# ANTIESTROGENS: STUDIES USING AN IN VITRO ESTROGEN-RESPONSIVE UTERINE SYSTEM*

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SUMMARY: Three antiestrogens have been tested in vitro for their capacity to affect the cytosol binding and nuclear uptake of 17β-estradiol ($E_2$) and induction of the synthesis of a specific uterine protein (IP). CI-628 competes with $E_2$ for binding sites, inhibits IP induction, and is itself much less effective than $E_2$ in promoting the IP response; U-11,100A is a binding competitor and a weak inducer of IP synthesis, but does not antagonize the $E_2$-induced IP response. Dimethylstilbestrol is an effective inhibitor of $E_2$ binding, elicits a high IP response, but does not antagonize $E_2$-induced IP synthesis. Higher concentrations of antiestrogens are required to inhibit nuclear binding of $E_2$ than expected from their relative binding ability to cytosol.

Considerable interest in antiestrogens has developed because of their present and potential clinical importance (1-3) and because of the variety of possible mechanisms by which they might antagonize biochemical and physiological responses to estrogens. Studies on their mechanism of action have been complicated by methods of assay entailing in vivo administration of estrogen and antiestrogen (4-7) with the inherent difficulties in clearly defining the parameters of the system (compound concentrations, metabolism, clearance, and quantitation of response). The fact that many antiestrogens are also estrogenic (6,8) has caused further complications. The defined in vitro estrogen-responsive uterine system that we have developed, in which physiological concentrations of estrogens will induce specific protein synthesis (9,10), seemed to be a particularly appropriate system for evaluating the actions of several known antiestrogens because it allows one to correlate, under clearly defined conditions, a biological response (Induced Protein, or IP, synthesis) with the intensively studied estrogen binding and translocation phenomena (3,11). The studies described here report the effects of three nonsteroidal antiestrogens, two of the clomiphene type (CI-628, originally called CN-55, 945-27, abbreviated CI; and U-11,100A, also known as nafoxidine, abbreviated

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UA) and one of the stilbestrol type (dimethylstilbestrol, abbreviated DMS), on the induction of IP synthesis and on the binding of estradiol ($E_2$) to uterine cytosol and nuclei. Our results demonstrate that while these three antiestrogens all compete with $E_2$ for uterine binding sites, they act quite differently in promoting or inhibiting the induction of IP synthesis.

## MATERIALS AND METHODS

Incubation of uteri in vitro and measurement of IP synthesis. Uteri were excised from 21 to 24 day old Holtzman rats and incubated (37°, 95% $O_2$ - 5% $CO_2$ atmosphere) in Eagle's HeLa medium (Difco; 3 uteri/2 ml) containing designated concentrations of antiestrogen, either CI, kindly supplied by Dr. J. Reel, Parke-Davis Co.; UA, kindly supplied by Dr. A. Nathan, Upjohn Co.; or trans-α,α'-DMS purchased from Gallard Schlesinger (Carle Place, N.Y.). After incubation with antiestrogen or vehicle control, nonradioactive $E_2$ (Mann Research) was added directly to the incubation flask for a further 1 hr incubation period. Uteri were then transferred to a new flask containing radioactive amino acid (either 20μCi/ml of L-[4,5-$^3$H] leucine (2.0 Ci/mmole) or 5μCi/ml of L-[$^{14}$C] leucine (316mCi/mmole), Schwarz Bioresearch) and 28μg/ml of actinomycin D (Sigma) and allowed to incorporate amino acid for 2 hr at 37° for determination of IP synthesis. The methods used for double isotope labeling of uterine proteins, for preparation and polyacrylamide gel electrophoresis of uterine soluble proteins, and for quantitation of IP synthesis were exactly as described previously (9).

Inhibition of nuclear $^3$H-$E_2$ binding by antiestrogens. After exposure to antiestrogen or vehicle control as above, uteri were incubated with radioactive $E_2$ ($^3$H-$E_2$; 17β-estradiol-6,7-$^3$H(N), 47 Ci/mmole, New England Nuclear) to determine total binding and with $^3$H-$E_2$ plus a 100-fold excess of unlabeled $E_2$ to determine non-specific binding. Uteri were homogenized (3 uteri/ml) in iced 0.01 M Tris HCl - 0.0015 M EDTA, pH 7.4 at 0° (TE buffer), and bound $^3$H-$E_2$ in the washed 800 x g nuclear fraction was determined by ethanol

extraction as previously described (12).

Competition of antiestrogens with $^3H$-$E_2$ for binding to cytosol. Aliquots of uterine cytosol (50 µl; 2 uteri/ml in TE buffer; 226,000 x g, 45 min) were added to a mixture of 10 µl of 7 x $10^{-8}$ M $^3H$-$E_2$ in TE buffer and 10 µl of various concentrations of cold competitor (in 1:1 dimethylformamide: TE buffer), to give final incubations that were $10^{-8}$ M in $^3H$-$E_2$, from $10^{-9}$ to $10^{-4}$ M (twelve concentrations in duplicate) in competitor, and 1.4 uteri/ml. After 24 hr at 0°, the free steroids were adsorbed by dextran-charcoal [10 µl of 0.5% Dextran C (Mann)-5% Norite A (Sigma) in TE buffer] for 1 hr, 0°, and aliquots were counted after centrifugation at 800 x g, 30 min. The relative binding ability of the competitors is taken as the ratio of concentrations of cold $E_2$/competitor required to inhibit 50% of specific $^3H$-$E_2$ binding (13).

## RESULTS AND DISCUSSION

Experiments with the antiestrogen CI indicated that exposure of uteri to the antiestrogen prior to addition of $E_2$, can significantly diminish both the magnitude of IP induction and the amount of nuclear-bound $E_2$ (Table I). At the highest CI concentration employed, a greater than 70% inhibition of induction and binding was seen. At this concentration, CI itself is only a weak inducer. Figure 1 demonstrates that the effect of CI is to inhibit specifically the induction of IP synthesis while synthesis of general proteins is not affected. Simultaneous administration of high concentrations of CI (6 x $10^{-5}$M) with the $E_2$ (2.5 x $10^{-8}$M) for a 1 hr incubation period was less effective than pretreatment with antiestrogen alone in inhibiting IP induction (only 20% inhibition). Further, transfer of uteri to media containing CI (6 x $10^{-5}$M) for a 1 or 2 hr incubation after a 5 min exposure to 5 x $10^{-8}$M $E_2$ failed to produce any diminution or reversal of the IP response.

Similar studies with the compound UA also indicated pretreatment to be the most effective regimen. While increasing concentrations of UA progressively inhibit nuclear $E_2$ binding, there is no detectable inhibition of the IP response (Table II). UA itself is able to induce some IP synthesis, but only in the higher concentration range.

Table I. Effect of CI-628 (CI) on $^3$H-estradiol ($E_2$) binding to nuclear receptor, and induction of IP synthesis.

| | CI Pretreatment | | | CI alone |
|---|---|---|---|---|
| CI conc. (M) | CI/$E_2$ ratio ($E_2$=2.5 x $10^{-8}$M) | $^3$H-$E_2$ binding** (Nuclear) | IP induction** | IP induction*** |
| 5 x $10^{-5}$ | 2000 | 22 ± 7* | 25 ± 8* | 9 ± 6* |
| 1 x $10^{-5}$ | 400 | 53 ± 8 | 65 ± 15 | 9 ± 2 |
| 2 x $10^{-6}$ | 80 | 99 ± 7 | 108 ± 12 | 0 |
| 5 x $10^{-7}$ | 20 | 111 ± 15 | 103 ± 11 | 0 |
| 1 x $10^{-7}$ | 4 | 114 ± 11 | 127 ± 19 | 0 |

*All values expressed as percent of control ± standard error of the mean.

**3 uteri/2 ml Eagle's HeLa medium, 37°. 90 min with CI, $E_2$ added during the last 60 mins. Three or 4 determinations from 2 experiments.

***3 uteri/2 ml Eagle's HeLa medium, 37°. 90 min with CI alone. Three determinations from 2 experiments.

Table II. Effect of U-11,100A (UA) on $^3$H-estradiol ($E_2$) binding to nuclear receptor, and induction of IP synthesis.

| | UA Pretreatment | | | UA alone |
|---|---|---|---|---|
| UA conc. (M) | UA/$E_2$ ratio ($E_2$=2.5 x $10^{-8}$M) | $^3$H-$E_2$ binding** (Nuclear) | IP induction** | IP induction*** |
| 5 x $10^{-5}$ | 2000 | 8 ± 2* | 100 ± 16* | 44 ± 2* |
| 1 x $10^{-5}$ | 400 | 32 ± 6 | 109 ± 17 | 18 ± 5 |
| 2 x $10^{-6}$ | 80 | 74 ± 3 | 105 ± 7 | 9 ± 5 |
| 5 x $10^{-7}$ | 20 | 79 ± 7 | 114 ± 15 | 0 |
| 1 x $10^{-7}$ | 4 | 91 ± 3 | 93 ± 14 | 0 |

*All values expressed as percent of control ± standard error of the mean.

**3 uteri/2 ml Eagle's HeLa medium, 37°. 90 min with UA, $E_2$ added during the last 60 mins. Three determinations from 2 experiments.

***3 uteri/2 ml Eagle's HeLa medium, 37°. 90 min with UA alone. Three or 4 determinations from 2 experiments.

Table III presents the effects of DMS pretreatment on $^3$H-$E_2$ binding and IP induction. Even at very high concentrations, DMS does not antagonize the

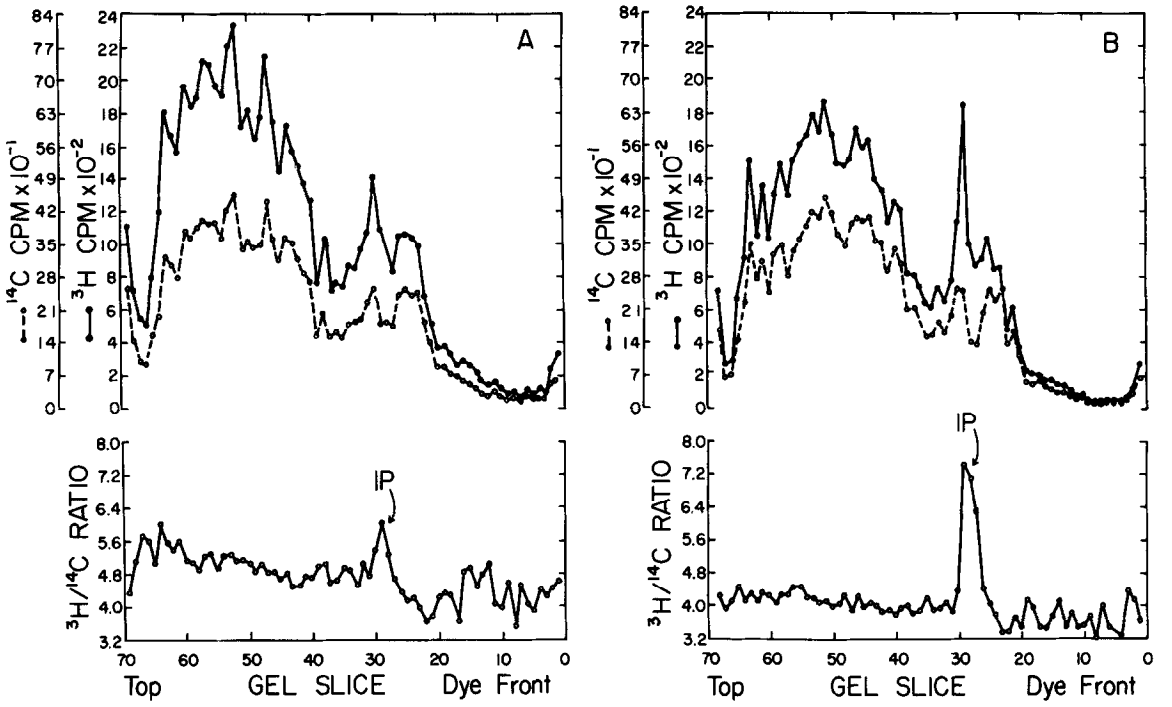


Fig. 1. Electrophoretic distribution on polyacrylamide gels of uterine soluble proteins synthesized in vitro following a 30 min in vitro pretreatment in Eagle's HeLa medium with (A) 5 x 10$^{-5}$M CI or (B) antiestrogen control vehicle (water) prior to the addition of 2.5 x 10$^{-8}$M 17β-estradiol or ethanol (0.24%; estradiol control) for a further 60 min incubation at 37°. Uteri were then allowed to incorporate labeled leucine ($^{3}$H for estradiol treated and $^{14}$C for non-estradiol treated uteri) into protein for 2 hr at 37° in the presence of 28 µg/ml actinomycin D. Uteri were homogenized and the supernatant fraction of centrifuged homogenates was separated by polyacrylamide gel electrophoresis. The radioactivity and $^{3}$H/$^{14}$C ratio in each gel slice were determined.

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$E_2$-induced IP synthesis, although it is an effective competitor of $E_2$ binding. DMS itself is a strong inducer at all concentrations tested (1 x 10$^{-7}$ to 5 x 10$^{-5}$M); its effect alone can account nearly completely for the full IP response observed in cases in which $E_2$ binding is inhibited.

Studies of competitive binding of these three antiestrogenic compounds to rat uterine cytosol indicated the binding ability of DMS, UA, and CI, relative to $E_2$, to be 17%, 8%, and 8% respectively. Similar relative binding abilities have been reported for UA (3%) using calf uterine cytosol (14) and for CI (1%) and DMS (16%) using rabbit uterine cytosol (13). Comparison of

Table III. Effect of dimethylstilbestrol (DMS) on $^{3}$H-estradiol ($E_2$) binding to nuclear recptor, and induction of IP syntehsis.

| | DMS Pretreatment | | | DMS alone |
|---|---|---|---|---|
| DMS conc. (M) | DMS/$E_2$ ratio ($E_2$=2.5 x $10^{-8}$M) | $^{3}$H-$E_2$ binding** (Nuclear) | IP induction** | IP induction*** |
| 5 x $10^{-5}$ | 2000 | 3 ± 1* | 116 ± 24* | 102 ± 14* |
| 1 x $10^{-5}$ | 400 | 10 ± 2 | 148 ± 7 | 117 ± 2 |
| 2 x $10^{-6}$ | 80 | 12 ± 1 | 152 ± 18 | 94 ± 13 |
| 5 x $10^{-7}$ | 20 | 37 ± 2 | 157 ± 16 | 89 ± 12 |
| 1 x $10^{-7}$ | 4 | 78 ± 2 | 130 ± 9 | 105 ± 4 |

*All values expressed as percent of control ± standard error of the mean.

**3 uteri/2 ml Eagle's HeLa medium, 37°. 90 min with DMS, $E_2$ added during the last 60 mins. Two or 3 determinations from 2 experiments.

***3 uteri/2 ml Eagle's HeLa medium, 37°. 90 min with DMS alone. Two determinations from 1 experiment.

these cytosol binding values with the concentrations of CI, UA, and DMS (from Tables I, II and III) required for inhibition of $^{3}$H-$E_2$ uptake by the whole uterus into uterine nuclei (Table IV), indicates that several fold (2-33x) higher concentrations of antiestrogens are required for nuclear binding inhibition as compared with inhibition of binding to cell-free cytosol. This apparent discrepancy may be explained by one of the following hypotheses: 1) Sites filled with antiestrogen are less readily translocated to the nucleus than $E_2$-filled sites and that during the 1 hr estrogen treatment at 37°, antiestrogen dissociates from some of these sites which then become available for translocation by $E_2$; and/or 2) The rate of entry of the antiestrogens into uterine cells is slower than that of $E_2$. More direct answers would be possible if radioactive antiestrogens were available.

These studies at the molecular level indicate that there are distinct differences in the behavior of the three compounds. As agonists, both CI and UA are unable to evoke maximum levels of IP induction even at the highest

Table IV. Concentrations of antiestrogens required for 50% inhibition of $^{3}$H-estradiol binding to nuclear and cytosol sites.

| Antiestrogen | Nuclear * | Cytosol ** | Nuclear/Cytosol Ratio |
|---|---|---|---|
| CI | $1 \times 10^{-5}$ M | $3 \times 10^{-7}$ M | 33 |
| UA | $6 \times 10^{-6}$ M | $3 \times 10^{-7}$ M | 20 |
| DMS | $3 \times 10^{-7}$ M | $1.5 \times 10^{-7}$ M | 2 |

* 50% nuclear binding inhibition determined as in Tables I, II, and III. Note that these values are overestimates of antiestrogen nuclear binding affinity as they are determined after a 30 min pretreatment period with antiestrogen alone prior to estradiol exposure.

** 50% cytosol inhibition = estradiol concentration/relative binding ability of antiestrogen. See Materials and Methods for details.

concentrations tested, while DMS is a potent agonist capable of evoking a maximum response. Terenius (7) has noted similar behavior of CI and UA in in vivo assays of uterotrophic activity in mice, although he finds that high doses of DMS are required to evoke even a minimal uterotrophic effect. As an antagonist of $E_2$ binding to cytoplasmic or nuclear receptor, DMS is considerably more effective than CI and UA; however, as an antagonist of $E_2$-induced IP synthesis, only CI is active, while in uterotrophic assays, all three compounds have a demonstrable antiestrogenic effect.

In terms of an early, specific estrogen-induced response, no single relationship appears to hold between the intrinsic activity of these compounds and their ability to suppress nuclear binding of $E_2$ and the $E_2$-induced IP response. It is clear that although DMS, CI, and UA are all able to suppress estrogen induced growth, the molecular bases of their activity may be quite different and deserve further study.

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