Journal of Steroid Biochemistry, 1973, Vol. 4, pp. 687–695. Pergamon Press. Printed in Great Britain

THE INFLUENCE OF SYNTHETIC ANTI-ESTROGENS ON THE BINDING OF TRITIATED ESTRADIOL-17β BY CYTOSOLS OF HUMAN UTERUS AND HUMAN BREAST CARCINOMA

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(*Received* 20 *April* 1973)

SUMMARY

The cytosol fractions of human uterus and human breast carcinoma were incubated with tritiated estradiol-17β alone or in the presence of non-steroid anti-estrogens. Some anti-estrogens (CN-55945, clomiphene, ICI-46474, U-11100A) inhibited the binding of estradiol-17β by the tissue fractions if they were present in at least 20-fold excess over the steroid. Other anti-estrogens tested (ICI-47699, MRL-37, U-11555A, dl-norgestrel, d-norgestrel, MER-25, RMI-4613) did not markedly influence the binding of estradiol even if they were present in 1000-fold excess. Evidence is presented which may indicate that in contrast to estriol which acts as competitive inhibitor for the estradiol-17β-binding, the anti-estrogens CN-55945, clomiphene, ICI-46474 and U-11100A influence the estradiol-17β-receptor interaction by allosteric (apparent) competition.

INTRODUCTION

THE EFFECT of an anti-estrogen, Upjohn – 11100A [1] in preventing estradiol-17β uptake both *in vivo* and *in vitro* has been used as a criterion for the recognition of a "target tissue" [2]. In the rat it appears to prevent the association of estradiol-17β with the specific binding sites in those tissues. Other estrogen antagonists have also been shown to compete with estradiol-17β for receptors in target organs: Clomiphene (MRL-41) diminished the uptake of estradiol-17β by rat uterus *in vivo* [3]; MER-25 [4] inhibited the uptake of estradiol-17β by the mouse uterus *in vivo* [5]. An extensive study of the influence of anti-estrogens on the interaction *in vitro* between estradiol-17β and tissue slices of mouse uterus has been published [6]. This report included not only synthetic anti-estrogens of the triphenylethylene (clomiphene) and diphenyldihydronaphthalene-types (U-11100A) and related compounds but also steroids and isomers of stilbestrol and hexestrol.

Similar studies in human tissues have been confined mainly to the specificity of steroids for the estrogen receptor of uterus [7, 8] and breast carcinoma [9]. As far as we know, only one report on the influence of non-steroid anti-estrogens (clomiphene and U-11100A) on the binding of estradiol-17β by human breast carcinomas has been published [10]. In view of the suggestion that anti-estrogens might be useful as therapeutic agents for some patients with breast carcinomas [10] and the possible implications for the treatment of other estrogen-dependent malignancies (endometrial carcinoma) the influence of 12 anti-estrogens on the binding of tritiated estradiol-17β by cytosols of human breast carcinoma and human uterus was studied. The cytosols were incubated with tritiated estradiol-17β alone or in the presence of anti-estrogens or non-radioactive estradiol-17β.

If an anti-estrogen competed with the tritiated estradiol-17β for binding sites on the receptor the binding of the tritiated estradiol-17β decreased. Inhibition of the binding of tritiated estradiol-17β by non-radioactive estradiol-17β served as a measure of the receptor activity.

MATERIALS AND METHODS

Tissues. Human uterine tissue was obtained at hysterectomy. Three of the four specimens showed secretory changes in the endometrium; the fourth had a proliferative type, fairly inactive endometrium. Of the three breast tumours two were infiltrating duct carcinomas and one a scirrhous carcinoma.

[6,7-^{3}H]-estradiol-17β. SA. 40 Ci/mmol was purchased from Radiochemical Centre, Amersham, England.

Estradiol-17β was purchased from Sigma Chemical Co. St. Louis, U.S.A.

Anti-estrogens. All anti-estrogens were gifts from the manufacturers. CI-628 (CN-55945) i.e. 1-[2-(p-[α-(p-methoxyphenyl)-β-nitrostyryl]phenoxy) ethyl] pyrrolidine monocitrate supplied by Parke-Davis and Co; U-11100A, i.e. 1-[2-(p-[3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl]phenoxy) ethyl]-pyrrolidine hydrochloride, and U-11555A, i.e. 2-[p-(6-methoxy-2-[p-methoxyphenyl] inden-3-yl)phenoxy]hydrochloride supplied by Upjohn Pty. Ltd; d-norgestrel and dl-norgestrel, i.e. 13β-ethyl-17α-ethynyl-17β-hydroxygon-4-en-3-one and Wy7095, i.e. 1-estradiol supplied by Wyeth Pharmaceuticals Pty. Ltd; MER-25, i.e. 1-[p-2-diethylaminoethoxyphenyl]-1-phenyl-2-p-methoxyphenyl ethanol, MRL-37, i.e. 1-[p-2-diethylaminoethoxyphenyl]-1-phenyl-2-p-methoxyphenyl ethane, WSM-4613 (RMI 4613), i.e. 1-(p-2-diethylaminoethoxyphenyl)-1-p-chlorophenyl-2-p-chlorophenyl ethanol and clomiphene citrate, i.e. 1-[p-(β-diethylaminoethoxy) phenyl]-1,2-diphenyl-2-chloroethylene supplied by Richardson-Merrell Pty. Ltd.; ICI 46474, i.e. trans-isomer of 1-[p-β-dimethylaminoethoxyphenyl-1,2-diphenyl-but-1-ene] and ICI 47699, i.e. cis-isomer of same compound, supplied by ICI Australia Ltd. In addition, a new non-steroid estrogen P 1496, i.e. (3S, 7ξ)-3,4,5,6,7,8,9,10,11,12-Decahydro-7,14,16-trihydroxy-3-methyl-1H-2-benzoxatetradecin-1-one supplied by Sandoz Australia Pty. Ltd. was tested.

Preparation of tissue fractions

The tissues were put on ice immediately after removal. Blood, adipose tissue and other unwanted adjoining tissues were removed from the specimens as far as macroscopically possible. The tissues were then immersed in twice their weight of ice-cold Tris buffer pH 8·0, homogenized and the cytosol isolated as described [8].

Incubation of tissue extracts with tritiated estradiol in the absence and presence of anti-estrogens.

The cytosol was diluted 1–10 with Tris buffer pH 8·0. One ml aliquots were incubated with 2×10^{-13} moles tritiated estradiol-17β for 30 min at 25°. The reaction mixture was then cooled in ice water and excess free and non-specifically bound tritiated estradiol-17β removed by adsorption to dextran-coated charcoal as described [8]. The receptor-bound tritiated estradiol-17β was estimated by measuring the remaining radioactivity in the incubation medium after the charcoal treatment. This result represents the control (= 100%).

The influence of the anti-estrogens and of non-radioactive estradiol-17β on the binding of tritiated estradiol-17β was determined by incubating the cytosols with

the tritiated estradiol-17β in the presence of $0{\cdot}184 \times 10^{-12}$ moles to $368{\cdot}0 \times 10^{-12}$ moles of non-radioactive estradiol-17β or antiestrogen.

RESULTS

The influence of various antiestrogens on the binding of tritiated estradiol-17β by human uterus cytosol (Table 1) and by human breast carcinoma cytosol (Table 2) is similar for both tissues. The order of efficiency as inhibitors of estradiol binding is the same and the inhibitions (in % of control) observed with the anti-estrogens are comparable for both tissues. The antiestrogens fall into two groups on the basis of their influence on the estradiol-receptor interaction: Some do not affect the binding of tritiated estradiol-17β by cytosols of uterus or breast carcinoma even in concentrations 1000-fold higher than that of the estradiol-17β. This group comprises RMI-4613, MER-25, d-norgestrel, dl-norgestrel, U-11555A, MRL-37 and possibly ICI-47699. The other antiestrogens, i.e. CN-55945, clomiphene, ICI-46474, U-11100A have only weak affinities towards the estrogen receptors when compared with estradiol-17β or other C_{18}-steroids [8, 9], but at concentrations above $3{\cdot}68 \times 10^{-9}$ M (equivalent to about 1000 pg estradiol) they clearly begin to inhibit the binding of estradiol-17β by the cytosols. These antiestrogens are, therefore, comparable to group 4 of the steroids (steroids that compete only at high concentrations with estradiol-17β for binding sites [8, 9]); in their affinity for the receptors (Fig. 1.).

Table 1. Influence of increasing concentrations of anti-estrogens on the binding of tritiated estradiol-17β by human uterus cytosol (patients HAR, early secretory endometrium and JOY, disintegrating secretory endometrium). Results are in % of control (binding in the absence of anti-estrogens). For comparison, binding results in the presence of non-radioactive estradiol-17β and a synthetic estrogen (P-1496) are also given. Each result is the mean of 3 replicates with the exception of the control which represents the mean of 5 replicates

Compound	Inhibition in % of control Concentration of anti-estrogens $\times 10^{-9}$ M						
	0·184	0·368	0·736	3·68	18·4	36·8	184·0
1. Estradiol	73·9	62·0	45·9	25·0	–	–	–
2. P-1496	92·6	84·4	70·4	40·8	18·1	12·5	9·1
3. CN-55945	94·0	97·0	104·0	99·0	52·9	50·6	20·3
4. clomiphene	101·0	103·0	95·0	101·0	81·0	76·5	42·8
5. ICI-46474	111·5	113·2	114·2	110·8	94·5	84·8	52·7
6. U-11100A	94·0	96·6	101·5	103 0	93·0	88·4	54·1
7. ICI-47699	91·8	95·0	95·6	98·3	96·1	93·4	88·4
8. MRL-37	93·0	90·5	90·8	101·5	105·0	105·0	90·5
9. U-11555A	96·5	98·0	102·0	96·8	95·0	97·4	92·4
10. dl-norgestrel	101·0	102·6	101·0	105·0	109·0	104·1	107·2
11. d-norgestrel	101·6	104·6	101·0	103·1	109·2	106·0	100·5
12. MER-25	104·2	109·0	109·8	108·5	104·1	106·5	106·0
13. RMI-4613	93·9	91·3	93·6	88·9	96·1	95·0	92·0
mean 3–6	100·1	102·5	103·7	103·5	80·4	75·1	42·5
standard deviation	± 8·27	± 7·74	± 7·98	± 5·16	± 19·27	± 17·06	± 15·62
mean 7–13	97·4	98·7	99·1	100·3	102·1	101·1	96·7
standard deviation	± 4·84	± 6·98	± 6·34	± 6·39	± 6·23	± 5·60	± 7·74

Table 2. Influence of increasing concentrations of anti-estrogens on the binding of tritiated estradiol-17β by human breast carcinoma cytosols (patients GIA, RIC). Results are in % of control (binding in the absence of anti-estrogens). For comparison, binding results in the presence of non-radioactive estradiol-17β and a synthetic estrogen (P. 1496) are also given. Each result is the mean of 3 replicates with the exception of the control which represents the mean of 5 replicates.

Compound	Inhibition in % of control Concentration of anti-estrogens $\times 10^{-9}$ M							
	0·184	0·368	0·736	3·68	18·4	36·8	184·0	368·0
1. Estradiol	71·2	56·6	41·0	24·5	17·2	–	–	–
2. P-1496	79·6	85·5	90·1	47·3	32·6	–	–	–
3. CN-55945	85·5	89·2	83·2	82·4	55·2	42·6	–	18·4
4. clomiphene	–	–	92·8	88·7	87·3	64·8	37·0	–
5. ICI-46474	–	–	98·0	94·2	88·5	81·5	32·7	–
6. U-11100A	99·0	94·0	77·0	99·0	92·4	64·8	–	25·8
7. ICI-47699	–	–	92·6	88·6	87·9	92·4	65·0	–
8. MRL-37	–	–	91·0	95·1	87·9	87·5	77·5	
9. U-11555A	94·1	95·1	101·0	92·5	95·3	90·6	–	37·5
10. dl-norgestrel	119·1	116·0	106·1	107·9	95·9	92·8		55·3
11. d-norgestrel	112·0	125·5	109·9	112·0	107·4	–	–	–
12. MER-25	–	–	104·1	100·3	102·6	102·7	100·0	–
13. RMI-4613	–	–	99·5	91·0	101·5	103·3	105·7	–
mean 3–6	92·3	91·6	87·8	91·1	80·9	63·4	34·9	22·1
standard deviation	± 9·55	± 3·39	± 9·43	± 7·15	± 17·24	± 15·96	± 3·04	± 5·23
mean 7–13	108·4	112·2	100·6	98·2	96·9	94·9	87·1	46·4
standard deviation	± 12·88	± 15·55	± 6·91	± 8·90	± 7·41	± 6·56	± 19·09	± 12·59

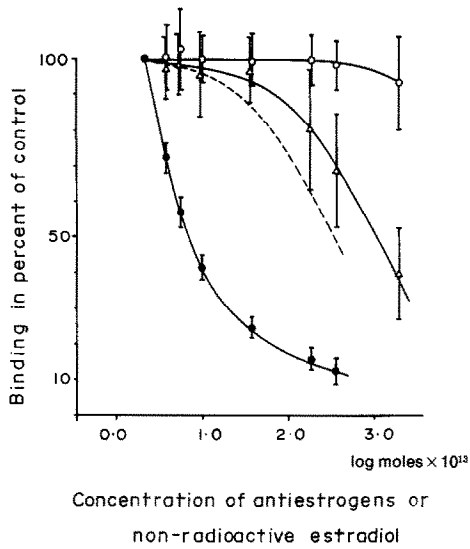

Fig. 1. Inhibition of the binding of tritiated estradiol by antiestrogens. On the abscissa the logarithm of the amount of antiestrogens is plotted. At point 0·0 (= control) the binding of tritiated estradiol in the absence of antiestrogen is shown (= 100%). On the ordinate is given the binding in per cent of control. The vertical bars show the standard deviations from the means. ●–● estradiol; △–△ antiestrogens 3–6 (results taken from Tables 1 and 2 (+ ○–○ antiestrogens 7–13 (result taken from Tables 1 and 2). The broken line represents the influence of group 4 steroids (steroids that compete only at high concentrations with estradiol for binding sites on the receptor) and was taken from Refs [8 and 9].

As the effect of the antiestrogens on the estradiol-receptor binding was observed only at relatively high concentrations, additional experiments were performed in order to determine if the antiestrogens were in fact competing for the estradiol-binding sites or if the inhibition was due to other influences. In these experiments the concentrations of both tritiated estradiol-17β and of inhibitors were varied, and the results presented in a double reciprocal plot of 1/bound estradiol over 1/total estradiol. In formal analogy to the equations used in enzyme kinetics [11] it should then be possible to distinguish between competitive, uncompetitive and non-competitive inhibitions [12]. The results for one antiestrogen (CN – 55945) are shown on Fig. 2. For comparison a similar plot of results obtained from incubations with tritiated estradiol and estriol, is given on Fig. 3. The pattern obtained for both estriol and CN-55945 is characteristic for competitive inhibition: The presence of the inhibitor affects only the slope while the intercept remains the same. In the plot the results obtained with the highest estradiol concentration ($3{\cdot}91 \times 10^{12}$ moles^{-1}) were ignored as it was known from Scatchard plots that at this concentration binding was no longer due to the high-affinity receptor sites only. For clarity of presentation the results of some of the inhibitor concentrations only are shown on the figures, but the influence of inhibitor concentrations of $0{\cdot}184 \times 10^{-12}$M, $0{\cdot}368 \times 10^{-12}$ M, $0{\cdot}734 \times 10^{-12}$ M, $3{\cdot}684 \times 10^{-12}$ M, $18{\cdot}374 \times 10^{-12}$ M, $36{\cdot}774 \times 10^{-12}$ M and $183{\cdot}77 \times 10^{-12}$ M was tested. Due to the fact that only high concentrations of the antiestrogen CN-55945 had an effect on estradiol-binding, the lines for the low concentrations (up to $3{\cdot}68 \times 10^{-12}$ M) were indistinguishable from the control on Fig. 2 while on Fig. 3 even the lowest estriol

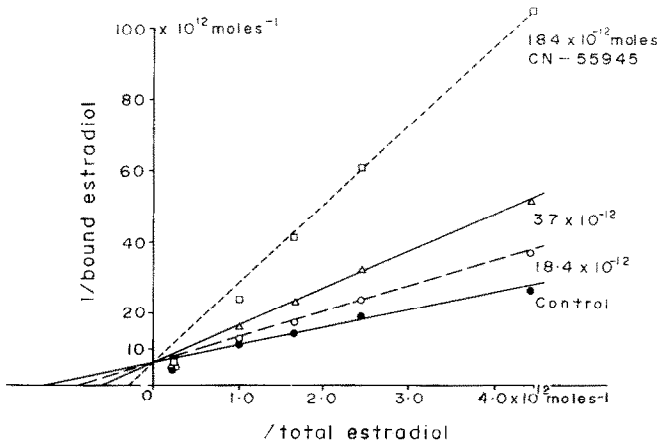

Fig. 2. Influence of antiestrogen CN-55945 on the binding of tritiated estradiol to uterine cytosol receptor (specimen HAR). Aliquots of the cytosol were incubated with varying amounts of antiestrogen ($0{\cdot}184 \times 10^{-12}$ moles to $183{\cdot}77 \times 10^{-12}$ moles) and varying amounts of estradiol ($0{\cdot}22 \times 10^{-12}$ moles to $3{\cdot}90 \times 10^{-12}$ moles) and the results presented in a double reciprocal plot.

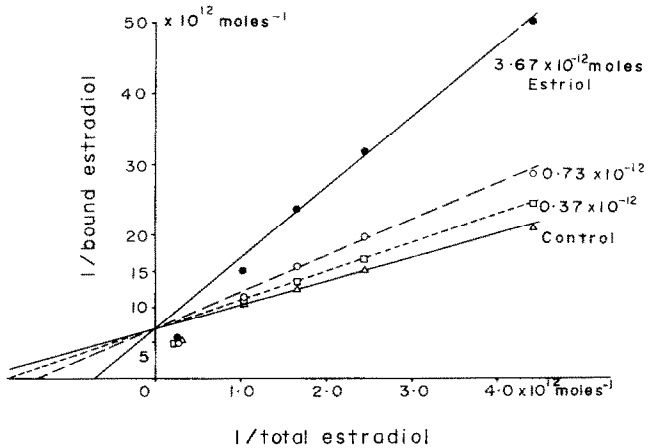

Fig. 3. Influence of estriol on the binding of tritiated estradiol to uterine cytosol receptor (specimen HAR). Aliquots of the cytosol were incubated with varying amounts of estriol ($0{\cdot}37 \times 10^{-12}$ moles to $3{\cdot}67 \times 10^{-12}$ moles) and with varying amounts of estradiol ($0{\cdot}22 \times 10^{-12}$ moles to $3{\cdot}90 \times 10^{-12}$ moles), and the results were presented in a double reciprocal plot.

concentration (not shown) changed the slope of the line significantly compared with that of the control.

The inhibitors could also react with the receptor at a second site and change the conformation of the receptor and thus influence the binding of the estradiol (allosteric inhibitor). In this case the double reciprocal plot may be indistinguishable from the competitive inhibition type where the inhibitor binds to the same site as the estradiol. A plot of 1/bound estradiol versus the inhibitor concentration will be different however [12]. In competitive inhibition straight lines are obtained while in allosteric inhibition (apparent competition) hyperbolic curves are the

result. Plotting the results in this way, shows that estriol inhibition (Fig. 4) is due to competition for the primary binding site on the receptor, while the inhibition by the anti-estrogen (ICI-46474) is probably caused by allosteric changes (Fig. 5). Curves similar to those in Fig. 5 were obtained when the influence of clomiphene and CN-55945 on estradiol binding was tested. In the same way it was demonstrated that the influence of the synthetic estrogen P-1496 on the binding of estradiol-17β was not achieved by competitive inhibition but due to allosteric inhibition.

DISCUSSION

Comparison of our results obtained on human tissues with those reported for animal tissues shows that there is a general agreement. In the mouse and the rat [6] all anti-estrogens tested inhibited the binding of estradiol to slices of uterus tissue, but the inhibitory potencies ranged over several orders of magnitude. The most efficient inhibitor was clomiphene followed by U-11100A, CN-55945 and ICI-46474. These four compounds were also most active in our experience: they inhibited the binding of 2×10^{-13} moles estradiol in concentrations above $3{\cdot}68 \times$

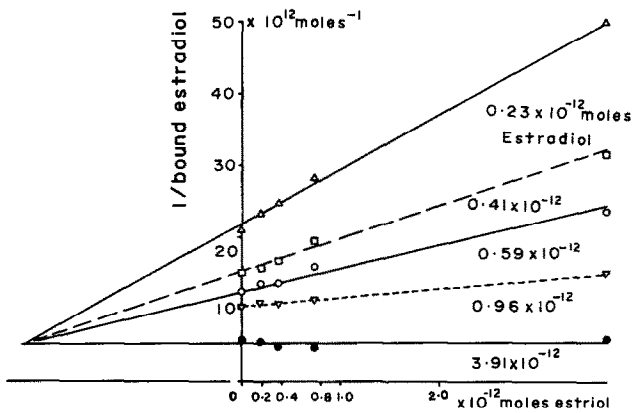

Fig. 4. Influence of estriol on the binding of tritiated estradiol to uterine cytosol receptor. Details as on Fig. 3. The reciprocal of the bound estradiol was plotted vs the amount of estriol.

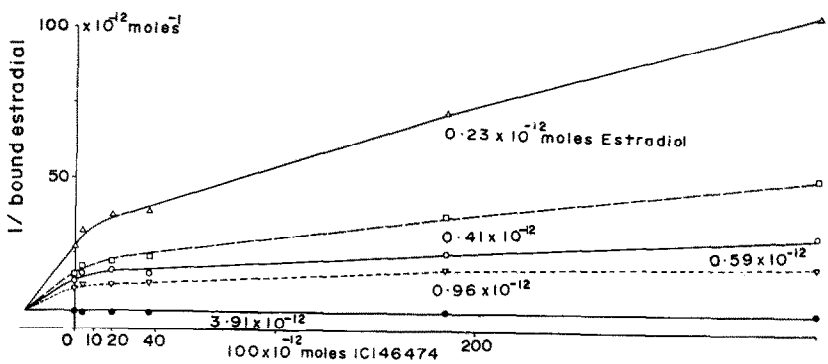

Fig. 5. Influence of antiestrogen ICI-46474 on the binding of tritiated estradiol to uterine receptor. Details as on Fig. 2. The reciprocal of the bound estradiol was plotted vs the amount of ICI-46474.

10^{-9} M. It appears that in contrast to mice CN-55945 is more potent than clomiphene in inhibiting estradiol binding in human uterine tissue and human breast carcinoma tissue, but the difference may also be due to different methodology (tissue slices versus tissue cytosol). The fact that Terenius found some inhibition of estradiol binding even with those antiestrogens which in our experiments had no influence, may be due to the much higher antiestrogen concentration used by him (up to 300,000 ng antiestrogens/ml, to inhibit the binding of 1 ng of estradiol; we used a maximum of about 75 ng antiestrogens to inhibit the binding of 0·058 ng estradiol).

For one antiestrogen, U-11100, it has been shown that its influence on estradiol binding is due to a reversible competitive inhibition [13]. When our results for some of the antiestrogens (CN-55945, clomiphene, ICI-46474) were presented in a double reciprocal plot a pattern typical for competitive inhibition was obtained. However, a plot of the reciprocal of the bound estradiol over the antiestrogen concentrations showed a deviation from straight lines. This indicates that there may be separate binding sites for estradiol and the antiestrogens tested (allosteric inhibition).

From studies on the structure-affinity relationship it had been concluded previously [8] that attachment of estradiol to the receptor probably occurs via two groups, the phenolic hydroxyl group on C-3 and an oxygen function on ring *D*. Visualizing a flexible affinity site for estradiol-17β on the receptor, binding may occur by initial attachment of the C-3 phenolic hydroxyl group to a highly specific centre facilitating the attraction of the C-17β hydroxyl function to a less specific binding centre thereby securing a second attachment. This interaction may induce a stereospecific configurational change in the receptor activating it to carry out its biological function.

It is somewhat more difficult to define a mechanism by which the antiestrogens CN-55945, Clomiphene, ICI-46474 and U-11100A can compete directly for this same binding site. The molecular structures of these four compounds are very similar, the aromatic-N-ethylether system being a common feature in all. Two of these antiestrogens (CN-55945 and U-11100A) have a methylether grouping on a second aromatic ring. Binding may occur in the first instance through the N-ethylether by attraction to the more specific centre. A second interaction may involve the aromatic methylether (in the case of CN-55945 and U-11100A). Alternatively, for effective competition by these four antiestrogens a second point of interaction may be unnecessary and added binding strength may be contributed by the shape of these considerably rigid molecules.

This is supported to some extent by the inability of the antiestrogens RMI-4613, MER-25 and MRL-37, which have similar but more flexible structures, to compete as efficiently for the estradiol binding centre. Further indication of the stereochemical dependence of binding is given by the marked difference in inhibition observed between ICI-46474 and its cis-isomer. The difference in inhibiting capabilities displayed by U-11100A and U-11555A may be explained through an alteration in distances (about 9·6 Å in U-11100A and about 10·6 Å in U-11555A) between the two apparently active functions—the N-ethylether and the methylether groupings. Both the lack of aromaticity in the A-ring and possible steric hindrance in the region of the C-17β hydroxyl group could contribute largely to eliminate the binding of d-norgestrel and dl-norgestrel.

This direct competition for the estradiol binding site, however, seems unlikely

in the light of previous results which showed that derivative formation on the phenolic hydroxyl group of estradiol (ethers, esters) all but abolished the binding to the receptor [8]. It appears more likely that CN-55945, clomiphene, ICI-46474 and U-11100A are bound to a different site. This induces changes on the receptor site for estradiol which in turn leads to a decreased affinity for estradiol (allosteric inhibition). This interpretation is supported by the results shown on Fig. 5, but more detailed studies are necessary to confirm the suggestion.

The synthetic estrogen P-1496 is comparable to group 3 steroids (including estrone, estriol, 16-epiestriol, 17-epiestriol, 16α-estradiol, 17α-estradiol [8]) in its affinity for the estradiol receptor. P-1496 is a very flexible molecule with two phenolic hydroxyl groups on one end of the molecule and an alcoholic hydroxyl group at the other end. Dreiding models show that P-1496 can easily obtain a configuration in which the alcoholic hydroxyl group and either one of the phenolic hydroxyl groups are equidistant to those in estradiol-17β. It came as a surprise, therefore, that the plot of 1/bound estradiol-17β vs the P-1496 concentration gave hyperbolic curves indicative of allosteric competition rather than competitive inhibition. In the light of this finding it may be useful to re-examine the influence of stilbestrol, hexestrol and various steroids [8] on the binding of estradiol-17β by target tissue to determine if there are more instances of allosteric inhibition where competition had been assumed so far.

There is a possibility that the antiestrogens affect the enzymatic activity of the dehydrogenase system (which transforms estradiol-17β to estrone) rather than estradiol binding. This enzyme is found mainly in the endometrium and is of a low activity in the myometrium. Furthermore, it appears to be rapidly and irreversibly inactivated at temperatures above 0° [14]. It is, therefore, unlikely that our results could be interpreted in this way.

ACKNOWLEDGEMENT

This study was aided by grants from the A. Yeldham and M. Raine Medical Research Foundation, University of Western Australia and from the Cancer Council of Western Australia. T. R. is holder of a Saw Medical Research Fellowship, University of Western Australia.

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