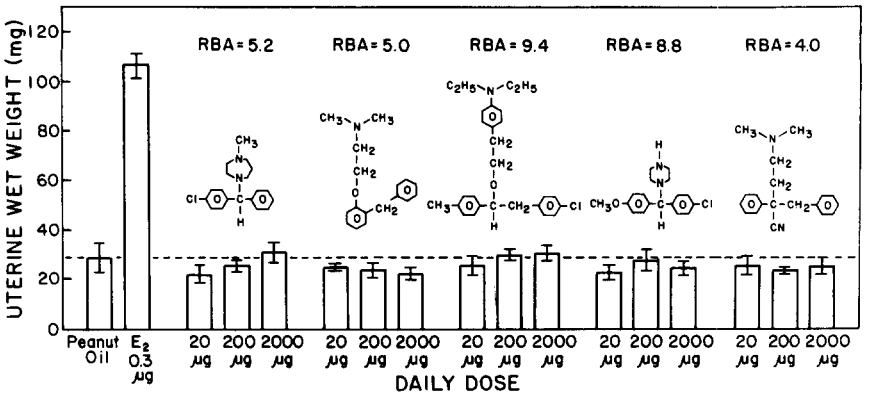


Fig. 5. Uterotrophic activities of the compounds from Table 2 in ovariectomized adult ICR mice. Uterine weight tests were performed as described in Materials and Methods. The RBA values in the figure are for mouse liver antiestrogen binding sites. All compounds were administered in two sub-doses at 8:00 a.m. Values shown are the mean ± S.E.M.; there were five or six mice per group.

experiments indicated that the relative binding affinities of a number of antiestrogens for AEBS were very similar in liver and uteri (data not shown). The equilibrium dissociation constant (K_D) of tamoxifen for mouse liver antiestrogen binding sites was determined by saturation analysis [22] to be 3.5 nM, and the concentration of binding sites was 6.5 pmoles/mg of 12,000 *g* supernatant protein (data not shown). The relative binding affinities of tamoxifen, 4-hydroxytamoxifen, MER-25, and two other non-steroidal antiestrogen compounds to estrogen receptors and to antiestrogen binding sites are listed in Table 1. Tamoxifen and 4-hydroxytamoxifen have RBAs for antiestrogen binding sites 11 and 6 times greater, respectively, than MER-25; similarly, the RBAs of tamoxifen and 4-hydroxytamoxifen are at least 42 and 2200 times as great, respectively, than MER-25 for estrogen receptor. Nafoxidine and keoxifene, two other non-steroidal antiestrogens that are uterotrophic in mice ([3], S.D. Lyman and V. C. Jordan, unpublished observation), have RBAs for antiestrogen binding sites and for estrogen receptors in the same range as tamoxifen and 4-hydroxytamoxifen. These data suggested that the anti-uterotrophic effects of MER-25 in mice might be mediated through antiestrogen binding sites since this compound, unlike the others listed in Table 1, has a very low (if any) affinity for estrogen receptors. Therefore, we decided to screen compounds with a structural resemblance to the triphenylethylene

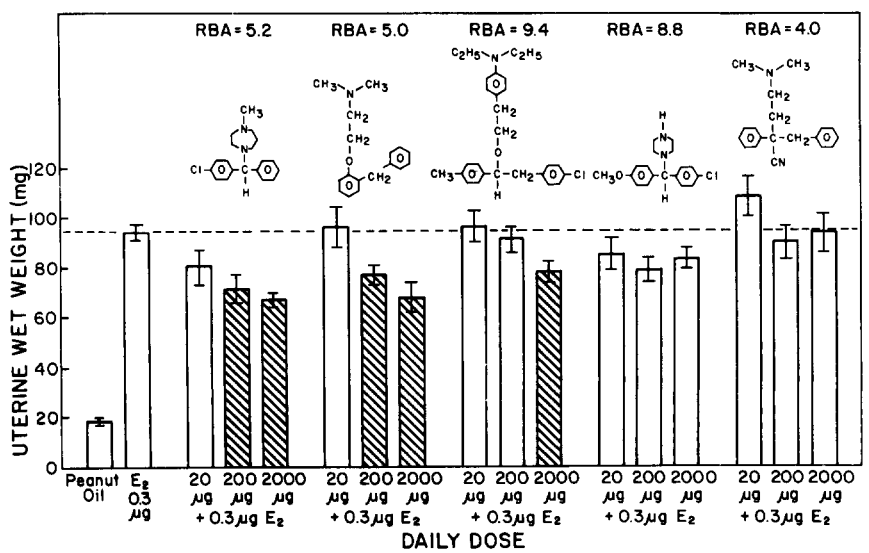


Fig. 6. Antiuterotrophic activities (vs estradiol) of the compounds from Table 2 in ovariectomized adult ICR mice. Uterine weight tests were performed as described in Materials and Methods. The RBA values in the figure are for mouse liver antiestrogen binding sites. All compounds were administered in two sub-doses at 8:00 a.m. and 8:00 p.m. except for estradiol (E_2) which was given only at 8:00 a.m. Values shown are the mean ± S.E.M.; there were three to five mice per group. The hatched bars indicate those groups with mean uterine weights significantly below the estradiol-treated group ($P < 0.01$).

derivatives listed in Table 1 in order to find other compounds, that, like MER-25, bind to antiestrogen binding sites but do not bind appreciably to estrogen receptors; these compounds would then be tested to determine if they were uterotrophic or antiuterotrophic in mice.

Uterotrophic and antiuterotrophic tests with potential antiestrogens. Five compounds, selected on the basis of their structural resemblance to the side chains of the compounds in Table 1, were found to meet the binding criteria outlined above; their structures and RBAs for estrogen receptor and antiestrogen binding sites are listed in Table 2. The structures of a variety of compounds that did not binding to antiestrogen binding sites with any appreciable affinity are given in Fig. 4 to illustrate the specificity of this binding. Like MER-25, none of the compounds illustrated in Table 2 were uterotrophic in the 3-day ovariectomized mouse uterine weight assay (Fig. 5). When tested for their antiestrogenic properties against estradiol, three of these compounds appeared to inhibit the estradiol-induced increase in uterine weight in a dose-dependent manner, whereas two other compounds were not antiuterotrophic (Fig. 6). However, mice treated with those compounds that appeared to be antiuterotrophic were found to weigh less than control mice, suggesting that these compounds were acting through a toxic mechanism. When the data in Fig. 6 were recalculated as a uterine wet weight/body weight ratio, none of the five compounds in Table 2 were significantly antiuterotrophic ($P < 0.01$, data not shown). Since MER-25 can inhibit the uterotrophic response to tamoxifen as well as estradiol (Fig. 2), we tested the capacity of these five compounds to inhibit the tamoxifen-stimulated increase in uterine weight (Fig. 7). None of these compounds were able to inhibit the uterotrophic response to tamoxifen ($P < 0.01$); in contrast, MER-25, at a dose of 2000 μg/day, inhibited the uterotrophic response to 25 μg tamoxifen by 87% (Fig. 3). Taken together, these data indicate that these five test compounds in Table 1 are not truly antiestrogenic, and suggest that binding to antiestrogen binding sites does not mediate the antiuterotrophic activity of MER-25.

Uptake of [^{3}H]*estradiol and* [^{3}H]4-*hydroxytamoxifen* in vivo. The compounds listed in Table 2 were tested for their capacity to block the uptake of [^{3}H]estradiol into mouse uterus, liver, and heart (Fig. 8). None of these compounds that have an extremely low affinity for estrogen receptors (Table 2) and are neither uterotrophic nor antiuterotrophic (Figs. 5–7) were able to cause a significant decrease in the uptake of [^{3}H]estradiol by the uterus. In contrast to these results, estradiol, 4-hydroxytamoxifen, and MER-25, which are either uterotrophic or antiuterotrophic *in vivo* (Fig. 2), were able to inhibit the uptake of radioactivity by the uterus. Although MER-25 had a low affinity for estrogen receptors *in vitro* (Table 2), these data suggest that the compound is metabolized *in vivo* to a derivative with a higher affinity for estrogen receptors (see Discussion). None of the tested compounds altered the uptake of radioactivity into tissues that contain little (liver) or no (heart) estrogen receptors.

The compounds listed in Table 2 were also tested for their capacity to inhibit the binding *in vivo* of [^{3}H]4-hydroxytamoxifen by antiestrogen binding sites in mouse uterus, liver, and heart (Fig. 9). In this experiment, mice were administered 1 mg estradiol (to fill estrogen receptors) along with the test

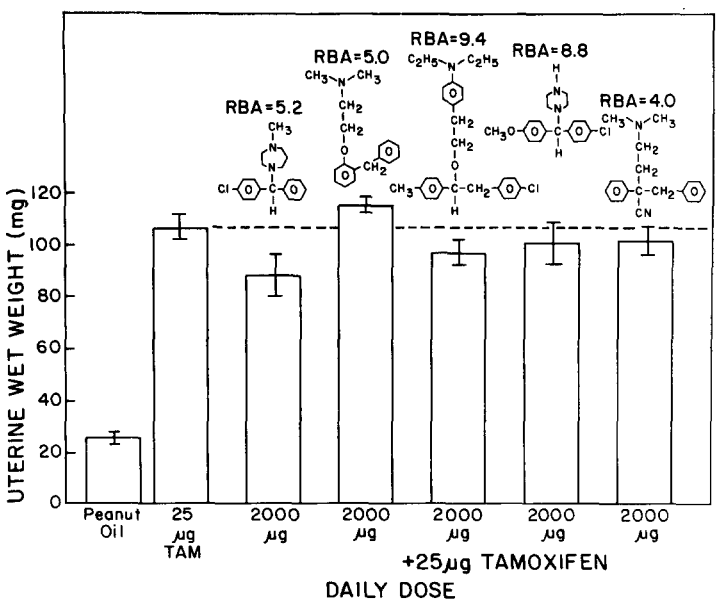
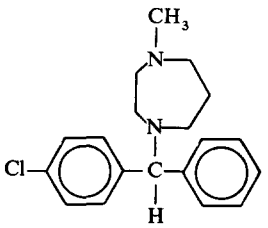
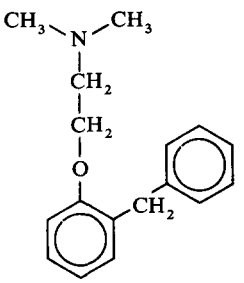
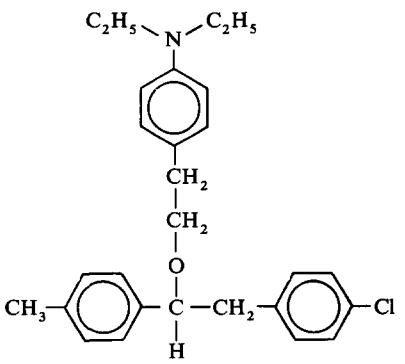
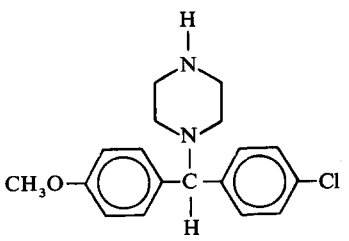
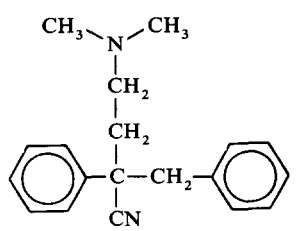


Fig. 7. Antiuterotrophic activities (vs tamoxifen) of the compounds from Table 2 in ovariectomized adult ICR mice. Uterine weight tests were performed as described in Materials and Methods. The RBA values in the figure are for mouse liver antiestrogen binding sites. All compounds were administered in two sub-doses at 8:00 a.m. and 8:00 p.m. except for tamoxifen (TAM) which was given only at 8:00 a.m. Values shown are the mean ± S.E.M.; there were give or six mice per group.

Table 2. Relative binding affinities (RBA) of a group of compounds (with similar side chains as the compounds in Table 1) for mouse liver antiestrogen binding sites (AEBS) and for mouse uterus estrogen receptor (ER)*

Compound	Mouse liver AEBS RBA (TAM = 100)	Mouse uterus ER RBA (E_2 = 100)
	5.2	<0.06
	5.0	<0.06
	9.4	<0.06
	8.8	<0.06
	4.0	<0.06

* Binding assays were performed as described in Materials and Methods.

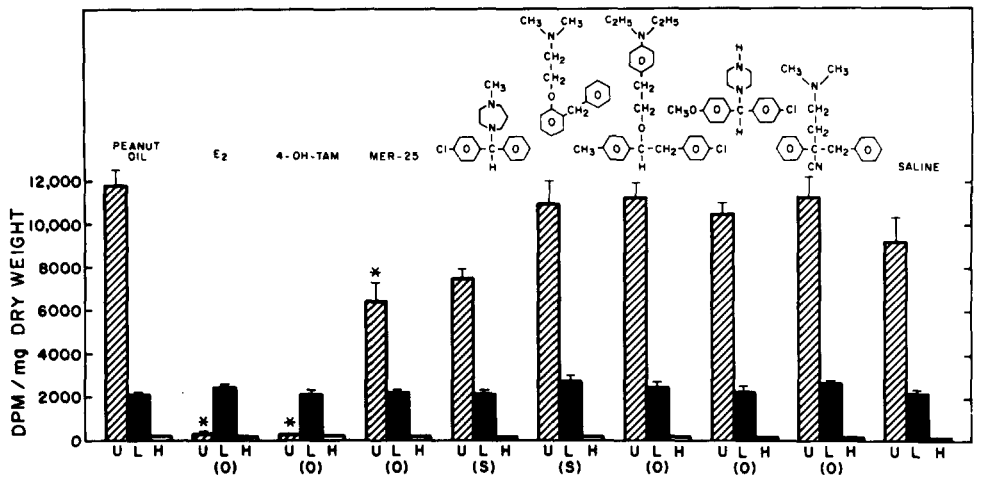


Fig. 8. Effects of the compounds in Table 2 on the uptake of [^{3}H]estradiol by mouse uterus (U), liver (L), or heart (H). Test compounds (1 mg) were administered in either peanut oil (O) or saline (S) as denoted below the figure: 2 hr later the mice were given 10 μCi [^{3}H]estradiol and then killed 4 hr after administration of the radioactivity (see Materials and Methods). There were five mice per group. Values shown are mean ± S.E.M.; no error bar indicates error too small to be illustrated. The asterisks denote those groups that were significantly ($P < 0.01$) lower than the appropriate control group (oil or saline).

compounds; [^{3}H]4-hydroxytamoxifen (10 μCi; 92 ng) was injected 2 hr later, and the mice were killed 4 hr after administration of the radioactivity. Almost all of the test compounds were able to reduce significantly the uptake of [^{3}H]4-hydroxytamoxifen by the uterus; however, only MER-25 and unlabeled 4-hydroxytamoxifen were able to reduce the uptake of radioactivity by liver. Uptake of radioactivity into heart, which has much lower levels of antiestrogen binding sites than either liver or uterus [8], was similar in all groups. There are many more antiestrogen binding sites in a 1.2 g liver than in a 25 mg uterus; we presume that the test compounds in Table 2 can inhibit the uptake of [^{3}H]4-hydroxytamoxifen by uterus, but not liver, because they fill a greater percentage of binding sites in uterus than in liver.

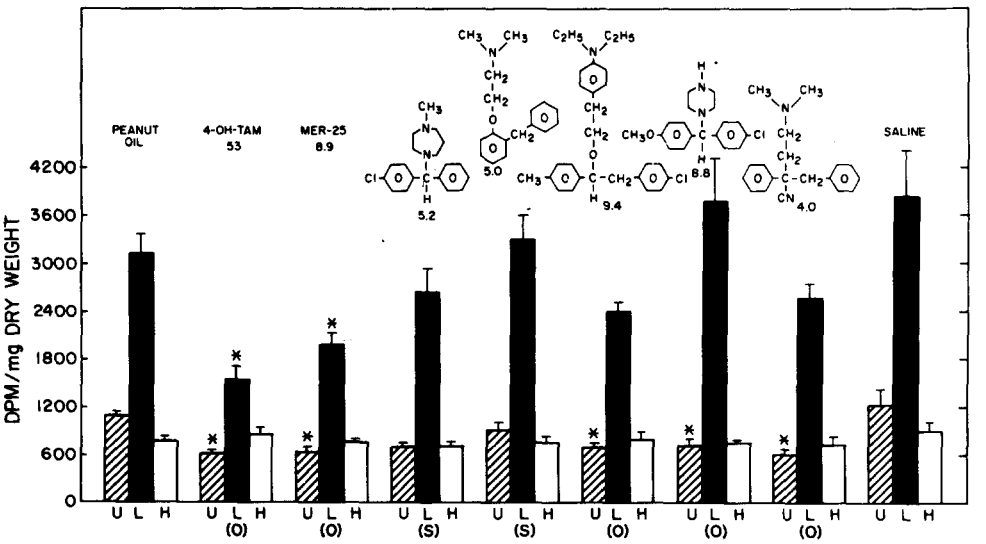
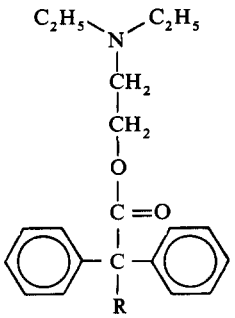
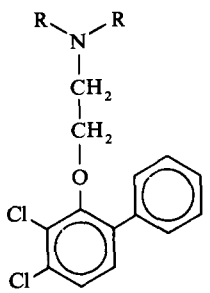
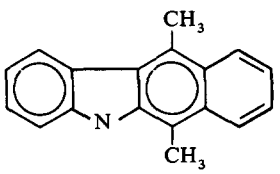
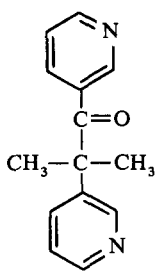


Fig. 9. Effects of the compounds in Table 2 on the binding of [^{3}H]4-hydroxytamoxifen by antiestrogen binding sites in mouse uterus (U), liver (L), or heart (H). Mice were given 1 mg estradiol (in 0.1 ml peanut oil) and then administered 1 mg of the test compounds in either oil (O) or saline (S) as denoted in the figure. Two hours later the mice were given 10 μCi [^{3}H]4-hydroxytamoxifen and then killed 4 hr after administration of the radioactivity (see Materials and Methods). Numbers in the figure indicate the binding affinities of the compounds for mouse liver antiestrogen binding sites (see Table 2). There were five mice per group; values shown are mean ± S.E.M. The asterisks denote those groups that were significantly ($P < 0.01$) lower than the appropriate control group (oil or saline).

Table 3. Relative binding affinities of a number of cytochrome P-450 inhibitors for mouse liver antiestrogen binding sites*

Compound		Mouse liver AEBS RBA TAM = 100
	R = C_3H_7 (SKF-525A) R = H (Adiphenine) R = OH (Benactyzine)	1.1 0.5 <0.8
	R = CH_3 (Lilly 18947) R = H (DPEA)	<0.4 0.8
	Ellipticine	<0.4
	Metyrapone	<0.4

* Binding assays are performed as described in Materials and Methods.

Possible identity of antiestrogen binding sites. If antiestrogen binding sites do not mediate the biological activity of antiestrogens, then the question remains as to what is the function of these sites. We considered the possibility that AEBS may be one or more species of cytochrome P-450 because both cytochrome P-450 and AEBS are localized to the microsomal fraction of the cell [8, 23] and the highest concentrations of P-450 and AEBS are found in the liver [8, 24]. Therefore, we measured the RBAs of a number of cytochrome P-450 inhibitors for antiestrogen binding sites (Table 3). Several of these compounds (SKF-525A, DPEA) that are structurally related to the tamoxifen and MER-25 side chains did bind to antiestrogen binding sites, albeit with a low affinity, whereas compounds that were not structurally related (ellipticine, metyrapone) did not bind. Table 4 summarizes our findings on the diverse classes of drugs that bind to AEBS.

DISCUSSION

Our data support the concept that the uterotrophic effects of both estrogens and antiestrogens in mice

Table 4. Classes of drugs that bind to mouse liver antiestrogen binding sites*

Class	Example	RBA
Estrogens	*cis*-Tamoxifen	45.7
Antiestrogens	MER-25	8.9
Anticholinergics	Adiphenine	0.5
Antilipemics	Triparanol	14.0
Antihistamines	Phenyltoloxamine	5.0
Tranquilizers	Trifluoperazine	24.4
Drug metabolism inhibitors	SKF-525A	1.1

* Binding assays were performed as described in Materials and Methods. The relative binding affinity (RBA) of tamoxifen for antiestrogen binding sites is operationally defined as 100. Although it has antiestrogenic properties in other species, tamoxifen is estrogenic in mice [3], whereas the *cis* isomer of tamoxifen is estrogenic in both mice and rats [2].

are mediated through the estrogen receptor. Estradiol, tamoxifen and 4-hydroxytamoxifen all bind to the mouse uterine estrogen receptor *in vitro* [6] and these compounds also block the uptake of [^{3}H]estradiol *in vivo* [25, 26]. Mühlbock and Van Maurik [17] and Quarmby *et al.* [18] have shown that the pituitary is not required for mice to mount a uterotrophic response to estrogens; similarly, we have demonstrated that hypophysectomy does not diminish the uterotrophic response to the antiestrogens tamoxifen and 4-hydroxytamoxifen. These data suggest that both estrogens and antiestrogens act directly on target tissues in the mouse.

The idea that estradiol, tamoxifen, and 4-hydroxytamoxifen exert their uterotrophic effects via a common mechanism is supported by the fact that MER-25 blocks the uterotrophic response (in a dose-dependent manner) to all three of these compounds (Fig. 2). The dose of MER-25 required to inhibit the uterotrophic effects of tamoxifen, 4-hydroxytamoxifen, and estradiol, is 3 or 4 orders of magnitude greater than the doses of estrogen and antiestrogen, respectively; this is consistent with the low relative binding affinity of MER-25 for the estrogen receptor (Table 1, [3, 17]). The affinity of MER-25 for the estrogen receptor may possibly be increased by aromatic ring hydroxylation *in vivo* in a manner analogous to that already described for the conversion of tamoxifen to 4-hydroxytamoxifen [6]; addition of the hydroxyl group would be expected to increase the affinity by 50- to 100-fold. This idea is supported by the fact that WSM-4613, a compound that is nearly identical to MER-25 and has the same affinity for the estrogen receptor, is not antiuterotrophic in mice [3]. Unlike MER-25, however, WSM-4613 cannot be hydroxylated *in vivo* as described above because of a para-Cl group on the aromatic ring. In addition, MER-25 was capable of inhibiting the uptake of [^{3}H]estradiol by the uterus *in vivo* (Fig. 8). This suggests that hydroxylation of MER-25 *in vivo* is necessary for the compound to exert its antiuterotrophic effects.

Since tamoxifen and MER-25 are structurally related, the question remains why tamoxifen is uterotrophic in mice whereas MER-25 is antiuterotrophic. We believe that the key difference between these compounds is the absence of the ethylene double bond from MER-25; without the double bond, the three aromatic rings are not locked into a fixed conformation and may rotate freely in space. This freedom of rotation enables MER-25 to form a complex with the estrogen receptor such that the ligand–receptor complex is not able to bind properly to genetic regulatory elements in the nucleus and thereby stimulate transcription of estrogen-inducible genes. This concept is supported by data indicating that another antiestrogen that does not have this ethylene linkage (e.g. MRL-37) is only weakly uterotrophic in mice and can slightly inhibit the response to estradiol [3], whereas compounds with a fixed geometry for these aromatic rings (enclomiphene, U-11,555A, nafoxidine [3], or LY 117018 [11]) are all uterotrophic in mice.

Our data suggest that the antiuterotrophic effects of MER-25 in mice are not mediated through antiestrogen binding sites. A number of compounds that had similar binding affinities as MER-25 for ER and AEBS were found in our screening program (Table 2); despite their similar affinities, none of these compounds were uterotrophic in mice (Fig. 5), nor were they antiuterotrophic (Fig. 6 and see Results). Binding affinity for the estrogen receptor appears to be the key factor that distinguishes the biological activity of tamoxifen and MER-25 from these other compounds. This idea is supported by the fact that MER-25 inhibits the uptake of [^{3}H]estradiol into estrogen target tissues, whereas the other compounds in Table 2 with binding affinities for AEBS similar to MER-25 are not inhibitory. Thus, our data support the conclusion of Katzenellenbogen *et al.* [27, 28] namely that antiestrogen binding sites do not appear to mediate the biological activity of antiestrogens, although they may indirectly affect antiestrogen action by altering the distribution and pharmacokinetics of these compounds. Indeed the wide variety of drugs (Table 4) that interact with AEBS argues that the name "antiestrogen" binding site is a misnomer, and the sites probably are generally involved in the tissue binding of several drug classes.

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