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DIFFERENTIAL DEPLETION OF CYTOPLASMIC HIGH AFFINITY OESTROGEN RECEPTORS AFTER THE *in vivo* ADMINISTRATION OF THE ANTIOESTROGENS, CLOMIPHENE, MER-25 AND TAMOXIFEN

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1 The *in vivo* actions of the oestrogen antagonists clomiphene, MER-25 and tamoxifen upon the cytosol oestrogen receptors prepared from amygdala, hypothalamus, pituitary and uterus of rats were studied 24 h after drug administration.

2 There was a dose-related depletion of cytosol oestrogen receptors. However, the uterine and pituitary receptors were consistently affected at a lower dose than were those from the brain.

3 The ratios of the combined central ED_{50} to the combined peripheral ED_{50} were clomiphene 169 > MER-25 19.2 > tamoxifen 2.13.

4 The receptor changes were not related to biological activity monitored by serum luteinizing hormone levels and uterotrophic response.

5 The possible role of these drug effects in the induction of ovulation and future developments are discussed.

Introduction

The formation of an oestrogen-protein complex in the cytoplasm of target organs is widely accepted to be a prerequisite for many of the actions of oestrogen. The receptor complex is translocated to the nucleus from where the subsequent biochemical events are directed (Jensen & DeSombre, 1973; Gorski & Gannon, 1976). Antioestrogenic drugs also promote receptor translocations although in this case the subsequent receptor dynamics distinguish between agonist and antagonist drugs. In the uterus it has been shown that oestrogens promote resynthesis of the receptor thus making more available for further tissue stimulation. Antioestrogens do not stimulate cytoplasmic receptor resynthesis and the administration of these drugs results in continued depletion of the cytosol receptor concomitant with the persistence of the antioestrogenic receptor complex in the nucleus (Clark, Anderson & Peck, 1973; Clark, Peck & Anderson, 1974; Katzenellenbogen & Ferguson, 1975). The oestrogen antagonists are used for the treatment of mammary carcinoma and female infertility (Cole, Jones & Todd, 1971; Lunan & Klopper, 1975). The drug interactions in peripheral tissues are well documented but there is a paucity of data as to how ovulation is induced by the antioestrogens; it has been suggested that they act upon the CNS and anterior pituitary to promote the ovulatory pattern of gonadotrophin secretion (Kistner, 1976). The present experiments examine some *in vivo* actions of three antioestrogens used for the induction of ovulation upon the central and peripheral cytoplasmic oestrogen high affinity receptors (HAR) in rat tissues. A preliminary account of some of this work has been published (Morris & Kurl, 1976).

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Methods

Adult female Sprague-Dawley rats (300–400 g) bilaterally ovariectomized 3 to 6 weeks previously were used. Twenty four hours after drug administration, 2 to 3 rats were decapitated and blood collected for serum preparation. The tissues (amygdala, hypothalamus, pituitary and uterus) were placed in ice cold homogenization medium (0.01 M phosphate buffer, pH 7.3, containing 0.25 M sucrose and 0.1 M 2-mercaptoethanol). Except for uterus, tissue dissection, homogenization, cytosol preparation and the determination of the number of oestrogen receptors and their affinity for oestradiol-17β was carried out as described by

Ginsburg, Greenstein, Maclusky, Morris & Thomas (1974). Uteri were pulverized after freezing in liquid nitrogen and then homogenization completed as previously described. Homogenates were centrifuged at 105,000 *g* for 1 h at 4°C. The cytosol was removed and used immediately. The cytosol was incubated to equilibrium with [^{3}H]-oestradiol-17β (4×10^{-9} to 2×10^{-10} M) with or without a 20 fold excess of diethylstilboestrol. After incubation, bound was separated from free [^{3}H]-oestradiol by the use of 5×60 mm columns of Sephadex LH_{20} maintained at 4°C. The radioactivity in the eluate was determined by liquid scintillation counting at a mean efficiency of 40%. The external standard channel ratio method was used for quench correction. The assayed concentrations of specifically bound oestradiol were used to construct plots (Scatchard, 1949) from which the equilibrium dissociation constant (K_D) of the binding reaction and the saturation binding capacity of the cytosol and hence the number of available sites were determined. After the administration of high doses of the antioestrogen, reliable Scatchard plots could not be constructed because of the low level of [^{3}H]-oestradiol binding in the cytosol. Therefore to obtain an estimate of the saturation binding capacity (n) the specifically bound oestradiol was determined for the two highest [^{3}H]-oestradiol concentrations (approximately 4 and 2×10^{-9} M) and (n) estimated from this value. K_D could not be determined for these cytosols.

Luteinizing hormone (LH) was measured by radioimmunoassay of the serum samples which had been stored at −15°C until required. LH estimation was performed in a single assay on serum samples at three

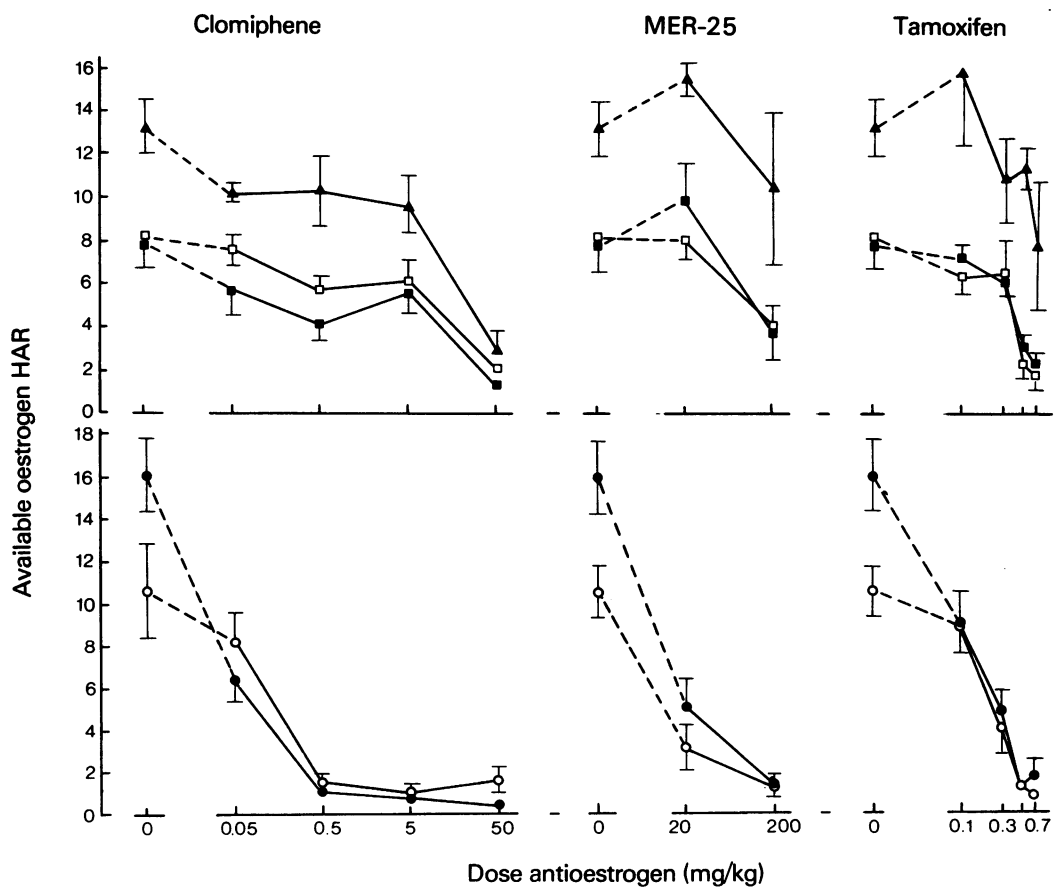

Figure 1 The abundance of cytoplasmic oestrogen high affinity receptors 24 h after administration of anti-oestrogens to the ovariectomized rat. (▲) Amygdala; (□) hypothalamus—anterior; (■) hypothalamus—middle ($\times 10^{-9}$); (○) uterus ($\times 10^{-11}$) and (●) pituitary ($\times 10^{-10}$), mean values are shown, number of determinations = 3 to 6; vertical lines show s.e. mean. For some values the s.e. mean is smaller than the symbol. Significance between control and test drug was calculated by the Mann-Whitney U test (two tailed).

dilutions employing antisera, purified hormone for radoiodination, and standard reference preparations kindly supplied by the American NIAMD programme. LH was iodinated with I^{125} using the chloramine T method (Greenwood, Hunter & Glover, 1963) and assayed in phosphate buffered saline (0.01 M) containing 1% bovine serum albumin.

[2,4,6,7 ^{3}H]-oestradiol-17β obtained from the Radiochemical Centre, Amersham, England or NEN Chemicals Dreieich W. Germany ($\simeq$100 Ci/mmol) was purified and stored as described previously (Ginsburg *et al.* 1974).

The antioestrogens used were tamoxifen (*trans*-1-(*p*-α-dimethylaminoethyoxy phenyl)-1,2-diphenylbut-1-ene)citrate) provided by ICI, MER-25 (ethamoxytriphetol 1(*p*-2 diethylaminophenyl)-1 phenyl-2 *p* methoxyphenyl ethanol) and–clomiphene (*cis*-triethyl amino-2(*p*(2-chloro-1.2 diphenylvinyl)phenoxy) monocitrate) provided by Merrel National Laboratories.

The drugs were administered intraperitoneally dissolved in arachis oil (1 ml/kg) except for the highest dose of each drug when a suspension in arachis oil was used. The doses (mg/kg) refer to the base.

Results

Oestrogen cytoplasmic high affinity receptors

The total numbers of available cytoplasmic HARs in the dissected tissues 24 h after the administration of the antioestrogens are presented in Figure 1. The pattern of receptor changes and the level of significance are similar when expressed in this manner or per unit weight.

Clomiphene significantly depleted the brain (amygdala, anterior and middle hypothalamus) HAR population only at a dose of 50 mg/kg ($P < 0.0.2$). In contrast the HARs in the peripheral tissues were lowered at 0.05 mg/kg, significantly only in the pituitary ($P < 0.01$) but highly significantly in both tissues at 0.5 mg/kg ($P < 0.01$) when less than 10% of the cytoplasmic HAR population was detectable. MER-25 lowered the pituitary and uterine HAR levels at 20 mg/kg ($P < 0.02$) whereas the brain HAR remained unaffected until the dose was raised 10 fold when both the hypothalamic HAR ($P < 0.05$) and the amygdaloid (NS) HAR were reduced. Tamoxifen treatment produced similar patterns of depletion but the separation of the brain and peripheral dose-response curves was not so marked as that found for the other antioestrogens. Tamoxifen 0.3 mg/kg depleted the peripheral HARs ($P < 0.01$) but those centrally located remained unchanged; 0.7 mg/kg significantly depleted both hypothalamic HARs ($P < 0.01$) and amygaloid tissue although only with hypothalamus was a significant change demonstrated.

It is obvious from these results that the potency of these compounds in affecting the receptor changes differs with respect to dose and also to the tissue under investigation. The results are summarized in Table 1 by comparing the dose of antioestrogen required to deplete the tissue HARs by 50% (ED_{50}). As only limited data are available at the low doses of the antioestrogens, some pituitary and uterine values have been extrapolated from the graphs, assuming that the log dose-response curves for the various antioestrogens are parallel.

Lower doses of antioestrogens were required to produce a similar receptor depletion in the pituitary and uterus than in the central nervous tissues. Clomiphene was the most potent drug investigated in promoting peripheral HAR depletion, closely followed by tamoxifen and then MER-25. In contrast tamoxifen was the most potent drug upon the brain HARs > clomiphene > MER-25. The ratio peripheral:brain

Table 1 Approximate ED_{50} (mg/kg) of the ability of the antioestrogens to induce oestrogen high affinity receptor (HAR) depletion

Tissue	*Clomiphene*	*Tamoxifen*	*MER-25*
Amygdala	14	>0.7	>200
Hypothalamus—anterior	17 (169)	0.42 (49)	190 (2,400)
—middle	11	0.39	200
Pituitary	0.034* (1)	0.13 (2.3)	9* (125)
Uterus	0.13	0.23	11*
Ratio of combined central ED_{50}: combined peripheral ED_{50}	169	2.13	19.2

Potency ratio compared to clomiphene (peripheral) in parentheses.
* Denotes extrapolated values.

ED_{50} is useful to clarify these observations being 169 for clomiphene but only 2.13 for tamoxifen. The amygdaloid HARs were the most resistent to the drug effect; it was not possible to estimate ED_{50}s for this tissue after MER-25 and tamoxifen.

Dissociation constants

Table 2 lists the apparent equilibrium dissociation constants (K_D) between [^{3}H]-oestradiol and the tissue cytosol HARs, in the control rats and 24 h after the high doses of the antioestrogens. Control values were similar to, but slightly larger than those already reported (Ginsburg, Maclusky, Morris & Thomas 1977). The majority of K_Ds were not significantly different from the control values, however at high doses the results tended to be elevated and the variance increased, becoming significant only in the pituitary and uterus after MER-25 and in the hypothalamus (front) after tamoxifen.

The estimates of the K_D of the pituitary and uterine cytosols after the higher dose of MER-25 (200 mg/kg) are smaller than the values obtained with 20 mg/kg; however, this difference is not significant. The data suggest that after the higher doses of the antioestrogens the K_D are greater. The lack of significant changes after some of the treatments probably reflects the difficulty in obtaining the results when the cytosol binding sites are severely depleted. Further experiments are necessary to examine this phenomenon.

Tissue response to antioestrogens

The oestrogen antagonists have been reported to possess agonist activity therefore uterine weight and serum LH concentration were measured as indices of peripheral and central mediated oestrogen responses.

Uterine weight

No significant uterotrophic response was demonstrated for tamoxifen or MER-25. Clomiphene at the lower doses increased uterine weight but the highest dose tested was not active in this respect (Table 3).

Serum luteinizing hormone concentration

No treatment significantly altered the serum LH concentration from control values which were 1599 ± 491 ng/ml LH RP-1, $n = 23$.

Table 2 Apparent dissociation constants $K_D \times 10^{-10}$ M 24 h after the high doses of the antioestrogens

	Hypothalamus				
Antioestrogen	*Front*	*Middle*	*Amygdala*	*Pituitary*	*Uterus*
Control	2.95 ± 0.43	3.47 ± 0.57	2.75 ± 0.71	3.14 ± 0.81	6.32 ± 1.33
Tamoxifen					
0.5 mg/kg	7.61 ± 0.38*	5.5 ± 1.69	8.56 ± 1.92	8.44 ± 3.71	7.92 ± 2.33
Clomiphene					
5 mg/kg	4.39 ± 1.95	3.62 ± 0.56	3.55 ± 0.33	2.53 ± 0.66	11.67 ± 3.74
MER-25					
20 mg/kg	6.48 ± 1.89	7.89 ± 3.33	4.75 ± 0.51	12.1 ± 1.97*	18.9 ± 3.9*
200 mg/kg	6.33 ± 2.55	4.57 ± 2.71	4.89 ± 2.71	8.87 ± 1.18*	7.09 ± 2.07

Values are mean ± s.e. mean. $n = 4$ to 6.
* Significance of difference v control, $P < 0.05$. Student's t test (two tailed).

Table 3 Uterine weight (mg/100 g bodyweight) 24 h after clomiphene (mg/kg bodyweight)

Control	*0.05*	*0.5*	*5*	*50*
41 ± 3.8 (9)	50 ± 2.5 (6)	54 ± 3.7 (12)	54 ± 3.6 (14)	45 ± 2.6 (11)
Significance of difference v control (Student's t test)	(NS)	(0.02)	(0.02)	(NS)

Number of determinations in parentheses.

Discussion

The non steroidal antioestrogenic drugs such as those used in this study have been shown to compete with oestrogen for the target tissue receptor system both *in vivo* and *in vitro* (Skidmore, Walpole & Woodburn, 1972; Clark *et al.*, 1973; Katzenellenbogen & Ferguson, 1975; Major, Green & Heald, 1976; Ginsburg *et al.*, 1977; Roy & Wade, 1977). However the mechanism by which the antioestrogens inhibit the oestrogenic response is unknown. An important discovery was made when it was demonstrated that the antioestrogens, nafoxidine, CI 628 and clc
a long term uterine cytosol receptor depletion which could be correlated with the antagonism of an oestrogenic response (Clark *et al.*, 1974). The present study confirms uterine cytosol depletion and in addition extends the observations to another antioestrogen, tamoxifen and also to the pituitary and central nervous tissues. Previous studies of the effect of the antioestrogens upon the uptake of [^{3}H]-oestradiol into the target areas of the brain are inconsistent. Workers have reported that either the antioestrogens interact with only the peripheral tissues (Perry, Dipasquale, Ferguson, Pozzi & Rassaert, 1973; Whalen & Gorzalka, 1973; Chazal, Faudon, Gogan & Rotsztyn, 1975), or both peripheral and central tissues (Maurer & Woolley, 1971; Luttge, Gray & Hughes, 1976; Roy & Wade, 1977). The experiments described here show that there is a dose-related depletion of the cytosol HAR in all tissues but at low doses the brain HAR are not affected, findings which help to clarify the anomalous results. The amygdaloid HARs appear most resistant to the drugs. Morris (1976) also found that the amygdaloid HARs were not as sensitive to oestradiol-17β when compared to other brain oestrogen receptor systems so that there may be a differential sensitivity not only between peripheral and central receptors but within the brain itself.

The results can be explained by the suggestion that the oestrogen receptor system differs in its mode of action from tissue to tissue. Indeed it has been shown that the accumulation of the nuclear oestrogen receptor complex is delayed in the hypothalamus (Fox, 1977) and that its cytosol receptors are not depleted to the same extent by *in vivo* oestradiol as those found in peripheral tissues (Morris, 1976). It is possible however that drugs may be excluded from the CNS by the blood brain barrier, a phenomenon well established for certain drugs (Day, 1977). Clomiphene, MER-25 and oestradiol-17β bind *in vitro* with identical affinities to the CNS and peripheral HARs (Ginsburg *et al.*, 1977) results which would support the latter hypothesis, but one cannot discount the possibility that the cellular concentrating mechanisms may differ in the intact tissue.

It is of interest that the *in vivo* potency of the antioestrogens in these experiments to produce the uterine HAR depletion (e.g. clomiphene, ED_{50}: MER-25 ED_{50}; 1:125) is related to the *in vitro* binding affinity (clomiphene K_D:MER-25 K_D; 1:100 from Ginsburg *et al.*, 1977) a situation already established for the oestrogenic steroids (Korenman, 1969; Terenius, 1971, Müller & Wotz, 1977) and as would be predicted if receptor occupation is related to the biological effect. The relative low affinity of the antioestrogens for the HAR may explain the change in the apparent K_D for oestradiol-17β found when the HARs were assayed after high doses of antioestrogens. The unbound drug may have been redistributed during the preparation of cytosol so that competition between MER-25 and [^{3}H]-oestradiol-17β occurred during the *in vitro* assay. A similar change in affinity has been noted after *in vivo* oestradiol-17β (Morris, 1976).

An attempt was made to correlate some of the biological effects of the drugs with the cytosol HAR changes. Tamoxifen and MER-25 were not uterotrophic whereas clomiphene produced a bell shaped dose-response curve, results similar to those of other workers (Roy, Greenblatt & Mahesh, 1964; Harper & Walpole, 1967; Mayer, 1973) and clearly not related to the receptor changes (Figure 1). In this respect it may have been more pertinent to examine the changes in the accumulation of nuclear receptor complex but as the drugs are found bound to two different sites in the nucleus (Rinehart, Ruh & Ruh, 1977) such associations may be difficult to make. High doses of these drugs have been demonstrated to inhibit the ovulatory surge of gonadotrophins (Shirley, Wolinsky & Schwartz, 1968; Labhsetwar, 1970) and sexual behaviour (Roy & Wade, 1977) which are oestrogen-dependent processes. In the latter study inhibition of [^{3}H]-oestrogen uptake into the brain tissue was also seen but when low doses were used which only inhibit pituitary [^{3}H]-oestrogen uptake, no behavioural changes were observed. Many studies have demonstrated two distinct actions of the antioestrogens on plasma gonadotropin concentrations, low doses inhibiting pituitary gonadotrophin secretion (Roy, Mahesh & Greenblatt, 1965; Nagel, Baeir & Taubert, 1970; Schally, Carter, Parlow, Arimura, Bowers & Holtkamp, 1970; Mayer, 1973). It is tempting to speculate that the stimulatory and inhibitory actions are correlated to the induced HAR changes in the pituitary and brain respectively and that the different experimental conditions e.g. dosage regime, endocrinological status, account for the failure. Further experiments are in progress to elucidate this point.

In summary the results demonstrate that the antioestrogens, clomiphene, MER-25 and tamoxifen produce long term cytosol HAR depletion in the CNS and pituitary, similar to that found for the uterus.

It has also been shown that *in vivo*, higher doses of the drugs are required to produce depletion of the HAR when compared to the peripheral tissues. The experimental data suggest that the two antioestrogens most extensively used for the treatment of female infertility, clomiphene and tamoxifen, possess different capabilities for penetration into the brain and also differ in their agonist property. If the rat data presented here can be extrapolated to the human situation, then clinical pharmacological profiles should differ and a more rational prognosis of drug treatment in female infertility may be possible. Similarly when these drugs are used for the treatment of peripheral conditions such as mammary carcinoma, the careful choice of drug and/or dosage required may also lead to a significant improvement in therapy with the existing drugs. Finally, the development of antioestrogenic or oestrogenic drugs with effects specific to certain target tissues may provide the key to even more rational and selective therapy in this area.

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