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ABSENCE OF CORRELATION BETWEEN ANTIESTROGENIC ACTIVITY AND BINDING AFFINITY FOR THE ESTROGEN RECEPTOR

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SUMMARY

We have compared the binding to the estrogen receptor (R) of different androgens and antiestrogens with their antiestrogenic activities on uterine growth. We found that estradiol (E_2)[1] and hydroxytamoxifen, a potent antiestrogen, displayed the same affinity for R. Conversely, androgens which have a much lower affinity for R and a much higher dissociation rate than E_2, behave at high doses as full estrogens, with no significant antiestrogenic activity. We conclude that there is no correlation between the dissociation rate from R and the antiestrogenic activity of R ligands and that one cannot discriminate between estrogen and antiestrogen ligands by simply evaluating their in vitro binding to the cytosol R.

Antiestrogens are compounds which bind to the cytosol R, translocate it to the nucleus but prevent further effect of E_2 (1). The triphenylethylene derivatives like tamoxifen are currently used in breast cancer treatment. Their molecular mechanism of action, although extensively studied, is still unknown. It has been proposed that antiestrogens dissociate more rapidly from the R than estrogens do. Consequently, they would be unable to maintain the receptor activation leading to full estrogenic effect (2) (3). In favor of this hypothesis, it was shown that the short acting antiestrogens

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The following compounds were used :

Dimethylstilbestrol (DMS) : 2,3 bis p-hydroxyphenyl but-2-ene.
Estradiol (E_2) : 3,17β-dihydroxy-1,3,5 (10)-estratriene.
Tamoxifen (tam) (ICI 46,474) : trans-1(p-dimethylamino ethoxyphenyl)-1,2-diphenyl but-1-ene.
Hydroxytamoxifen (OH tam) : trans-1(p-dimethylamino ethoxyphenyl)1(p-hydroxyphenyl)2-phenyl but-1-ene.
5α-dihydrotestosterone (DHT) : 17β-hydroxy-5α-androstane-3-one.
5α-androstane-3β,17β-diol (3β Adiol).
Δ5 androstene-3β,17β-diol (Δ5 Adiol).

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estriol (4), dimethylstilbestrol (5) and 11α-hydroxyestradiol (3) as well as the long acting antiestrogens, tamoxifen (6) and CI 628 (7) were dissociating more rapidly from R than E_2 or synthetic estrogens.

The purpose of this work was to verify the validity and generality of this hypothesis by evaluating the affinity, the rate of dissociation and the antiestrogenic activity of other R ligands such as androgens and hydroxytamoxifen, an antiestrogen metabolite.

MATERIAL AND METHODS

$(6,7\text{-}^3H)$ estradiol (45-60 Ci/mmole) and $(1\alpha,2\alpha\text{-}^3H)$ 5α-androstane-3β,17β-diol (43 Ci/mmole) were purchased from CEA (France), (^3H)tamoxifen (19.5 Ci/mmole) was a gift from Dr Patterson (ICI Laboratories, England). Estradiol, DHT and 5α androstane-3β,17β-diol were provided by Roussel UCLAF (France), tamoxifen and hydroxytamoxifen were kindly given by ICI Laboratories. The purity of these products was checked periodically by thin-layer chromatography (5). Calf uterine cytosol was prepared in Tris-HCl 10 mM, EDTA 1.5 mM buffer, pH 7.4 and the receptor bound ligand was assayed by the dextran-coated charcoal technique (DCC), as described previously (5). Competition, exchange and kinetic experiments were performed using the dextran-coated charcoal assay as described previously (6)(8)(9) and in the text. Radioactivity was counted in 10 ml of toluene scintillation mixture + 3 ml ethanol with a counting efficiency of 20-25 %. Protein concentrations were evaluated according to their absorption at 280 and 260 nm. The uterotrophic and antiuterotrophic activities of DHT, tam and OH tam were evaluated on immature 20 day old rat uterus (10).

RESULTS

1. Hydroxytamoxifen, a strong antiestrogen, binds to the estrogen receptor with a high affinity and a low dissociation rate.

We examined the relative binding affinity for R of OH tam, tam, E_2 and DHT using competitive experiments with $(^3H)E_2$. Fig. 1 shows that the relative binding affinity of OH tam was similar to that of E_2, while it was 100 fold lower for tam and considerably lower for DHT. The relative competitive efficiency of OH tam did not markedly vary according to the time of incubation at 0° with $(^3H)E_2$. In contrast, it decreased drastically for tam as already observed with other classical antiestrogens (9). This suggested that the dissociation rate of OH tam was similar to that of E_2 and much slower than that of tam. The dissociation rate of these ligands was evaluated indirectly by measuring the rate of exchange on the R of different non radioactive ligands by $(^3H)E_2$. This rate was similar for OH tam and E_2 at 0 and 20°, while it

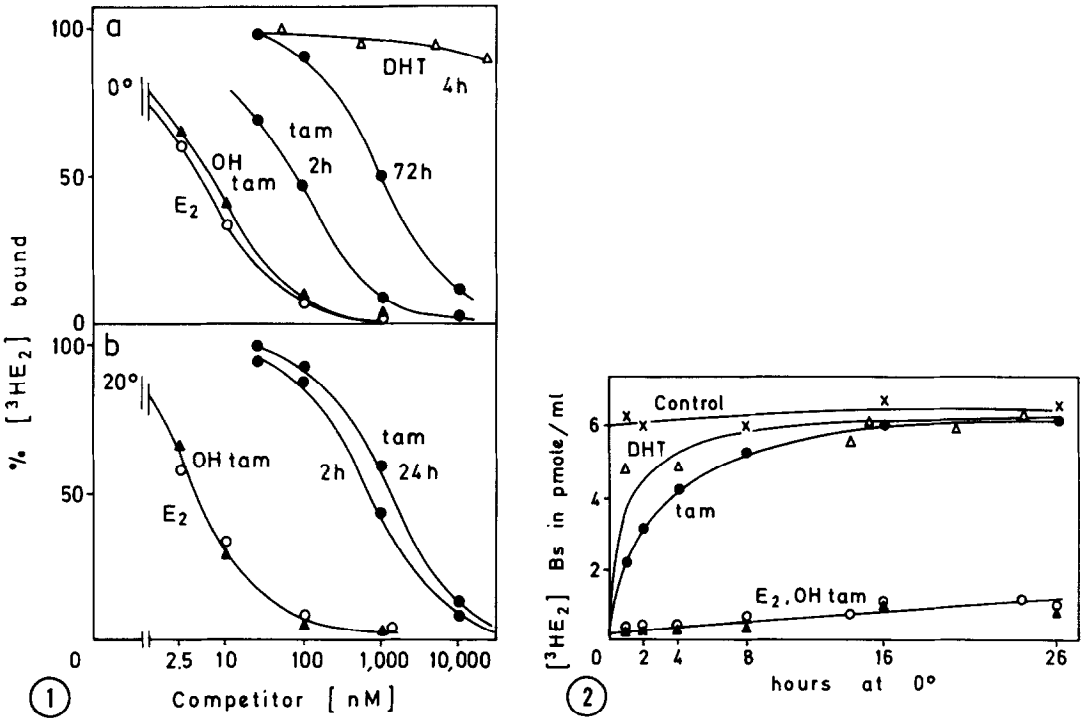

Figure 1 : Relative binding affinities of tam, E_2, OH tam and DHT on the estrogen receptor at 0° and 20° : Calf uterine cytosol was incubated at 0° (a) or 20° (b) with 5 nM $(^3H)E_2$ and increasing concentrations of estradiol (o), hydroxytamoxifen (▲), tamoxifen (●) and dihydrotestosterone (Δ) for 2 to 72 h. The $(^3H)E_2$ binding was assayed by charcoal at 0° as described previously (9). The incubation times are only indicated when results were significantly different for the different times.

Figure 2 : Rate of exchange of tam, OH tam, DHT and E_2 : Calf uterine cytosol was incubated 4 h at 0° with the following non radioactive ligand : E_2 (10 nM) (o), tam (30 nM) (●), OH tam (10 nM) (▲) or DHT (5 µM) (Δ). The stability of the R was controlled in parallel by incubating the cytosol with $(^3H)E_2$ 10 nM ± E_2 1 µM for 4 h at 0° (X). The samples were then treated by charcoal suspension for 30 min at 0° to adsorb the unbound and some non specifically bound ligand. $(^3H)E_2$ 10 nM ± E_2 1 µM (for non specific binding) were then added to the supernatant for the indicated periods of time. The specifically bound $(^3H)E_2$ was then assayed by charcoal as described in (8).

was higher for tam and much more rapid for DHT (Fig. 2). The rate of liberation of the sites occupied by OH tam or E_2 after adsorbtion of the released non radioactive ligand with charcoal were also similar . Table 1 summarizes the binding characteristic of the different ligands studied by us here and previously (6)(8)(9). The half time of exchange, as determined with non radioactive ligands, is related to the dissociation rate constant (k-) as measured directly with (^3H) ligands. The dissociation rate cons-

Table 1

Ligand	K_D (nM) 0°	RBA 0° 2-4 h	RBA 0° ≥24 h	RBA 20° 24 h	k-(sec^{-1}) 0°	Half exchange in hours 0°	Half exchange in hours 20°
E_2 (6)	0.2	1	1	1	1.3×10^{-6}	120	∿2.9
OH Tam	—	2.9	1.5	1.08	—	120	∿3.4
Tam (6)	1.7	30	279	398	1.1×10^{-4}	2.5	≤ 0.4
DMS (5)	0.2	21	—	—	7.8×10^{-6}	—	
3β Adiol (8)	17	42	1,360	—	3.4×10^{-4}	1	
DHT (8)	—	1,700	—	—	—	< 1	

Equilibrium and kinetic constants for the cytosol R : The equilibrium dissociation constants (K_D) were determined by Scatchard plot using $[^3H]$labelled compounds. The relative binding affinities (RBA) were obtained from present and previous (5)(6)(8) works by competition with $[^3H]E_2$ according to Korenman (2). The dissociation rate constant (k-) was evaluated with an excess of unlabelled ligand (1 to 5 µM) as described in (5). The half time of exchange corresponding to 50 % of exchange with $[^3H]E_2$ was obtained from fig. 2. Results for OH tam are from fig. 1 and 2.

tant was actually much higher for androgens like 3β Adiol (8) and for tam (6) than for E_2 (Table 1). However, the antiestrogens are not always dissociating more rapidly from R than the estrogens agonist For instance, the dissociation rate of dimethylstilbestrol, a short acting antiestrogen, is slower than that of 3β Adiol, an estrogen agonist. Moreover, the dissociation rate constant of OH tam is probably similar to that of E_2 since the rate of exchange of the two ligands was identical at 0° and similar at 20°. These results also indicated that the high affinity of OH tam was mainly due to its slow dissociation rate from R and that in this respect, its in vitro binding on the cytosol R was not different to that of E_2. In contrast, OH tam has been shown to be a potent antiestrogen in the rat uterus system (11) and confirmed by us, in the rat uterus (Fig. 3) and breast cancer cell line (in preparation).

2. Androgens bind to the estrogen receptor with a low affinity and very rapid dissociation rate but display no or very little antiestrogenic activity.

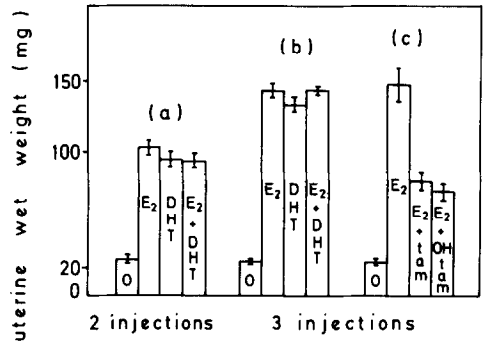

Figure 3 : Relative antiestrogenic effects of DHT, tam and OH tam : Immature female rats received two (a) or three (b and c) daily injections of oil (o), 10 mg DHT (DHT), 2.5 µg E_2 alone (E_2) or together with 10 mg DHT (E_2 + DHT), 20 µg tam (E_2 + Tam), 20 µg OH tam (E_2 + OH tam). Uterine wet weight were determined 24 h after the last injection. Each value represents the mean ± SD obtained from 5 to 10 uteri.

DHT at pharmacological doses interact weakly with R, translocate it into the nucleus (12) and inducesestrogenic effects(13) (14). With tritiated 3β Adiol and Δ5 Adiol we have recently characterized more directly the interaction of androgens with R (8). The binding characteristics of these androgens are similar to those of classical antiestrogens as shown in Table 1. Both typesof ligands are very rapidly dissociating from R and their affinity for R is much lower than that of E_2. Since androgens are also known to induce the regression of mammary tumors, we anticipated that they would display antiestrogenic activities by interacting with R. To demonstrate this, we evaluated the antiestrogenic activity of high doses of DHT on uterine growth. After two or three daily injections of DHT, we could not demonstrate any inhibition of the E_2 induced increase of rat uterine wet weight, while tam and OH tam were very efficient under the same conditions (Fig. 3). After 5 daily injections of DHT (1 mg), the E_2 increase of uterine wet weight was inhibited but not significantly ($p = 0.2$). We concluded that the antiestrogenic activity of DHT as evaluated on uterine weight was nil or very low. On the other hand, the same stimulation of uterine wet weight was obtained with DHT or with E_2 (Fig. 3). We have previously shown (14) that the uterotrophic effect of DHT was mainly due to the occupation and nuclear translocation of the R sites and that DHT was a full agonist for R. Other results support this conclusion. In the MCF_7 cell line, androgens were able to induce the progesterone receptor and the nuclear

processing of R (15). The ultrastructural modifications of the endometrial nuclei also differed after DHT or tam administration (16). We show here that pharmacological doses of androgens are not only able to activate R in order to give a full estrogenic activity, but are also inefficient to prevent E_2 action on uterus growth.

3. General Discussion :

In this paper we present two series of evidences which indicate the absence of correlation between the dissociation rate and affinity of a ligand and its antiestrogenic activity. It has been proposed that ligands which dissociate rapidly from R are antiestrogens (2) (3) while compounds which form stable complexes with R are estrogens. By contrast, we show here that the low affinity R-androgen complex is less efficient that the high affinity R-OH tam complex in preventing E_2 action. Moreover, an estrogen agonist like E_2 and an antagonist like OH tam present the same *in vitro* binding characteristics to R. Similar results have recently been found by Nicholson et al (17) in the rat uterus and mammary tumors. There are other hydroxylated metabolites of triarylethylene antiestrogens such as the H-1285 (18) and 9411 X 27 (19) compounds that also display a very high affinity for R. The *in vivo* responsibility of these hydroxylated derivatives in the antagonist activity of antiestrogens is currently studied. An allosteric type model (20) has been proposed according to which an hormone antagonist would be less active than the hormone to induce or stabilize the activated form of the receptor. This does not seem to be the case for the estrogen receptor since all antiestrogens studied so far are fully able to induce the nuclear translocation of R which is one of the earlier test for R activation. Without excluding the previous hypothesis we therefore propose another mechanism of antiestrogenic action according to which the blockade of E_2 action by antiestrogen would be located beyond the nuclear translocation step. The fact that the R nuclear translocation induced by androgens and antiestrogens results in quite different effects supports this last hypothesis. Androgens are full estrogen agonist (8) (13) (14) as long as their concentration is high enough to occupy a sufficient number of R sites. Conversely, the slowly dissociating OH tam is antagonist even though the totality of R can be translocated to

the nucleus. Whatever the mechanisms are in the chromatin this paper shows in practice that the in vitro binding characteristics to R are not reliable to specify whether a ligand will be agonist or antagonist.

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