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MONOHYDROXYTAMOXIFEN : AN ANTIOESTROGEN WITH HIGH AFFINITY FOR THE CHICK OVIDUCT OESTROGEN RECEPTOR

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SUMMARY. The monohydroxylated derivative of tamoxifen (a non-steroidal triaryl ethylene antioestrogen) shows an apparent affinity (Ki = 0.2 nM) for the chick oviduct oestrogen receptor which is higher than that of oestradiol itself, and ∿ 10 times higher than that of tamoxifen. Administered in vivo with oestradiol benzoate, it inhibited the increase of tissue growth, progesterone receptor content, ornithine decarboxylase activity (ODC), and ovalbumin and conalbumin synthesis, and also inhibited the oestradiol induced increase of ODC in vitro. It did not display any oestrogenic effect by itself. We conclude that anti-oestrogenic action may be exhibited by a molecule with higher affinity binding to the oestrogen receptor than oestradiol itself. Metabolic studies demonstrated that the antioestrogenic action of tamoxifen is not due to its prior conversion to monohydroxytamoxifen.

Tamoxifen has been found to act as an antioestrogen with no detectable agonist activity in the chick oviduct (1). This model offers considerable advantages for the study of the mechanism of oestrogen action compared to the mammalian systems where all known antioestrogens are also partial agonists (2-4). In the rat, monohydroxytamoxifen (OH-T), a metabolite of tamoxifen was isolated (5) and showed an affinity for the uterus oestrogen receptor which was nearly as high as that of oestradiol (2,6). The affinity of tamoxifen was ∿ 100 times lower than that of oestradiol (2,7). In vivo the effects of OH-T and tamoxifen are similar ; however, smaller doses of OH-T are needed to produce the oestrogenic and antioestrogenic effects (2).

Earlier experiments showed that OH-T had a very high affinity for the chick oviduct oestrogen receptor (8). It was, therefore, of interest to examine the antioestrogenic properties of OH-T in the chicken magnum and to determine whether the antioestrogenicity of tamoxifen (1) is due to its prior conversion to OH-T.

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MATERIALS AND METHODS

Chemicals : [^{3}H]tamoxifen (19,4 Ci/mmol), unlabelled tamoxifen and OH-T were gifts of ICI, England. DL-[1-^{14}C] ornithine hydrochloride (40-60 mCi/mmol), [^{3}H]oestradiol (85-110 Ci/mmol) and [^{3}H]progesterone (80-110 Ci/mmol) were from Radiochemical Centre Amersham, England, and the unlabelled steroids from Roussel-Uclaf, Romainville, France. All other chemicals were of analytical grade.

Animals : Chickens were used which had been oestrogen-primed (10 daily injections of 1 mg oestradiol benzoate per chicken), and subsequently withdrawn (4-6 weeks). For *in vivo* experiments compounds were injected in propylene glycol. After decapitation magnum portion of the oviduct was removed and placed on ice before homogenisation or *in vitro* incubation. For studies of receptor-ligand interactions *in vitro*, 0.5 M NaCl extract of laying hen magnum purified nuclei was prepared as described previously (9). Endogenous ligand was removed by a 2 h incubation at 30°C with 0.25 % charcoal (10).

Methods : All techniques used have been described in detail elsewhere : determinations of oestrogen receptor (10), progesterone receptor (11) ; ornithine decarboxylase activity (12) ; relative rates of ovalbumin and conalbumin synthesis (13) and DNA (14). Oestradiol was measured by radioimmunoassay using a highly specific antibody produced by Institut Pasteur, Paris ; the procedure employed was as in (15), except that the bound/free steroid separation was carried out by charcoal adsorption.

RESULTS

Receptor binding affinity : Competition experiments (Fig. 1) showed that the affinity of OH-T for the oestrogen receptor was $\sim$ 1.8 times higher than that of oestradiol in the NaCl (0.5 M) extract of purified hen's magnum nuclei. The affinity of OH-T was $\sim$ 1.3 times higher than that of oestradiol for the cytosol receptor of withdrawn chicks, and the same number of binding sites was found for both ligands (as well as for tamoxifen) in nuclear extracts and in cytosol (data not shown).

In vivo effects : The effects of oestradiol benzoate and OH-T administered alone or simultaneously to withdrawn chicks were determined by evaluation of the following parameters : magnum wet weight, progesterone receptor content, ODC activity and relative rate of synthesis of two secretory proteins, ovalbumin and conalbumin. When administered with oestradiol benzoate OH-T displayed a dose-dependent inhibitory activity (Fig. 2), while given alone it had no effect on any of these parameters (data not shown). At 10 mg/kg, it completely inhibited the oestrogen increase of total receptor concentration, and, given alone, it caused the translocation of oestrogen receptor to the nucleus and prevented

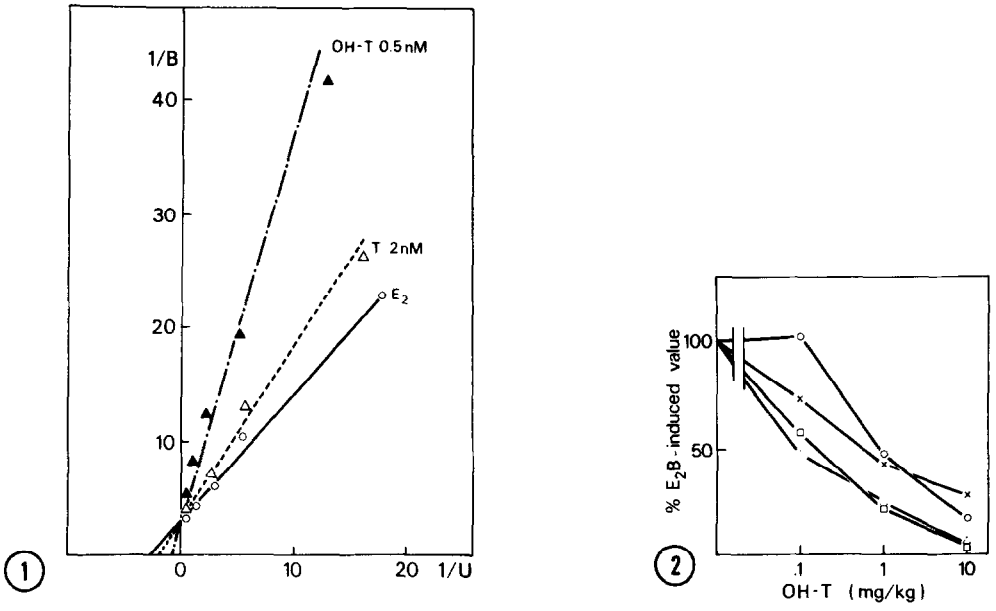


Figure 1. Competition of OH-T with [^{3}H]oestradiol for the chick oviduct nuclear receptor. The charcoal-dextran treated extract of purified nuclei from laying hen magnum was incubated for 24 h at 0°C with 0.1-10 nM [^{3}H]oestradiol in the presence (▲--▲) or absence (o—o) of 0.5 nM OH-T. Bound hormone was separated from unbound by charcoal adsorption and the supernatant was counted ; the data are represented according to Lineweaver-Burk (17). Calculated Kd for oestradiol is 0.36 nM and Ki for OH-T is 0.2 nM. For comparison tamoxifen (2 nM) competition is shown (Δ--Δ) (Ki = 2.7 nM).

Figure 2. Inhibition of oestrogen effects in vivo by OH-T : dose dependence. Chicken (groups of 3) were given 1 mg of oestradiol benzoate (E_2b) together with various doses of OH-T (0, 0.1, 1 or 10 mg/kg). The induction of conalbumin synthesis at 4 h (x--x), ornithine decarboxylase activity at 4 h (□--□), ovalbumin synthesis at 16 h (Δ--Δ) and progesterone receptor level at 16 h. (o--o) were determined. The values obtained in the untreated chicken (identical to those in chicken given OH-T alone) have been subtracted.

the replenishment of the cytoplasmic receptor for at least 24 h (Table I).

In vitro effects : The induction by oestrogens of ODC in the chicken oviduct in vitro (16) was used to test the antioestrogenicity of OH-T and of tamoxifen. Both compounds inhibited the oestrogen effect (OH-T being more efficient than tamoxifen) and were unable to induce enzyme activity (Table II).

Metabolic studies : In vivo and in vitro experiments with [^{3}H]tamoxifen were carried out to test the possibility that the antioestrogenic effects of tamoxifen might in part be due to its conversion to OH-T. In vivo experiments (Fig. 3) indicated a relatively slow elimination of tamoxifen from circulation (half-life of about 20 h) and no OH-T was detected in the serum. Similar ana-

TABLE I

Withdrawn chicks (groups of 3) were injected with 1 mg/kg of oestradiol benzoate (E_2b) and with varying doses of OH-T. They were killed 24 h later and the oestrogen receptor concentration measured in cytoplasmic and nuclear fractions of the magnum. Results are expressed as femtomoles of [^{3}H]oestradiol bound per mg of DNA.

Compound injected	Magnum wet weight (mg)	Oestrogen receptor content		
		Cytoplasm	Nuclei	Total (cytoplasm plus nuclei
none	87	970	240	1,210
E_2b	260	1,820	1,020	2,840
E_2b + OH-T 0.5 mg/kg	140	1,460	1,400	2,860
E_2b + OH-T 1 mg/kg	113	1,380	1,140	2,520
E_2b + OH-T 10 mg/kg	97	580	680	1,260
OH-T 10 mg/kg	93	560	620	1,180

TABLE II

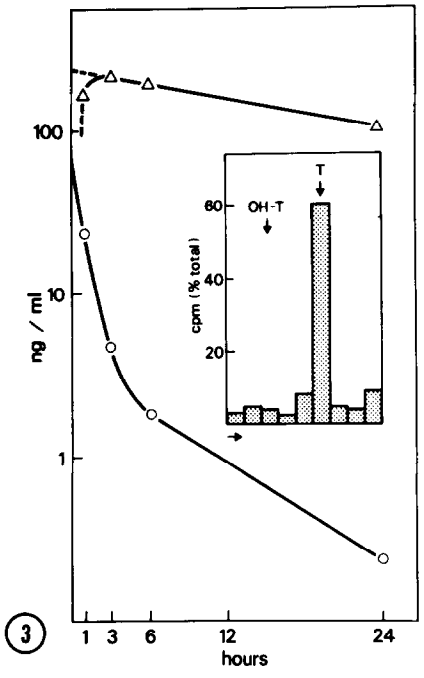
Inhibition of oestradiol induction of ornithine decarboxylase activity (ODC) in vitro by OH-T and tamoxifen (T).

Pieces of withdrawn magnum tissue were randomized and incubated in MEM (2 ml per vial contains about 50 mg tissue) for 2 h at 37°C. Tissue was then homogenized and ODC activity measured. The data represented means of 3 independent experiments. The mean value induced by oestradiol (20 nM) was 42 pmol CO_2 liberated per h/µg DNA, and the results are expressed as % of oestradiol induced activity.

Compounds added	ODC	Compounds added	ODC
E_2 + OH-T 200 nM	74	E_2 + T 200 nM	102
E_2 + OH-T 2 µM	0	E_2 + T 2 µM	23
OH-T 2 µM	0	T 2 µM	0

lysis of bile collected 24 h after injection of tamoxifen showed that ∿ 20 % of the radioactivity migrated as OH-T, suggesting that, as in the rat (2), OH-T can be formed from tamoxifen. This metabolite appears to be predominantly excreted and not resorbed into the blood.

The efficiency of tamoxifen as an antioestrogen may result from its longer persistence in the blood, compared to that of oestradiol or oestradiol ben-



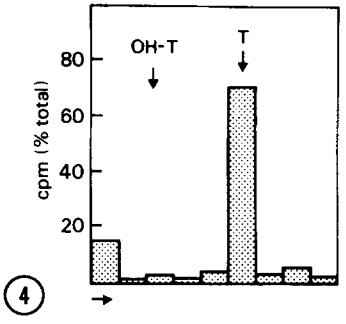


Figure 3. Metabolism of [^{3}H]tamoxifen and of oestradiol benzoate in the chicken in vivo.

1. Chicks were given a mixture of [^{3}H]tamoxifen (0.25 mCi) and unlabelled tamoxifen (10 mg/kg) ; blood samples were taken from the wing vein at 1, 3, 6 and 24 h, serum radioactivity was determined and the concentration of tamoxifen was calculated on the basis of specific activity of the injected mixture (open triangles). An aliquot (0.5 ml) of serum sample taken at 6 h. was extracted with ether (2 x 5 ml), dried and chromatographed on silica gel thin layer plate in benzene : triethylamine mixture (9 : 1). Sections (1 cm) of the track were scraped and counted (inset).

2. Chicks received 1 mg oestradiol benzoate/kg and blood samples were taken from the wing vein at 0, 1, 3, 6 and 24 h, serum was extracted with ethylacetate (2 x 5 ml) and oestradiol was determined by radioimmunoassay. The data (open circles) represent means of 2 chicks.

Figure 4. Metabolism of [^{3}H]tamoxifen in vitro by chicken magnum. Pieces of withdrawn chicken magnum tissue were incubated for 2 h at 37°C in MEM containing 2 µM [^{3}H]tamoxifen. The incubation medium and the cytoplasmic fraction of the tissue were extracted by ether and the nuclear fraction by acetone. Extracts were run on t.l.c. in benzene : triethylamine (9 : 1). Sections (1 cm) of the plate were scraped and counted. Only the results obtained with the nuclear fraction are shown ; the same pattern was found with the cytoplasm and incubation medium (except for lack of polar material on the origin in the case of medium). Migration is indicated by an arrow ; the positions of OH-T and tamoxifen (T) are shown. The data are means of two experiments.

zoate. To test this hypothesis, we injected oestradiol benzoate and carried out a radioimmunoassay of serum extracts using a specific antibody to oestradiol (15). The elimination of the immunoreactive oestrogen from the blood (Fig. 3) was rapid compared to that of tamoxifen and proceeded in two stages characterized by half-lives of ∿ 1 h and 6 h. Since the antibody displayed very little cross-

reactivity with oestradiol benzoate (1 ng of oestradiol benzoate was equivalent to 3.5 pg of oestradiol), the values obtained apparently represented the serum concentration of oestradiol liberated by progressive hydrolysis of the ester. These results support the hypothesis in vivo tamoxifen exerts a strong anti-oestrogenic effect by virtue of its long half-life.

The in vivo experiments, however, do not exclude that tamoxifen might be metabolized within the oviduct tissue itself. Therefore, the metabolism of [^{3}H]tamoxifen was studied in vitro under the same conditions as ODC induction (see Table I). The results showed that, for any fraction tested, most of the radioactive material displayed the mobility of [^{3}H]tamoxifen and no measurable radioactivity was detected in the OH-T region of the chromatogram (Fig. 4).

DISCUSSION

Introduction of a hydroxyl group at C-4 of tamoxifen increases considerably the affinity of the compound for the oestrogen receptor in the chick oviduct (8) as in the rat uterus (2). In fact, OH-T binds more tightly to the chick oviduct receptor than oestradiol. It is, therefore, important to note that this hydroxylated compound lacks any detectable oestrogenic activity in this system.

The similar number of binding sites calculated from competition experiments for [^{3}H]oestradiol binding to the receptor suggests that the same sites of the receptor molecule are involved in binding both oestradiol and antioestrogens, and that the antioestrogen effects result from a competitive mechanism. Until now, all known antioestrogens showed lower binding affinities than oestradiol for the oestradiol receptor. OH-T in the chick oviduct is the first compound displaying antioestrogenic activity which has a higher binding affinity than oestradiol for the receptor.

Since there is a $\sim$ 10-fold difference in the affinities of OH-T and tamoxifen for the oestrogen receptor, it could be that the action of tamoxifen is in fact due to its prior conversion to the monohydroxylated derivate. Our stu-

dies on metabolism of [^{3}H]tamoxifen excluded this possibility : no [^{3}H]OH-T could be detected by thin layer chromatography in the serum following [^{3}H]tamoxifen injection, nor in the tissue or medium following incubation with [^{3}H]tamoxifen. Moreover, tamoxifen inhibited the in vitro increase in ODC activity by oestradiol under conditions where no conversion to OH-T occured.

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REFERENCES

1. Sutherland, R., Mester, J., and Baulieu, E.E. (1977) Nature 267, 434-435.
2. Jordan, V.C., Dix, C.J., Naylor, K.E., Prestwich, C.T., and Rowsby, L. (1978) J. Toxicol. Environ. Health 4, 363-390.
3. Katzenellenbogen, B.S., and Ferguson, E.R. (1975) Endocrinology 97, 1-12.
4. Horwitz, K.B., Koseki, Y., and Mac Guire, W.L. (1978) Endocrinology 103, 1742-1758.
5. Fromson, J.M., Pearson; S., and Bramah, S. (1973) Xenobiotica 3, 693-710.
6. Rochefort, H., Garcia, M., Borgna, J.L. (1979) Biochem. Biophys. Res. Commun. 88, 351-357.
7. Capony, F., and Rochefort, H. (1978) Mol. Cell. Endocrinol. 11, 181-198.
8. Mester, J., Seeley, D., Catelli, M.G., Binart, N., Geynet, C., Sutherland, R., and Baulieu, E.E. (1979) J. Ster. Biochem. 11, 307-313.
9. Best-Belpomme, M., Mester, J., Weintraub, H., and Baulieu, E.E. (1975) Eur. J. Biochem. 57, 537-547.
10. Sutherland, R., and Baulieu, E.E. (1976) Eur. J. Biochem. 70, 531-541.
11. Mester, J., and Baulieu, E.E. (1977) Eur. J. Biochem. 72, 405-414.
12. Pegg, A.E., Williams-Ashman, H.G. (1968) Biochem. J. 108, 533-539.
13. Palmiter, R.D., Oka, T., and Schimke, R.T. (1971) J. Biol. Chem. 246, 724-737.
14. Burton, K. (1956) Biochem. J. 62, 315-323.
15. Abraham, G.E. (1969) J. Clin. Endocrinol. Metab. 29, 866-870.
16. Cohen, S., O'Malley, B.W., Stastny, M. (1970) Science 170, 336-338.
17. Lineweaver, H., and Burk, D. (1934) J. Am. Chem. Soc. 56, 658-666.