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Antiestrogenic effect of trifluoperazine in mice*

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The molecular mechanism of action of the non-steroidal antiestrogens is unknown. A precise understanding of how antiestrogens exert their biological actions is, however, complicated by the fact that these drugs have opposing effects in rodent and avian species. For example, tamoxifen (1-(4-β-dimethylaminoethoxyphenyl)1,2 diphenylbut-1-ene) (Fig. 1) is a complete estrogen antagonist in the chicken oviduct [1], a partial estrogen agonist/antagonist in the rat uterus [2], and a full estrogen agonist in the mouse uterus [3].

It is believed that the biological effects of tamoxifen are mediated through binding to estrogen receptors, since tamoxifen competes with estradiol for the estrogen receptor *in vitro* [4] and inhibits the uptake of [^{3}H]estradiol into an estrogen target tissue, the uterus, *in vivo* [5]. This view has been challenged because a new class of binding sites was discovered in uterine cells that bound tamoxifen (and other antiestrogens) with high affinity, but did not bind estradiol [6, 7]. These sites, referred to as antiestrogen binding sites, have since been shown to exist in virtually all body tissues [8]. To complicate matters even further, Lam has demonstrated recently that tamoxifen is an inhibitor of the activation of cAMP phosphodiesterase activity by calmodulin [9], suggesting that some effects of antiestrogens may be calmodulin mediated.

Calmodulin is thought to play an important role in regulating cell proliferation [10]. One of the most potent inhibitors of calmodulin action is the phenothiazine tranquilizer trifluoperazine, which has been shown to have antiproliferative effects against human breast cancer cells *in vitro* [11]. Since tamoxifen inhibits the proliferation of breast cancer cells *in vitro* [12] and there are marked structural similarities between trifluoperazine and tamoxifen (Fig. 1), it is possible that some of the effects are mediated by similar mechanisms. Indeed, Sudo *et al.* [7] have shown that antiestrogens with a methyl-substituted piperazine ring on the side chain, like that found in trifluoperazine, have a very high affinity for antiestrogen binding sites. We therefore decided to study the ability of trifluoperazine to inhibit the binding of antiestrogens to antiestrogen binding sites *in vivo* and *in vitro*. We have also examined the binding of trifluoperazine to estrogen receptors because there is a report [13] that phenothiazines compete with estradiol for estrogen receptors. Finally, we have determined whether the interaction of trifluoperazine with antiestrogen binding sites or the estrogen receptor can be correlated with the uterotrophic and antiuterotrophic actions of the trifluoperazine in ovariectomized mice. Although trifluoperazine appeared to inhibit estradiol-stimulated increases in uterine weight, we suggest that the effect occurs because the tranquilizing activity of the drug results in a decrease in food and water intake. Restriction of the diet produced an "antiestrogenic" effect upon estradiol-stimulated increases in uterine weight.

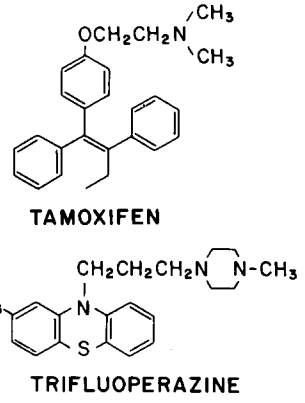

Fig. 1. Formulae of tamoxifen and trifluoperazine.

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, as were the *trans*-isomers of tamoxifen and 4-hydroxytamoxifen. [6,7-^{3}H]17β-Estradiol (sp. act. 52 Ci/mmole; 95% radiochemically pure) was purchased from Amersham, Arlington Heights, IL. MER-25 (ethamoxytriphetol) was a gift of Merrell Dow Pharmaceuticals, Cincinnati, OH. Trifluoperazine dihydrochloride and 17β-estradiol were purchased from the Sigma Chemical Co., St. Louis, MO..

Animals. Female mice (outbred ICR strain) were purchased from Sasco/King Animal Laboratories, Oregon, WI. All animals were given food (Wayne Mouse Breeder Blox, Allied Mills, Chicago, IL) and water *ad lib.*, unless otherwise noted. In one experiment, mice were given a liquid diet (Mouse Diet No. 0514, Bio Serv Inc., Frenchtown, NJ) by gavage in place of food and water.

Uterine weight tests. Adult female ICR mice (8 or 10 weeks old) were ovariectomized under ether anesthesia and used 8 days later. Estradiol was dissolved in peanut oil as previously described [14]; trifluoperazine was not soluble in peanut oil and was dissolved in saline (0.85% NaCl). All injections were given subcutaneously 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.

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 the experiment with [^{3}H]4-hydroxytamoxifen, mice were injected with 1 mg 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 after injection of the radioactivity, and their uteri were removed and weighed. The uteri were dried (22°) for 7 days, weighed, and burned in a tissue oxidizer; the resulting ^3H_2O was quantitated by liquid scintillation spectrometry.

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

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[10 mM Tris, pH 7.4 (4°), 1.5 mM Na_2EDTA, 5 mM monothioglycerol].

Antiestrogen binding site assays (AEBS). Mice were killed by cervical dislocation. Livers were homogenized with a glass–Teflon homogenizer in TEA buffer at a concentration of 40 mg wet weight tissue/ml TEA. Homogenates were centrifuged at 12,000 *g* for 30 min (4°), and the supernatant fractions were used for AEBS determination. Pooled tissues from two or three mice were used in each experiment.

Our method was modified from the procedure of Sudo *et al.* [7]. The 0.5-ml reaction volume was comprised of 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), and 485 μl of 12,000 *g* liver supernatant fraction in TEA buffer. The 12,000 *g* liver supernatant fraction was preincubated with estradiol for 30 min (4°) to fill estrogen receptor sites. 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 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 relative binding affinities (RBAs) of the test compounds were calculated as described below for the estrogen receptor.

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.

Our assay was a modification of the procedure of Jordan and Gosden [14]. 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 200 *g* for 10 min to pellet the charcoal. An aliquot of the supernatant fraction was removed, and the radioactivity in it was quantified by liquid scintillation spectrometry. Nonspecific binding was calculated using 1 μM 17β-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 RBA of the test compound was calculated as previously described [14].

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

Results and discussion

The relative binding affinities of trifluoperazine and several triphenylethylene derivatives for mouse uterus cytosolic estrogen receptors and for mouse liver antiestrogen binding sites are listed in Table 1. Tamoxifen and 4-hydroxytamoxifen are uterotrophic in mice [3, 15], whereas MER-25 is antiuterotrophic in this species [16]. All three triphenylethylene derivatives bound to antiestrogen binding sites, whereas only tamoxifen and 4-hydroxytamoxifen bound to estrogen receptors with any appreciable affinity. Trifluoperazine bound to antiestrogen binding sites with an affinity about one-fourth that of tamoxifen; however, this compound did not compete with [^{3}H]estradiol for binding to estrogen receptors *in vitro*. These data suggest that any biological effects of trifluoperazine *in vivo* will not be estrogen receptor mediated. However, many antiestrogens are metabolized *in vivo* to phenolic derivatives that have a much higher affinity for estrogen receptors than the parent compound [4, 17]. Since phenothiazines have been reported [13] to compete with estradiol *in vivo* for rat brain estrogen receptors, we considered the possibility that trifluoperazine could also be metabolized *in vivo* to a compound with a higher binding affinity for estrogen receptors. To test this idea, we determined the relative capacities of estradiol, trifluoperazine, MER-25, and 4-hydroxytamoxifen, to block the uptake of [^{3}H]estradiol into the uteri of ovariectomized mice (Table 2).

Table 1. Relative binding affinities (RBA) of several drugs for mouse liver antiestrogen binding sites (AEBS) and mouse uterus estrogen receptors (ER)*

Compound	Mouse liver AEBS RBA (TAM = 100)	Mouse uterus ER RBA (E_2 = 100)
Tamoxifen	100	2.5
4-Hydroxytamoxifen	53.0	131
MER-25	8.9	<0.06
Trifluoperazine	24.4	<0.06

* Binding assays were performed as described in Materials and Methods. Tamoxifen (TAM) is assigned an RBA of 100 for AEBS; similarly, estradiol (E_2) is assigned an RBA of 100 for ER.

Estradiol and 4-hydroxytamoxifen were very good inhibitors of [^{3}H]estradiol binding in uterus, as would be expected from their binding affinities for estrogen receptor determined *in vitro* (Table 1). MER-25 also inhibited [^{3}H]estradiol uptake by the uterus; this is most likely a result of metabolism of this drug *in vivo* to a compound with a higher affinity for estrogen receptors. Trifluoperazine did cause a significant ($P < 0.05$) reduction in [^{3}H]estradiol binding in the uterus, suggesting that this drug may be metabolized *in vivo* to a compound with a higher affinity for estrogen receptors than the parent drug.

The capacity of the compounds listed in Table 1 to inhibit the binding of [^{3}H]4-hydroxytamoxifen to mouse uterus antiestrogen binding sites was also determined. In addition to the test compounds, mice were also given 1 mg estradiol at time zero to fill estrogen receptors (see Materials and Methods). MER-25 and unlabeled 4-hydroxytamoxifen caused significant decreases in the binding of [^{3}H]4-hydroxytamoxifen in uterus ($P < 0.01$), as did trifluoperazine ($P < 0.05$). These data suggest that all of these compounds bind to antiestrogen binding sites *in vivo*.

We next tested trifluoperazine in ovariectomized mice to determine if this compound was either uterotrophic or antiuterotrophic *in vivo*. When the data were calculated as the mean uterine wet weight, trifluoperazine was not uterotrophic in mice at doses up to 2000 μg/day (Fig. 2). However, if the data were calculated as the uterine wet weight/body weight ratio, then trifluoperazine did appear to be significantly uterotrophic ($P < 0.01$) at the two highest doses. This is because mice given 200 or 2000 μg trifluoperazine/day are so heavily sedated that they eat and drink very little during the 3-day treatment period; as a

Table 2. Effects of various drugs on the binding of [^{3}H]estradiol and [^{3}H]4-hydroxytamoxifen by the uteri of ovariectomized mice *in vivo**

Compound	[^{3}H]Estradiol (dpm/mg dry weight uterus)	[^{3}H]4-Hydroxytamoxifen (dpm/mg dry weight uterus)
Peanut oil	11,900 ± 700	1,100 ± 60
4-Hydroxytamoxifen (PO)	230 ± 10†	600 ± 60†
MER-25 (PO)	6,600 ± 800†	640 ± 60†
Estradiol (PO)	300 ± 60†	ND‡
Saline	9,200 ± 1,200	1,200 ± 200
Trifluoperazine (S)	5,700 ± 1,300§	580 ± 100§

* Test compounds (1 mg) were administered in either peanut oil (PO) or saline (S) as indicated by the groupings above. To measure the binding of [^{3}H]4-hydroxytamoxifen by antiestrogen binding sites, mice also received 1 mg estradiol at zero time to fill estrogen receptors. Two hours after the initial injection(s), mice received 10 μCi of either [^{3}H]estradiol (53 ng) or [^{3}H]4-hydroxytamoxifen (92 ng), and 4 hr after injection of radioactivity mice were killed and their uteri were removed. See Materials and Methods for quantitation of tissue radioactivity. Values are expressed as mean ± S.E.M.

† Significantly less than vehicle alone ($P < 0.01$).

‡ Not determined.

§ Significantly less than vehicle alone ($P < 0.05$).

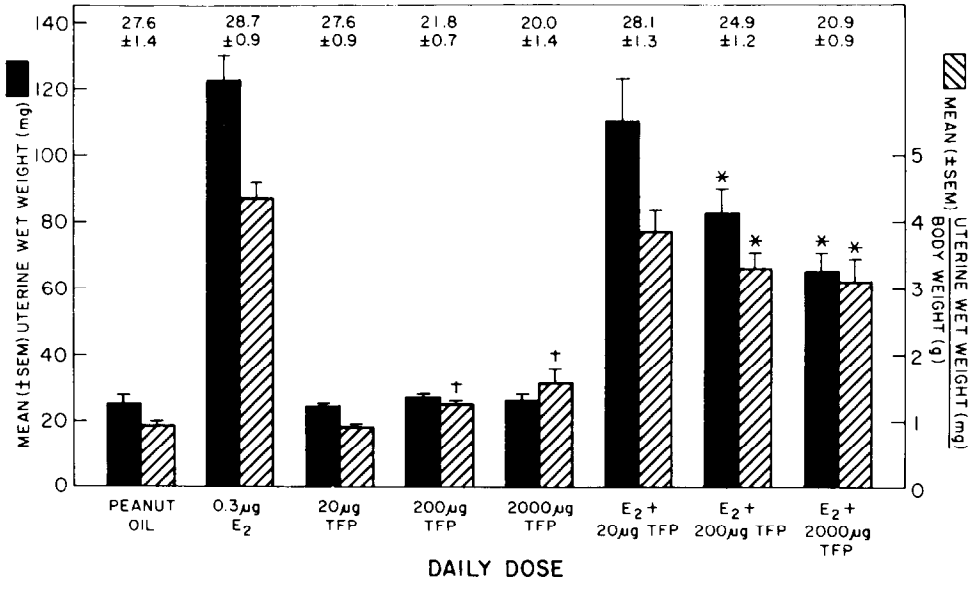

Fig. 2. Effect of trifluoperazine (TFP) on uterine weight and on the uterine weight/body weight ratio in ovariectomized mice. Animals were administered the indicated daily dose of estradiol (E_2) or TFP (administered in two sub-doses) for 3 days, killed on day 4, and their body weights and uterine weights were recorded. There were five or six mice/group; numbers in the figure are the mean ± S.E.M. body weights of mice in that group. Key: (*) significantly less than E_2 control ($P < 0.01$); and (†) significantly greater than peanut oil control ($P < 0.01$).

result of this, the mice will lose an average of 23% of their body weights during this time (see body weights listed in Fig. 2).

Trifluoperazine, at doses of 200 or 2000 μg/day, also appeared to be antiestrogenic in mice whether the data was calculated as the mean uterine wet weight, or as the uterine wet weight/body weight ratio (Fig. 2). This could have been a true antiestrogenic effect; alternatively, it may have been a result of the reduced food and water intake by these mice. To determine if the antiestrogenic effect of trifluoperazine was a result of food deprivation, we tested the effect that dietary restrictions had on the uterotrophic response to estradiol and to estradiol plus trifluoperazine (Fig. 3). Animals injected with estradiol or estradiol plus trifluoperazine had similar uterotrophic response (and body weights) if food was restricted for 3 days. Furthermore, mice given a liquid diet by gavage (in an attempt to maintain their body weights) had similar uterine response to estradiol and to estradiol plus trifluoperazine. However, in animals given free access to food and water, the mice treated with estradiol plus trifluoperazine had significantly lower ($P < 0.01$) uterine weights (and body weights) than mice

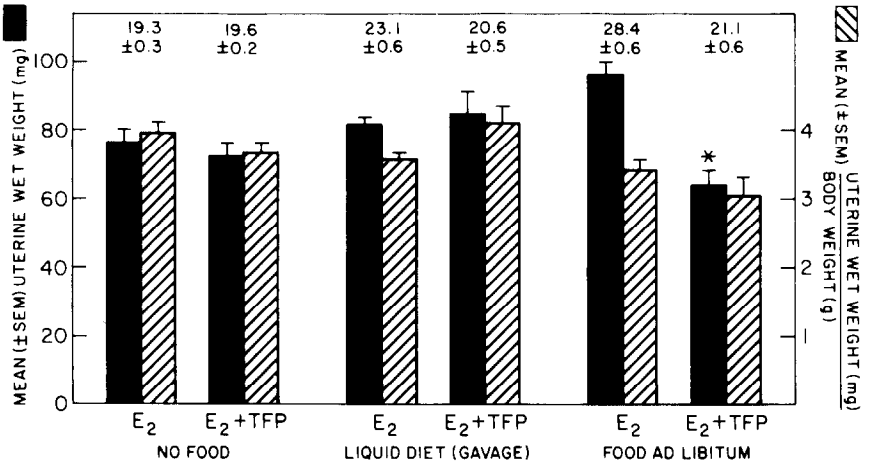

Fig. 3. Effect of diet on the capacity of trifluoperazine to inhibit the uterotrophic response of ovariectomized mice to estradiol. Animals were given daily injections of either 0.3 μg E_2 plus 2 mg TFP (administered in two sub-doses) for 3 days, killed on day 4, and their body weights and uterine weights were recorded. There were four to ten mice/group; numbers in the figure are the mean ± S.E.M. body weights of mice in that group. Key: (*) significantly less than E_2 control ($P < 0.01$).

treated with estradiol alone. It should be noted in this experiment that dietary restriction alone caused a significant decrease ($P < 0.01$) in the uterine response to estradiol (i.e. compare "No Food" with "Food *ad libitum*") and trifluoperazine did not cause further reduction in estradial-stimulated uterine weight in animals with restricted diets. Thus, although trifluoperazine appeared to inhibit the binding of [^{3}H]estradiol to the mouse uterus, the anti-uterotrophic effect of trifluoperazine in mice probably results from the fact that mice treated with this drug do not eat or drink as much as control mice, and the diet restriction leads to a decreased uterotrophic response.

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