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DIFFERENTIAL BINDING OF ANTIESTROGENS BY RAT UTERINE AND CHICK OVIDUCT CYTOSOL

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SUMMARY

Analysis of the interactions of two synthetic estrogen antagonists, tamoxifen and CI 628, with rat uterine and chick oviduct cytosol revealed significant differences in the antiestrogen binding properties of these tissues. In the rat uterus CI 628, tamoxifen and estradiol were bound to a similar number of saturable binding sites and estradiol could completely inhibit the binding of tritiated antiestrogens to these sites. In contrast, high affinity, saturable antiestrogen binding sites in chick oviduct were present at three times the concentration of estradiol binding sites and estradiol could only partially inhibit the binding of tritiated antiestrogens to these sites. It is concluded that antiestrogens bind to the estrogen receptor in both tissues and that chick oviduct has an additional saturable antiestrogen binding site distinct from the classical estrogen receptor site.

INTRODUCTION

Tamoxifen (1) and CI 628 (2) belong to a group of structurally related synthetic compounds commonly referred to as the non-steroidal antiestrogens. When tested in the immature rat uterotrophic assay these compounds act as partial estrogen agonists and partial antagonists (3,4). In contrast, studies with the immature chick oviduct have illustrated that tamoxifen (5) and a number of other structural analogues (Sutherland & Foo, unpublished data) are pure estrogen antagonists with no agonist activity. Thus the non-steroidal anti-estrogens demonstrate different modes of action in these two estrogen target tissues. In order to account for these differences in molecular terms, and to understand more clearly the modes of action of these compounds, we have initiated a systematic study of antiestrogen action in these two experimental systems. This paper describes experiments where the binding of tamoxifen and CI 628 to cytoplasmic preparations from rat uterus and chick oviduct was

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studied. Such studies revealed the presence of a novel, high affinity, saturable antiestrogen binding site in chick oviduct cytosol which is distinct from the classical estradiol receptor site.

MATERIALS AND METHODS

Materials. Tritiated estradiol (85-110 Ci/mmole) and tritiated CI 628 (18 Ci/mmole) were obtained from the Radiochemical Centre, Amersham, U.K. Unlabelled estradiol-17β was from Sigma and unlabelled CI 628 was a gift of Warner Lambert/Parke Davis, U.S.A. Tritiated tamoxifen (19.5 Ci/mmole) and unlabelled tamoxifen base were gifts of ICI Pharmaceuticals, U.K.

Cytosols. Uteri were from 21-23 day old Hooded rats. Oviducts were from 4-6 weeks estrogen withdrawn White Leghorn chicks (6). Tissues were homogenized in 10mM Tris-HCl, 1.5 mM EDTA buffer, pH 7.4 containing 0.5mM dithiothreitol. Cytosols were prepared as previously described (6). The homogenization volume was 1:25 (w/v) for rat uteri and 1:10 for chick oviducts.

Determination of Binding Parameters. In order to derive estimates of protein bound (B) and unbound (U) ligand over a wide range of ligand concentrations, 100 µl of cytosol was incubated with 50 µl tritiated ligand and 50 µl unlabelled ligand for 16 hr at 4°C. Tritiated estradiol was used at a final concentration of 0.25 nM and the antiestrogens at final concentrations of 1.25 nM in the reaction mixture. Unlabelled ligand concentrations were in the range 0.25-500 nM for estradiol and 1.25 nM - 2 µM for CI 628 and tamoxifen. Following incubation B and U were separated by charcoal adsorption, an aliquot of the supernatant was removed, counted in a liquid scintillation spectrometer using external standardization, and the molar concentrations of B and U calculated (6). The binding parameters i.e. the dissociation constant (K_d) and the binding capacity (C) of the saturable binding component and an estimate of the non-specific binding (K_{ns}), were derived from the molar concentrations of B and U using a non-linear, least squares regression analysis as previously described (7).

Competition Studies. To determine the ability of estradiol to compete for saturable antiestrogen binding sites and vice versa tritiated ligand at the concentrations used above, was incubated with cytosol and increasing concentrations of estradiol or antiestrogen for 16 hr at 4°C. B and U were separated by charcoal adsorption and the data plotted as percent tracer bound versus log of the ligand concentration. The relative binding affinities of CI 628 and tamoxifen with respect to estradiol were calculated from the amount of ligand required to displace 50% of the tracer estradiol (8).

RESULTS

Binding of Estradiol, CI 628 and Tamoxifen to Rat Uterine Cytosol.

Analysis of the data derived from saturation analysis studies using rat uterine cytosol, revealed that the measured values of B and U for all three ligands could be fitted most appropriately to a model consisting of one high affinity, saturable binding component and non-specific binding (7). This is illustrated in Fig. 1 where the binding data are plotted according to the

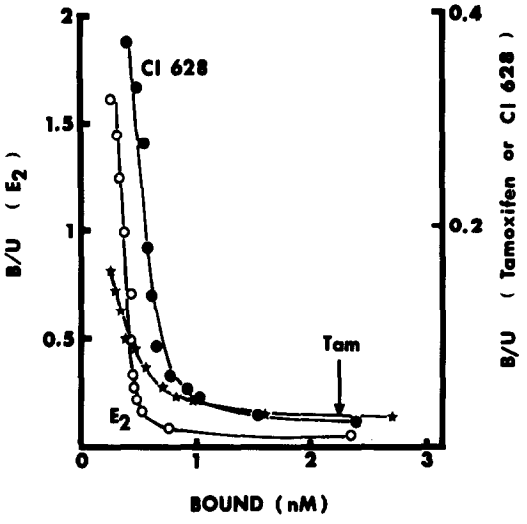

Fig. 1. BINDING OF ESTRADIOL, CI 628 AND TAMOXIFEN TO RAT UTERINE CYTOSOL.

Data are plotted according to Scatchard (9) and the calculated regression lines are shown. Estradiol (O), CI 628 (●) tamoxifen (★).

Scatchard transformation (9) and the calculated non-linear regression lines are shown. Replicate estimates of the binding parameters showed that the three ligands were bound to a similar number of saturable binding sites in rat uterine cytosol but with appreciably different affinities (Table 1). CI 628 was bound

TABLE 1

MEAN ($\pm$ SEM) PARAMETERS FOR ESTRADIOL, CI 628 AND TAMOXIFEN BINDING TO RAT UTERINE AND CHICK OVIDUCT CYTOSOL.

LIGAND	TISSUE	Kd (nM)	C (nM)	Kns
ESTRADIOL	RAT	0.17 $\pm$ 0.02	0.99 $\pm$ 0.07	0.039 $\pm$ 0.005
	CHICK	0.07 $\pm$ 0.02	0.19 $\pm$ 0.01	0.037 $\pm$ 0.004
TAMOXIFEN	RAT	2.54 $\pm$ 0.37	1.08 $\pm$ 0.08	0.040 $\pm$ 0.004
	CHICK	9.82 $\pm$ 1.96	0.66 $\pm$ 0.24	0.085 $\pm$ 0.043
CI 628	RAT	1.03 $\pm$ 0.18	1.53 $\pm$ 0.11	0.022 $\pm$ 0.003
	CHICK	1.90 $\pm$ 0.72	0.61 $\pm$ 0.17	0.039 $\pm$ 0.002

The binding parameters are defined in Materials and Methods. Binding capacity, C, is expressed as nmole/litre of cytosol which is diluted 2 fold in the incubation medium i.e. the final dilutions are 1:50 (w/v) amd 1:20 for rat uterus and chick oviduct, respectively. Non-specific binding, K_{ns}, is a unitless parameter (7).

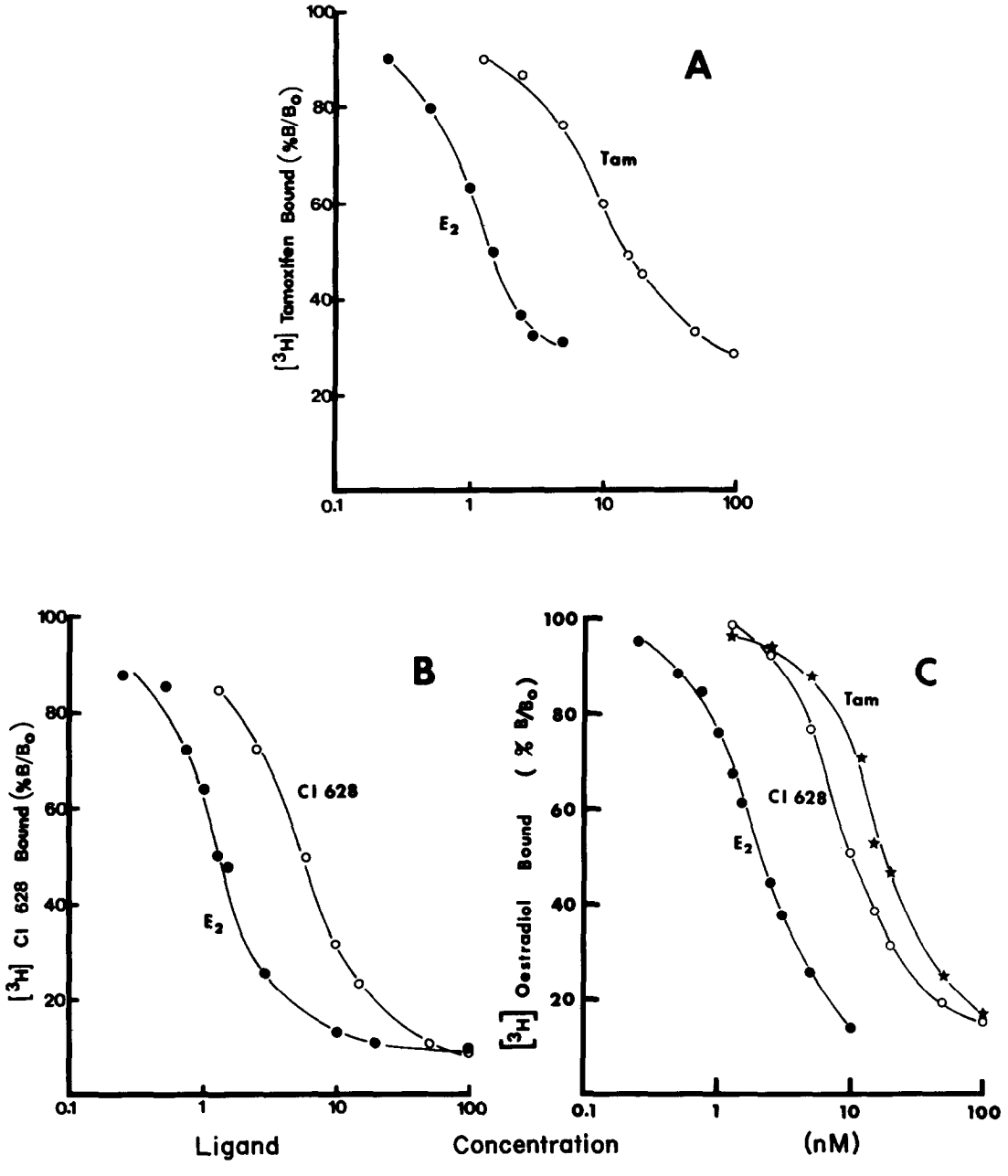

Fig. 2. COMPETITION OF ESTRADIOL, CI 628 AND TAMOXIFEN FOR ESTROGEN AND ANTIESTROGEN BINDING SITES IN RAT UTERINE CYTOSOL.

A. Estradiol (●) and tamoxifen (○) inhibition of tritiated tamoxifen binding.
B. Estradiol (●) and CI 628 (○) inhibition of tritiated CI 628 binding.
C. Estradiol (●), CI 628 (○) and tamoxifen (★) inhibition of tritiated estradiol binding.

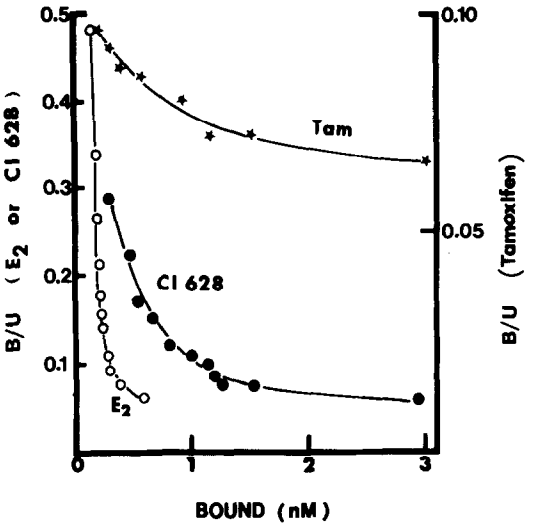

Fig. 3. BINDING OF ESTRADIOL, CI 628 AND TAMOXIFEN TO CHICK OVIDUCT CYTOSOL.

Data are plotted according to Scatchard (9) and the calculated regression lines are shown. Estradiol (O), CI 628 (●), tamoxifen (✱).

6 times less tightly than estradiol and tamoxifen 15 fold less tightly. Non-specific binding (K_{ns}) of tamoxifen and estradiol was very similar whilst that for CI 628 was 2 fold lower (Table 1).

Competition studies revealed that CI 628 and tamoxifen could completely inhibit the binding of trititated estradiol to its saturable binding site with CI 628 being the more potent inhibitor (Fig. 2C). Similar competition experiments, employing tritiated antiestrogens and unlabelled estradiol, demonstrated that estradiol could completely inhibit the binding of labelled CI 628 and tamoxifen to their saturable binding sites (Fig 2). Such data are compatible with the two antiestrogens binding to the high affinity, saturable estrogen receptor site of rat uterine cytosol.

Binding of Estradiol, CI 628 and Tamoxifen to Chick Oviduct Cytosol.

When the chick oviduct cytosol binding data for the three ligands were analysed, the measured B and U values were again fitted most appropriately by a model consisting of one saturable binding component and non-specific binding (Fig. 3). Replicate estimates demonstrated that both tamoxifen and CI 628 were bound to a

similar number of saturable binding sites but these sites were present at approximately three times the concentration of the saturable estradiol binding sites (Table 1). CI 628 had a 5 fold higher affinity for these sites than did tamoxifen (Table 1). Values for non-specific binding of CI 628 and estradiol were similar and were about 50% of the values seen with tamoxifen (Table 1).

Competition studies illustrated that unlabelled CI 628 and tamoxifen could completely inhibit the binding of estradiol to its saturable binding site with CI 628 being the more potent inhibitor (Fig. 4C). Further competition experiments using tritiated antiestrogens revealed that unlabelled estradiol could only partially inhibit the binding of these compounds to their saturable binding sites in chick oviduct cytosol (Fig. 4).

DISCUSSION

The results presented here confirm earlier observations that the synthetic non-steroidal antiestrogens bind to cytoplasmic estrogen receptor sites in estrogen target tissues (10-12). More importantly, our data also demonstrate the existence of an additional, hitherto undefined, high affinity, saturable antiestrogen binding site in chick oviduct which does not bind estradiol. The evidence for the existence of such a site is substantial. Firstly, direct binding studies with tritiated antiestrogens revealed that CI 628 and tamoxifen were bound to saturable binding sites which were present in three fold excess over saturable estrogen binding sites (Fig. 3, Table 1). However, it should be noted that the estimates of total antiestrogen binding sites derived from the computer analysis include antiestrogen binding to estrogen receptor sites and thus the concentration of specific antiestrogen binding sites i.e. those that do not bind estradiol is only twice that of the estrogen receptor. Secondly, competition experiments in which unlabelled estradiol could only partially inhibit tritiated antiestrogen binding to its saturable binding sites (Fig. 4) support the existence of a population of saturable antiestrogen binding sites distinct from the classical estrogen receptor site.

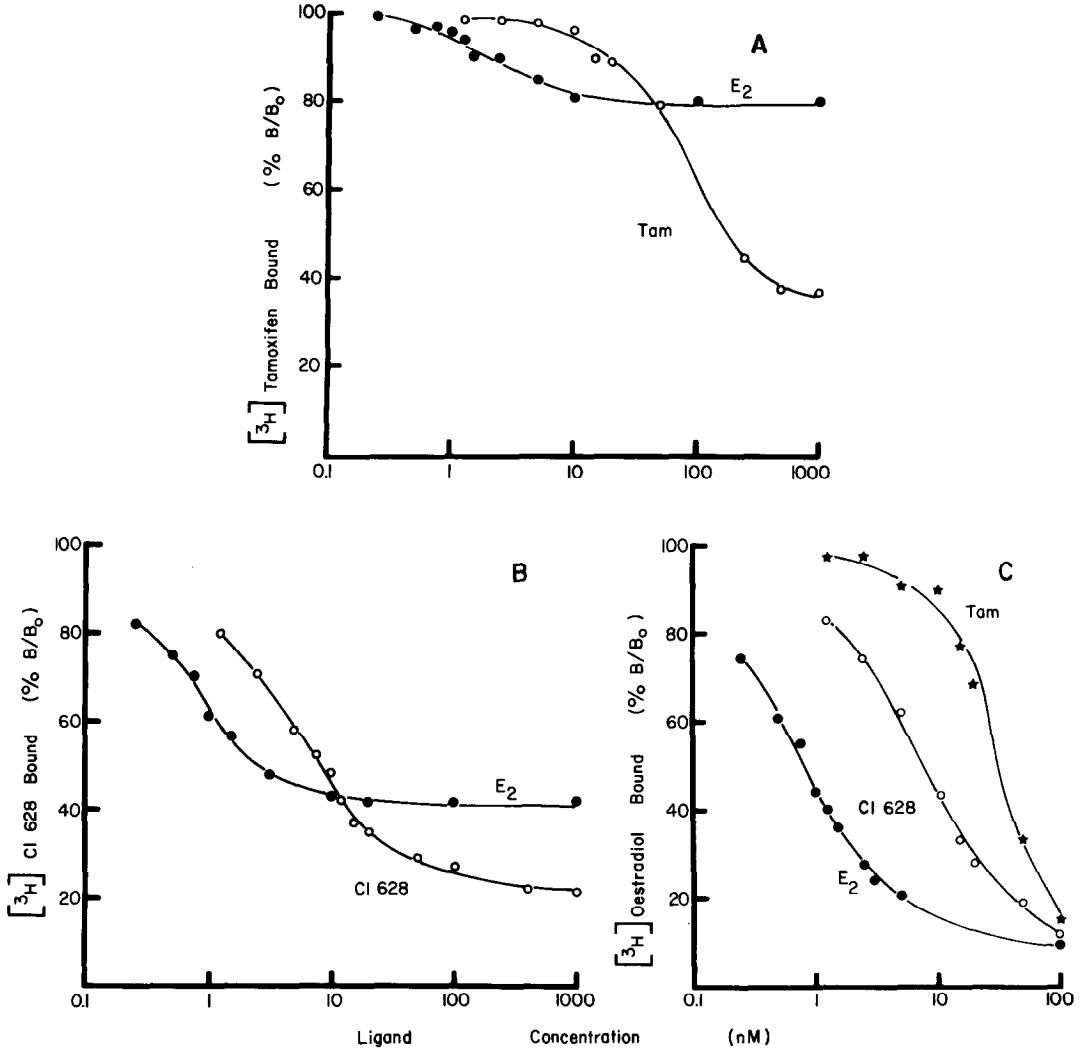

Fig. 4. COMPETITION OF ESTRADIOL, CI 628 AND TAMOXIFEN FOR ESTROGEN AND ANTIESTROGEN BINDING SITES IN CHICK OVIDUCT CYTOSOL.

A. Estradiol (●) and tamoxifen (○) inhibition of tritiated tamoxifen binding.
B. Estradiol (●) and CI 628 (○) inhibition of tritiated CI 628 binding.
C. Estradiol (●), CI 628 (○) and tamoxifen (✱) inhibition of tritiated estradiol binding.

The conclusion that antiestrogens are bound to three components in chick oviduct cytosol i.e. the estrogen receptor, the specific antiestrogen site and non-specific binding, while our data are best fitted by a model consisting of one saturable binding component and non-specific binding requires further

explanation. The ability of the computer analysis (7) to resolve different binding components is related to their relative affinities for the ligand and their degree of saturation at the lowest ligand concentrations. Under the present experimental conditions we were unable to distinguish the affinities of the antiestrogens for the two saturable binding components i.e. the estrogen receptor and the specific antiestrogen binding site, and thus the K_d values presented in Table 1 are intermediate estimates of the true K_{ds} of these two saturable binders. In this regard it is interesting to note that the apparent K_{ds} of CI 628 and tamoxifen relative to estradiol are significantly higher in the chick than in the rat (Table 1). If the structure and specificity of the estrogen receptor site is not markedly different between rat uterus and chick oviduct, this may indicate that the K_{ds} of CI 628 and tamoxifen for the specific antiestrogen binder, are significantly higher than the respective K_{ds} for binding to estrogen receptor sites. Should this be the case it may be possible to resolve the binding data into a three component model when estimates of B and U at lower degrees of saturation i.e. lower ligand concentrations, are available.

At present we have no information on the role of this novel, specific antiestrogen binding site in the modulation of antiestrogenic action in the chick oviduct. However, recent experiments have shown that this type of binding site is not confined to chick oviduct but is present in a number of estrogen target tissues (Sutherland, Murphy, Foo, Green and Whybourne, to be published). The affinity and concentration of this specific antiestrogen binding site are such that it may play a significant role in the intracellular binding of non-steroidal antiestrogens and may regulate the amount of antiestrogen available for estrogen receptor binding. A more detailed understanding of the functions and properties of this site requires further experimentation and this is currently being undertaken in this laboratory.

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