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Structure–activity relationships of calmodulin antagonism by tryphenylethylene antiestrogens

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The triphenylethylene antiestrogen tamoxifen is widely used in the treatment of human breast cancer because of its growth inhibiting activity on tumor cells [1]. The competition of tamoxifen for intracellular estrogen receptor seems to interfere with estrogen-regulated processes related to cell growth [2]. However, the antiestrogen-induced inhibition of the growth of some estrogen receptor-negative breast cancer cells [3] suggests the possibility of additional mechanisms of action of this drug. This is also supported by the findings of high affinity specific binding sites for triphenylethylene antiestrogens, which are distinct from estrogen receptors, in several experimental models [4–6]. Calmodulin (CaM)* is a ubiquitous protein that binds calcium ions and is thereby activated to regulate several enzymes and physiological cellular processes [7]. Some evidence suggests a relationship between neoplastic cell transformation and growth and CaM-mediated events. Transformation of cells to malignancy appears to be one general mechanism causing a specific increase in the intracellular content of CaM [7]. Furthermore, CaM has been reported to play an important role in initiation of DNA synthesis, as studied using CaM antagonists [7]. Finally, several CaM antagonists possess antitumor activity *in vivo* [8]. Tamoxifen has been recently reported to antagonize CaM in the activation of cyclic AMP-phosphodiesterase (cAMP-PDE) system [9], but the structure–activity relationship of this antagonism has not been studied. Since several modifications of the tamoxifen molecule influence its growth inhibiting potency on breast cancer cells [3], we have investigated the ability of several triphenylethylene derivatives of antagonizing CaM in the activation of cAMP–PDE.

Materials and methods

Materials. Hog brain CaM was obtained from Boehringer (Mannheim, F.R.G.). Activator-deficient bovine brain cAMP-PDE, 5′-nucleotidase from *Crotalus atrox* venom, cAMP and estradiol were purchased from Sigma Co. (St Louis, MO). Tamoxifen (ICI 46,474, *trans*-1-(4-dimethylaminoethoxyphenyl)1,2-diphenylbut-1-ene), 4-hydroxytamoxifen (ICI 79,280), 3,4-dihydroxytamoxifen (ICI 77,307), *N*-desmethyltamoxifen (ICI 55,548), Metabolite A (ICI 46,929, 1-(4-dimethylaminoethoxyphenyl)1-(hydroxy)-2-diphenylbutane), nafoxidine (U11,100A, 1-[2 - (*p* - (3,4 - dihydroxy - 6 - methoxy - 2 - phenyl - 1-naphtyl)-phenoxy)ethyl]pyrrolidine hydrochloride) were kindly provided by ICI Pharma (U.K.).

CaM-dependent cAMP-PDE activity assay. CaM-dependent cAMP-PDE activity was assayed according to the method of Theo [10] in a reaction mixture (0.9 ml) containing 72 mM Tris, 36 mM imidazole, 18 mM magnesium acetate (pH 7.5), 0.9 mM cAMP, 8 mU cAMP-PDE, 0.1 U 5′-nucleotidase, 0.1 mM $CaCl_2$ and 0.1 μg CaM, in the presence or in the absence of different concentrations of the triphenylethylene derivatives or estradiol, dissolved in dimethylformamide (final concentration 1%), in order to determine the concentration of the inhibitor giving 50% inhibition of CaM-dependent cAMP-PDE activity (IC_{50}). The 1% dimethylformamide concentration did not affect significantly the enzyme activity. Incubation was carried out at 30° for 30 min and then stopped by adding 0.1 ml 55% trichloroacetic acid. The inorganic phosphate liberated in the reaction mixture was determined by the method of Ames [11]. The basal activity of cAMP-PDE (CaM-independent) was determined in the absence of CaM.

Results and discussion

Figure 1 shows the structure of the compounds used in this study for antagonizing CaM activity on the cAMP-PDE system.

Tamoxifen was an effective antagonist of CaM in the activation of cAMP-PDE (IC_{50} 4.4 ± 0.4 (S.D.) μM) (Fig. 2A and Table 1), while it did not affect the CaM-independent activity of cAMP-PDE up to 10^{-4} M concentrations (Fig. 2B). Several structural modifications of the antiestrogen molecule affect to a different extent the antagonistic potency on CaM activity. The effect of several hydroxylated derivatives on both CaM-dependent and -independent cAMP-PDE activity is shown in Figs. 2A and B and Table 1. CaM-independent cAMP-PDE activity was not affected by metabolite A and 4-hydroxytamoxifen up to 10^{-4} M concentrations, while 5×10^{-5} M and higher concentrations of 3,4-dihydroxytamoxifen significantly decrease enzyme activity. The saturation and hydroxylation of the ethylene chain interconnecting the phenyl rings dramatically affects the antagonism of CaM-mediated activation of cAMP-PDE, since metabolite A slightly decreased the CAM-activated enzyme activity (IC_{50} 95 ± 9(S.D.) μM). The hydroxylation of other regions of the antiestrogen molecule (i.e. phenyl ring) also decreases the antagonistic potency, although to a lesser extent compared to metabolite A. The concentration of 4-hydroxytamoxifen giving 50% inhibition of CaM-dependent cAMP-PDE activity was 8.2 ± 1.6 (S.D.) μM. When lower concentrations of 3,4-dihydroxytamoxifen than those inhibiting CaM-independent cAMP-PDE activity were investigated for antagonizing CaM-activated cAMP-PDE, an intermediate potency was observed compared to 4-hydroxytamoxifen and metabolite A (IC_{50} 15.6 ± 1.9(S.D.) μM). The aminoether side chain of tamoxifen does not seem to play a role in the control of the antagonism of CaM-dependent activation of cAMP-PDE, since no significant difference was observed between *N*-desmethyltamoxifen and tamoxifen (Fig. 2A and Table 1). In contrast, *N*-desmethyltamoxifen significantly decreased CaM-independent cAMP-PDE activity, although at high concentration (Fig. 2B). No significant difference between nafoxidine and tamoxifen was observed in antagonizing both CaM-dependent and -independent cAMP-PDE activity (Fig. 2 and Table 1).

The enzyme assay used in this study was a two-enzyme coupled system, cAMP-PDE and 5′-nucleotidase. In order to study whether the inhibitory effect of some of the triphenylethylene derivatives on CaM-indepedent cAMP-PDE described above was due to an effect on cAMP-PDE or 5′-nucleotidase, we have studied the effect of these antiestrogens on the hydrolysis of 5′AMP by purified 5′-nucleotidase. None of the compounds we have used in this study was able to inhibit 5′-nucleotidase activity up to 10^{-4} M concentrations (not shown).

Some of the structural characteristics of triphenylethylene antiestrogens are related to those of estrogens, since both classes of compounds are known to bind to

* Abbreviations used: CaM, calmodulin; cAMP-PDE, cyclicAMP-phosphodiesterase.

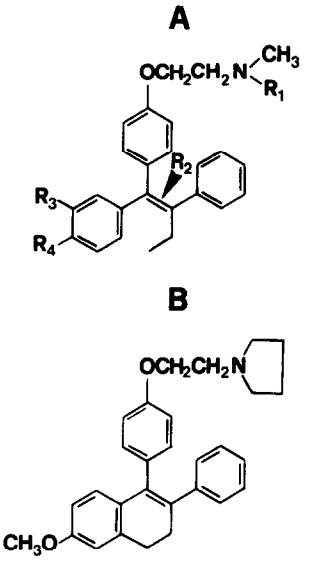

Fig. 1. Structure of triphenylethylene antiestrogens used in this study. Substitutions of R for each derivatives are: (A) tamoxifen (R_1, CH_3; R_3 and R_4, H), N-desmethyltamoxifen (R_1, H; R_3 and R_4, H), metabolite A (R_1, CH_3; R_2, CHOH–CH; R_3 and R_4, H), 4-hydroxytamoxifen (R_1, CH_3; R_3, H; R_4, OH), 3,4-dihydroxytamoxifen (R_1, CH_3; R_3 and R_4, OH); (B) nafoxidine.

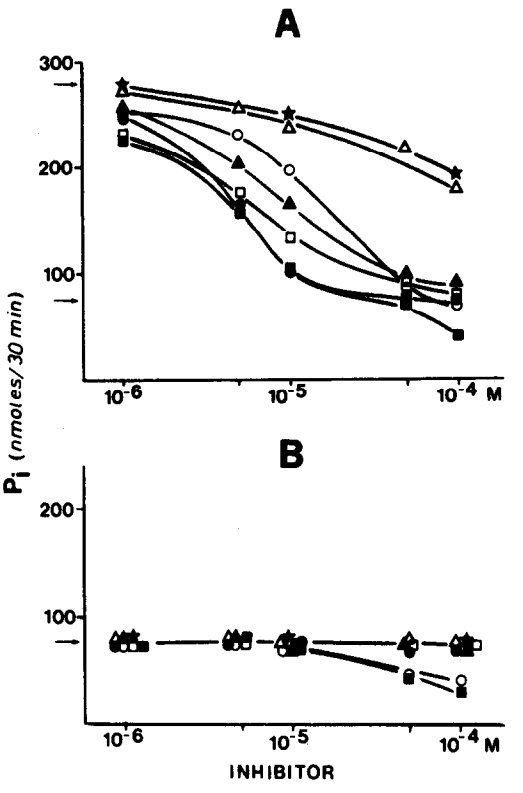

Fig. 2. Effect of different molar concentration of tamoxifen (●), metabolite A (△), 4-hydroxytamoxifen (▲), 3,4-dihydroxytamoxifen (○), N-desmethyltamoxifen (■), nafoxidine (□) and estradiol (★) on CaM-dependent (A) and CaM-independent (B) cAMP-PDE activity. (A) The activity of cAMP-PDE in the absence of the inhibitor and in the presence of CaM was 280 nmoles P_i/30 min (top arrow), while CaM-independent cAMP-PDE activity in the absence of inhibitor was 80 nmoles P_i/30 min (bottom arrow). (B) The arrow indicates the CaM-independent cAMP-PDE activity in the absence of the inhibitor. The results shown are the mean values from 3–5 experiments.

Table 1. Concentration of different triphenylethylene antiestrogens giving 50% inhibition (IC_{50}) of CaM-dependent cAMP-PDE activity

Drug	IC_{50} (± S.D.) μM	N	P
Tamoxifen	4.46 ± 0.4	4	
N-desmethyltamoxifen	3.40 ± 1.4	4	>0.05
Nafoxidine	4.50 ± 0.7	4	>0.05
4-Hydroxytamoxifen	8.20 ± 1.6	5	<0.01
3,4-Dihydroxytamoxifen	15.60 ± 1.9	5	<0.01
Metabolite A	95.00 ± 9	3	<0.01
Estradiol	≥100	3	

P values correspond to the statistical comparisons between IC_{50} of each drug versus that of tamoxifen (calculated using the Student's t-test). The results shown are the mean values (± S.D.) from N experiments.

estrogen receptor [1]. Consequently, we have investigated the effect of estradiol on CaM-activated cAMP-PDE activity. Interestingly, estradiol although at very high concentration, was able to inhibit CaM-dependent cAMP-PDE activity (IC_{50} > 100 μM), while it did not affect CaM-independent enzyme activity (Fig. 2 and Table 1).

The results reported in this paper suggest that the hydroxylation of the antiestrogen molecule represents the main structural modification affecting the ability to antagonize CaM activity. This is in agreement with the data reported by other authors who suggest that the general structural characteristics of CaM-inhibitory drugs are represented by a large hydrophobic region and a side chain amino group [12]. The decrease by hydrophobicity of the triphenylethylene group (hydrophobic region) consequent to its hydroxylation, could result in the decrease of the CaM antagonistic potency we have reported in this study. In conclusion, the different CaM antagonist potencies of the triphenylethylene derivatives we have described in this study, could permit a new approach to the study of the possible involvement of CaM-antiestrogen interactions in mediating the complex actions of these drugs on breast cancer cells.

In summary, structural modification of the triphenylethylene antiestrogen molecule affect to a different extent the drug-induced inhibition of calmodulin-activated cyclicAMP phosphodiesterase activity. The pattern of antagonistic potency was: tamoxifen = N-desmethyltamoxifen = nafoxidine > 4-hydroxytamoxifen > 3,4-dihydroxytamoxifen > metabolite A (IC_{50} (means ± S.D.): 4.4 ± 0.4 μM, 3.4 ± 1.4 μM, 4.5 ± 0.7 μM, 8.2 ± 1.6 μM, 15.6 ± 1.9 μM, 95 ± 9 μM, respectively). High concentrations (≥50 μM) of N-desmethyltamoxifen and 3,4-dihydroxytamoxifen were also able to inhibit calmodulin-independent cyclicAMP-phosphodiesterase activity. Our data suggest that hydroxylation of the antiestrogen molecule represents the main structural modification decreasing its calmodulin antagonistic potency.

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