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Binding Characteristics of Novel Nonsteroidal Antiestrogens to the Rat Uterine Estrogen Receptors

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Tamoxifen (TAM), the only antiestrogen currently available for the endocrine therapy of breast cancer behaves as a mixed agonist/antagonist of estrogen action, thus limiting its therapeutic potential. We report the binding characteristics of a novel series of nonsteroidal antiestrogens to the rat uterine estrogen receptor. As measured by competition studies, the affinity of EM-652, the active metabolite of the prodrug EM-800, for the estrogen receptor is 7–11 times higher than that of 17β-estradiol (E_2), ICI 182780, and hydroxy-tamoxifen (OH-TAM), the active metabolite of Tamoxifen. EM-652 is 20× more potent than ICI 164384 and Droloxifene while it is 400 times more potent than Toremifene in displacing [^{3}H]E_2 from the rat uterine estrogen receptor. On the other hand, the prodrug EM-800 and Tamoxifen have respectively 150-fold and 410-fold less affinity for the estrogen receptor than the pure antiestrogen EM-652. No significant binding of EM-652, EM-800, TAM or OH-TAM was observed to the rat uterine progesterone receptor at concentrations up to 10 000 nM except for TAM that caused a 50% displacement of labeled R5020 at 4000 nM. No significant binding of EM-652 or EM-800 was observed on the rat ventral prostate androgen receptor or the rat uterine progesterone receptor. The present data demonstrate the high affinity and specificity of the new antiestrogen, EM-652, for the rat uterine estrogen receptor. The antiestrogen EM-652 thus becomes the compound having the highest known affinity for the estrogen receptor. Due to its unique potency and its pure antiestrogenic activity already demonstrated in many systems, this antiestrogen could well offer an important advance for the endocrine therapy of breast cancer, uterine cancer, and other estrogen-sensitive diseases in women.

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INTRODUCTION

Since estrogens are well recognized to play a major role in the development and growth of human breast cancer [1], considerable attention has been given to the mechanisms of action involved and to the development of inhibitors of estrogen action at the target cell level. The existing ablative procedures, either surgical or medical, do not permit complete elimination of estrogens [2–4]. Consequently, antiestrogens or compounds blocking estrogen action at the receptor level, remain the most rational approach for the therapy of estrogen-sensitive breast cancer.

Unfortunately, until very recently, no compounds with pure antiestrogenic activity were available. The only antiestrogen widely available for the endocrine therapy of breast cancer, namely the triphenylethylene derivative Tamoxifen (TAM) shows mixed estrogenic and antiestrogenic activities which are species-, cell-, and even gene-tissue specific [5–11]. Although TAM has a weak affinity for the estrogen receptor (ER), as compared with E_2, it is metabolized in peripheral tissues into, among other metabolites, the *trans*-4-hydroxylated form which is a much stronger antiestrogen and has greater affinity for the ER than its precursor [12–14]. Nevertheless, the metabolically activated antiestrogen, e.g. *trans*-4-monohydroxytamoxifen (OH-TAM), has estrogenic activity in various tests comparable to that found with Tamoxifen [15].

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We have thus synthesized a series of benzopyrans which are pure and highly potent antiestrogens in human breast and uterine cancer cells in the most representative *in vitro* and *in vivo* model systems [16–18]. In order to gain more information on the characteristics of these new compounds, we now report the binding characteristics of EM-652, the active metabolite of EM-800 to the rat uterine estrogen and progesterone receptors as well as on the rat ventral prostate androgen receptor. Comparison is made with the affinity of other antiestrogen to the rat uterine ER.

MATERIALS AND METHODS

Chemicals

Trans-4-monohydroxytamoxifen (OH-TAM) [α-(4β-N-dimethylaminoethoxy) phenyl-hydroxy-α'-ethyl-transtilbene] was generously provided by Dr. D. Salin-Drouin, Besins-Iscovesco, Paris, France. EM-343 ((*d,l*) 7-hydroxy-3-(4'-hydroxyphenyl)-4-methyl-2-(4''-(2'''-piperidinoethoxy)phenyl)-2H-benzopyran), EM-651 ((−)-7-hydroxy-3-(4 -hydroxyphenyl)-4-methyl-2-(4''-(2'''-piperidinoethoxy)phenyl)-2H-benzopyran), EM-652 ((+)-7-hydroxy-3-(4'-hydroxyphenyl)-4-methyl-2-(4''-(2'''-piperidinoethoxy)phenyl)-2-H-benzopyran), EM-762 ((*d,l*)-7-pivaloyloxy-3-(4'-pivaloyloxyphenyl)-4-methyl-2-(4''-(2'''-piperidinoethoxy)phenyl)-2H-benzopyran), EM-800 ((+)-7-pivaloyloxy-3-(4'-pivaloyloxyphenyl)-4-methyl-2-(4''-(2'''-piperidinoethoxy)phenyl)-2H-benzopyran) and EM-776 ((−)-7-pivaloyloxy-3-(4'-pivaloyloxyphenyl)-4-methyl-2-(4''-(2'''-piperidinoethoxy)phenyl)-2H-benzopyran) (Fig. 1) were synthesized by the medicinal chemistry division of our laboratory as described [17]. Droloxifene, ICI 164384 and ICI 182780 were also synthesized in our laboratory. Dexamethasone (DEX), Tamoxifen, and diethylstilbestrol (DES) were purchased from Sigma (St. Louis, MO) while 17β-estradiol (E_2), estrone (E_1), estriol (E_3), testosterone (testo), dihydrotestosterone (DHT), triamcinolone acetonide (TAC), and progesterone (Prog) were purchased from Steroids (Pawling, NY). Toremifine was generously provided by Schering-Plough Research Institute (NJ).

[2, 4, 6, 7 ^{3}H] 17β-estradiol ([^{3}H]E_2) (S.A. 111.6 Ci/mmol), [17α-methyl-^{3}H] promegestone ([^{3}H]R5020) (S.A. 86.2 Ci/mmol) and [17α-methyl-^{3}H] methyltrienolone ([^{3}H]R1881) (S.A. 86.0 Ci/mmol) were obtained from New England Nuclear (Lachine, Que.).

Tissue preparation

Female and male Sprague–Dawley rats (Crl: CD(SD)Br) weighing 200–300 g were obtained from Charles-River (St. Constant, Que.). The rats were gonadectomized under general anesthesia (Isoflurane) and killed by cervical dislocation 24 h later. The uteri and ventral prostates were rapidly removed, dissected free from adhering tissue and frozen on dry-ice. Uteri and prostates were kept at −80°C until assayed.

All subsequent steps were performed at 0–4°C. Uteri and prostates were homogenized in 10 (uteri) or 5 (prostates) vol (wt/vol) of buffer A (25 mM

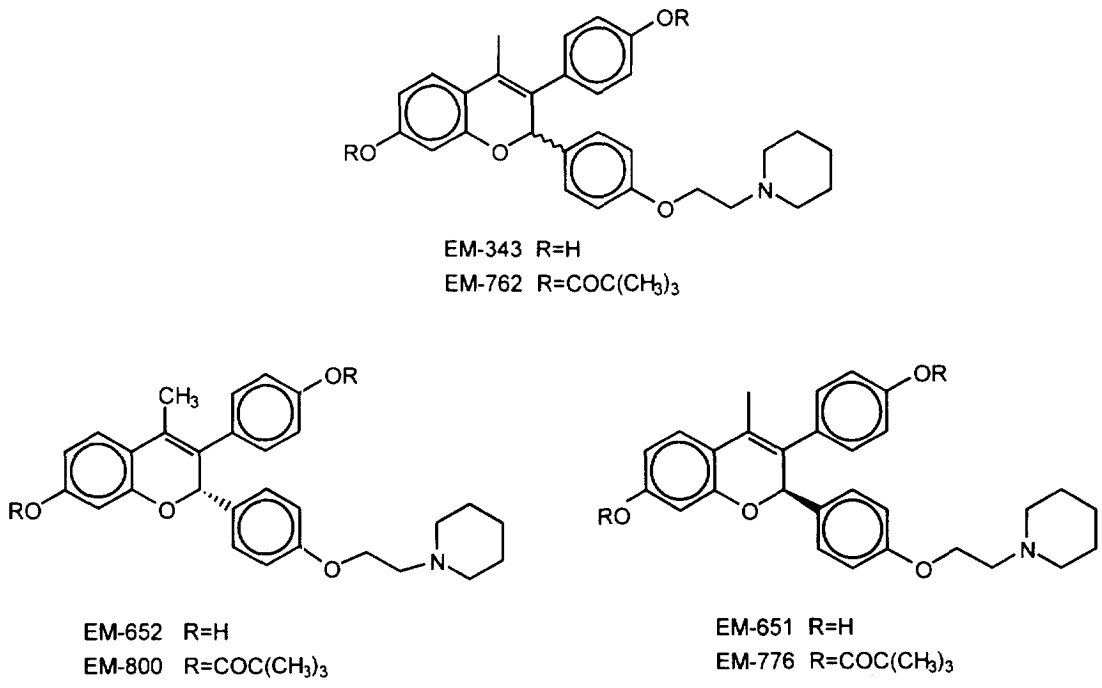

Fig. 1. Structure of EM-652, EM-800 and related benzopyrans.

Tris–HCl, 1.5 mM EDTA disodium salt, 10 mM α-monothioglycerol, 10% glycerol, and 10 mM sodium molybdate, pH 7.4), using a Polytron PT-10 homogenizer (Brinkman Instruments, Canada) at a setting of 5 for three periods of 10 s, with intervals of 10 s for cooling. The homogenate was then centrifuged at 105 000 × *g* for 60 min in a Beckman L5-65 ultracentrifuge (Fullerton, CA). The protein concentration of the cytosol fraction was measured according to the method of Bradford [19], using bovine serum albumin as standard.

Progesterone (PR) and estrogen receptor (ER) assays

Estrogen and progesterone binding was measured using the dextran-coated charcoal adsorption technique as described previously [20, 21]. Briefly, the radioactive steroids ([^{3}H]E_2 and [^{3}H]R5020) solubilized in ethanol were diluted into buffer A. Aliquots of uterine cytosol preparation (0.1 ml) were then incubated with 8 nM [^{3}H]R5020 or 5 nM [^{3}H]E_2 (~200 000 cpm, 0.1 ml) in the presence or absence of the indicated concentrations of unlabeled compounds (0.1 ml, prepared in buffer A containing 10% ethanol) for 16–18 h at 0–4°C for PR and 3 h at room temperature for ER, respectively. In Fig. 3(B), cytosol preparation was incubated 2 h at room temperature in a buffer containing 2.5% dimethylformamide (instead of 3 h in a buffer containing 10% ethanol). Dexamethasone (150 nM) was added for the PR assay in order to saturate glucocorticoid receptors. Unbound steroids were then separated by incubation for 15 min at 0–4°C with 0.3 ml 0.5% Norit-A and 0.05% Dextran T-70 in buffer B (1.5 mM EDTA disodium salt, 10 mM monothioglycerol, and 10 mM Tris–HCl, pH 7.4) and centrifuged at 3000 × *g* for 15 min. Aliquots of the supernatant (0.3 ml) were removed for radioactivity measurement. After the addition of 10 ml Formula-989 scintillation liquid (New England Nuclear-DuPont), the radioactivity was measured in a Beckman counter at a counting efficiency of 62%.

Androgen receptor assay

Androgen binding was measured using the hydroxylapatite assay (HAP) [22]. In brief, the radioactive steroid [^{3}H]R1881 solubilized in ethanol was diluted into buffer A. Aliquots of prostate cytosol preparation (0.1 ml) were then incubated with 8 nM [^{3}H]R1881 (0.1 ml, ~200 000 cpm) in the presence or absence of the indicated concentrations of unlabeled compounds (0.1 ml, prepared in buffer A containing 10% ethanol) for 16–18 h at 0–4°C. Triamcinolone acetonide (150 nM) was added in order to mask progesterone receptors. Unbound steroids were separated by incubation for 40 min at 0–4°C with 0.3 ml HAP prepared in buffer P (50 mM, Tris–HCl, 10 mM KH_2PO_4, pH 7.4) as follows: 10 g HAP were washed with buffer P until the supernatant reached a pH of 7.4 and then following centrifugation and decantation of the supernatant, 37.5 ml of buffer P were added. After incubation with HAP and 10 min of centrifugation at 1000 × *g*, the pellet was washed 3× with 1 ml buffer P. Thereafter, the radioactivity was extracted from the pellet by incubation at room temperature for 60 min with 1 ml EtOH. After centrifugation, the supernatant was decanted into a scintillation vial and the pellet was extracted again with ethanol. Thereafter, 10 ml Formula-989 scintillation liquid was added to pooled supernatant and the radioactivity was measured in a Beckman counter.

Statistical analyses and calculations

Apparent IC_{50} values were calculated using an iterative least square regression method [23]. Values are presented as means ± SEM of triplicate measurements. Where no bar is shown, the SEM was smaller than the symbol used.

RESULTS

Affinity of EM-652 and EM-800 for the rat uterine estrogen and progesterone receptors and for the rat prostatic androgen receptor

As illustrated in Fig. 2(A), EM-652 is 11× more potent than E_2 in displacing [^{3}H]E_2 from the rat uterine estrogen receptor, the IC_{50} values being calculated at 0.44 and 4.96 nM for EM-652 and E_2, respectively. The prodrug EM-800, on the other hand, caused a 50% displacement of 5 nM [^{3}H]E_2 at an IC_{50} value of 65.3 nM (13.2-fold lower affinity than E_2 and 148-fold lower affinity than EM-652).

Since the highly potent antiestrogen EM-652 is the active enantiomer of the racemic mixture EM-343, it is of interest to see in Fig. 2(B) that EM-343 inhibited [^{3}H]E_2 binding at an IC_{50} value of 1.07 nM while the inactive enantiomer EM-651 has only 2.2% the affinity of EM-652 at an IC_{50} value of 20.0 nM. On the other hand, the dipivaloate derivative of EM-343, EM-762, caused a 50% inhibition of 5 nM [^{3}H]E_2 binding at 138 nM while the inactive enantiomer EM-776 had only approximately 3.0% the affinity of EM-800 at an IC_{50} value of 2141 nM (Fig. 2(C), Table 1).

As illustrated in Fig. 2(D), EM-652 is approximately 8-fold more potent than hydroxy-tamoxifen in displacing 5 nM [^{3}H]E_2 binding, the respective IC_{50} values being calculated at 0.66 and 5.36 nM for EM-652 and OH-TAM, respectively (Table 2). On the other hand, Tamoxifen is approximately 410-fold less potent than the new antiestrogen EM-652 with a calculated IC_{50} value of 272 nM (Table 2). It is of interest to see in Fig. 3(A) that EM-652 is approximately 2.0-fold more potent than diethylstilbestrol (DES) in displacing [^{3}H]E_2 from the rat uterine estrogen receptor (IC_{50} values of 0.79 nM for EM-652 versus

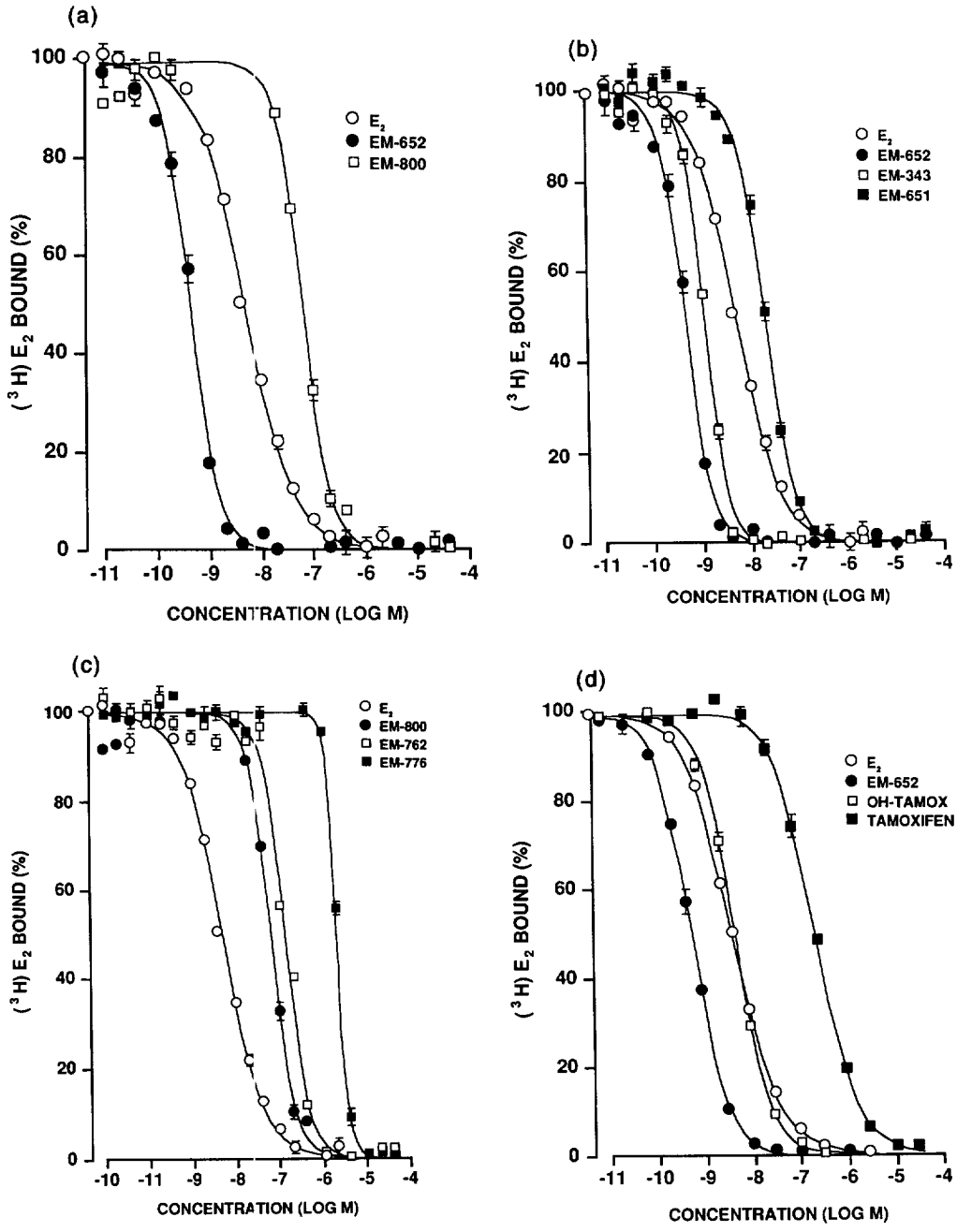

Fig. 2. Effect of increasing concentrations of (A) EM-652, 17β-estradiol (E_2), and EM-800; (B) EM-652, EM-343, E_2, and EM-651; (C) E_2, EM-800, EM-762, and EM-776; and (D) EM-652, E_2, OH-Tamoxifen, and Tamoxifen on [^{3}H]17β-estradiol binding to the rat uterine estrogen receptor. The incubation was performed with 5 nM [^{3}H]17β-estradiol (E_2) for 3 h at room temperature in the presence or absence of the indicated concentrations of unlabeled compounds.

1.31 nM for DES). IC_{50} values of 5.8, 130 and 129 nM were measured in the same experiment for E_2, estrone, and estriol. The androgens testosterone and dihydrotestosterone (DHT) had no significant effect on [^{3}H]E_2 binding under the experimental conditions used.

It can be seen in Fig. 3(B) that EM-652 is 7- to 8-fold more potent than E_2 and ICI 182780 in displacing [^{3}H]E_2 from the rat uterine estrogen receptor (IC_{50} values of 0.52, 4.13 and 3.59 nM for EM-652, E_2, and ICI 182780, respectively). ICI 164384 and Droloxifene are 21-fold less potent than EM-652 while Toremifene is 400× less potent than EM-652.

As illustrated in Fig. 4(A), the androgens DHT and testosterone caused a 50% inhibition of binding of 8 nM [^{3}H]R1881 to the rat ventral prostate andro-

Table 1. IC_{50} values of displacement of 5 nM [^{3}H]17β-estradiol from the rat uterine estrogen receptor by the new antiestrogens EM-652 and EM-800 and related compounds. The relative affinities of the various compounds are also indicated taking 100% as reference of binding by EM-652

Compound	IC_{50} value (nM)	IC_{50} EM-652/IC_{50} compound	
		%	fold difference
E_2	4.96	8.9	−11.3
EM-343	1.07	41	−2.4
EM-652	0.44	100	—
EM-651	20.0	2.2	−45
EM-762	138.0	0.3	−310
EM-800	65.3	0.7	−150
EM-776	2141	0.02	−4900

Table 2. IC_{50} values of displacement of 5 nM [^{3}H]17β-estradiol from the rat uterine estrogen receptor by the new antiestrogen EM-652 compared with Tamoxifen, and hydroxytamoxifen. The IC_{50} value of EM-652 is taken as 100%

Compound	IC_{50} value (nM)	IC_{50} EM-652/IC_{50} compound	
		%	fold difference
EM-652	0.66	100	—
OH-TAM	5.36	12	−8
TAM	272	0.25	−410

gen receptor at IC_{50} values of 7.1 and 49.5 nM, respectively, while E_2 and progesterone exerted similar effects at the much higher IC_{50} values of 1295 and 3875 nM, respectively. The antiestrogens EM-652, EM-800, Tamoxifen and OH-TAM exerted no significant effect up to 10 000 nM.

When the affinity of the same compounds was studied on the rat uterine progesterone receptor, IC_{50} values of 71, 1245, 2055 and 11 500 nM were measured for progesterone, E_2, DHT, and testosterone, respectively (Fig. 4(B)). Tamoxifen caused a 50% displacement of 8 nM [^{3}H]R5020 at 4000 nM while OH-TAM caused a 25% displacement at 30 000 nM. The antiestrogen EM-652 exerted a 50% inhibition at 22 500 nM while EM-800 had no significant effect up to the highest dose used, namely 30 000 nM.

DISCUSSION

The present data show that the highly potent antiestrogen EM-652 has 7–11-fold higher affinity than 17β-estradiol, hydroxytamoxifen and ICI 182780 for the rat uterine estrogen receptor. Since DES was the compound known so far to have the highest affinity for the estrogen receptor, the present data show that the novel antiestrogen EM-652 is the compound having the highest known affinity for the estrogen receptor.

In agreement with the present data, the steroidal antiestrogen ICI 182780 has been reported to have an affinity for the rat uterine estrogen receptor similar to E_2 and 4-hydroxytamoxifen [24, 25]. The present data also indicate that ICI 182780 has 3× more affinity for the estrogen receptor than ICI 164384, while relative binding affinities for the rat uterine ER of

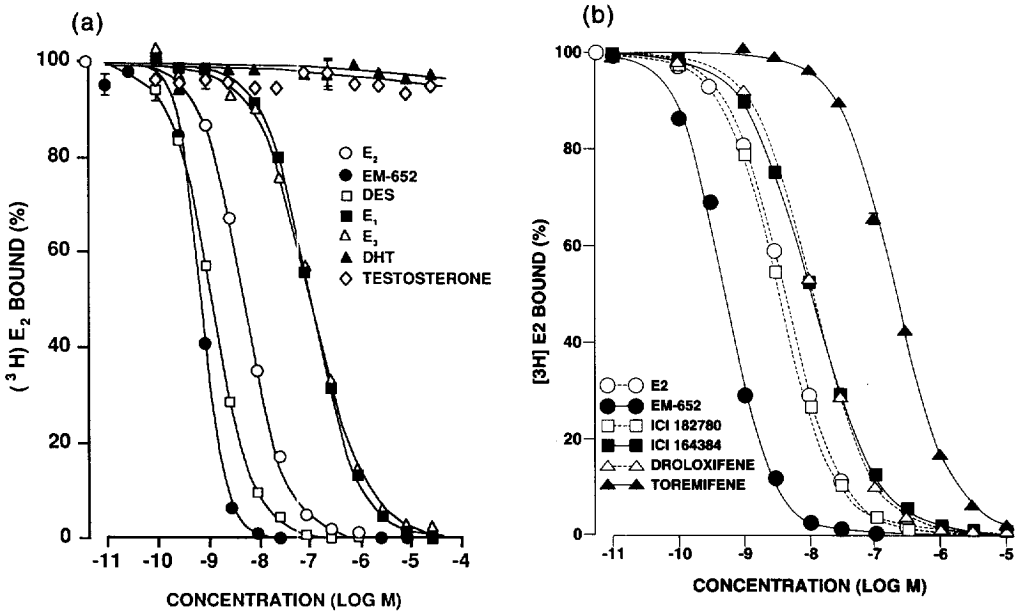

Fig. 3. Effect of increasing concentrations of (A) EM-652, DES, E_2, estrone (E_1), estriol (E_3), DHT and testosterone; and (B) EM-652, E_2, ICI 182780, Droloxifene, ICI 164384, and Toremifene on [^{3}H] 17β-estradiol binding to the rat uterine estrogen receptor. The incubation was performed with 5 nM [^{3}H] 17β-estradiol (E_2) for 3 h (A) or 2 h (B) at room temperature in the presence or absence of the indicated concentrations of unlabeled compounds.

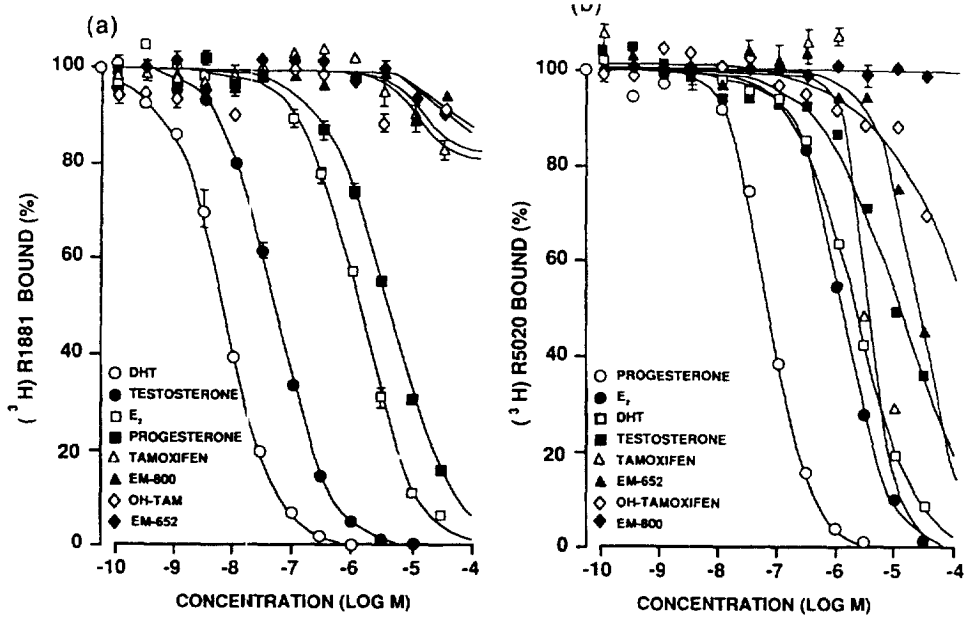

Fig. 4. (A) Effect of increasing concentrations of EM-652, EM-800, E_2, progesterone, testosterone, DHT, Tamoxifen and OH-TAM on the binding of [^{3}H]R1881 to the rat ventral prostate androgen receptor. The incubation was performed with 8 nM [^{3}H] R1881 for 16–18 h at 4°C in the presence or absence of the indicated concentrations of unlabeled compounds. (B) Effect of increasing concentrations of progesterone, E_2, DHT, testosterone, Tamoxifen, EM-652, OH-TAM and EM-800 on the binding of [^{3}H]R5020 to the rat uterine progesterone receptor. The incubation was performed with 8 nM [^{3}H]R5020 for 16–18 h at 4°C in the presence or absence of the indicated concentrations of unlabeled compounds.

0.89 and 0.19 have been measured for ICI 182780 and ICI 164384, respectively [25] compared to E_2.

4-OH-Toremifene has been found to have 17× more affinity than Toremifene for the ER while the ability of 4-OH-Toremifene to inhibit E_2-induced MCF-7 cell growth (~2 nM) has been estimated 100-fold higher than that of the prodrug Toremifene (~200 nM) [26]. The present data show that Toremifene has approximately 2% the affinity of E_2 for the estrogen receptor.

The steroidal antiestrogen *N*-butyl-2-[5-[4-(3,17β-dihydroxy-estra-1,3,5(10)-trien-11β-yl)-phenoxy]pentylthio]-*N*-methyl-acetamide (a 11β-amidoalkoxyphenyl estradiol derivative) has been found to have a relative binding affinity for the mouse uterine estrogen receptor of 38 compared to 100 for E_2 or 1/3 the affinity of E_2 [27]. In that system, OH-TAM had an affinity of 40% that of E_2. On the other hand, RU39411, a 11β-aryl derivative of E_2, showed a relative binding affinity of 140% relative to E_2 [28].

EM-652 was approximately 315-fold less potent than progesterone to displace [^{3}H]R5020 from the progesterone receptor. Since EM-652 exerts near-maximal or maximal antiestrogenic activity at 10 nM, the IC_{50} value measured at 22 500 nM is approximately 2250-time higher than the expected blood levels of the compound in women.

The mechanisms of action of antiestrogens in inhibiting breast cancer growth are multiple. Thus, in a first step, they compete with endogenous and/or exogenous estrogens at the level of the estrogen receptor and, secondarily they cause the loss of estrogen receptors [29, 30]. Moreover, recent data have shown that they also inhibit 17β-hydroxysteroid dehydrogenase (17β-HSD) activity, the enzyme that catalyses the reversible formation of 17β-estradiol from estrone [31–33]. Since the objective of estrogen blockade is to induce apoptosis or breast cancer cell death, a dose-dependent phenomenon, the availability of EM-652, the compound having the highest affinity for the ER, suggests the possibility of an improve therapy of estrogen-sensitive breast cancer.

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