ESTRADIOL DEPENDENT DECREASE OF BINDING INHIBITION BY ANTI-ESTROGENS (A POSSIBLE TEST OF RECEPTOR ACTIVATION)

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SUMMARY

The interaction of the uterine estrogen receptor (R) with three anti-estrogens, Dimethylstilbestrol[1], Tamoxifen and Nafoxidine, have been studied either indirectly by competitive experiments or directly using radioactive compounds. The affinity of these anti-estrogens for R was found much higher when determined directly without estradiol (E_2) than when evaluated by competitive experiments. In the presence of E_2, but not in its absence, the inhibitory activity of the anti-estrogens decreased slowly with time. The present report strongly suggests that R is transformed by E_2 into a form less sensitive to anti-estrogen. This "desensitization" of R to the estrogen antagonists is proposed as another in vitro test for the E_2 induced "activation" of its receptor.

Anti-estrogens are non steroidal compounds which inhibit the binding of E_2 to its specific receptor and its action (1). The inhibition is reversible and complete for high concentrations of any of the antagonists studied (2). However, these results having been obtained indirectly with non radioactive compounds, it has not yet been demonstrated that anti-estrogens bind directly to the estrogen receptor. In addition, the nature of the inhibition, either competition for the same site or heterotrope negative cooperativity for two different sites has not been elucidated. Finally, the apparent binding affinities of several anti - estrogens for the estrogen receptor as evaluated indirectly by using competitive experiments with $^3HE_2$ varied markedly in the litterature. For instance, DMS was 3 (3) to 50 (4) times less active than E_2 to compete on its receptor, and we found that these differences could be related to the length of the incubation time (5) (6).

In an attempt to elucidate the relationship between anti-estrogens and estrogen receptor, and to determine the real affinity of these drugs,

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1 The following compounds were used.

Dimethylstilbestrol (DMS) : α,α' dimethyl - 4 - 4' - stilbene - diol.
Estradiol (E_2) : 3,17β- dihydroxy - 1, 3, 5, (10) - estratrien.
Estrone (E_1) : 3, ol 17, one - 1, 3, 5 (10) - estratrien.
Tamoxifen (Tam) (ICI 46,474) : trans - 1(p - dimethylamino ethoxyphenyl) - 1,2 - diphenyl but - 1 - ene.
Nafoxidine (U 11,100A) : 1 - (2 - (p - 3,4 dihydro - 6 methoxy - 2 - phenyl - 1 - naphtyl) phenoxy) pyrolidine hydro-chloride.
Diethylstilbestrol (DES) : α,α' diethyl - 4 - 4' - stilbene - diol.

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for their receptors, we decided to study directly the interaction of anti-estrogens for their binding proteins by using ^{3}H anti-estrogens (7) and to compare the results with those obtained in competitive experiments.

MATERIAL AND METHODS

6-7 ^{3}H estradiol (45-60 Ci/mmole) was purchased to CEN (France), ^{3}H Diethylstilbestrol (11 Ci/mmole) from Amersham (England), ^{3}H Dimethylstilbestrol (60 Ci/mmole) was tritiated for us in position ortho of the phenyl by Drs Morgat and Fromageot (CEN, France) (7), ^{3}H Tamoxifen (19.5 Ci/mmole) tritiated in position ortho of the phenyl bearing the amino group, was a gift from Dr Patterson (ICI Laboratories, England). Non radioactive Dimethylstilbestrol was purchased from Gallard Schlesinger (USA) Estradiol 17β and Estrone were provided by Roussel Uclaf (France), Diethylstilbestrol, Tamoxifen and Nafoxidine by Merck (Germany), ICI Laboratories (England) and Upjohn Company (Kalamazoo, USA) respectively. The purity of these products was checked periodically by thin-layer chromatography as indicated elsewhere (7) (8). The specific radioactivity values of ^{3}H anti-estrogens were verified by isotopic dilution with the corresponding non-radioactive compound using the cytosol estrogen receptor. Calf uterine cytosol was prepared in TE or TE 50 buffers (Tris-HCl 10 mM or 50 mM, EDTA 1.5 mM, pH 7.4) as described previously (2). The bound radioactivity was separated by the dextran coated charcoal (DCC) (2) and the hydroxylapatite (HAP) (7) assays. The non-specific binding systematically evaluated in parallel in the presence of a 100 to 1,000 fold molar excess of unlabelled ligand was subtracted from the total binding (8). Kinetic studies were performed using the procedures described for the estradiol receptor (9) (7).

Protein concentrations were evaluated according to their absorption at 280 and 260 nm. Radioactivity was counted after DCC assay in 10 ml of toluene scintillation mixture + 3 ml ethanol and after HAP assay in 15 ml of Bray scintillation mixture. The counting efficiency of ^{3}H, evaluated by the channel ratio with external standard was 20-25 %.

RESULTS AND DISCUSSION

1. Competitive experiments : variation of the degree of inhibition by antiestrogens as a function of time.

When the uterine cytosol was incubated with $^{3}HE_2$ and a non radioactive anti-estrogen, the binding equilibrium was not reached before several days, the bound $^{3}HE_2$ increasing slowly up to 7 days. Conversely, in the absence of anti-estrogen, the binding equilibrium of $^{3}HE_2$ was apparently reached after 4 hours. This observation was previously made with Nafoxidine (fig. 1) (5) and then extended to Dimethylstilbestrol and Tamoxifen. We therefore concluded that the apparent affinities determined by competition after $\geq$ 24 h. incubation were

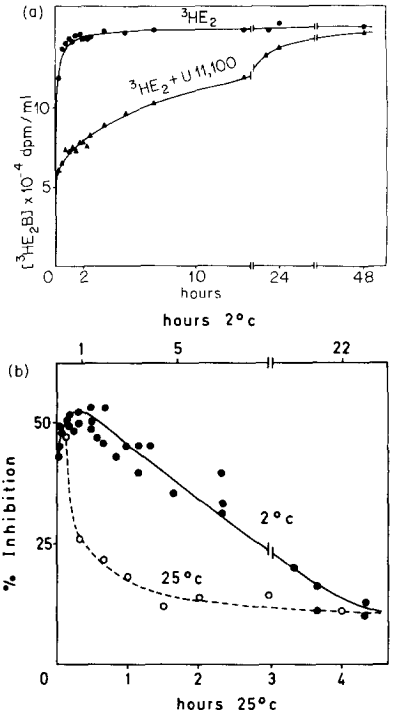

Fig. 1 a : Time course of $^3HE_2$ binding with (▲) or without (●) Nafoxidine (U 11,100) : calf uterine cytosol was incubated at 2°C with $^3HE_2$ (2 nM) and $U_{11,100}$ (20 nM) for increasing periods of time. The specifically bound $^3HE_2$ was then evaluated by DCC adsorption.

Fig. 1 b : Time dependent variation of the binding inhibition by Nafoxidine. The experiments of fig. 1 a are represented in percent of binding inhibition in taking the 100 % inhibition as the $^3HE_2$ binding obtained with a large excess of non radioactive E_2. Incubations of cytosol were also performed at 25° C.

generally over estimated. The extent of the inhibition by Nafoxidine, plotted according to the time of incubation, gave a dome shaped curve with a maximum plateauing between 30and 50min. at 2°C (fig. 1b). The equilibrium of the inhibited complex was reached much more rapidly at 25°C than at 2°C. Control experiments using labelled compounds have eliminated the possibility of a metabolism of either estradiol or antagonists. Since the competitive efficiencies of DMS (fig. 2) and Nafoxidine (not shown) were not altered by prolonged incubation of the cytosol in the absence of ligands, we concluded that the presence of E_2 was required during the incubation process and that these changes were not due to artifactual transformation(s) of cytosol entities including receptor.

The apparent Ki were then determined for several anti-estrogens following either 4 h or $\geqslant$ 2 days incubations. For all anti-estrogens studied, the

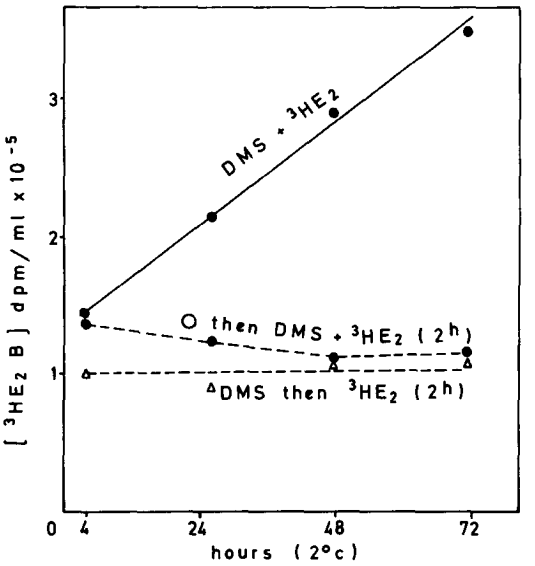

Fig. 2 : Requirement for estradiol. Calf uterine cytosol was incubated at 2°C with either DMS (500 nM) (-△-) or $^3HE_2$ (10 nM) + DMS (500 nM) (-●-) or without ligand (-●-) for the indicated periods of time. To the non labelled cytosol (-●-) (-△-) $^3HE_2$ (10 nM) was added for 2 additional hours. The specifically bound $^3HE_2$ was then evaluated by DCC adsorption.

Ki values were significantly higher after a long term than after a short term incubation (table 1). After 2 days incubation, the anti-estrogen was still active to inhibit totally the binding of ^3H estradiol, but at much higher concentrations. For DMS (fig. 3) and Nafoxidine (not shown), linear Dixon ([10]) plots were obtained suggesting classical competitive inhibition. The validity of the Dixon plot was controlled by using tritiated and unlabelled E_2 or tritiated and unlabelled DMS. In these cases, the inhibition was constant with time and the Ki values were similar to the K_D values determined directly. When plotting the Ki values obtained for DMS (fig. 4) or for Nafoxidine (not shown) in function of the time of incubation with ^3H E_2, we observed a progressive increase of Ki, without being able to conclude to an actual plateau at 7 days which would indicate an equilibrium.

To explain this variation of the apparent Ki and to choose the value that was more close to the real affinity of anti-estrogen for R, we had to decide between at least two possibilities : according to the first one, the association of both ligands to R would not have been synchronous, the non radioactive antagonist occupying the R binding sites more rapidly that ^3H estradiol. In this case, R would not have been modified during the incubation and the real Ki for the antagonist would be that determined after several days of incubation

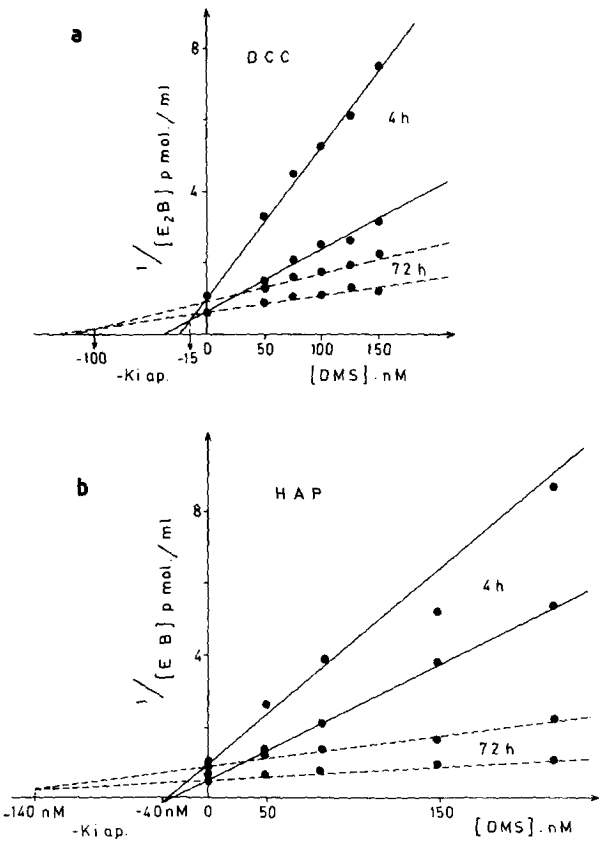

Fig. 3 : Determination of the apparent dissociation constant of DMS (Ki). Calf uterine cytosol was incubated at 2°C with $^3HE_2$ (1 nM and 2 nM) and DMS at the concentrations indicated in abscissa, for 4 hours (——) or 72 h (~ ~). The specifically bound $^3HE_2$ determined either by DCC (fig. 3a) or by HAP assay (fig. 3b) was represented according to Dixon (10). In both cases two apparent Ki values were obtained for the two periods of incubation.

at a time closer to the equilibrium time. The second possibility was that the equilibrium between R and the two ligands was rapidly reached, within a few hours, the large delay in the equilibrium time being explained by a transformation of one of the interacting molecules. To choose between these 2 hypothesis, the affinity of antagonist for R was determined directly in the absence of E_2 using labelled antagonists.

2. Direct studies with ^3H antiestrogens : R "desensitization" to antiestrogens.

The availability of ^3H DMS and ^3H Tamoxifen allowed us to study directly their interactions with the uterine cytosol receptor. In studies reported elsewhere (7), we could show that these drugs bound directly to R, since their binding specificities were similar to that of E_2 for its receptor, the sedimen-

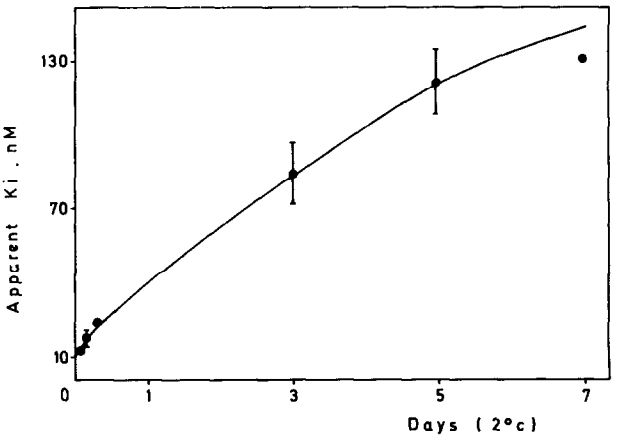

Fig. 4 : Variation of the apparent Ki of DMS in function of time. Different Ki values were obtained for DMS using Dixon plots as described in fig. 3, and represented according to the length of incubation at 2°C. The K_D value determined directly using ^3H DMS (fig. 5) was much closer to the Ki value extrapolated to time zero, than to the Ki value of the apparent plateau (> 5 d.).

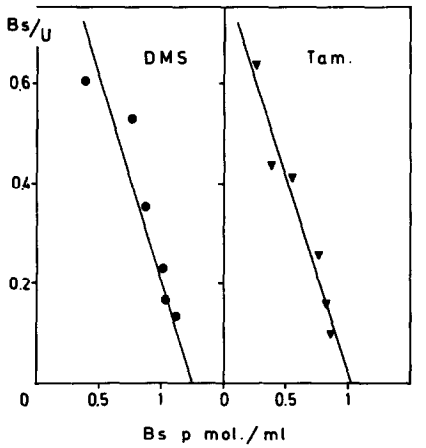

Fig. 5 : Scatchard plot of specifically bound ^3H DMS and ^3H Tamoxifen. The labelled anti-estrogens were separately incubated with calf uterine cytosol for 4 h at 2°C in the presence or absence of a 500 fold excess of non radio-active ligand. The specifically bound (Bs) ^3H antiestrogen was then determined by DCC adsorption and represented according to Scatchard (11). The concentration of unbound ligand (U) was obtained by difference between the total ^3H anti-estrogen and the bound anti-estrogen after charcoal assay.

Table 1

	Ki 2 - 6h	Ki ⩾ 2d	K_D
DMS	17 ± 5	103 ± 36	1.81 ± 0.7
Tamoxifen	a) 15 b) 72	230	1.23 ± 0.14
Nafoxidine	18 ± 2	100	N. I.

The dissociation constants of three antiestrogens with the estrogen R have been determined, either indirectly by competitive experiments (Ki) as indicated under fig. 3 or directly using ^3H labelled antagonist (K_D) (fig. 5). The mean ± SD are represented in nano molar concentrations. For DMS, the Ki values determined at 2 - 6 h and at > 2 days were significantly different (p value < 0.05). For Tamoxifen, the Dixon plot was non linear and gave 2 Ki values.

tation constant of the saturable binding protein was 8S in a sucrose gradient and the complex was translocated to the nucleus. At binding equilibrium (4 hours), Scatchard plots gave the number of specific binding sites which was slightly lower than that of E_2 binding sites (fig. 5) (7). The K_D values obtained from the slope of the curve were in the range of 1 to 2 nM indicating a very high affinity for R. Similar values were obtained by DCC and HAP assays and using partially purified receptor. These values were much closer to the Ki value extrapolated to time 0, than to the Ki value determined later (fig. 4) (Table 1). The first possibility dealing with an absence of binding equilibrium for the 2 ligands was therefore very unlikely. In addition, we have controlled that the specific (K_D) and non specific binding of ^3H DMS or $^3HE_2$ incubated separately did not vary between 4 h and 72 h incubation, indicating that neither the ligands nor their non saturable binding were modified by a prolonged incubation. These results including those of fig. 2, strongly suggested that R was transformed by E_2 into a form less sensitive to anti-estrogens.

3. General Discussion.

The mechanism and the reversibility of this E_2 induced transformation of R into a form less sensitive to the anti-estrogen have not been precised. Actually, Ki gives an estimate of the competing efficiency of the antagonist with regard to the hormone-receptor interaction and does not indicate directly

the affinity of the antagonist for R. Moreover, we ignore whether the hormone and the anti-hormone bind to the same site or to different sites of the receptor. Consequently, one can predict at least three mechanisms to explain the time and E_2 dependent increase of Ki : either the affinity of R for the antiestrogen is decreased, or its affinity for estradiol is increased, or the coupling mechanism between two mutually exclusive binding sites is altered. These possibilities are under investigation (8). We propose that the "desensitization" of R for anti-estrogen and its "activation" for nuclear translocation (12) are due to the same transformation of the receptor, whatever is its mechanism. This is based on the facts that both phenomena are induced by E_2, and that they appear simultaneously at 2°C and at 25°C (fig. 1b). Although the evidence is indirect to conclude to an identity between these two phenomena , we propose the following model : the receptor, in the absence of ligands, would be mainly present under an inactive form (Ri), sensitive to the anti-estrogen inhibition and having a low affinity for the nuclear receptor sites. E_2 would transform Ri into an activated form (Ra), less sensitive to anti-estrogens and having a higher affinity for the nuclear acceptor sites. The affinity of anti-estrogen for Ri could be either determined directly (K_D) by using labelled antagonists, or approached indirectly by using competitive experiments and extrapolating the Ki value to time zero (fig. 4). On the other hand, the affinity of anti-estrogen for Ra could be obtained after a prolonged incubation with E_2.

We propose here an additionnal *in vitro* criteria for the hormone induced activation of R which is the decrease of the competing efficiency of anti-estrogen for the R-$^3HE_2$ interaction. This test could be used both to evaluate R "activation" and to screen putative anti-estrogens. It gives also an explanation for the rescue activity of estradiol which has been described against the toxic effect of anti-estrogen (13). However, these results and those showing that anti-estrogens are able *in vitro* and *in vivo* to translocate the estrogen receptor to the nucleus (1) are not sufficient to understand the molecular mechanism of the anti-estrogenic activity of these drugs.

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