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SHORT COMMUNICATION

Antagonism of Estrogen Receptor and Calmodulin Association by Antiestrogens Is Not Dependent on an Interaction with Calmodulin

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ABSTRACT. Previously, two antiestrogenic estradiol derivatives (**3** and **4**) bearing the basic side chain of tamoxifen were shown to impede the binding of the estrogen receptor (ER) to calmodulin (CaM)-Sepharose. In this study, the interaction of these and related compounds with calmodulin was examined using the cyclic AMP phosphodiesterase (cAMP-PDE) assay. Neither of the steroids gave any significant inhibition of the calmodulin dependent cAMP-PDE activity up to a final concentration of 20 μM. For comparison, tamoxifen and nafoxidine produced IC_{50} values of 6.7 μM ± 1.0 and 7.4 μM ± 1.1, respectively. In addition, a comparison was made of the activity of some triphenylethylene derivatives against CaM dependent cAMP-PDE and the ER-CaM Sepharose assays, but no relationship was observed. Overall, these results demonstrate that inhibition of the ER-CaM association by various steroidal and triphenylethylene antiestrogens does not relate to antagonism of calmodulin function or their binding affinity for the estrogen receptor. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;2:241–244, 1997.

KEY WORDS. tamoxifen analogues; triphenylethylenes; steroidal antiestrogens; cAMP phosphodiesterase

Calmodulin, which is a calcium binding protein that is involved in many intracellular signalling pathways, has been shown to bind to the estrogen receptor [1, 2]. The triphenylethylene antiestrogen tamoxifen can prevent the formation of the ER-CaM§ complex [2]. Originally, tamoxifen was identified as a calmodulin antagonist by its inhibition of calmodulin dependent cyclic AMP phosphodiesterase [3]. Further work demonstrated a direct interaction of tamoxifen with calmodulin using TNS as a fluorescent probe [4] or tritiated tamoxifen aziridine in competitive binding experiments [5]. Structure–activity studies on a range of tamoxifen analogues showed that the dimethylaminoethoxy side chain of tamoxifen was essential for calmodulin antagonism [4, 6] and inhibition of the association of ER with calmodulin [2]. Interestingly, the basic side chain is required for the antiproliferative action of the drug [7, 8].

To produce steroidal antiestrogens, estradiol derivatives were synthesised with the basic side chain of tamoxifen in position 11β (RU 39411, **3**) or 7α (RU 45144, **4**) on the steroid nucleus (Fig. 1). Both compounds were potent antiestrogens and inhibited growth of human breast cancer cell lines [9, 10, 11]. Furthermore, **3** and **4** were as effective as tamoxifen in blocking the binding of ER to CaM-Sepharose [12]. We report here the inability of these steroidal compounds to inhibit the activity of calmodulin dependent cAMP-PDE and alter the fluorescence exhibited by the TNS + Ca^{2+}/calmodulin complex. In addition, we show that for selected antiestrogens there is no relationship between the inhibition of calmodulin function, antagonism of the interaction between ER and CaM, or binding affinity for the ER.

MATERIALS AND METHODS

Materials

[^{3}H]E_2 (~100 Ci/mmol) and [^{2}H]cAMP (~25 Ci/mmol) were purchased from Amersham International (Bucks, U.K.). RU 39411 (**3**) and RU 45144 (**4**) were provided by Dr. P. Van de Velde from Roussel Uclaf (Romainville, France). Tamoxifen (**1**) and 1-[2-(*p*-bromophenoxy)ethyl]-pyrrolidine (**5**) were obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Nafoxidine (**2**) was purchased from Sigma Chemical Co. (Poole, U.K.). Analogues of tamoxifen were synthesised by the following literature procedures: (**6**) [13], (**7**) [14], and (**8**) [15].

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§ *Abbreviations:* ER, estrogen receptor; E_2, estradiol; CaM, calmodulin; TNS, 2-*p*-toluidinyl-naphthalene-6-sulfonic acid; RBA, relative binding affinity.

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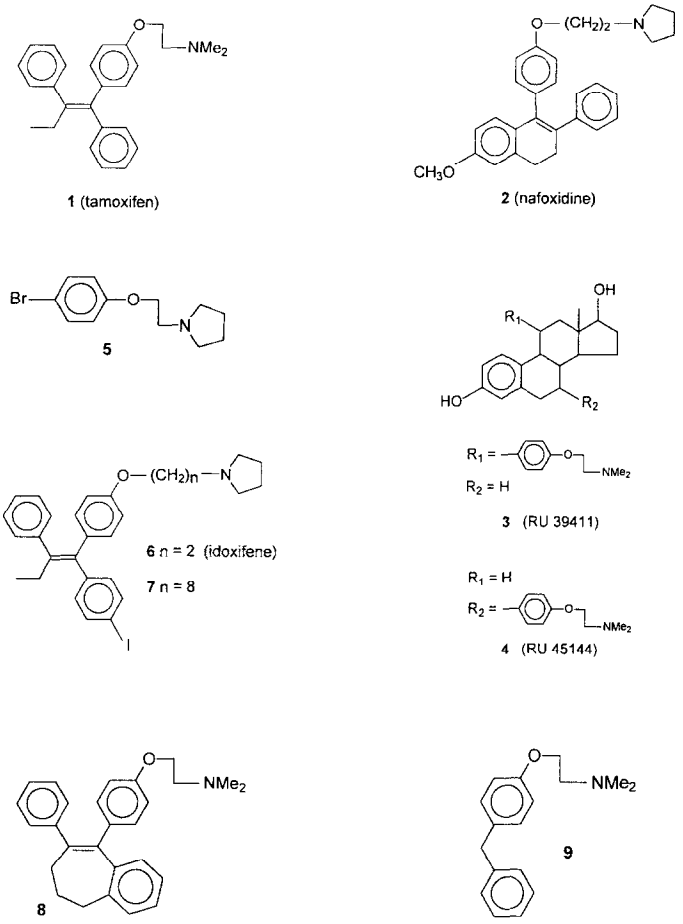

FIG. 1. Structures of compounds.

CaM-Sepharose Chromatography

The binding of [^{3}H]E_2 labeled ER to CaM-Sepharose was carried out as previously described [2, 12]. Test compounds were added to the [^{3}H]E_2 labeled rat uterine cytosol samples at the time of mixing with CaM-Sepharose to evaluate their ability to impede the association between ER and CaM. Bound [^{3}H]E_2-ER complexes were eluted with EDTA. The percentage inhibition is the difference in levels of radioactivity of the [^{3}H]E_2-ER complexes eluted from CaM-Sepharose with EDTA in the presence and absence of 1 μM of the test compounds.

Assays for Calmodulin Antagonism and Binding to ER

The calmodulin dependent and independent cAMP-PDE activities were assayed as previously described [6, 16]. The results are expressed as the concentration of inhibitor giving 50% inhibition of the calmodulin dependent cAMP-PDE (IC_{50}); each IC_{50} value is the mean ± SE of triplicate determinations. The fluorescence measurements were carried out as previously described [16]. All the compounds were tested at a final concentration of 0.5 and 1.0 μM for an interaction with the fluorescence produced by 20 μM TNS in the presence of calmodulin at 2 μM. The fluorescence of the basal samples (+1 mM EGTA) was subtracted from the test samples in order to obtain the relative fluorescence intensity. The estrogen receptor binding assays were carried out as described [13, 15], with the relative concentrations of E_2 and the test compound required to achieve 50% inhibition of [^{3}H]E_2 binding as the RBA: i.e. RBA = (IC_{50} for E_2)/IC_{50} for test compound) × 100.

RESULTS AND DISCUSSION

Previously, the steroidal compounds **3** and **4** and an analogue of the basic side chain, namely 1-[2-(*p*-bromophenoxy)ethyl]pyrrolidine (**5**), were found to block the binding of ER to CaM-Sepharose with a potency similar to tamoxifen (Table 1) [12]. It was suggested that the mechanism of action was via a direct interaction with calmodulin. In the present study, we have demonstrated that none of the aforementioned compounds inhibited the calmodulin dependent cAMP-PDE activity up to a final concentration of 20 μM (IC_{50} values of >20 μM in Table 1). Tamoxifen (**1**) and nafoxidine (**2**) produced a dose dependent inhibition of this enzyme with IC_{50} values of 6.7 and 7.4 μM, respectively. Using a similar assay method, MacNeil *et al.* [17] published values of 8.0 μM for tamoxifen and 7.7 μM for nafoxidine. Furthermore, none of the three compounds (**3**, **4**, and **5**) elicited an increase in fluorescence intensity of the TNS + Ca^{2+}/calmodulin complex that is characteristic of a direct interaction with calmodulin as demon-

TABLE 1. Comparison of calmodulin antagonism with inhibition of ER-CaM association and RBA for the ER

Compound	CaM antagonism inhibition of cAMP-PDE (IC_{50}, μM)*	ER-CaM association (% inhibition at 1 μM)	RBA for ER (E_2 = 100)
1	6.7 ± 1.1	53	1
2	7.4 ± 1.0	58	5
3	>20	55	33
4	>20	37	33
5	>20	53	0
6	1.5 ± 0.1	65	5
7	0.3 ± 0.05	37	0.05
8	>20	47	1
9	>20	40	0

As an indication of the variability of the ER-CaM association assay, the result with tamoxifen (1) of 53% inhibition at 1 μM has an SD of 3.3 after 10 different experiments (N = 10).

* None of the compounds inhibited the CaM independent activity of cAMP-PDE when assayed at final concentrations of 10 and 20 μM.

strated by the established calmodulin antagonists (Table 2). Previous studies have shown a good correlation between the TNS fluorescence assay and the CaM dependent cAMP-PDE assay [4, 16].

Idoxifene (6) and its analogue with an elongated basic side chain (7) are based on the triphenylethylene structure of tamoxifen, but were more potent calmodulin antagonists (4-fold and 22-fold, respectively) in the cAMP-PDE assay. This increase in potency was not reflected in their ability to impede the ER-CaM Sepharose association (Table 1). Although idoxifene was slightly better than tamoxifen in this regard, 7 was worse. Compounds 8 and 9 were inactive in the cAMP-PDE assay, but retained activity in the ER-CaM-Sepharose assay. The fused ring analogue of tamoxifen, 8, is interesting, because although it lacks calmodulin antagonism it retains the same relative binding affinity for the estrogen receptor and antitumour ability against MCF-7 cells as tamoxifen [15]. These structure–activity results demonstrate that the ability of various steroidal and non-steroidal antiestrogens to block the association of calmodulin to the estrogen receptor is not dependent on a direct interaction with calmodulin, but may be a feature of the compounds' binding to the estrogen receptor. However, a comparison of the RBA values for the compounds' binding to the ER with E_2 as a ligand showed no relationship to either antagonism of the ER-CaM association or inhibition of CaM-PDE. Therefore compounds like 5 and 9, which have no RBA, may be binding to a site on the ER that is distinct from the E_2 binding domain but crucial for the ER-CaM association. In support of this hypothesis, a specific antiestrogen binding domain was recently identified [18, 19]. On the other hand, the antiestrogens can span both sites, thereby blocking the binding of estrogen and calmodulin. Further investigations are under way in our laboratories to explore the relationship of calmodulin to the functioning of the estrogen receptor.

TABLE 2. Relative fluorescence intensity for the interaction of the compounds with calmodulin

Compound	% Fluorescence intensity relative to control	
	0.5 μM	1.0 μM
1	128	145
2	136	170
3	105	115
4	100	100
5	100	95

Each result is the mean of triplicate determinations that were within 10%.

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