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INTERACTION OF ESTROGEN-NITROSOUREA CONJUGATES WITH THE ESTROGEN RECEPTOR IN RAT UTERUS*

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Abstract—Several estrogen-nitrosourea conjugates have been synthesized with the aim of producing more selective cytotoxic agents. The conjugates were shown to compete with estradiol for binding to cytosolic estrogen receptor in rat uterus; the relative binding affinities for *N*-(2-chloroethyl)-*N'*-(3,17β-dihydroxyestra-1,3,5(10)-trien-17α-yl)methyl-*N*-nitrosourea (17α-CNU), *N*-(2-chloroethyl)-*N'*-(3-hydroxyestra-1,3,5(10)-trien-17β-yl)-*N*-nitrosourea (17β-CNU), and *N*-(2-chloroethyl)-*N'*-2,3-di(*p*-hydroxyphenyl)-pentanyl-*N*-nitrosourea (HEX-CNU) were 2, 0.4, and 0.2, respectively, using a binding affinity of 100 for estradiol. In the ligand exchange assay, cytosolic receptors preloaded with 17α-CNU and HEX-CNU were found to lose some of their estradiol (E_2) binding sites, suggesting that binding to estrogen receptor (ER) may be irreversible. An increase of nuclear accumulation of ER was observed in the presence of 17α-CNU and HEX-CNU. In the rat system, even at a 10,000-fold excess, these two agents failed to show any antagonism of the uterotrophic effect of E_2 *in vivo*. The low binding affinity and instability of these conjugates may account for their lack of antiestrogen activity. On the other hand, 17α-CNU at 100 or 1000 μg/day and HEX-CNU at 1000 μg/day demonstrated significant uterotrophic activity. This study did not resolve whether the stimulation of uterine growth was due to the parent estrogen-nitrosourea conjugate or to decomposition and/or metabolic products.

Many natural products have been used as carriers for cytotoxic agents in an attempt to enhance specificity of action. Since clinical studies have shown that about 50% of human breast cancers contain estrogen receptor protein, and 55–60% of such ER¶-positive patients respond to hormonal therapy [1], estrogen appears to be a good candidate as a carrier in this effort to synthesize more specific antitumor agents. We have synthesized several estrogen nitrosourea conjugates and have demonstrated that they possess antitumor activity against implanted rat mammary tumor [2]. The rationale for the synthesis of these compounds is to retain the critically positioned hydroxyl groups on the estrogen moiety so that binding of the conjugates to estrogen receptor is not compromised [3–6]. A specific objective is that these cytotoxic compounds may be taken up selectively by hormone responsive tumors containing estrogen receptors, thereby possibly increasing the therapeutic index of such agents.

Furthermore, since nitrosoureas decompose in aqueous solution and produce isocynates which are strong carbamoylating agents for nucleophilic groups on protein molecules [7, 8], we also investigated whether estrogen containing nitrosoureas might block the estrogen binding site on the receptor by forming a covalent linkage with the receptor and thus act as either estrogens or antiestrogens.

In this paper, we report the synthesis of a nitrosourea conjugate of a hexestrol derivative and describe the interaction of this and other estrogen nitrosourea conjugates with rat uterine estrogen receptor. The cytotoxic activity of these compounds against human breast cancer cells *in vitro* has been reported elsewhere [9].

MATERIALS AND METHODS

Materials. The following compounds were obtained from the source indicated: estradiol (Sigma); [2,3,6,7-^{3}H(N)]estradiol (101.7 Ci/mmol, New England Nuclear); charcoal, norit A (Sigma); dextran, clinical grade (Sigma); scintillation fluid PCS (BDH); Dulbecco's phosphate-buffered saline (Gibco); ethylenediamine-tetraacetic acid (EDTA, Fisher); and tris-hydroxymethylaminomethane (Tris, Sigma). All other chemicals were of the best available grade.

Chemical synthesis. *N*-(2-Chloroethyl)-*N'*-(3,17β-dihydroxyestra-1,3,5(10)-trien-17α-yl)methyl-*N*-

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¶ Abbreviations: [^{3}H]E_2, [2,3,6,7-^{3}H(N)]-17β-estradiol; ER, estrogen receptor; 17α-CNU, *N*-(2-chloroethyl)-*N'*-(3,17β-dihydroxyestra-1,3,5(10)-trien-17α-yl)methyl-*N*-nitrosourea; 17β-CNU, *N*-(2-chloroethyl)-*N'*-(3-hydroxyestra-1,3,5(10)-trien-17β-yl)-*N*-nitrosourea; HEX-CNU, *N*-(2-chloroethyl)-*N'*-2,3-di(*p*-hydroxyphenyl)-pentanyl-*N*-nitrosourea; and CCNU, *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea.

nitrosourea (17α-CNU) and *N*-(2-chloroethyl)-*N'*-(3-hydroxyestra-1,3,5(10)-trien-17β-yl)-*N*-nitrosourea (17β-CNU): syntheses of these two compounds and their corresponding amino steroid precursors have been described previously [2].

N-(2-Chloroethyl)-*N'*-2,3-di(*p*-hydroxyphenyl)-pentanyl-*N*-nitrosourea (HEX-CNU): 1-amino-2,3-di(*p*-hydroxyphenyl)-pentane as its hydrochloride was prepared according to the procedure of Lee *et al.* [10]. To a solution of 1-amino-2,3-di(hydroxyphenyl)-pentane hydrochloride (2 g, 6.5 nmol) in pyridine (20 ml) at 0°, (2-chloroethyl)-nitrosocarbamoyl azide [2] (1.15 g, 6.5 nmol) in ether (3 ml) was added dropwise with stirring. The mixture was stirred at 0° for 3 hr and then ice water was added followed by extraction with ether. The ether extract was washed with ice-cold 2 N HCl, 10% $NaHCO_3$ and saturated NaCl solution successively, dried over $MgSO_4$, and evaporated to dryness. Crystallization from ethanol–chloroform yielded HEX-CNU as a colorless crystal (1.7 g, 65% yield): m.p. 161–163° (dec.); i.r.: 3560 (OH), 3410 (NH), 1720 (C=O), 1525 (CNH) 1490 cm^{-1} (NO); PMR (d_6THF): (ppm) 53.30, 3.94 (2 triplets, $J = 7H_z$, 4H, A_2B_2, —N—CH_2CH_2Cl), 0.54 (triplet, $J = 7H_z$, 3H, CH_3—CH), 1.32 (m, 2H, CH_3—CH_2—CH—) 7.94 (phenolic-OH). Mass spec. 405 (M^+), 375 (M^+—NO), 297 (M^+ – HON=N—CH_2CH_2Cl).

Preparation of rat uterus cytosol. Immature female Sprague–Dawley rats (day 21–25) were decapitated, and their uteri were collected in ice-cold TEA buffer (0.01 M Tris–HCl, 0.0015 M EDTA, 0.02% sodium azide, pH 7.4). After the attached fatty tissue was trimmed off, the uteri were rinsed with fresh cold buffer and two uteri were homogenized in 1 ml of TEA buffer at 4° using a motor-driven all-glass conical tissue grinder. The homogenate was centrifuged at 180,000 *g* for 60 min. The supernatant (cytosol) was removed with a pipette and used freshly in all studies. Protein was determined by the method of Bradford [11] using Bio-Rad reagents (Missisauga, Ontario) and bovine serum albumin as standard.

Competitive binding assay. Competitive ER binding assay was performed using the dextran-coated charcoal (DCC) technique, as described by Katzenellenbogen *et al.* [4], except that immature Sprague–Dawley rats (day 21–25) were used. Briefly, various concentrations of drugs (diluted in 1:1 dimethylformamide:TEA buffer solution) and fixed concentrations of [^{3}H]E_2 were pipetted into the wells of a microtiter plate on ice. An aliquot of uterine cytosol was then added such that the concentration of [^{3}H]E_2 was 1×10^{-8} M and the concentration of dimethylformamide was 7%; the total volume was 70 μl. After 16 hr of incubation at 0–4°, 10 μl dextran-coated charcoal (5 g acid washed Norit-A, 0.5 g dextran in 100 ml of TEA) was added to each well and the plate was vortexed in a shaker at 4° for 0.5 hr. The microtiter plate was then centrifuged at 800 *g* for 15 min at 4°, and 50-μl aliquots were pipetted into 10 ml of scintillation counting fluid (1:1 PCS/xylene) for counting.

The concentration of competitor required to inhibit by 50% (IC_{50}) the binding to ER of [^{3}H]E_2, which has a high binding affinity, was determined for each agent. The relative binding affinity (RBA) is defined as the ratio of IC_{50} of unlabeled estradiol to that of the competitor:

$$\text{RBA} = \frac{(IC_{50})\text{estradiol}}{(IC_{50})\text{ competitor}} \times 100$$

Ligand exchange assay. The procedure described by Katzenellenbogen *et al.* [12] was used. Cytosol was incubated with one of these: 30 nM E_2, 1.5 μM 17α-CNU, 15 μM HEX-CNU or 15 μM CCNU at 0° for 16 hr. The unbound ligand was removed by DCC (10% v/v, 15 min) adsorption. After centrifugation (1000 *g*, 10 min), the charcoal-stripped cytosol was divided into two portions which were saturated with 30 nM [^{3}H]E_2 ("hot" exchange) or 30 nM [^{3}H]E_2 plus 3 μM E_2 ("hot plus cold" exchange). The incubation mixture was allowed to reach equilibrium (1 hr), and a portion was assayed by DCC adsorption to determine the concentration of specific binding sites that were unoccupied at the start of the assay (time 0). The above procedures were all performed at 0°. The mixture was warmed to 10°, and exchange was allowed to proceed for 30 hr. At this temperature, the cytosol preloaded with E_2 showed complete exchange after 20 hr. At different times, aliquots were taken and cooled to 0° and assayed by DCC adsorption. The specifically bound [^{3}H]E_2 at each time point was calculated by subtracting the non-specific binding (hot and cold) from the total binding (hot).

To determine the stability of ER at this temperature, two portions of cytosol were incubated with 30 nM [^{3}H]E_2 and 30 nM [^{3}H]E_2 plus 3 μM E_2 respectively. The incubation was carried out at 0° and 10° in parallel with the exchange assay. Aliquots were taken at different times to measure specific binding.

Nuclear fixation of estrogen receptor by estrogen-nitrosourea conjugates. Oophorectomy was performed on 2- to 3-month-old Sprague–Dawley rats; 3 days later, the uteri were excised. Two horns from each uterus were separated and incubated at 37°, for 1 hr in oxygenated Dulbecco's phosphate-buffered saline supplemented with 0.1% glucose, pH 7.4, in the presence of either drug (treated group) or vehicle (control). The final concentration of ethanol (vehicle) in the buffer was 1%. After incubation, the horns were homogenized separately in TEA buffer; the nuclear fraction (800 *g*, 10 min, pellet) and cytosol (115,000 *g*, 30 min, supernatant) were fractionated by centrifugation. The ER content in the cytosol and nuclei was determined by ligand exchange assay as described by Anderson *et al.* [13].

Assay of uterotrophic and anti-uterotrophic activity. Sprague–Dawley rats (20- to 22-days-old) were randomly sorted into groups of six to ten animals each. One group was injected subcutaneously with 0.1 μg estradiol in 0.1 ml sesame oil for 3 consecutive days to demonstrate the uterotrophic effect of estradiol. To test the uterotrophic or anti-uterotrophic effect of the nitrosoureas, either 1, 10, 100 or 1000 μg of drug was administered s.c. alone or simultaneously with 0.1 μg estradiol in 0.1 ml sesame oil for 3 consecutive days. The negative control group consisted of rats receiving only 0.1 ml of sesame oil. On day 4, the animals were weighed and then killed. Uteri were removed, blotted on a piece

of filter paper and weighed; the ratio of the uterine weight (mg) to body weight (g) was used as an index of drug effect.

RESULTS

Competitive binding assays. The structures of E_2, hexestrol, the three synthetic estrogen-nitrosourea conjugates, and CCNU are shown in Fig. 1. The competitive binding assay was utilized to determine whether or not the conjugates can bind to ER. The inhibition of binding of [^{3}H]E_2 to rat uterine cytosolic ER was determined at various concentrations of competitors (Fig. 2). The relative binding affinities for 17α-CNU, 17β-CNU and HEX-CNU were 2, 0.4, and 0.2 respectively (the relative binding affinity for E_2 is defined as 100).

By contrast, CCNU [*N*-(2-chloroethyl)-*N*-cyclohexyl-*N*-nitrosourea], which has a cyclohexyl instead of an estrogen moiety, could not inhibit [^{3}H]E_2 binding even at a concentration 10,000-fold higher than that of labeled ligand (data not shown).

In vitro *inactivation of ER by nitrosourea conjugates.* The competitive binding assay measures the relative binding affinity of the competitor to ER at equilibrium conditions. It does not provide information whether or not the binding is reversible. To gain more information about the nature of the binding of these nitrosoureas to ER, we performed the ligand exchange assay. In this assay and experiments described below, we used only 17α-CNU and HEX-CNU since they exhibited the highest and lowest binding affinity, respectively, for ER. Rat uterine cytosol ER was first loaded with either 17α-CNU,

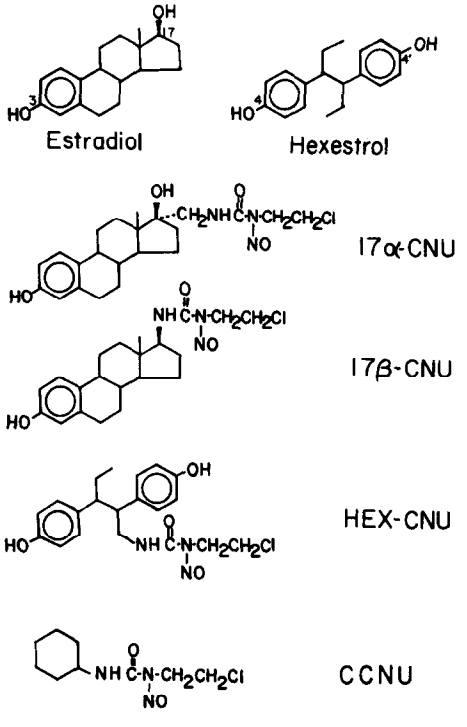

Fig. 1. Chemical structures of estrogens, CCNU, and estrogen-nitrosourea conjugates.

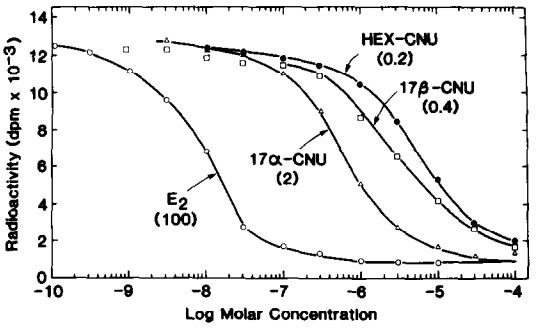

Fig. 2. Inhibition of [^{3}H]estradiol (E_2) binding to rat uterine cytosol receptors by estrogen-nitrosourea conjugates. Various concentrations of competitors were incubated with 1×10^{-8} M [^{3}H]E_2 in rat uterine cytosol (2 uteri/ml) for 16 hr at 4°. Dextran-coated charcoal was used to adsorb unbound ligands. The amount of radioactivity represents [^{3}H]E_2 bound to ER at the specified concentration of inhibitors. Numbers in parentheses are relative binding affinities (see text).

HEX-CNU or CCNU, and the ligand was then exchanged with [^{3}H]E_2. As shown in Fig. 3, in the cytosol preparation preloaded with 17α-CNU or HEX-CNU, there was a fraction of ER which could not be replaced by [^{3}H]E_2. The inactivation of ER amounted to 15% for 17α-CNU and 70% for HEX-CNU. For CCNU which could not bind to ER specifically, very little inactivation was observed. Under these experimental conditions, the E_2-receptor complex was stable for at least 46 hr, as shown by the stability test.

To determine whether this irreversible loss of receptor binding sites is specific or not, the following experiment was performed. The receptor was first preloaded with 30 nM [^{3}H]E_2 at 18° for 2 hr, followed by incubation with one compound: 30 nM E_2, 1.5 μM 17α-CNU, 15 μM HEX-CNU or 15 μM CCNU at 0° for 16 hr. The unbound E_2 and nitrosoureas were adsorbed by 0.5% dextran-coated charcoal. After centrifugation at 1000 *g* for 10 min, the supernatant fraction was adjusted to a final concentration of 30 nM [^{3}H]E_2 with or without 3 μM unlabeled E_2.

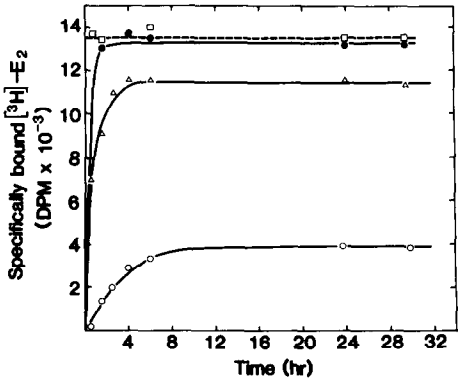

Fig. 3. Ligand exchange assay of rat uterine cytosol saturated with 1.5 μM 17α-CNU (△), 15 μM HEX-CNU (○) or 15 μM CCNU (●). The stability of ER (□) under the experimental conditions was determined as described in Materials and Methods.

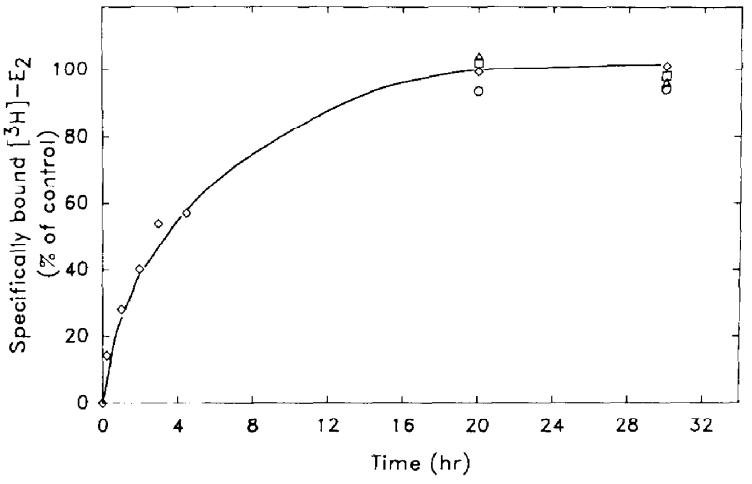

Fig. 4. Ligand exchange assay of rat uterine cytosol preloaded with 30 nM E_2 at 18° for 2 hr and then with either 1.5 μM 17α-CNU (△), 15 μM HEX-CNU (○) or 15 μM CCNU (□) at 0° for 16 hr. The exchange was complete after 20 hr at 10° for cytosol preloaded only with 30 nM E_2 (◇). The conditions of the experiment are described in the Results.

The exchange of radiolabeled with unlabeled E_2 was performed at 10° as in the previous experiment. Figure 4 shows that at the exchange temperature (10°), rat uterine cytosol ER preloaded with unlabeled estradiol completely exchanged with [^{3}H]E_2 in 20 hr. The cytosol preloaded with unlabeled E_2 and then treated with nitrosoureas before the exchange with radiolabeled E_2 showed no loss of binding sites at 20 or 30 hr.

Nuclear fixation of estrogen receptor by estrogen-nitrosourea conjugates. To test whether the estrogen nitrosourea conjugates interact with estrogen receptor in the cell, we performed experiments in which the distribution of ER in cytosol and nucleus was determined in the presence of these agents (Table 1). At a concentration of 1×10^{-7} M 17α-CNU, there was a significant increase of ER in the nucleus although there was no significant change in cytosolic ER content. At 1×10^{-5} M 17α-CNU, there was a significant decrease in cytosolic ER and a significant increase in nuclear ER. Similarly, 1×10^{-5} M HEX-CNU also induced a decrease in cytosolic ER and an enhancement of nuclear ER content. These experiments clearly illustrated that these estrogen-nitrosourea conjugates, like E_2, interact with uterine ER and cause a greater location of ER in the nucleus.

Effects of nitrosourea conjugates on rat uterine weight. The effects of nitrosourea conjugates on

Table 1. Nuclear fixation of ER in rat uterus by E_2, 17α-CNU and HEX-CNU

Condition*	ER content (% of control) Cytosol	Nuclei
Control	100.0 ± 11.7	100.0 ± 3.2
E_2 (1×10^{-7} M)	38.1 ± 9.9 ($P < 0.01$)	259.0 ± 7.0 ($P \ll 0.001$)
Control	100.0 ± 11.3	100.0 ± 13.1
17α-CNU (1×10^{-7} M)	114.2 ± 8.9 (NS)†	130.2 ± 7.1 ($P < 0.02$)
Control	100.0 ± 2.0	100.0 ± 23.1
17α-CNU (1×10^{-5} M)	76.7 ± 11.0 ($P < 0.001$)	373.8 ± 13.1 ($P < 0.001$)
Control	100.0 ± 18.2	100.0 ± 9.8
HEX-CNU (1×10^{-7} M)	111.0 ± 18.6 (NS)	119.0 ± 13.1 (NS)
Control	100.0 ± 11.5	100.0 ± 3.1
HEX-CNU (1×10^{-5} M)	46.1 ± 3.2 ($P < 0.02$)	303.0 ± 9.6 ($P < 0.01$)

* The two horns of each rat uterus were separated and incubated in phosphate buffer containing E_2 or drug at the concentration shown or with 1% ethanol as control. After 60 min at 37°, the cytosol and nuclei were fractionated by centrifugation. The ER content in these two fractions was determined by the dextran-coated charcoal adsorption method as described in Materials and Methods. Considerable inter-experimental variation was noted, so that a simultaneous control was performed within each experiment ER content (mean ± SE) in sixteen control determinations in the cytosolic fraction was 115.4 ± 17.9 fmol/mg protein and in open nuclear fraction was 162.5 ± 24.0 fmol/mg protein. The values in the table are expressed as percentage of control; each represents the mean ± SE of three to six determinations. The data were statistically evaluated by Student's two-tailed *t*-test.

† NS, no significance.

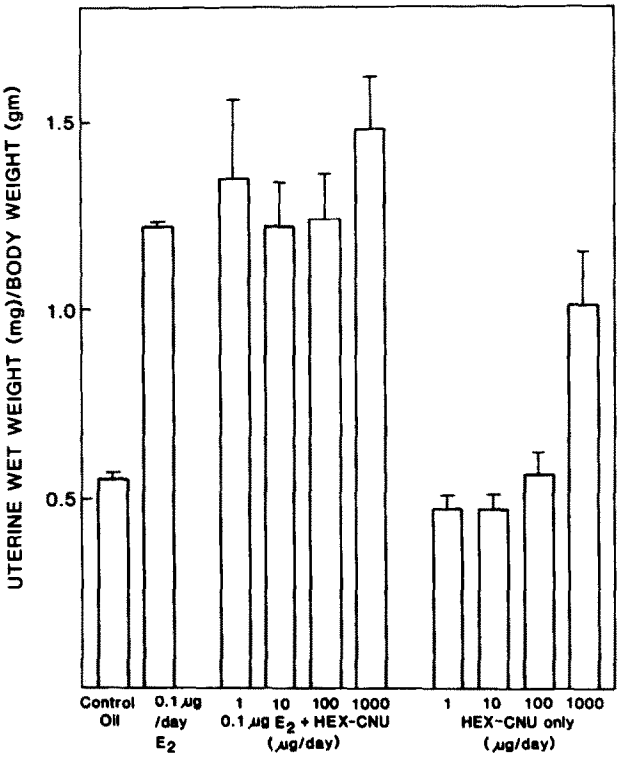

Fig. 5. Effect of administration of E_2 and/or HEX-CNU on uterine weight in immature rats. Twenty- to twenty-two-day-old female Sprague–Dawley rats were injected s.c. with E_2 (0.1 μg/day), HEX-CNU (1–1000 μg/day) or both for 3 consecutive days. The control group received the vehicle only (0.1 ml sesame oil). On day 4, the animals were killed, and their uteri were excised, blotted and weighed. Values represent the mean ± SEM of eight animals. Results were anlayzed by Student's two-tailed *t*-test. The increase in uterine weight in rats treated with estradiol either alone or with HEX-CNU was highly significant, compared to control rats receiving oil ($P \ll 0.001$). There was no significant difference in uterine weight in rats treated either with estradiol alone or with estradiol and HEX-CNU. In rats treated with HEX-CNU alone, uterine weight was significantly greater than that of controls receiving oil only in those treated with 1000 μg HEX-CNU ($P < 0.001$).

uterine weight in rats treated with drug for 3 consecutive days were evaluated (Figs. 5 and 6). The ratio of uterine weight (mg) to body weight (g) was used as an index of normalized uterine weight. Our results showed that neither 17α-CNU nor HEX-CNU possessed any antagonistic activity against the uterotrophic effect of 0.1 μg E_2, even at a dosage 10,000-fold higher than that of E_2. By contrast, 17α-CNU at a dosage of 100 and 1000 μg/day and HEX-CNU at 1000 μg/day each demonstrated a significant augmentation of uterine weight.

DISCUSSION

The competitive binding assay has been used to determine which structural factors are involved in ligand binding to ER. Our results demonstrated the importance of the free hydroxyl functional groups in the binding of estrogen to ER, as others have reported [3–6]. Among the three estrogen-nitrosourea conjugates studied, 17α-CNU had the highest RBA, in accord with other reports that substitution at the 17α position of E_2 has relatively little effect on binding affinity [3–6]. On the other hand, HEX-CNU possessed the lowest RBA despite the presence of two phenolic hydroxyl groups. This is probably due to the introduction of a bulky nitrosourea moiety into the mid-portion of the hexestrol molecule, thereby predisposing to more steric hindrance for binding.

To determine the nature of the interaction of nitrosourea with ER, we first loaded the cytosolic ER with nitrosourea and then studied the exchange of bound nitrosourea with radiolabeled E_2. The ligand exchange assay provided evidence that 17α-CNU and HEX-CNU were able to deactivate ER when the incubation temperature was raised to 10° even in the presence of E_2 excess; by contrast, the non-ER-binding nitrosourea, CCNU, was inactive. When the receptor was first preloaded with unlabeled E_2, followed by incubation with the nitrosoureas at 0°, full E_2 exchange could be restored. This indicated that the irreversible inactivation of ER by the two nitrosourea conjugates was not a non-specific reaction or due to the dilution of the specific activity of the tracer by any unadsorbed residual nitrosourea conjugates or their decomposition products. These results suggest that the two nitrosourea conjugates may bind to ER covalently so that, despite the high

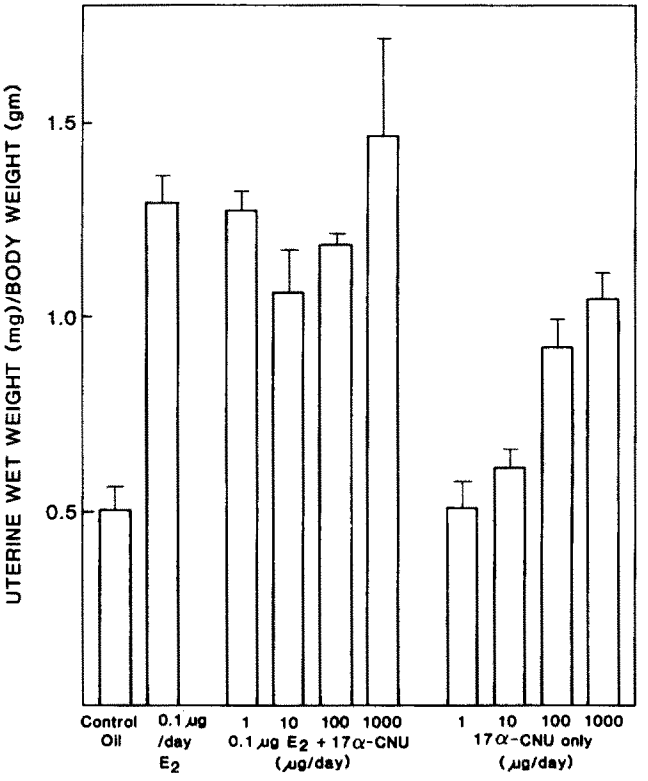

Fig. 6. Effect of administration of E_2 and/or 17α-CNU on uterine weight in immature rats. The experimental design has been described in the legend to Fig. 5. Values are expressed as the mean ± SEM of five or six animals. Uterine weight in rats treated with estradiol either alone or together with 17α-CNU was greater than that of control rats receiving oil, and the difference was statistically significant ($P \ll 0.001$). No difference was noted between rats treated with estradiol alone or with estradiol and 17α-CNU. In rats treated with 17α-CNU alone, uterine weight was significantly greater than that of control rats only in those treated with 100 μg ($P < 0.01$) or 1000 μg ($P < 0.001$) 17α-CNU per day.

affinity of E_2, the latter cannot displace the drugs from the conjugate–ER complex; binding of the nitrosourea to the receptor appeared to be a prerequisite for inactivation of ER. Since decomposition of the nitrosourea conjugate may yield an active isocyanate intermediate, which has strong carbamoylating activity, it is possible that this derivative may react with an amino group(s) at the binding site of ER and form covalent links to ER. However, direct evidence for this mechanism will require experiments using nitrosoureas with radiolabel on the estrogen moiety.

Furthermore, the abilities of 17α-CNU and HEX-CNU to deactivate ER *in vitro* suggest that they may act either as estrogens or antiestrogens. This prompted us to study the effects of these agents *in vivo* against the uterotrophic effects of E_2. However, our studies indicated that neither 17α-CNU nor HEX-CNU, which has potent ER-deactivating activity, exhibited any antiuterotrophic effect when administered to immature rats even at concentrations 10,000-fold higher than E_2. The half-life of each compound was determined in phosphate-buffered saline at pH 7.4, at 37°, and was found to be 110 min for 17α-CNU and 60 min for HEX-CNU [9]. The low binding affinity and low stability of these agents in aqueous buffer may be responsible for their ineffectiveness as antiestrogens *in vivo*.

One paradoxical observation was that the binding affinity of HEX-CNU for ER was about one order of magnitude lower than that of 17α-CNU, although the former was more potent than the latter in inactivating ER *in vitro* at 10°. There are at least two explanations for this observation: (1) although HEX-CNU is less stable, it may decompose while still associating with the binding site, thereby enabling the active isocyanate intermediate to react more effectively with ER at the binding site, and/or (2) the sites of substitution of the nitrosourea moiety on the estrogen molecule are different in these two conjugates. In the HEX-CNU molecule, the nitrosourea is attached to the hydrophobic mid-portion of the estrogen skeleton. By contrast, the nitrosourea group is attached to a region closer to the free hydroxyl group in 17α-CNU. Therefore, the isocyanate derivatives formed from the decomposition of these two agents may interact with different regions of the ER binding site. If the isocyanate group generated from HEX-CNU interacts with a region of the ER where amino groups are more accessible, the parent conjugate will be more potent in the inactivation of ER.

Interestingly, 17α-CNU at 100 and 1000 μg/day and HEX-CNU at 1000 μg/day demonstrated significant uterotrophic activity. The uterotrophic effects may be due to either covalent linkage of the agent to ER and hence formation of a permanent activated complex or to decomposition/metabolic products of these nitrosoureas. However, on the basis of our results we cannot distinguish whether the estrogenic effect is due to the parent nitrosourea conjugates or to decomposition products such as isocyanates.

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