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Estradiol derivatives bearing the side-chain of tamoxifen antagonize the association between the estrogen receptor and calmodulin

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Abstract—Calmodulin (CaM) is known to associate with the estrogen receptor (ER). The antiestrogen tamoxifen impedes this association suggesting that the latter would play an important role in CaM-dependent enzymatic catalyses. The ethoxyaminoalkyl side-chain of tamoxifen which confers antiestrogenicity appears to be involved in this antagonism. Antiestrogenic estradiol derivatives bearing the side-chain of tamoxifen in position 11β (RU 39 411) or 7α (RU 45 144) were tested for their potential antagonism towards the association between CaM and ER. According to molecular modelling studies, such graftings position the chain in an orientation corresponding to that found in tamoxifen. Both compounds impeded the binding of ER to CaM-Sepharose at the same concentrations as found with tamoxifen indicating smilar effectiveness. Steroidal analogs with or without a side-chain in a non-appropriate orientation failed to show this property. On the contrary, a non-conjugated side-chain analog antagonized the binding of the receptor indicating that the steroidal backbone of RU 39 411 and RU 45 144 did not play a major role in this regard. Since this free side-chain had been reported to be totally devoid of antiestrogenicity, one may consider that the steroidal backbone of these two antiestrogens partcipate to their antiproliferative activity. One may speculate that within the cell, ER should convey such compounds to CaM leading to a blockade of CaM-dependent catalyses. This hypothesis would also be relevant to the stilbene backbone of tamoxifen.

Antiestrogenic activity of triphenylkethylene and derivatives has been widely reported in the endocrinological literature [1]. Considerable attention has been given to the mechanism of action of such drugs since they were demonstrated to antagonize the development of breast cancers, especially those containing ER*. However, such drugs also usually display a weak estrogenic activity which may have undesirable consequences on treated patients. Of these drugs, tamoxifen is now the compound of reference in view of its high efficacy and lack of major side effects [2, 3].

The original view was that triphenylethylene antiestrogens compete with endogenous estradiol for binding to ER thereby blocking the expression of estrogen-dependent genes stimulating cell growth [1]. However, recent studies demonstrated that these drugs bind to a lot of macromolecules, some of which should also contribute to the antiproliferative activity in view of their involvement in signal transduction related to the action of growth factors. Along this line, the discovery that tamoxifen and analogs are competitive inhibitors of a calmodulin-dependent cyclic AMP phosphodiesterase [4–6] led to the finding of an association between tamoxifen and CaM [7, 8]. The additional observation of a correlation between the inhibitory activity of such compounds and their cytotoxicity in the MCF-7 breast cancer cell model [6] suggested that the tamoxifen-CaM association should be of prominent importance for the antitumor activity of the drug. This concept was reinforced by our observation that binding of tamoxifen to CaM impedes the association of the latter with ER [9], a property playing a role in the phosphorylation of the receptor [10, 11].

A common chemical property of antiestrogens is their ethoxyaminodialkyl side-chain which has been shown to confer their antiproliferative activity [12]. Interestingly, this chain is directly implicated in the antagonistic effect of these drugs towards the ER–CaM association: bisphenol (Formula in Fig. 1) a compound devoid of such a chain is ineffective in this regard [9].

In view of the structural analogy between triphenylethylene and steroid estrogens [13, 14] the Roussel Uclaf Co. promoted the synthesis of estradiol derivatives bearing the side-chain of tamoxifen in position 11β (RU 39 411) [15] or 7α (RU 45 144) [16] to produce steroidal antiestrogens (Formulas in Fig. 1). Molecular modeling studies suggested that such graftings would orient this chain in a position similar to that found in tamoxifen. These syntheses were crowned with success: both compounds displayed antiuterotrophic activity and produced growth inhibition of ER-positive human mammary cell lines [16–18].

RU 39 411 and RU 45 144 as well as two analogs devoid of antiuterotrophic activity were tested in our laboratory for their potential antagonism towards the CaM–ER association. The results of our investigation clearly demonstrated such an antagonism indicating that the grafting of a phenylethoxyaminodialkyl side-chain on molecules known to interact with ER may generate a receptor-mediated anti-CaM activity.

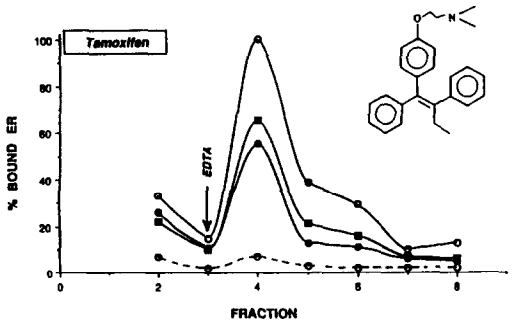
Materials and Methods

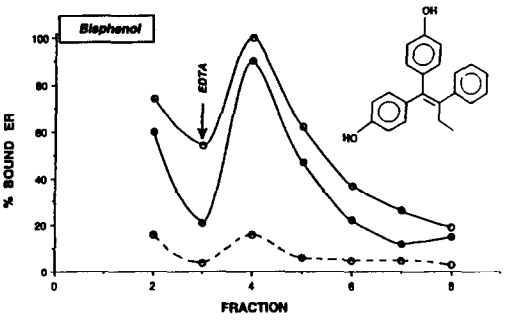
Reagents. [^{3}H]E_2 (86 Ci/mmol) was purchased from Amersham (U.K.). Unlabeled E_2 was from the Sigma Chemical Co. (St Louis, MO, U.S.A.). RU 39 411, RU 45 144 and RU 48 660 were provided by Dr P. Van de Velde from Roussel Uclaf (Romainville, France) and tamoxifen by Dr A. E. Wakeling from Zeneka (Macclesfield, U.K.). DMAEE was a gift of Dr Y. J. Abul-Hajj, University of Minnesota, Mineapolis, MN. 1-[2-(*p*-Bromophenoxy)ethyl] pyrrolidine was obtained from Aldrich Europe, Belgium.

Cytosol preparation. Uteri were dissociated from 25 day-old rats (IOPS Wistar from Iffa Credo, France). After removal they were washed and then homogenized in 10 mM phosphate buffer pH 7.4 containing 10 mM monothioglycerol and 10% glycerol (PTG buffer) by means of a whole glass homogenizer (4 mL buffer/1 g tisue).

* Abbreviations: CaM, calmodulin; DMAEE, 11β-[2-(*N*,*N*-dimethylamino)ethoxy]estradiol; E_2, estradiol; ER, estrogen receptor; PTG, 10 mM phosphate pH 7.4 containing 10 mM monothiolglycerol and 10% glycerol; PTG Ca^{2+}, PTG containing 1 μM $CaCl_2$.

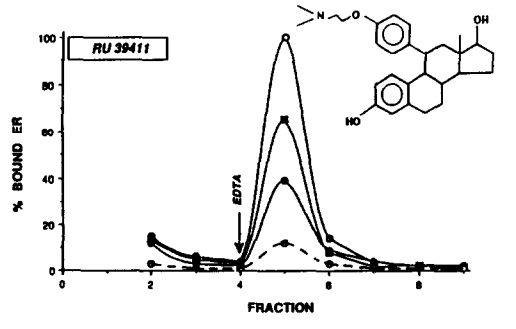
TRIPHENYLETHYLENES

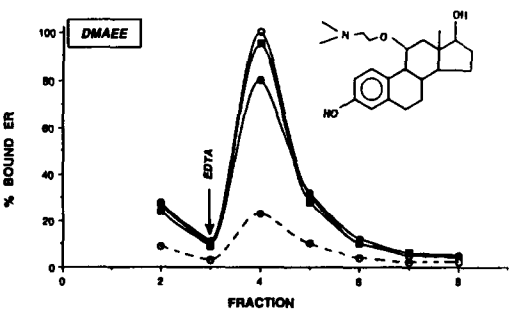




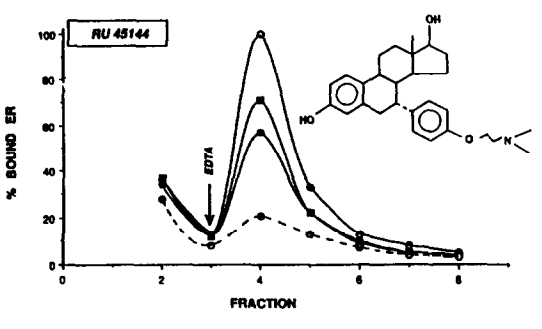
STEROIDS

11β derivatives





7α derivatives



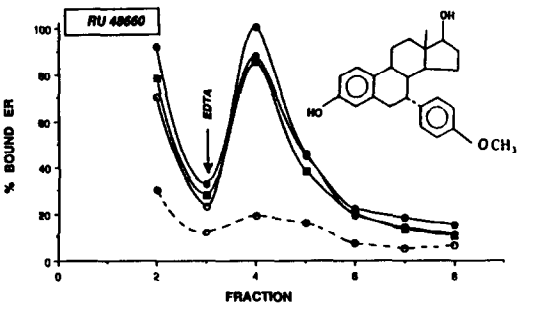


Fig. 1. Antagonism of investigated antiestrogens (tamoxifen, RU 39 411 and RU 45 144) and related compounds devoid of antiestrogenicity (bisphenol, DMAEE and RU 48 660) on the binding of $[^3H]E_2$ labeled ER to CaM-Sepharose. Each compound was added to $[^3H]E_2$ labeled cytosol to reach a final concentration of 10^{-7} (■) or 10^{-6} (●) M before its elution on CaM-sepharose; control (○). On the graph, the arrow indicates the beginning of the elution with EDTA. ER contents of each elution fraction is given by the difference between the radioactivity of the samples labeled with $[^3H]E_2$ alone (full line) and an excess of unlabeled E_2 (dotted line). Data are expressed as percentage of the highest level of radioactivity released with EDTA. The figure shows that antiestrogens solely impede the binding of ER to CaM-Sepharose.

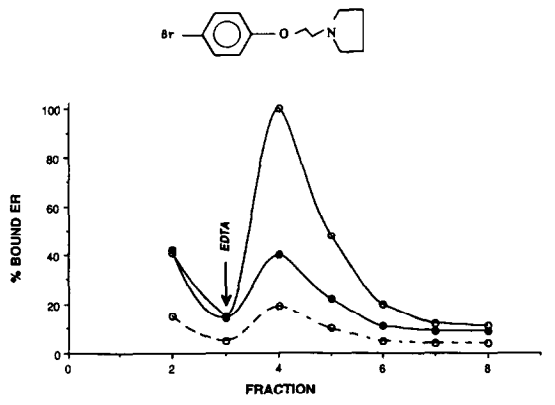


Fig. 2. Antagonism of an analog of the side-chain of investigated antiestrogens on the binding of [^{3}H]E_2 labeled ER to CaM–Sepharose. Experimental conditions and analysis of the data were conducted as in Fig. 1.

Cytosol was subsequently obtained by ultracentrifugation of the homogenate at 100,000 *g* for 1 hr and stored in liquid nitrogen until assay (1–2 weeks).

Labeling and treatment of cytosol for CaM–Sepharose chromatography. Cytosol samples were incubated for 1 hr at 0° with 1 nM [^{3}H]E_2 either in absence or in presence of a 200-fold excess unlabeled E_2. These samples were then treated with dextran-coated charcoal (0.5% charcoal, 0.05% dextran) to remove unbound steroids. Specifically bound [^{3}H]E_2 was measured from the difference in bound radioactivity between both samples. Radioactivity levels were measured by liquid scintillation counting.

CaM-Sepharose (1 mL) was equilibrated with PTG buffer containing 1 μM $CaCl_2$ (PTG Ca^{2+}) in a 1 × 10 cm column kept at 4° for all experiments. For each chromatographic run, 1 mL of [^{3}H]E_2 labeled cytosol (with or without an excess of unlabeled E_2) was mixed with this matrix, incubated overnight and eluted with 4 mL of PTG Ca^{2+} buffer to remove all unbound material. Bound [^{3}H]E_2 ~ ER complexes were subsequently extracted with PTG buffer containing 10 mM EDTA.

Tamoxifen and steroid antiestrogens were added to the [^{3}H]E_2 labeled cytosol samples at the time of mixing with the CaM–Sepharose for evaluating their ability to impede the association between ER and CaM; steroids devoid of antiestrogenicity as well as 1-[2-(*p*-bromophenoxy)ethyl]-pyrrolidine were processed identically.

Results and Discussion

As shown in Fig. 1, RU 39 411 and RU 45 144 impede the binding of ER to CaM–Sepharose at the same concentrations as tamoxifen indicating similar effectiveness (note that tamoxifen was previously shown as strong as the anti-CaM drug calmidazolium [9]). Since both compounds operated with [^{3}H]E_2 saturated ER, a receptor-mediated process should not be taken into account and a direct action on CaM must be considered. Interestingly, DMAEE [19] which differs solely from RU 39 411 by the lack of a phenyl group between the steroidal backbone and the ethoxyaminodialkyl side-chain was devoid of significant antagonistic activity. On the other hand, and as previously reported for bisphenol [9], the absence of this chain also suppressed the antagonistic activity of the compounds (RU 48 660 versus RU 45 144). Hence, both the phenoxy group and aminodialkyl residue [6] of the side-chain appear to be required for binding to CaM; the former group may interact with a few amino acids of the protein and/or orient the latter residue in an appropriate direction thereby allowing its interaction with other amino acids. This (these) interaction(s) should be relevant to the antiproliferative activity of the drugs in view of the lack of antiestrogenicity of DMAEE [19], RU 48 660 and bisphenol.

An analogue of the side-chain (1-[2-(*p*-bromophenoxy)ethyl]pyrrolidine) was tested to evaluate the importance of the steroidal backbone of RU 39 411 and RU 45 144 in their antagonistic activity towards the association between the ER and CaM (this analysis was also relevant to the stilbene part of tamoxifen). This compound impeded the binding of the receptor to CaM–Sepharose (Fig. 2) clearly indicating that the steroidal backbone did not play a major role in this regard. The fact that this analog was reported to be totally devoid of binding affinity for ER [20] was an additional proof that steroidal as well as triphenylethylenic antiestrogens operate at this level by interacting directly with CaM.

It is noteworthy that this free side-chain, as well as its analog *t*-butylphenoxyethyl diethylamine [21], were reported to be totally devoid of antiestrogenicity and anti-mammary tumor potency [20, 21]. One may therefore consider that binding to ER of the steroidal and stilbene backbone of antiestrogens participate to their antiproliferative activity. Indeed, one may speculate that within the cell, ER should convey such drugs to CaM leading to a blockage of CaM-dependent catalyses [22]. Such a hypothesis implicates a dissociation of the drug–ER complex with a transfer of the former to CaM. The fact that in cell culture, triphenylethylene antiestrogens usually display a relatively low binding affinity for ER (roughly 100–1000-fold less than E_2) [23], is surely not in contradiction with this hypothesis. Such a specific transport process of antiestrogens may explain why they produce a cytotoxic effect mainly on ER-positive cells [24]. Exchange experiments conducted with radiolabeled drugs in ER and CaM preparations may help to evaluate the validity of this hypothesis. Such investigations are now undertaken in our laboratory.

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REFERENCES

1. Lerner LJ and Jordan VC, Development of antiestrogens and their use in breast cancer: eight Cain memorial award lecture. *Cancer Res* **50**: 4177–4189, 1990.
2. Love RL, Tamoxifen therapy in primary breast cancer: biology, efficacy and side effects. *J Clin Oncol* **7**: 803–815, 1989.
3. Early breast cancer trialist collaborative group, Systemic treatment of early breast cancer by hormonal, cytotoxic or immunotherapy: results for tamoxifen. *Lancet* **339**: 6–13, 1992.
4. Lam HYP, Tamoxifen is a calmodulin antagonist in the activation of c-AMP phosphodiesterase. *Biochem Biophys Res Commun* **118**: 27–32, 1984.
5. Gulino A, Barrera G, Vacca A, Farina A, Ferretti C,

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Screponti J, Dianzanni MW and Fratti L, Calmodulin antagonism and growth inhibiting activity of triphenylethylene antiestrogens in MCF-7 human breast cancer cells. *Cancer Res* **46**: 6274–6278, 1986.

6. Rowlands MG, Parr IB, McCague R, Jarman M and Goddard PM, Variation of the inhibition of calmodulin dependent cyclic AMP phosphodiesterase among analogues of tamoxifen: correlation with cytotoxicity. *Biochem Pharmacol* **40**: 283–289, 1990.
7. Lopes F, Graca M, Vale P, and Carvahlo AP, Ca^{2+} dependent binding of tamoxifen to calmodulin isolated from bovine brain. *Cancer Res* **50**: 2753–2758, 1990.
8. Edwards KJ, Laughton CA and Neidle S, A molecular modeling study of the interactions between the antiestrogen drug tamoxifen and several derivatives, and the calcium-binding protein calmodulin. *J Med Chem* **35**: 2753–2761, 1992.
9. Bouhoute A, and Leclercq G, Antagonistic effect of triphenylethylenic antiestrogens on the association of estrogen receptor to calmodulin. *Biochem Biophys Res Commun* **184**: 1432–1440, 1992.
10. Castoria G, Miggliaccio A, Nola E and Aurichio F, *In vitro* interaction of estradiol receptor with Ca^{++}-calmodulin. *Mol Endocrinol* **2**: 167–174, 1988.
11. Miglaccio A, Rotondi A and Aurichio F, Calmodulin-stimulated phosphorylation of the 17β-estradiol receptor on tyrosine. *Proc Natl Acad Sci USA* **81**: 5921–5925, 1984.
12. Jordan VC and Gosden B, Importance of the alkylaminoethoxy side-chain for estrogenic and antiestrogenic actions of tamoxifen and trioxifene in the mature rat uterus. *Mol Cell Endocr* **27**: 291–306, 1982.
13. Pons M, Michel F, Crastes de Paulet A, Gilbert J, Miquel J-F, Précigoux G, Hospital M, Ojasoo T and Raynaud J-P, Influence of new hydroxylated triphenylethylene (TPE) derivatives on estradiol binding to uterine cytosol. *J Steroid Biochem* **20**: 134–145, 1984.
14. Duax WL, Molecular details of receptor binding and hormonal action of steroid derived from X-ray crystallographic investigations. *J Steroid Biochem* **15**: 41–47, 1981.
15. Teutsch JG, Torelli V, Deraedt R and Philibert D, 19-Norsteroids substituted at C-11 and C-2. Fr Demande FR 2,528,434 (11/06/1982). *Chemical Abstracts* **101**: 23815 n, 1984.
16. Nique F, Nédelec L, Bouton MM and Philibert D, New 7-aryl-substituted, 19-norsteroids useful as proliferative, antiestrogens, and/or estrogens, and their preparation, use as medicines, and pharmaceutical compositions. Eur. Patent Appl. EP 280,618 (31/08/1988). *Chemical Abstracts* **110**: 115181 s, 1989.
17. Nédelec L, Bouton MM, Nique F, Teusch G, Van de Velde P and Philibert D, 11β-Aminoalkoxyphenylestradiols, a new series of potent anticstrogens. *9th Int Symp J Steroid Biochem, Las Palmas, Spain,* Abstract 34 P.
18. Gottardis MM, Jiang S-Y, Jeng M-H and Jordan VC, Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. *Cancer Res* **49**: 4090–4093, 1989.
19. Quian X and Abul-Hajj Yj, Synthesis and biological activities of 11β-Substituted estradiol as potential antiestrogens. *Steroids* **55**: 238–241, 1990.
20. Leclercq G, Devleeschouwer N and Heuson JC, Guidelines in the design of new antiestrogens and cytotoxic-linked estrogens for the treatment of breast cancer. *J Steroid Biochem* **19**: 75–85, 1983.
21. Sheen YY, Simpson DM and Katzenellenbogen BS, An evaluation of the role of antiestrogen-binding sites in mediating the growth modulatory effects of antiestrogens: studies using t-butylphenoxyethyl diethylamine, a compound lacking affinity for the estrogen receptor. *Endocrinology* **117**: 561–564, 1985.
22. Rochefort H, Do antiestrogens and antiprogestins act as hormone antagonists or receptor-targeted drugs in breast cancer? *Trends Pharmacol Sci* **8**: 126–128, 1987.
23. Stoessel S and Leclercq G, Competitive binding assay for estrogen receptor in monolayer culture. *J Steroid Biochem* **25**: 677–682, 1986.
24. Bardon S, Vignon F, Montcourrier P and Rochefort H, Steroid receptor-mediated cytotoxicity of an antiestrogen and an antiprogestin in breast cancer cells. *Cancer Res* **47**: 1441–1448, 1987.